

Regulation of Hypoxia-inducible Factor 1 by Glucose Availability under Hypoxic Conditions

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Hypoxia-inducible transcription factor 1 (HIF-1), consisting of HIF-1 α and HIF-1 β subunits, regulates the expression of a variety of genes involved in diverse adaptive processes in response to hypoxia. While oxygen availability regulates HIF-1 α by proteolytic degradation, some growth factors regulate HIF-1 α by protein synthesis in part through mammalian target of rapamycin complex 1 (TORC1) pathway. We herein report the role of nutrient availability on the regulation of HIF-1. A reduced availability of glucose, not amino acids, results in a decrease of the expression of HIF1-dependent genes and HIF-1 α protein in response to hypoxia. HIF-1 α mRNA expression was not significantly suppressed and DMOG, an inhibitor for proteasomal degradation of HIF-1 α , did not induce HIF-1 α protein expression under hypoxia combined with glucose depletion. In comparison to the effect in the presence of glucose, glucose depletion under hypoxia induced a much stronger activation of the AMP-dependent kinase pathway and phosphorylation of eIF2 α , and nearly complete inhibition of the TORC1 pathway. These findings imply that the reduced availability of glucose under hypoxia downregulates HIF-1 in part through the inhibition of HIF-1 α mRNA translation, which is occasionally observed in pathophysiological situations such as ischemic diseases.

Oxygen deprivation or hypoxia is induced in tissues when oxygen supply relatively fails to meet the demand of cells. This situation is observed in several pathophysiological conditions where blood flow decreases or the demand of oxygen supply relatively increases in tumor formation. It is also observed in physiological conditions such as muscle exercise. Hypoxia is often accompanied with a limited supply of nutrients, including glucose and amino acids. Organisms or individual cells are able to monitor changes in their environments and adapt themselves to the limited supply of oxygen and nutrients.

Hypoxia-inducible transcription factor 1, HIF-1, has been identified by the studies of adaptation to hypoxia. HIF-1, consisting of the oxygen-regulated HIF-1 α subunit and the constitutively expressed HIF-1 β subunit, regulates the expression of a variety of genes involved in diverse adaptive processes such as angiogenesis, erythropoiesis or glycolysis

(24,26). Under normoxic conditions, HIF-1 α is a short lived-protein because of its continuous proteolysis via the ubiquitin-proteasome pathway (9,23). In contrast, reduced oxygen availability induces HIF-1 α accumulation through the relaxation of its degradation. HIF-1 α degradation is regulated via recognition and modification by the product of the von Hippel-Lindau tumor suppressor gene, pVHL, which acts as an E3 ubiquitin ligase for HIF-1 α (13,22). The interaction of HIF-1 α and pVHL requires oxygen-dependent hydroxylation on proline residues of HIF-1 α , thereby HIF-1 α accumulates only under hypoxic conditions (8).

HIF-1 α is also regulated by growth factors or hormones, such as heregulin, IGF1, or insulin via protein synthesis of HIF-1 α in a cell-type specific manner (3,19,25). Several studies using inhibitors to Phosphoinositide 3-kinase (PI3-K) and mammalian target of rapamycin (mTOR) have implicated the PI3-K and mTOR signaling pathways in the upregulation of HIF-1 α (25). PI3-K phosphorylates the 3'-OH position of the inositol ring in inositol phospholipids, which then activates 3'-phosphoinositide-dependent-kinase-1 (PDK1). PDK1 directly phosphorylates and activates p70 S6 kinase in a synergistic manner with mTOR (12). The Ser/Thr protein kinase, mTOR signals through two physically distinct multiprotein complexes called TOR complex 1 and 2 (TORC1 and TORC2) (27). TORC1 contains the polypeptides raptor and LST8, and the ability of raptor to properly present its substrates, such as eukaryotic initiation factor 4E binding protein 1 (4EBP1) and p70 S6 kinase, to the mTOR catalytic domain is essential for the translation of a fraction of mRNA (6,14). mTOR-dependent signals are also required for HIF-1 α induction by hypoxia, as well as by growth factors in some cells since mTOR inhibitor rapamycin interferes with the induction of HIF-1 α (10). In addition to being regulated by insulin or IGF1, the TORC1 pathway is regulated by nutrients, since the phosphorylation and/or activation of 4EBP1 and p70 S6 kinase are severely inhibited by depletion of amino acids and to a lesser extent by depletion of glucose (5). Therefore, mTOR plays a crucial role in the nutrient- and oxygen- sensitive signaling pathways.

In the present study, we set out to examine the role of nutrients in the regulation of HIF-1 in response to hypoxia. Our results demonstrated that the induction of HIF-1 α by hypoxia requires the availability of glucose, not amino acids in HEK293 cells and HeLa cells, at least in part through the regulation of mRNA translation.

