Loss of HIF-1α impairs GLUT4 translocation and glucose uptake by the skeletal muscle cells

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DIABETES MELLITUS IS BECOMING epidemic throughout the world. Approximately 90–95% of people who are diagnosed with diabetes have type 2 diabetes, which is characterized by insulin resistance and pancreatic β-cell failure (21). Three specific abnormalities contribute to hyperglycemia in type 2 diabetes: impaired insulin secretion, increased hepatic glucose production, and decreased insulin-stimulated uptake of glucose in peripheral tissues (21). Skeletal muscle is the largest reservoir for peripheral glucose disposal in response to insulin. Binding of insulin to the heterotetrameric receptors on the surface of the skeletal muscle cells results in insulin receptor substrate-1 (IRS-1) phosphorylation and IRS-1-associated phosphatidylinositol 3-phosphate kinase activation. This affects downstream effectors such as protein kinase B (Akt), which activates the translocation of the glucose transporter 4 (GLUT4) to the plasma membrane to import glucose into the cells (12). Therefore, GLUT4 traffic is thought to be a critical determinant of insulin sensitivity of the skeletal muscle. Indeed, clinical observations demonstrated a reduction in insulin-stimulated GLUT4 translocation rather than an altered total GLUT4 content in the skeletal muscle of subjects with type 2 diabetes (5). GLUT4 is stored in intracellular vesicles associated with the small G protein Rab, which organizes intracellular membrane traffic (7). Recent studies have suggested that the ability of Rab to traffic GLUT4 storage vesicles to the cell surface is regulated by Rab-GTPase-activating proteins (Rab-GAPs). A candidate of Rab-GAP regulating GLUT4 vesicle traffic is Akt substrate of 160 kDa (AS160). Insulin-stimulated Akt phosphorylates AS160 to inactivate its Rab-GAP activity, leading to an increase in GTP loading of Rab on GLUT4 vesicles and subsequently to promotion of GLUT4 vesicle traffic (23). The precise mechanism underlying AS160-mediated regulation of the GLUT4 vesicle transport, however, is still elusive.

The hypoxia-inducible factor (HIF)-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits (28). Upon cellular exposure to hypoxic conditions, HIF-1 is induced to activate transcription of genes encoding erythropoietin (9), vascular endothelial growth factor (VEGF) (4), glycolytic enzymes (22), glucose transporters (29), and plasminogen activator inhibitor-1 (14). The detailed mechanism for activation of HIF-1 by lack of oxygen has recently been determined (25). Under normoxic conditions, HIF-1α is targeted by the von Hippel-Lindau protein (pVHL) for ubiquitylation and rapid proteasomal degradation. pVHL binding is mediated through hydroxylation of specific prolyl residues in HIF-1α protein catalyzed by 2-oxoglutarate- and Fe(II)-dependent dioxygenases designated HIF prolyl hydroxylases. The hypoxic condition decreases the prolyl hydroxylation of HIF-1α, resulting in release of pVHL and stabilization of HIF-1α protein (17). Stabilized HIF-1α then translocates to the nucleus where it dimerizes with HIF-1β to bind to the hypoxia response element of target genes and thereafter recruits nuclear co-factors such as CBP/P300 to elicit a transactivation of the target gene (11).

In addition to this classical mode of activation of HIF-1α by hypoxia, evidence suggests that various cellular stimuli such as cytokines, metabolites in the tricarboxylic acid cycle such as fumarate, reactive oxygen species, and various kinase cascades activate HIF-1α even under normoxic con-
conditions (6, 8, 18, 20). Therefore, it is highly plausible that HIF-1 plays critical roles in regulation of cellular functions not only under hypoxic conditions but also in conditions such as metabolic disturbance and inflammation. Insulin has been shown to induce HIF-1α and its target gene expression in certain cells under normoxic conditions, indicating an involvement of HIF-1α in mediating a part of insulin actions (30). The role of HIF-1α in signal transduction in skeletal muscle cells by insulin, however, is largely unknown.

Here we investigated the role of HIF-1α in insulin-stimulated glucose uptake in the skeletal muscle cells and demonstrated an implication of HIF-1α in regulation of GLUT4 translocation to the cellular membrane, which may indicate a previously unknown aspect of HIF-1α in determination of glucose metabolism in the skeletal muscles.

