AMP-activated protein kinase (AMPK) functions as an energy sensor to provide metabolic adaptations under the ATP-deprived conditions such as hypoxia. In the present study, we considered a role of AMPK in the adaptive response to hypoxia by examining whether AMPK is involved in the regulation of hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor that is critical for hypoxic induction of physiologically important genes. We demonstrate that hypoxia or CoCl2 rapidly activated AMPK in DU145 human prostate cancer cells, and its activation preceded the induction of HIF-1 alpha expression. Under these conditions, blockade of AMPK activity by a pharmacological or molecular approach significantly attenuated hypoxia-induced responses such as HIF-1 target gene expression, secretion of vascular endothelial growth factor, glucose uptake, and HIF-1-dependent reporter gene expression, indicating that AMPK is critical for the HIF-1 transcriptional activity and its target gene expression. Its functional requirement for HIF-1 activity was also demonstrated in several different cancer cell lines, but AMPK activation alone was not sufficient to stimulate the HIF-1 transcriptional activity. We further present data showing that AMPK transmits a positive signal for HIF-1 activity via a signaling pathway that is independent of phosphatidylinositol 3-kinase/AKT and several mitogen-activated protein kinases. Taken together, our results suggest that AMPK is a novel and critical component of HIF-1 regulation, implying its new roles in oxygen-regulated cellular phenomena.

**Abstract**

AMPK is a heterotrimeric enzyme consisting of a catalytic subunit (α) and two regulatory subunits (β and γ), plays a critical role as an energy sensor in these responses (reviewed in Refs. 1–3). In response to nutritional or environmental stress factors that deplete intracellular ATP, AMPK is activated by allosteric binding of AMP (4, 5) and by phosphorylation by a still uncharacterized upstream AMPK kinase (6). Once activated, AMPK minimizes further ATP consumption by suppressing ATP-consuming anabolic pathways as well as activating ATP-generating catabolic pathways. The physiological or stress conditions known to activate AMPK include exercise (7–9), nutritional starvation (10), heat shock (11), oxidative stress (12), and ischemia/hypoxia (3, 13–15).

Similar to the intracellular energy status, cellular oxygen concentration is precisely regulated in mammals to maintain cellular function and integrity. The reduced oxygen availability also initiates a series of adaptive responses, and many of these are mediated by HIF-1, a heterodimeric protein consisting of HIF-1α (19) and HIF-1β subunits (20), and both subunits contain cofactors including CBP/p300, SRC-1, or TIF2, reflecting the complex nature of its regulatory mechanisms (16–18).

**RESULTS**

AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells.


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**Introduction**

The energy status of the cell plays a crucial role for cell survival, and exposure of eukaryotic cells to metabolic stresses that accompany the depletion of intracellular ATP triggers specific and systemic adaptive responses. AMP-activated protein kinase (AMPK), a heterotrimeric enzyme consisting of a catalytic subunit (α) and two regulatory subunits (β and γ), plays a critical role as an energy sensor in these responses (reviewed in Refs. 1–3). In response to nutritional or environmental stress factors that deplete intracellular ATP, AMPK is activated by allosteric binding of AMP (4, 5) and by phosphorylation by a still uncharacterized upstream AMPK kinase (6). Once activated, AMPK minimizes further ATP consumption by suppressing ATP-consuming anabolic pathways as well as activating ATP-generating catabolic pathways. The physiological or stress conditions known to activate AMPK include exercise (7–9), nutritional starvation (10), heat shock (11), oxidative stress (12), and ischemia/hypoxia (3, 13–15).

Similar to the intracellular energy status, cellular oxygen concentration is precisely regulated in mammals to maintain cellular function and integrity. The reduced oxygen availability also initiates a series of adaptive responses, and many of these are mediated by HIF-1, a heterodimeric protein consisting of HIF-1α (19) and HIF-1β subunits (20), and both subunits contain cofactors including CBP/p300, SRC-1, or TIF2, reflecting the complex nature of its regulatory mechanisms (16–18).

Hypoxia leads to the activation of AMPK because of failure to generate sufficient ATP required for cellular functions (13–15). Thus, under the hypoxic conditions, AMPK and HIF-1 initiate various adaptive responses in response to two different cellular parameters, namely the decreased ATP level and the reduced oxygen level, respectively. Although the relationship between AMPK and HIF-1 had never been examined, there seems to be a possibility that a part of the signals transduced by the lowered energy level and by the reduced oxygen level may be interlinked at the molecular level; AMPK and HIF-1 exhibit similar effects on anaerobic glucose metabolism as recent studies demonstrated that hypoxia-induced glucose uptake (26) and hypoxia-stimulated glycolysis (14) are also mediated by AMPK. Thus, in the present study, we explored the possibility that AMPK is involved in the regulation of HIF-1. Although most of the currently identified substrates of AMPK are metabolic enzymes, a growing body of evidence demonstrated that AMPK is also implicated in the regulation of gene expression, and there are indeed several examples of transcription factors and cofactors that are directly phosphorylated and regulated by AMPK (27). Here, by using a pharmacological and molecular approach, we demonstrate that AMPK activity is critical for the HIF-1 transcriptional activity and its target gene expression in several cancer cell lines, implying a novel role of AMPK in cancer pathogenesis as well as in oxygen-regulated cellular physiology. Moreover, we also show that AMPK transmits its positive signal to HIF-1 via a signaling pathway that is independent of PI 3-kinase/AKT and MAP kinase pathways. To our knowledge, this is the first report demonstrating that AMPK is involved in the regulation of the oxygen-regulated gene expression.