MATERIALS AND METHODS

Reagents and antibodies. Dulbecco's Phosphate-Buffered Saline (D-PBS) composed of 0.1 g/l CaCl₂, 0.2 g/l KCl, 0.2 g/l KH₂PO₄, 0.047 g/l MgCl₂, 8 g/l NaCl, and 1.15 g/l Na₂HPO₄ was purchased from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM), Earle's Balanced Salt Solution (EBSS) and Dulbecco's Modified D5030 Eagle's Medium Base (D5030) were purchased from SIGMA-Aldrich (St. Louis, MO). DMEM without amino acids, which contains all ingredients of DMEM except for amino acids, was made by mixture of vitamin, Sodium Hydrogen Carbonate and 4.5 g/l glucose in EBSS (designated as EBSS-derived medium). DMEM without glucose, which contains all ingredients of DMEM except for glucose, was made by mixture of L-glutamine, Sodium Hydrogen Carbonate and Phenol Red in D 5030 medium (designated as D5030 medium). Anti-HIF-1 α antibody was purchased from BD Biosciences (Rockville, MD) and anti-HIF-1 β antibody was from Novus Biologicals, Inc (Littleton, CO). Anti-phospho-Acetyl-CoA Carboxylase (Ser79) antibody, anti-phospho-AMPK- α (Thr172) antibody, anti-phospho-S6 ribosomal protein (Ser235/236) antibody, anti-S6 ribosomal protein antibody, anti-phospho-eIF2 α (Ser51) antibody and anti-eIF2 α antibody were

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purchased from Cell Signaling Technologies (Beverly, MA). Compound C was purchased from Merck KGaA (Darmstadt, Germany) and Dimethylallyl glycine (DMOG) was purchased from Alexis Biochemicals (San Diego, CA).

Cell culture and treatments. HEK293 cells and HeLa cells were maintained in DMEM with 10% fetal calf serum (FCS). Confluent cells were washed once with D-PBS and incubated in DMEM, D5030 medium, EBSS-derived medium or D-PBS for 2 h under either normoxic or hypoxic conditions without FCS. Hypoxic conditions (95% N₂, 5% CO₂, and less than 0.1% O₂) were achieved by using an anaerobic jar equipped with AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan). When the cells were treated with Compound C, the cells were pretreated with the indicated concentrations of compound C for 30 min, then transferred into the indicated media with the same concentration of Compound C. When the cells were treated with DMOG, the cells were incubated in D5030 medium with 1mM DMOG under normoxic or hypoxic conditions for 2 h.

Cell lysis and immunoblotting. The cell treatments were terminated by one wash with ice cold PBS and the cells were extracted in buffer H (10 mM HEPES-KOH, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 20 mM NaF, 20 mM β -glycerophosphate, 5 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride), followed by centrifugation at 2,000 rpm for 5 min at 4°C. The supernatants were used as crude cytosolic fractions. The pellets were resuspended in sample buffer, followed by sonication and the supernatants were used as crude nuclear fractions. The nuclear and cytosolic fractions were separated by SDS-PAGE, transferred onto PVDF membranes, and then the membranes were immunoblotted with the indicated antibodies and visualized by using the ECL method.

RT-PCR and Quantitative analysis of mRNAs. Total cellular RNA was isolated from the cells with the use of an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) and real-time quantitative RT-PCR analysis was performed using an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). The primer set used for human HIF-1 α was purchased from QIAGEN and human 18S ribosomal RNA was used as an internal standard. The primers used for detection of VEGF, GLUT1 and β actin by RT-PCR were as follows: human VEGF, 5'-aaggaggag ggcagaatcat-3' (sense) and 5'-atctgcatggtgatgttggga-3' (antisense); human GLUT1, 5'-cttactgtcgtgtcgtgt-3' (sense) and 5'-tgaagagttcagccacgatg-3' (antisense); human β actin, 5'- cctggcaccagcacaat-3' and 5'-gccgatccacacggagtact-3' (antisense).

Measurement of intracellular ATP. The cells were lysed in solution A (0.5 M Perchloric Acid, 1mM EDTA) and centrifuged at 3,000 rpm for 10 min at 4°C. The supernatants were collected and neutralized, then, intracellular ATP levels were measured using the ATP Determination Kit (Molecular Probes, Inc, Eugene, OR).

RESULTS

The effects of the withdrawal of nutrients on the regulation of HIF-1 in response to hypoxia. As a first step for defining the role of nutrients on hypoxia-inducible HIF-1, HEK293 cells were incubated in DMEM or D-PBS under normoxic or hypoxic conditions for 2 h without FCS and the induction of HIF-1 α in the nucleus was analyzed by immunoblotting (Fig. 1A). HIF-1 α was induced when the cells were incubated in DMEM without FCS under hypoxic conditions for 2 h. In contrast, when the cells were incubated in D-PBS, the induction of HIF-1 α in the nucleus was severely inhibited. The inhibitory effect on HIF-1 α induction was, however, reversible as media was exchanged from D-PBS to DMEM without FCS and thereafter the accumulation of HIF-1 α was recovered (lane 6).