**Glossary**

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>AS160</td>
<td>Akt substrate of 160 kDa</td>
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<tr>
<td>CA-HIF-1α</td>
<td>Constitutively active mutant of HIF-1α</td>
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<tr>
<td>CHX</td>
<td>Cycloheximide</td>
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<tr>
<td>C2C12ΔHIF-1α</td>
<td>Myoblast in which HIF-1α is knocked down</td>
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<tr>
<td>EDL muscle</td>
<td>Extensor digitorum longus muscle</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible factor-α</td>
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<tr>
<td>HIF-1αΔ397-409</td>
<td>Heterozygote of HIF-1α gene knockout mice</td>
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<tr>
<td>KHB buffer</td>
<td>Krebs-Henseleit bicarbonate buffer</td>
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<tr>
<td>KRP buffer</td>
<td>Krebs-Ringer phosphate buffer</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAS</td>
<td>Phospho-Akt substrate motif</td>
</tr>
<tr>
<td>pVHL</td>
<td>von Hippel-Lindau protein</td>
</tr>
<tr>
<td>Rab-GAP</td>
<td>Rab-GTPase-activating protein</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short-hairpin RNA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>2-DG</td>
<td>2-Deoxyglucose</td>
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**METHODS**

**Reagents and antibodies.** All chemicals were obtained from Sigma (St. Louis, MO), unless otherwise noted. Human recombinant insulin was purchased from MP Biomedicals (Fountain Parkway, Solon, OH). The anti-mouse HIF-1α antibody was purchased from Novus Biologicals (St. Louis, MO). Anti-GLUT4 and myogenin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against phospho-Akt substrate motif, AS160, TBC1D1, phosphorosine, Akt, phospho-Ser373 on Akt, insulin receptor β, and Rab11 were from Cell Signaling Technology (Danvers, MA). Anti-HIF-1α hydroxy P564 rabbit polyclonal antibody was from Rockland (Gilbertsville, PA). Mouse monoclonal anti-α-tubulin antibody was from Upstate Biotechnology (Lake Placid, NY).

**Cell culture.** C2C12 myoblast (RIKEN Tsukuba) was maintained in DMEM containing 10% FCS and 1% penicillin-streptomycin at 37°C in 5% CO2. After the cells had reached confluence, differentiation was induced by growing the cells in 5% horse serum DMEM for 5 days. Differentiation was confirmed morphologically and by expression of myogenin. Before each assay, the cells are serum-starved for 12 h in DMEM containing 0.2% bovine serum albumin (BSA) instead of the serum.

**Glucose uptake assay.** After serum starvation for 4 h, C2C12 myotubes were washed with Krebs-Ringer phosphate buffer (KRP buffer): 25 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1.2 mM KH2PO4, 2.5 mM MgSO4, 5 mM NaHCO3, and 0.1% BSA and incubated with KRP buffer for 3 h. Next the cells were treated for 30 min with or without 100 nM insulin. After the treatment, the cells were incubated in KRP buffer containing 5 mM 2-deoxyglucose and 1 μCi/ml 2-deoxy-D-[3H]glucose (2-DG) (Moravek Biochemicals) for 5 min. The reaction was terminated by adding ice-cold PBS, and the cells were dissolved in 1 N NaOH. The radioactivity was measured using a liquid scintillation counter. Nonspecific uptake was determined by incubating the cells in the presence of absence of 5 μM cytochalasin B (Sigma).

**Preparation of cell fractions.** Preparation of plasma membrane fraction was carried out as described elsewhere (19) with minor modification. Briefly, differentiated cells were washed twice with PBS and scraped in ice-cold HEPES-EDTA-sucrose (HES) buffer containing 5 mM EDTA, 250 mM sucrose, 1 tablet 15 ml of complete mini protease inhibitor cocktail tablet, 10 mM NaF, 1 mM Na3VO4, and 10 mM HEPES adjusted to pH 7.4. The cells were homogenized at 4°C using a Dounce homogenizer. The homogenate was centrifuged for 15 min at 8,000 g. The pellet was resuspended in 1 ml of HES buffer and layered on a 1.12 M sucrose cushion. After a 60-min centrifugation at 100,000 g, the top layer was taken off, and HES

**Knockdown of HIF-1α in C2C12 myoblast.** To knock down the HIF-1α, C2C12 myoblasts were stably transfected with HIF-1α short-hairpin RNA (shRNA) expression plasmid. The sequences of the two complementary oligonucleotides to transcribe the shRNA are the following: sense, 5′-GATCGCCGATAGTGTAGTTTCAATTCAGGAGATTTAGAACTACATATCGTGGTTTGGAAA-3′; antisense, 5′-AGCTTCTCCAAAAACACTGATGTGTCTAATAATCTTGAAATTTGAAACATATCACATCGGCG-3′. Those oligonucleotides were ligated into pSilencer U6 neo vector (Life Technologies, Carlsbad, CA) and transfected into C2C12 myoblast with TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI). The transfected cells were selected by using G418 sulfate (Promega, Madison, WI) for several days to eliminate cells that were not transfected.

**Constitutively active mutant of HIF-1α.** The constitutively active mutant of HIF-1α (CA-HIF-1α) is a chimeric protein consisting of amino acid 1 to 396 of HIF-1α and VP-16 activation domain to escape from the oxygen-dependent degradation domain (16). The pcDNA3/HIF-1α/VP-16 vector was transfected into C2C12 myoblast with TransIT-LT1 transfection reagent.