**RESULTS**

AMPK Is Rapidly Activated in Response to Hypoxia or CoCl2 in DU145 Cells—We first determined the kinetics of AMPK activation in DU145 human prostate cancer cells exposed to hypoxia or CoCl2 (Fig. 1). Because of the hypoxia-mimicking effect, CoCl2 has been used extensively to study the hypoxic signaling pathway. Throughout the present study, the hypoxic effect was investigated under 1% O2, and 100 µM CoCl2 was used as a hypoxia-mimetic reagent. DU145 cells were incubated under the hypoxic conditions or treated with CoCl2 at the normoxic conditions for the indicated times, and AMPK activity was directly measured by an immune complex substrate, serine 79 of acetyl-CoA carboxylase-α (ACC-α), which is the best characterized phosphorylation site by AMPK (32), as assessed by immunoblotting with an antibody specific for the phosphorylated serine 79 of ACC-α (Fig. 1B). The total amount of ACC-α...
AMPK α subunit was not changed during the experimental conditions (Fig. 1B), thereby revealing a tight correlation between the actual AMPK activity and the phosphorylation level of ACC-α Ser79. HIF-1α expression was detected as early as 0.5 and 2 h exposure to hypoxia or CoCl2, respectively, whereas the expression level of HIF-1β was not altered (Fig. 1B). The AMPK activation and HIF-1α induction by hypoxia occurred at a similar time point, whereas AMPK activation apparently preceded HIF-1α induction in CoCl2-treated cells.

FIG. 1. Time-dependent effects of hypoxia or CoCl2 on AMPK activity and HIF-1 expression level in DU145 cells. DU145 cells were exposed to hypoxia (1% O2) or CoCl2 (100 µM) for the indicated period. A, AMPK was immunoprecipitated with AMPK-α antibody, and in vitro activity assay was performed using SAMS peptide as a substrate. The results represent the means ± S.E. for two independent assays in duplicate. B, total cell extracts were prepared under the identical conditions and subjected to Western blot assay using anti-phosphospecific ACC Ser79 (P-ACC), anti-ACC (ACC), anti-AMPK α (AMPK-α), anti-HIF-1α (HIF-1α), and anti-HIF-1β (HIF-1β) antibodies.

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AMPK Is Required for HIF-1 Transcriptional Activity—The temporal profiles of AMPK activity and HIF-1α expression under the hypoxic condition prompted us to investigate whether AMPK is required for HIF-1 activity and its target gene expression. To this end, we have taken a pharmacological and molecular approach to inhibit the AMPK activity (Fig. 2), and then we examined its subsequent effect on the HIF-1-dependent gene expression. Recently, a potent and selective small molecule AMPK inhibitor was identified and named compound C by Zhou et al. (33). Consistent with the reported concentration, pretreatment of DU145 cells with 20 µM compound C for 30 min almost completely prevented the hypoxia- or CoCl2-induced AMPK activation as indicated either by ACC-α Ser79 phosphorylation level (Fig. 2A, upper panel, 1st to 5th lanes) or by a direct enzyme activity assay (Fig. 2B), suggesting that this inhibitor could be used as a powerful tool to study the role of AMPK. However, compound C has not been intensively characterized yet, so we also attempted to confirm our observations by using molecular approaches to rule out any nonspecific effects of compound C. To this end, we generated the inactive form of AMPK α1 subunit (α1-DN) by replacing Asp157 with alanine because this mutant was reported to exert a dominant negative effect over the endogenous AMPK (29). To ensure a high level expression, we further developed the recombinant adenovirus, which delivers c-Myc-tagged AMPK α1 wild type (Ad-α1WT) and AMPK α1 dominant negative DNA (Ad-α1DN). Following infection with Ad-α1WT or Ad-α1DN, the expression of each form was monitored by Western blotting using c-Myc antibody (Fig. 2A, lower panel). Ad-α1DN also effectively blocked the hypoxia- or CoCl2-induced phosphorylation of ACC-α Ser79 (Fig. 2A, upper panel, 6th to 10th lanes) as well as an endogenous AMPK activity (Fig. 2B). Total amount of ACC was essentially the same under each condition (Fig. 2A, middle panel). Because the formation of a trimeric subunit complex is necessary for an optimal AMPK
activity (34), it is known that overexpression of wild type α subunit does not exert any positive effect on an endogenous AMPK activity. Consistent with this report (34), Ad-α1WT had little effect on the hypoxia-induced AMPK activity (Fig. 2).