To elucidate the mechanism why HIF-1 α induction was inhibited in D-PBS, the induction of HIF-1 α was examined in cells incubated in the media lacking amino acids alone (EBSS-derived medium) or glucose alone (D5030 medium) (Fig. 1B). HIF-1 α was clearly induced by hypoxia even when the cells were incubated in EBSS-derived media, in which amino acids alone were removed from DMEM. In sharp contrast, when the cells were incubated in D5030 media in which glucose alone was removed from DMEM, the HIF-1 α induction by hypoxia was severely inhibited to the level where the cells were incubated in D-PBS. On the other hand, HIF-1 β was not influenced by the removal of amino acids and/or glucose in the same experiments. To confirm the role of glucose availability on HIF-1 α induction by hypoxia, the cells were incubated in media containing decreasing amounts of glucose under hypoxic conditions for 2 h and induction of HIF-1 α was examined (Fig. 1C). The induction of HIF-1 α was reduced by decreasing the concentration of glucose in media in a dose dependent manner; HIF-1 α expression was significantly reduced when glucose concentration was decreased to 0.5 g/l and became undetectable when the glucose concentration was less than 0.125 g/l. Since the withdrawal of glucose and oxygen from media should induce a depletion of ATP in living cells, we also measured the intracellular ATP levels in the same conditions (Table 1A). The intracellular ATP levels declined to 78% after incubation in DMEM under hypoxic conditions for 2 h. When glucose concentrations were decreased, the ATP levels declined more severely in a dose dependent manner.

To examine whether the decrease of HIF-1 α protein expression has any influence on the expression of HIF-1-dependent genes, the induction of VEGF and GLUT1 mRNAs was analyzed by RT-PCR (Fig. 1D). Whereas VEGF and GLUT1 mRNAs were clearly induced in response to hypoxia in DMEM, the inductions of these mRNAs under hypoxia were severely inhibited by the depletion of glucose. These results indicated that glucose depletion under hypoxia caused inhibitions of the expression of HIF-1-dependent genes as well as HIF-1 α protein.

The effects on the expression of HIF-1 α mRNA and the degradation of HIF-1 α protein. To explore the mechanism through which hypoxia-inducible HIF-1 α protein expression is inhibited by the depletion of glucose, we first examined the effect on HIF-1 α mRNA (Fig. 2A). In comparison to the mRNA level of HIF-1 α in the presence of glucose under hypoxia, glucose depletion under hypoxia appeared to decrease the mRNA level slightly. However, this marginal inhibitory effect of glucose depletion on HIF-1 α mRNA cannot fully explain the remarkable decrease in the amount of HIF-1 α protein and VEGF and GLUT1 mRNAs as described above.

Since the HIF-1 α protein is subject to rapid degradation through the pVHL-mediated ubiquitin-proteasome pathway, we next examined the effect of DMOG, a competitive inhibitor for HIF-1 α prolyl-hydroxylase (Fig. 2B). Whereas treatment of the cells with DMOG induced accumulation of HIF-1 α even when the cells were incubated in D5030 medium under normoxic conditions, the induction of HIF-1 α was not observed in the presence of DMOG in D5030 medium under hypoxia. Thus, these results suggested that the inhibition of HIF-1 α protein expression is not caused by degradation of HIF-1 α protein or inhibition of mRNA expression but by suppression of mRNA translation under hypoxia combined with glucose depletion.

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FIG. 1. Effects of the withdrawal of nutrients on hypoxia-induced HIF-1 α .