**Immunoblot.** Whole cell extracts were prepared as described previously (16). The proteins were separated in 8% SDS-polyacrylamide gels and blotted to polyvinylidene fluoride membranes. The membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) with 5% dry milk. Primary antibodies were incubated overnight at 4°C in TBS with 1% milk. Corresponding secondary antibodies conjugated with horseradish peroxidase (GE Healthcare, Freiburg, Germany) were applied in TBS containing 1% milk. To visualize the blots, the ECL detection system was used according to the manufacturer’s instructions. For loading normalization, the amount of α-tubulin was determined by reprobing the membrane with anti-α-tubulin antibody.

**Immunoprecipitation.** For immunoprecipitation, the whole cell lysate was incubated with anti-AS160 antibody (Cell Signaling Technology) and incubation buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X, 1 mM DTT, 50 mM β-glycerophosphate, 50 mM NaF, 1 mM NaVO4, and proteinase inhibitor cocktail) with rotation for 1 h at 4°C. Fifty percent slurry of protein G- Sepharose beads was then added to the mixture and rotated for 30 min at 4°C. Bound immune-complexes were washed three times with incubation buffer and eluted by boiling in 2× SDS loading buffer for 5 min. Immunoblots were probed with anti-phospho-(Ser/Thr) Akt substrate antibody (Cell Signaling Technology).

**Glucose uptake assay.** After serum starvation for 4 h, C2C12 myotubes were washed with Krebs-Ringer phosphate buffer (KRP buffer): 25 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1.2 mM KH2PO4, 2.5 mM MgSO4, 5 mM NaHCO3, and 0.1% BSA and incubated with KRP buffer for 3 h. Next the cells were treated for 30 min with or without 100 nM insulin. After the treatment, the cells were incubated in KRP buffer containing 5 mM 2-deoxyglucose and 1 μCi/ml 2-deoxy-D-[3H]glucose (2-DG) (Moravek Biochemicals) for 5 min. The reaction was terminated by adding ice-cold PBS, and the cells were dissolved in 1 N NaOH. The radioactivity was measured using a liquid scintillation counter. Nonspecific uptake was determined by incubating the cells in the presence of absence of 5 μM cytochalasin B (Sigma).
Fig. 1. Insulin induces expression of hypoxia-inducible factor (HIF)-1α in C2C12 myotubes under normoxic conditions. A: insulin-induced expression of HIF-1α in C2C12 myotubes. C2C12 myotubes were serum starved for 12 h and treated with 100 nM insulin for the indicated time period. The cell lysates were subjected to immunoblot analysis for HIF-1α expression. B: C2C12 myotubes were treated with insulin at the indicated concentration for 30 min. C: densitometric analysis of the HIF-1α protein levels in response to insulin. HIF-1α protein levels relative to loading control (α-tubulin; α-tub) are presented as means ± SD from 4 independent experiments. *Statistical significance P < 0.05 between 2 groups as indicated. D: insulin does not affect the prolyl hydroxylation of HIF-1α in C2C12 myotubes. Myotubes were serum starved for 12 h and treated with or without 10 μM MG-132 for 2 h and then 100 nM insulin was added for 30 min. The cell lysates were subjected to immunoblot analysis for hydroxylated HIF-1α expression. E: densitometric analysis of the hydroxylated HIF-1α levels relative to loading control (α-tubulin) are presented as means ± SD from 4 independent experiments. *Statistical significance P < 0.05 between 2 groups as indicated. F: cycloheximide did not alter the level of HIF-1α stabilized by MG-132. Myotubes were treated with 10 μM MG-132 for 2 h and then cultured with cycloheximide (CHX; 50 mM) for 10 min. G: densitometric analysis of the HIF-1α protein levels after MG-132/CHX treatment relative to loading control (α-tubulin) are presented as means ± SD from 4 independent experiments. *Statistical significance P < 0.05 between 2 groups as indicated. H: inhibition of protein synthesis abrogates insulin-dependent HIF-1α expression. C2C12 myotubes were treated with 100 nM insulin (Ins) for 30 min and then CHX (50 mM) was added for 10 min. Expression of HIF-1α was detected by immunoblot. I: densitometric analysis of the HIF-1α protein levels in response to insulin and CHX. HIF-1α protein levels relative to loading control (α-tubulin) are presented as means ± SD from 4 independent experiments. *Statistical significance P < 0.05 between 2 groups as indicated. J: HIF-1α mRNA expression after stimulation with insulin. After serum starvation, C2C12 myotubes were treated with or without 100 nM insulin for 30 min, and mRNA levels were determined by real-time PCR. Four independent experiments were performed, and means ± SD of mRNA levels relative to the cells with nontreated control are presented. Statistical difference was not significant.
buffer was added to the volume of 10 ml and then centrifuged for another 60 min at 100,000 g to pellet the plasma membrane fraction. The supernatant from the first centrifugation (15 min at 8,000 g) step was centrifuged for 20 min at 41,000 g, and the resulting supernatant was centrifuged for 75 min at 180,000 g to pellet the low-density microsomal (LDM) fraction.