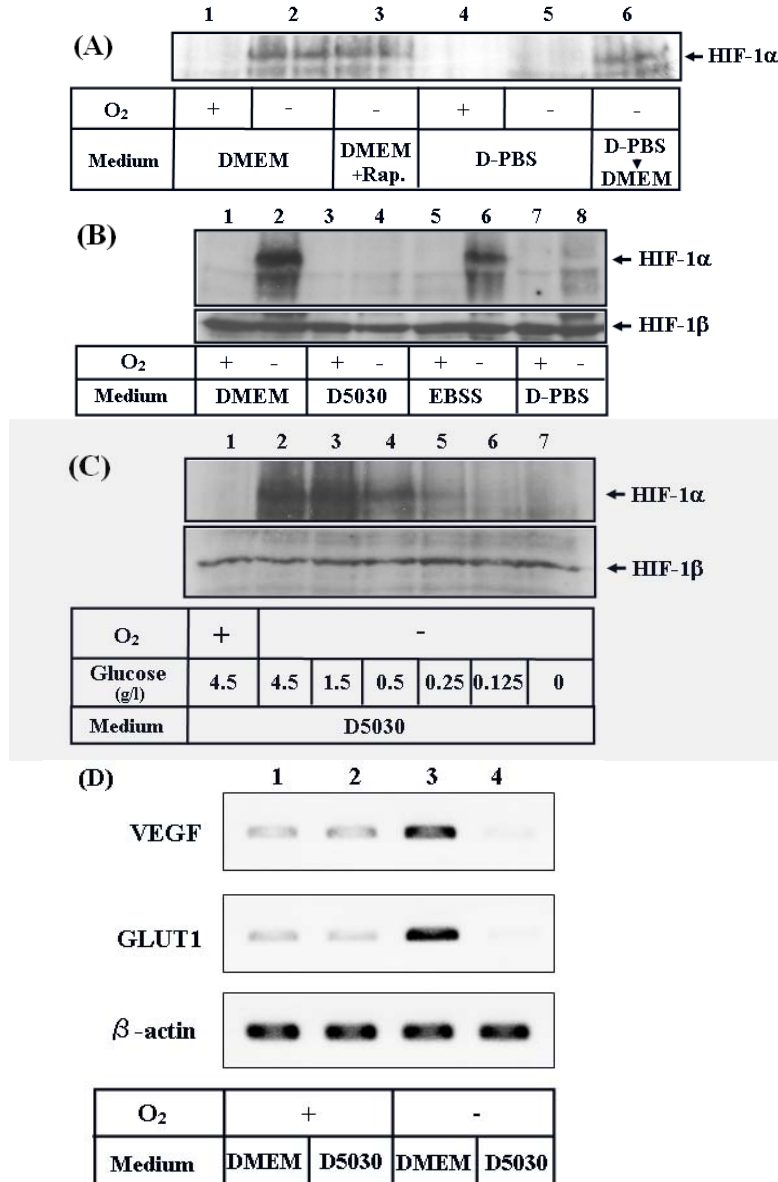
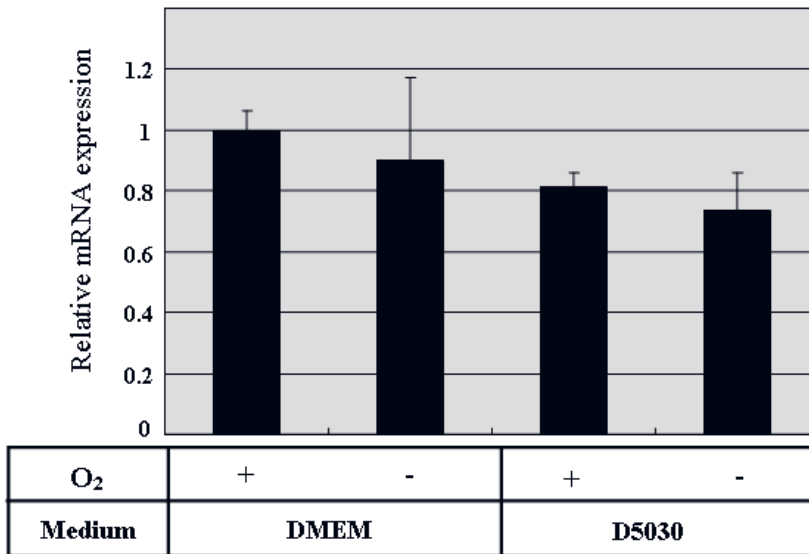


FIG. 1. (A) HEK293 cells were incubated in DMEM or D-PBS without FCS under normoxic or hypoxic conditions for 2 h. Some cells (lane 3) were treated with rapamycin (200 nM) during the incubation. In lane 6, after incubation in D-PBS under hypoxic conditions for 2 h, media was changed to DMEM and the cells were incubated under hypoxic conditions for an additional 2 h. Nuclear extracts were analyzed by immunoblotting with the anti-HIF-1 α antibody. (B) HEK293 cells were incubated in DMEM (lanes 1 and 2), D5030 media (lanes 3 and 4), EBSS-derived media (lanes 5 and 6) or D-PBS (lanes 7 and 8) under normoxic or hypoxic conditions for 2 h. Nuclear fractions were analyzed by immunoblotting with the indicated antibodies. (C) HEK293 cells were incubated in D5030 media containing indicated concentrations of glucose under normoxic or hypoxic conditions for 2 h. Nuclear extracts were analyzed by immunoblotting with the indicated antibodies. (D) HEK293 cells were incubated in DMEM or D5030 media under normoxic or hypoxic conditions for 2 h. Total mRNAs were prepared from the cells and RT-PCR was performed as described in Materials and methods.

(A)



(B)

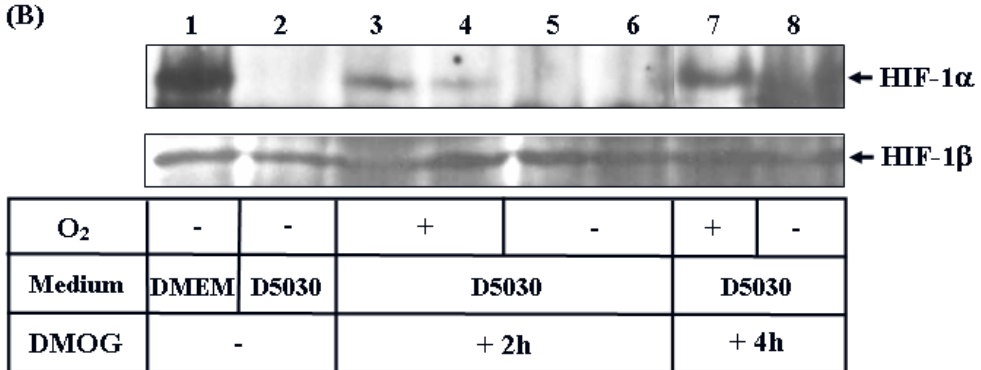


FIG. 2. mRNA expression of HIF-1 α and effects of DMOG on HIF-1 α expression.