Quantitative PCR analyses. Total RNA was isolated with RNeasy (QIAGEN, Hilden, Germany). An equal amount of total RNA was reverse transcribed into cDNA with oligo(dT) primers using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). For quantification of mRNA expression, real-time PCR was performed using β-actin as an internal standard on the basis of TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). All of the samples were divided in three aliquots, and the average of the relative C<sub>t</sub> value from the aliquots was representative for one assay run.
Animals and measurement of 2-deoxy-d-glucose uptake in ex vivo experiment. Heterozygotes of HIF-1α gene knockout mice (HIF-1α+/−) (26) were generously provided by Dr. Shuhei Tomita (Tottori University). HIF-1α+/− mice and control littermate mice were subjected to overnight fasting before assay. Extensor digitorum longus (EDL) muscles were isolated and incubated in Krebs-Henseleit bicarbonate (KHB) buffer (10 mM HEPES, pH 7.4, 114 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.2 mM CaCl₂, and 24 mM NaHCO₃) containing 8 mM glucose and 32 mM mannitol at 30°C for 1 h with shaking. The buffers were continuously gassed with 95% O₂ and 5% CO₂. Insulin (100 nM) was added to the culture and incubated for 30 min. After the treatment the muscles were rinsed for 10 min in KHB buffer containing 40 mM mannitol and then incubated for 20 min in KHB buffer with 4 mM 2-deoxy-d-[3H]glucose (1.5 μCi/ml) and 36 mM α-[1-14C]mannitol (0.2 μCi/ml) (GE Healthcare). Glucose uptake was stopped by washing the muscles in ice-cold KHB buffer, freezing in liquid N₂, and lysing in 1 N NaOH. The radioactivity was determined in a liquid scintillation counter. The uptake of 2-deoxy-d-[3H]glucose was expressed as 3H counts per milligram of the lysed muscle. Nonspecifically trapped radioactivity was estimated from the amount of α-[1-14C]mannitol in the tissue. All animal experiments were conducted according to institutional guidelines for animal care and welfare.

Statistical analysis. All data are presented as means ± SD from the experiments with several repetitions as indicated. Comparisons between two groups were analyzed by unpaired t-test. One-factor analysis of variance followed by the Bonferroni/Dunn test were carried out to determine significant differences in multiple comparisons. P values <0.05 were considered significant.

RESULT

Insulin stimulates HIF-1α protein expression in skeletal muscle cells. Previous studies have shown that insulin stimulates HIF-1α expression in a variety of cell lines irrespective of oxygen concentration. We thus examined expression of HIF-1α protein in C2C12 myotubes cultured in the presence of insulin. As shown in Fig. 1A, the expression of HIF-1α protein in C2C12 myotubes, even under normoxic conditions, increased with treatment by 100 nM insulin for 30 min and remained elevated for at least 2 h. The effect of insulin became evident at 10 nM by fourfold induction compared with the basal state and increased to ninefold induction at 100 nM (Fig. 1, B and C). To determine the mechanism underlying insulin-mediated upregulation of HIF-α in myotubes, we first tested hydroxylation status of HIF-α. Hydroxylation of specific proline residues (Pro⁴⁰² and Pro⁵⁶⁴) of HIF-α is a key modification for polyubiquitylation and proteasomal degradation. As shown in Fig. 1D, with an aid of the proteasome inhibitor MG-132, HIF-1α hydroxylated at those prolyl residues escaped from proteasomal degradation to be visualized by immunoblot with antihydroxylated HIF-1α antibodies. Addition of insulin did not reduce the level of prolyl hydroxylation of HIF-1α (Fig. 1, D and E), indicating that insulin has no effect on the biochemical modification of HIF-1α linked to protein stability. Next, we examined the effect of the protein synthesis inhibition on the HIF-1α protein level. Treatment with the proteasome inhibitor MG-132 for 2 h caused protein stabilization and an accumulation of HIF-1α protein in C2C12 myotubes (Fig. 1, F and G). Further incubation with the protein synthesis inhibitor cycloheximide (CHX) for 10 min scarcely altered the level of HIF-1α stabilized by MG-132 (Fig. 1, F and G). In contrast, HIF-1α induced by the treatment with 100 nM insulin was prone to CHX treatment to be rapidly decreased to the basal level (Fig. 1, H and I), indicating that insulin enhances HIF-1α expression via a mechanism distinct from that by the protein stabilizer MG-132. On the other hand, mRNA of HIF-1α was not changed in the same time course of the insulin treatment (Fig. 1J). Taken together, insulin increases HIF-1α expression in C2C12 myotubes under normoxic conditions via upregulation of protein synthesis mechanisms.

Insulin-dependent GLUT4 translocation and glucose uptake are reduced in HIF-1α knockdown C2C12 myotubes. To explore the role of HIF-1α in insulin action in skeletal muscle cells, we generated C2C12 myoblasts in which HIF-1α is knocked down (C2C12ΔHIF-1α) by a stable transfection with HIF-1α shRNA expression plasmids. C2C12ΔHIF-1α myoblasts grow normally with the same culture medium as the wild-type C2C12 cells (data not shown). In wild-type C2C12 myotubes, HIF-1α protein was modestly detected under normoxic conditions (21% O₂), and massive accumulation of HIF-1α protein was observed under hypoxic conditions (1% O₂) (Fig. 2A, lane 2 compared with lane 1). In contrast, in C2C12ΔHIF-1α myotubes, basal expression of HIF-1α under normoxic conditions was scarcely detectable, and the HIF-1α protein induction by exposure to hypoxia was largely reduced compared with wild-type myotubes (Fig. 2A, lane 4 compared with lane 2). Semi-quantitative determination of HIF-1α expression by densitometric analysis of immunoblots revealed that C2C12 myotubes are capable of about 25-fold induction of HIF-1α in response...
to hypoxia, whereas C2C12ΔHIF-1α myotubes show only about twofold induction under hypoxic conditions (Fig. 2B), indicating that expression of HIF-1α in C2C12ΔHIF-1α myotubes is severely compromised to 10% of that in wild-type myotubes. Expression of myogenin in C2C12ΔHIF-1α cells after induction of differentiation showed a certain delay in the first 3 days compared with wild-type cells (Fig. 2C). The myogenin level in C2C12ΔHIF-1α cells caught up with that in the wild-type cells at 4 days after induction and remained at similar levels to the wild-type cells afterward. Moreover, micrographs of hematoxylin and eosin staining of the culture demonstrated formation of fused and multinucleated syncytial myotubes by C2C12ΔHIF-1α myoblasts at 5 days after exposure to differentiation medium (Fig. 2D). The percent differentiation was determined by the ratio of the multinucleated (>2) cells in the total nuclei. The mean differentiation percentage in C2C12ΔHIF-1α cells was 78%, and that of wild-type C2C12 cells was 80% (Fig. 2E). Therefore, we conclude that C2C12ΔHIF-1α cells similarly differentiate as wild-type cells to myotubes at least 5 days after induction of differentiation and thus decided to use 5 days or later myotubes in all experiments. Next we determined the glucose uptake rate of the cells treated with insulin by monitoring the influx of 2-DG. Upon the treatment with 100 nM insulin for 30 min, wild-type C2C12 cells increased uptake of 2-DG by about twofold compared with the basal level (Fig. 2F, lane 2 compared with lane 1). In contrast, C2C12ΔHIF-1α showed impaired insulin-stimulated upregulation of 2-DG uptake (Fig. 2F, lane 4 compared with lane 3), indicating that HIF-1α plays an important role in regulation of insulin-induced uptake of the glucose in skeletal muscle cells.

It has been shown that translocation of GLUT4 from the intracellular vesicle to the plasma membrane is a critical step of insulin-induced glucose uptake in skeletal muscle cells. Indeed, accumulation of GLUT4 in the plasma membrane fraction of the wild-type C2C12 cells was observed 30 min after insulin treatment (Fig. 2, G and H, lane 2 compared with lane 1). On the other hand, in C2C12ΔHIF-1α myotubes, translocation of GLUT4 to the plasma membrane was severely inhibited, even under treatment with insulin (Fig. 2, G and H, lane 4). The content of insulin receptor β, a marker of plasma membrane fraction, was similar in both wild-type and C2C12ΔHIF-1α myotubes, indicating appropriate preparations of the plasma membrane fraction in both wild-type and C2C12ΔHIF-1α myotubes (Fig. 2G). Reciprocally, the amount of GLUT4 protein in the intracellular membrane fraction (LDM fraction) of wild-type cells was decreased with insulin stimulation, whereas that of C2C12ΔHIF-1α cells was not observed (Fig. 2G). On the other hand, the mRNA level of GLUT4 in C2C12ΔHIF-1α was equivalent to that of wild-type C2C12 cells (Fig. 2G). GLUT1 is a mediator of insulin-independent glucose uptake. We confirmed that the expression of GLUT1 mRNA was comparable both in wild-type cells and C2C12ΔHIF-1α (Fig. 2J).

Taken together, HIF-1α in skeletal muscle cells plays pivotal roles in regulation of insulin-stimulated glucose uptake via determination of GLUT4 translocation to the plasma membrane of the cells.