(A) HEK293 cells were incubated and total mRNAs were prepared as described in Fig. 1D, and HIF-1 α mRNA expression was analyzed by quantitative real-time PCR as described in Materials and methods. (B) HEK293 cells were incubated in DMEM (lane 1) or D5030 media without (lane 2) or with 1mM DMOG for 2 h (lanes 3-6) or 4 h (lanes 7 and 8). For the last 2 h, some cells (lanes 1, 2, 5, 6 and 8) were incubated under hypoxic conditions for 2 h. Nuclear extracts were analyzed by immunoblotting with the indicated antibodies.

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Effects of Glucose (A) and (B) 2DG on ATP Contents in HEK293 Cells (% of control)

(A)			(B)		
O ₂	Glucose (g/l)	ATP (%)	O ₂	2DG (mM)	ATP (%)
+	4.5	100	+	0	100
-	4.5	78 ± 1.9	-	0	81.2 ± 0.8
-	1.5	109.3 ± 5.2	-	2.75	86.1 ± 0.5
-	0.5	69.4 ± 3.4	-	5.5	89.3 ± 0.5
-	0.25	66.8 ± 1.4	-	11	63.4 ± 1.9
-	0.125	40.1 ± 2.3	-	22	42.1 ± 3.0
-	0	19.1 ± 1.7	-	44	20.7 ± 1.1
-			-	88	6.3 ± 0.1

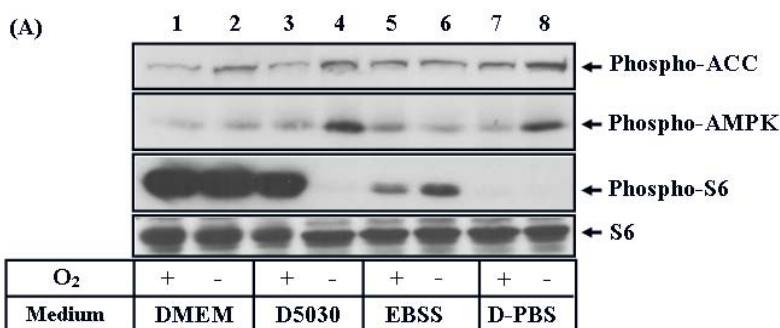
Table 1. HEK293 cells were treated as described in Fig. 1C (Table A) or Fig. 3B (Table B) and intracellular ATP concentrations were measured as described in the Materials and methods. The results were normalized to the measurements of ATP in normoxic cells in DMEM and are shown as the means +/- S.E..

Roles of translational regulation. We next set out to explore the possible involvement of translational regulation. We first focused on the AMPK and mTOR pathways because these pathways are regulated by glucose and amino acids, and they play important roles in mRNA translation. We examined the regulation of these pathways by monitoring the phosphorylation of AMPK, its substrate acetyl-CoA carboxylase (ACC), and ribosomal protein S6 (Fig. 3A). The phosphorylation of AMPK and ACC slightly increased when the cells were incubated in DMEM under hypoxic conditions for 2 h. The increases in the phosphorylation of AMPK and ACC were enhanced by the removal of glucose under hypoxic conditions to similar levels obtained when the cells were incubated in D-PBS under hypoxia. When compared to the ATP levels in cells (Table 1), this observation appeared consistent with the notion that AMPK is activated by the increase of AMP/ATP ratio; AMPK was slightly activated when the intracellular ATP levels was decreased to about 80% in the presence of glucose under hypoxia, whereas AMPK was highly activated when the ATP level was decreased to about 20% in the absence of glucose under hypoxia. On the other hand, the removal of amino acids had no additional effect on AMPK and ACC phosphorylation under hypoxic conditions. As expected, the phosphorylation of S6, which is physiologically induced by the activation of p70 S6 kinase via TORC1, was severely inhibited by removal of amino acids alone but not by glucose alone, under normoxic conditions. In contrast, the incubation in hypoxia without glucose severely inhibited the phosphorylation of S6 to similar levels observed when the cells were incubated in D-PBS under hypoxia.