Phosphorylation of AS160 is inhibited in HIF-1α knockdown C2C12 myotubes. To elucidate the mechanism of regulation of insulin-stimulated glucose uptake by HIF-1α, we analyzed the key components of signal transduction by insulin in the myotubes. Phosphorylation of IRS-1 is a critical step in the initial phase of insulin signaling. As shown in Fig. 3A, treatment with insulin resulted in tyrosine phosphorylation of IRS-1 both in wild-type C2C12 and C2C12ΔHIF-1α myotubes (Fig. 3A, lanes 2 and 4). The basal level of the IRS-1 Ser[512] phosphorylation in C2C12ΔHIF-1α myotubes was lower than the wild-type cells; however, insulin induced phosphorylation of IRS-1 Ser[512] to the same degree as the wild-type cells (Fig. 3B, lanes 2 and 4). Another key event of Akt phosphorylation upon treatment with insulin in C2C12ΔHIF-1α myotubes was the same level as in wild-type myotubes (Fig. 3, C and 3D, lanes 2 and 4). AS160 is a Rab-GAP known to be involved in GLUT4 translocation downstream of insulin stimulation. Upon phosphorylation by activated Akt, AS160 loses Rab-GAP activity to permit translocation of the vesicles containing GLUT4 to the plasma membrane (23). Immunoprecipitation assays demonstrated insulin-dependent phosphorylation of AS160 in wild-type C2C12 myotubes (Fig. 3, E and F, lane 2 compared with lane 1). Contrary, in C2C12ΔHIF-1α myotubes phosphorylation of AS160 by insulin treatment was significantly reduced, whereas AS160 expression level was similar to that of wild-type myotubes (Fig. 3, E and F, lane 4 compared with lane 2). Another Rab-GAP, TBC1D1, is also a substrate of Akt known to mediate GLUT4 translocation by insulin stimulation (23). In C2C12ΔHIF-1α myotubes phosphorylation of TBC1D1 by insulin treatment was also significantly reduced compared with wild-type C2C12 myotubes, although the expression level of TBC1D1 was quite similar to that of wild-type myotubes (Fig. 3, G and H, lane 4 compared with lane 2). On the other hand, AS160 is also employed as a molecular switch in AMP-activated protein kinase (AMPK)-dependent GLUT4 translocation in skeletal muscle. In agreement with this insight, glucose uptake by the AMPK activator 5′-aminoimidazole-4-carboxamide ribonucleoside (AICAR) was impaired by HIF-1α knockdown in C2C12 myotubes (Fig. 4A, lane 4 compared with lane 2). Similarly, phosphorylation of AS160 by AICAR treatment was significantly reduced in C2C12ΔHIF-1α myotubes (Fig. 4, B and C, lane 4 compared with lane 2). Accumulation of GLUT4 in the plasma membrane fraction and the reciprocal reduction of GLUT4 in the LDM fraction of the wild-type C2C12 cells were induced by AICAR (Fig. 4, D and E, lane 2 compared with lane 1). Contrary, in C2C12ΔHIF-1α myotubes AICAR-mediated translocation of GLUT4 to the plasma membrane from the LDM fraction was severely inhibited (Fig. 4, D and E, lane 4). Given these results, we conclude that HIF-1α plays an important role in regulation of AS160 phosphorylation leading to GLUT4 translocation and thus is a critical determinant for both insulin- and AMPK-dependent glucose uptake in skeletal muscle.

Phosphorylation of AS160 and GLUT4 trafficking are increased in cells expressing constitutively active HIF-1α. To further address the role of HIF-1α in AS160 regulation, we employed CA-HIF-1α. Fusion of the transactivation domain of the HERPES simplex virus-derived transcription factor VP-16 to the NH2-terminus of HIF-1α resulted in hypoxia-independent constitutive transcriptional activity (Fig. 5A).

C2C12 expressing this CA-HIF-1α shows elevated expression of the HIF-1α target gene, VEGF, even under normoxic conditions, indicating that CA-HIF-1α cells contain enhanced function of HIF-1 (Fig. 5B).
Interestingly, CA-HIF-1α cells show higher AS160 phosphorylation comparable to the insulin-stimulated level, even in the absence of insulin (Fig. 5, C and D). Consistent with this observation, GLUT4 localization in the plasma membrane fraction in CA-HIF-1α cells was observed as well in the absence of insulin (Fig. 5, E and F), indicating that HIF-1 activity might be involved in the regulation of AS160 phosphorylation and subsequent GLUT4 translocation.