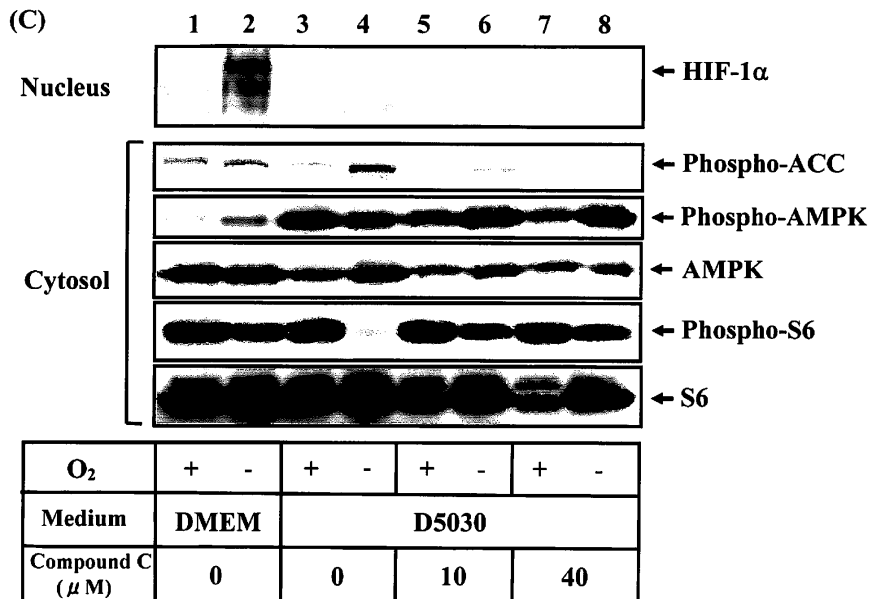
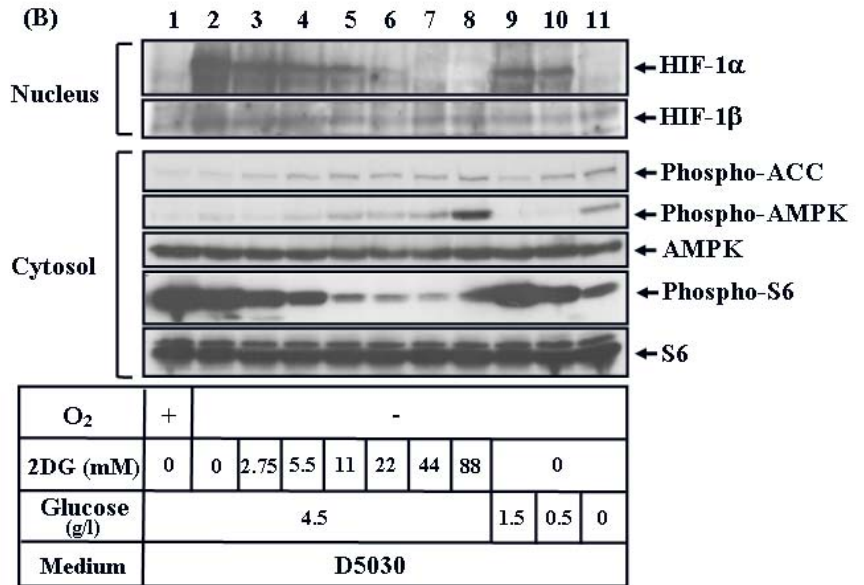
We also examined the effect of the glucose analogue, 2-Deoxy-D-glucose (2DG), which competitively inhibits glucose metabolism, thereby blocking ATP synthesis (Fig. 3B). The cells were incubated in DMEM containing 4.5 g/l glucose with increasing concentrations of 2DG under hypoxic conditions for 2 h. The immunoblot findings showed that HIF-1 α induction by hypoxia was reduced by incubation with 2DG in a dose dependent manner; the HIF-1 α induction was significantly inhibited at 11 mM and became undetectable at more than 22 mM. Intracellular ATP levels also declined by the presence of 2DG (Table 1B). The decrease of ATP in the cells induced the activation of AMPK in a dose dependent manner as shown by the phosphospecific immunoblot for AMPK and ACC. In contrast, the TORC1 pathway was reciprocally inhibited as shown by the phosphospecific immunoblot for S6. These results raise the possibility that AMPK activation or concomitant inhibition of the TORC1 pathway might be involved in the inhibition of HIF-1 α induction through the inhibition of HIF-1 α mRNA translation.

To examine this possibility, we used compound C, an AMPK inhibitor (Fig. 3C). Consistent with the inhibition of AMPK by compound C, phosphorylation of ACC was inhibited in a dose dependent manner. On the other hand, AMPK phosphorylation was not affected by treatment with compound C, because compound C inhibits AMPK activity by competitive binding to AMPK with ATP but does not inhibit the upstream kinase toward Thr172 on AMPK. In sharp contrast, the S6 phosphorylation that was abolished by incubation without glucose under hypoxia was clearly recovered by treatment with compound C. This is consistent with the idea that p70 S6 kinase was inhibited by active AMPK in the absence of glucose under hypoxia, and that the inhibition of AMPK by compound C resulted in the rescue of the p70 S6 kinase activity (11,15). In contrast to the effect on p70 S6 kinase, HIF-1 α induction was not rescued by the treatment of the cells with compound C. Although these results cannot rule out the possibility of TORC1 and AMPK pathway involvements in the regulation of HIF-1 α in the glucose-depleted conditions, another important mechanism could be expected. Therefore, we then examined the effect on the phosphorylation of eIF2 α . The phosphorylation of eIF2 α is known to be rapidly induced as a part of the endoplasmic reticulum (ER) stress responses through the pathways known as unfolded protein response (UPR) and this event has been implicated in the inhibition of mRNA translation in response to cellular stress including hypoxia (16). Incubation of the cells in DMEM under hypoxia slightly induced phosphorylation of eIF2 α , however the extent of phosphorylation was greatly enhanced by the depletion of glucose from media under hypoxia, whereas the effect of the depletion of amino acids alone was scarce (Fig. 4A). A similar effect was also observed in HeLa cells (Fig. 4B). The depletion of glucose from media significantly suppressed the induction of HIF-1 α in response to hypoxia. As observed in the case of HEK293 cells, the phosphorylation of eIF2 α and AMPK was significantly enhanced while the phosphorylation of S6 was severely inhibited by the depletion of glucose under hypoxia.