Preconditional induction of endogenous HIF-1α by 2,2'-dipyridyl increased phosphorylation of AS160. A decrease in Fe (II) availability by treatment with iron chelators such as 2,2'-dipyridyl is known to inhibit HIF-3-prolyl hydroxylases to stabilize HIF-1. Consistently, treatment with 2,2'-dipyridyl induces HIF-1α in C2C12 myotubes even under normoxic conditions (Fig. 6A). Of note, such preconditional induction of endogenous HIF-1α by 2,2'-dipyridyl increased AS160 phosphorylation comparable to the insulin-stimulated level (Fig. 6, A and B, lane 3 compared with lane 2). Interestingly, although phosphorylation of AS160 was upregulated, translocation of GLUT4 to the plasma membrane was not increased by 2,2'-dipyridyl (Fig. 6C). Although an underlying mechanism of this inconsistency is elusive, we may speculate iron chelation might directly or indirectly affect GLUT4 translocation machinery.

Insulin-stimulated glucose uptake is impaired in the skeletal muscle of HIF-1α−/− mice. To clarify the physiological relevance of HIF-1α-dependent regulation of insulin action, we determined glucose uptake by the skeletal muscles of HIF-1α−/− mice. To this end, EDL muscles were isolated from the wild-type mice and HIF-1α−/− mice to carry out ex vivo culture. After washes to remove serum factors, each muscle was incubated with or without insulin and then 2-DG uptake...
was monitored. Insulin-stimulated 2-DG uptake was increased up to ~2.5-fold above basal levels in wild-type mice. In excellent agreement with in vitro analysis, HIF-1α+/− mice demonstrated significantly reduced uptake of 2-DG under insulin-stimulated conditions (Fig. 7A). Consistently, as we found in C2C12 myotube culture experiments, phosphorylation of AS160 in the skeletal muscles isolated from HIF-1α+/− mice was reduced compared with specimens from wild-type animals (Fig. 7, B and C, lanes 2 and 4).

In conclusion, HIF-1α essentially operates in regulation of glucose uptake by the skeletal muscle and thus plays a crucial role in maintenance of glucose disposal and metabolism.

**DISCUSSION**

In the present study, we demonstrated in cultured skeletal muscle cells that the expression level of HIF-1α is a critical determinant in insulin-induced glucose uptake; knockdown of HIF-1α resulted in severe reduction in insulin-stimulated glucose uptake. In the skeletal muscle of HIF-1α+/− mice, similar reduction of glucose uptake in response to insulin was observed; therefore, HIF-1α-mediated regulation of glucose uptake stimulated by insulin could be important in the context of organ physiology. Interestingly, we demonstrated that insulin induces HIF-1α expression under normoxic conditions not via inhibition of protein degradation but via protein translation-dependent mechanisms in skeletal muscle cells. The induction of HIF-1α was a relatively early event after insulin stimulation (30 min), and we could not preclude the possibility that upregulated HIF-1α is implicated in concurrent augmentation of the insulin action. On the other hand, phosphorylation of Akt has been shown to occur in a shorter time course of 1–5 min after exposure to insulin (2), suggesting that insulin-induced HIF-1α may not always contribute to the cellular response induced by insulin. In such case, HIF-1α may participate in insulin-
independent cellular events. Indeed, loss of HIF-1α compromised insulin-independent glucose uptake by AMPK activator in the skeletal muscle cells as well. Insulin-mediated induction of HIF-1α, therefore, may constitute a regulatory mechanism either in insulin-dependent or independent glucose metabolism in the skeletal muscles.

We demonstrated that loss of HIF-1α in skeletal muscle cells resulted in reduction of insulin-mediated phosphorylation of AS160 and translocation of GLUT4 to the plasma membrane. AS160 is a Rab-GAP that regulates the activity of Rab proteins linked to the control of intracellular vesicle transport to the plasma membrane (15). In the unstimulated state, AS160 hydrolyses GTP to generate inactive GDP-bound Rab. Upon phosphorylation by Akt, AS160 is inactivated to permit Rab to return to an active GTP-bound state, thereby initiating GLUT4 exocytotic trafficking (23). Therefore, phosphorylation of AS160 is believed to be prerequisite to GLUT4 translocation and glucose uptake by insulin. Interestingly, insulin-stimulated AS160 phosphorylation is shown to be impaired in skeletal muscle of type 2 diabetic patients (13). Moreover, AS160 carrying mutations in the phosphorylation site by Akt behaves in a dominant-interfering manner and inhibits GLUT4 translocation (24). Reduction of phosphorylation of AS160 in C2C12 may resemble this situation, thus resulting in impairment of GLUT4 translocation and decreased glucose uptake. Contrary, previous studies have demonstrated AS160 phosphorylation is upregulated under hypoxic conditions (3, 10). Although the level of HIF-1α was not examined in those studies, it might be possible that cellular HIF-1α content correlates with phosphorylation status of AS160. In support of this hypothesis, introduction of CA-HIF-1α into C2C12 myocytes led to a phosphorylation of AS160 and subsequent GLUT4 translocation in the absence of insulin. Similarly, preconditional induction of endogenous HIF-1α by the iron
Fig. 6. Preconditional induction of endogenous HIF-1α increases phosphorylation of AS160. A: induction of HIF-1α in C2C12 myotubes by treatment with 100 μM 2,2′-dipyridyl for 4 h and phosphorylation status of AS160. Whole cell lysates were subjected to immunoblot with HIF-1α antibody or to immunoprecipitation by anti-AS160 antibodies and control IgG followed by immunoblot with anti-PAS antibodies. B: the ratio of AS160 phosphorylation to total AS160 was determined by densitometry of the bands and is presented as means ± SD from 3 independent experiments. *Statistical significance P < 0.05 between 2 groups as indicated. C: detection of GLUT4 trafficking to the plasma membrane. After serum starvation for 12 h, cells were treated with 100 μM 2,2′-dipyridyl for 4 h and then with 100 nM insulin for 30 min. GLUT4 levels in the PM fraction and LDM fraction were detected by Western blot with anti-GLUT4 antibodies. The contents of IRβ or Rab11 were confirmed as markers for PM or LDM, respectively.

Fig. 7. Glucose uptake by the skeletal muscle of HIF-1α+/− mice. A: the isolated extensor digitorum longus (EDL) muscle strips of WT mice and heterozygote of HIF-1α gene knockout mice (HIF-1α+/−) were cultured and incubated with or without 100 nM insulin for 30 min at 30°C. Then uptake of 2-deoxy-D-[3H]glucose was carried out, and the radioactivity was measured. The error bars represent means ± SD (n = 4). *Statistical significance P < 0.05 between 2 groups as indicated. B: insulin-stimulated phosphorylation of AS160. The isolated EDL muscle strips of WT and HIF-1α+/− mice were cultured and incubated with or without 100 nM insulin for 5 min. Immunoprecipitated muscle lysate with anti-AS160 antibodies (IP: AS160) or with control IgG (IP: IgG) was subjected to immunoblot analysis with anti-PAS antibodies. C: the ratio of AS160 phosphorylation to total AS160 was determined by densitometry of the bands and is presented as means ± SD from 3 independent experiments. *Statistical significance P < 0.05 between 2 groups as indicated.
chelator, 2,2′-dipyridyl also led to a phosphorylation of AS160 in the absence of insulin. These results suggest that the presence of HIF-1α is important for determination of AS160 phosphorylation status.

The mechanism underlying HIF-1α-mediated upregulation of AS160 phosphorylation is still elusive. The most widely recognized functional aspect of HIF-1α is a transcriptional regulation, and so far evidence demonstrating kinase activities in HIF-1α has not been found. Consistently, we could not find physical interaction between HIF-1α and AS160 in the present study (data not shown). Therefore, it is unlikely that HIF-1α directly affects the phosphorylation process of AS160. Given the fact that the introduction of the constitutively active form of HIF-1α and/or activation of endogenous HIF-1α by a hypoxia mimetic enhanced phosphorylation of AS160, we may speculate that an indirect mode that a HIF-1 target gene product regulates phosphorylation of AS160 e.g., via an allosteric mechanism.

AS160 also subjects to phosphorylation by AMPK (2). AMPK is a metabolic fuel gauge in eukaryotes for sensing changes in the AMP-to-ATP ratio. Recent studies have shown that AMPK phosphorylates AS160 to induce GLUT4 translocation and subsequent glucose uptake in the skeletal muscles (10). In the present study, loss of HIF-1α abolished AMPK-mediated phosphorylation of AS160 and following glucose uptake by the AMPK activator AICAR in the myotube. Therefore, HIF-1α might be implicated in glucose uptake under conditions such as energy starvation in the skeletal muscles. Moreover, AMPK-mediated AS160 phosphorylation and GLUT4 translocation are also involved in contraction/exercise-induced glucose uptake by the skeletal muscles (15); thus, HIF-1α might play a role in glucose uptake by exercising muscles as well. Along with this insight, we previously reported that HIF-1α protein and vascular endothelial growth factor gene expression are upregulated by a single bout of joint-extension exercise in human skeletal muscles (1). Such induction response had been supposed, at least in part, to contribute to increases in capillary growth and blood flow for adaptation to oxygen demands in exercising muscles. Besides such aspect, regulation of phosphorylation of AS160 and glucose uptake by HIF-1α may provide an additive interpretation concerning the potential role of induced HIF-1α in energy metabolism in exercising skeletal muscles.

A mainstay in the treatment of diabetic patients is a management of the hyperglycemia. Clinical studies such as the United Kingdom Prospective Diabetes Study showed that strict blood glucose control is essential to decrease the likelihood of vascular complications in patients with type 2 diabetes (27). Enormous medical challenges, therefore, have been raised to overcome the defect in glucose metabolism in the patients. Intervention to augment glucose disposal in peripheral tissues may be of an appreciation as promising tools for the treatment overcame the defect in glucose metabolism in the patients. Intervention to augment glucose disposal in peripheral tissues may provide a window to develop a novel anti-diabetic strategy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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