FIG. 3. Effects on AMPK and mTOR pathways.



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(A) HEK293 cells were treated as described in Fig. 1B and cytosolic fractions were analyzed by immunoblotting with the indicated antibodies. (B) HEK293 cells were incubated in D5030 media containing the indicated concentrations of 2DG or glucose under normoxic or hypoxic conditions for 2 h. The nuclear and cytosolic fractions were analyzed by immunoblotting with the indicated antibodies. (C) HEK293 cells were pretreated with the indicated concentrations of compound C (lanes 5 and 6; 10 μM, lanes 7 and 8; 40 μM) or DMSO (lanes 1-4) for 30 min, then the cells were incubated in DMEM or D5030 media containing compound C or DMSO under normoxic or hypoxic conditions for 2 h. The nuclear and cytosolic fractions were analyzed by immunoblotting with the indicated antibodies.

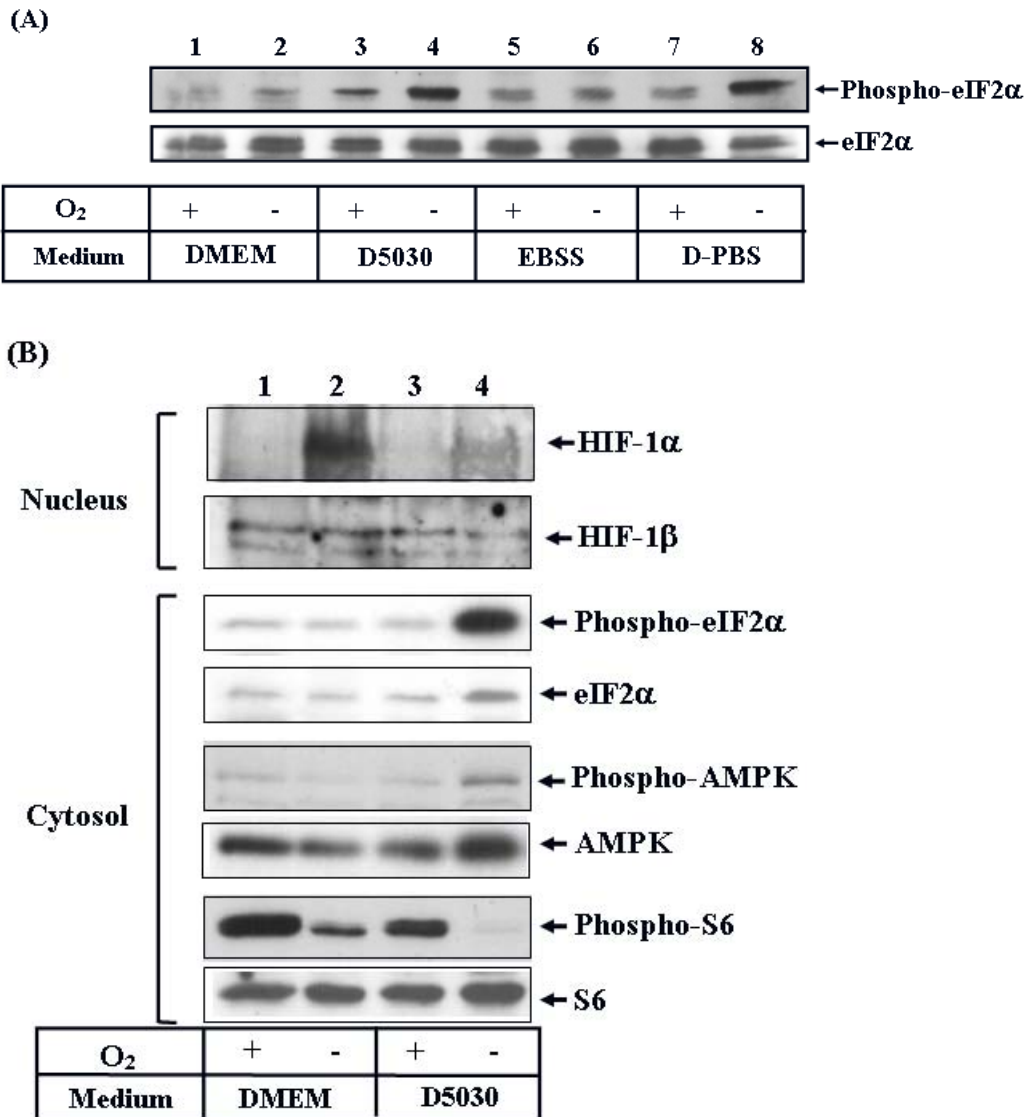


FIG. 4. Effects on the phosphorylation of eIF2 α .

(A) HEK293 cells were incubated as described in Fig. 1B and the cytosolic fractions were analyzed by immunoblotting with the indicated antibodies. (B) HeLa cells were incubated in DMEM or D5030 media under normoxic or hypoxic conditions for 2 h. Nuclear and cytosolic fractions were analyzed by immunoblotting with the indicated antibodies.

DISCUSSION

When cells are exposed to stresses that lack oxygen or nutrients, HIF-1 and/or mTOR respond to the change in the environment and modify their own effectors. Importantly, deprivation of oxygen and nutrients often occurs simultaneously in mammalian tissues, as both oxygen and nutrients are supplied from blood flow. Therefore, we examined the

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regulation of HIF-1 by hypoxia in the context of supply of nutrients. Our results demonstrated that the availability of glucose controls HIF-1 in response to hypoxia in HEK293 cells and HeLa cells. Although the depletion of glucose in combination with hypoxia causes a severe amount of stress to the cells, the cellular response described herein is a part of physiological program, not just cell damage. This is demonstrated by the results that the replacement of glucose-depleted media with glucose-containing media reversed the HIF-1 α response to hypoxia and that AMPK, ACC and S6 show more dramatic changes of phosphorylation in response to hypoxia in glucose-depleted media than in glucose-containing media. Moreover, the finding that the treatment of the cells with compound C in glucose-depleted media under hypoxic conditions reversed the phosphorylation of S6 indicates the ability of these cells to respond to the stimuli under such severe conditions.

By the removal of glucose or the addition of 2DG under hypoxic conditions, the expression of HIF-1 α declines in parallel with a decrease of ATP levels in the cells; when the intracellular levels of ATP decreased to ~60%, HIF-1 α induction was remarkably reduced, and when the ATP levels decreased to ~40%, HIF-1 α induction was nearly undetectable. Interestingly, when the cells were incubated in media with a modest decrease in glucose (1.5 g/l) or low concentration of 2DG (2.75 mM), intracellular ATP slightly increased under hypoxic conditions. This could be explained if the energy consumption is suppressed by a slight inhibition of the mTOR pathway as we have previously reported (4).

How then is HIF-1 α induction blocked by the removal of glucose or decrease in cellular ATP? A real-time PCR analysis demonstrates that it is not mainly mediated by the suppression of transcription of HIF-1 α mRNA. Lu *et al.* reported that the accumulation of HIF-1 α under normoxic conditions was inhibited by the depletion of glucose, and pyruvate prevents the degradation of HIF-1 α (21). Although the exact mechanism underlying HIF-1 α accumulation by pyruvate in normoxic conditions remains undetermined, they proposed a possible inhibitory action of pyruvate at the steps involving pVHL-mediated proteasomal degradation. In our case, it is also expected that glucose depletion under hypoxia causes a loss of pyruvate, thereby inhibiting the HIF-1 α protein expression via the activated degradation process for HIF-1 α . We cannot completely rule out this possibility however, the findings that DMOG failed to protect the HIF-1 α induction in D5030 medium under hypoxic conditions suggest another mechanism.

Several recent reports have indicated that protein synthesis is inhibited under hypoxia by changes in the rate of mRNA translation. The inhibition of mTOR effectors such as p70 S6 kinase and 4EBP1 has been implicated in this process (1,2). The involvement of translation initiation factor eIF2 α has been also reported (16,20). The phosphorylated form of eIF2 α , which is induced by several cellular stresses including hypoxia as a part of ER stress, inhibits mRNA translation by inhibiting an active ternary complex formation of eIF2, GTP and Met-tRNA through sequestering the exchange factor eIF2B. In the present study, we confirmed the activation of the AMPK pathway and the ER-stress pathway as well as inhibition of the mTOR pathway in response to hypoxia. Moreover, it is notable that the depletion of glucose remarkably enhances these responses. This suggests that hypoxia induces a severer inhibition of global protein synthesis if it is accompanied with a limited supply of glucose. It has been already reported that AMPK is strongly activated in wild-type and HIF-1 α -null mouse embryo fibroblasts following exposure to hypoxia in glucose-free medium (17). Our results using HEK293 cells and HeLa cells are consistent with their findings and support the idea that the ER-stress pathway or UPR plays a more important role in the inhibition of HIF-1 α mRNA translation. In addition to the regulation of global protein synthesis, another possibility is that HIF-1 α mRNA is specifically regulated

in response to glucose depletion under hypoxia. HIF-1 α mRNA has been reported to contain an internal ribosomal entry site (IRES) at the 5'UTR which thus enables HIF-1 α to be expressed in such a condition like hypoxia that cap-dependent translation is suppressed (18). If this is the case then, there might be a mechanism that responds to glucose depletion under hypoxia and leads to inhibition of IRES-dependent translation of HIF-1 α . We have previously reported that a small GTPase Rac1 is activated in response to hypoxia and required for the induction of HIF-1 α (7). The expression of the active form of Rac1, however, did not rescue HIF-1 α in the glucose depleted conditions under hypoxia (data not shown).

Although HIF-1 plays an important role in the organism or cells, thus adapting themselves to changes in their environment in response to hypoxia, a low availability of glucose restricts this program. In other words, hypoglycemia, if it occurs in a specific organ or throughout the body, then it interferes with the response to hypoxia. However, these components and their regulation in this system still remain to be elucidated.

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