FIG. 2.
Effects of compound C and expression of AMPK dominant negative form on hypoxia- or CoCl2-induced phosphorylation level of ACC-α Ser79 and endogenous AMPK activity. DU145 cells were either pretreated for 30 min with 20 µM compound C, a pharmacological inhibitor of AMPK, or infected with Ad-α1WT or Ad-α1-DN at 100 plaque-forming units per cell and incubated for an additional 24 h. Then these cells were further exposed to hypoxia (1% O2) or CoCl2 (100 µM) for 1 h. Under these conditions, the phosphorylation level of ACC-α Ser79 (P-ACC), total amount of ACC (ACC), and the expression level of the recombinant c-Myc-tagged wild type or dominant negative form of AMPK α (c-myc) was analyzed by Western blot analysis using an anti-phosphospecific ACC-α Ser79 antibody, an antibody for ACC, and c-Myc antibody, respectively (A). The AMPK activity was also measured under these identical conditions, and the results represent the means ± S.E. for two independent assays in duplicate (B). Null, adenovirus with no exogenous gene; WT, Ad-α1WT; DN, Ad-α1-DN.

To examine the role of AMPK in adaptive responses to hypoxia, we first examined the effect of AMPK inhibition on HIF-1 target gene expression such as VEGF and GLUT1 (Fig. 3). DU145 cells were pretreated with 20 µM compound C for 30 min and exposed to hypoxia or CoCl2 for 8 h. The mRNA level of VEGF165 and GLUT1 was then analyzed by semi-quantitative RT-PCR. Each stimuli distinctively increased VEGF165 and GLUT1 transcript level, and compound C pretreatment dramatically attenuated this increase (Fig. 3, left panels). The compound C effect was specifically associated with HIF-1 target gene expressions, as the mRNA level of GAPDH and β-actin remained constant under all conditions, thus representing the specificity of the drug. Moreover, essentially identical results were obtained when AMPK activity was abrogated by Ad-α1DN (Fig. 3, right panels).

FIG. 3.
AMPK inhibition blocks the hypoxia-induced VEGF165 and GLUT1 mRNA expression. DU145 cells were identically treated as described in Fig. 2 legend either by compound C (left panels) or by Ad-α1WT and Ad-α1DN infection (right panels). After exposure to hypoxia (1% O2) or CoCl2 (100 µM) for 8 h, total RNA was extracted from these cells, reverse-transcribed, and subjected to semi-quantitative RT-PCR using specific primers for VEGF165, GLUT1, GAPDH, and β-actin genes. The amplified cDNA was analyzed on 1% agarose gel. Null, adenovirus with no exogenous gene; WT, Ad-α1WT; DN, Ad-α1-DN.

In accordance with the transcript level (Fig. 3), the secreted VEGF protein amount, as measured by a commercial ELISA kit, increased ~3–4-fold in culture media of DU145 cells that were exposed to hypoxia or CoCl2 for 24 h (Fig. 4A). Under these conditions, pretreatment with 20 µM compound C significantly abrogated the hypoxia- or CoCl2-induced VEGF secretion as shown in Fig. 4A. Likewise, the hypoxia- or CoCl2-induced glucose uptake was also attenuated by compound C pretreatment (Fig. 4B). In DU145 cells, GLUT1 is a major isoform of glucose transporters, so it seems reasonable to consider that this result also reflects the GLUT1 mRNA level shown in Fig. 3. Taken together, our results (Figs. 3 and 4) indicate that AMPK activity is necessary for the hypoxia-induced VEGF165 and GLUT1 gene expression. Under these conditions (Fig. 4), cell viability was not significantly affected by compound C (data not shown).
AMPK inhibitor prevents the hypoxia-induced VEGF secretion and glucose uptake. A, DU145 cells were pretreated with 20 µM compound C for 30 min and then exposed to hypoxia (1% O2) or CoCl2 (100 µM) for 24 h. Then the culture media were collected, and the level of secreted VEGF was measured using a commercially available VEGF ELISA assay kit. B, under the identical conditions, 2-deoxy-D-[3H]glucose was added to culture media for 10 min at the end of hypoxia or CoCl2 exposure period, and glucose uptake was measured as described under "Experimental Procedures." Results are the means ± S.E. of at least six determinations.

To determine whether AMPK modulates VEGF or GLUT1 transcription by HIF-1-dependent mechanism, we transfected DU145 cells with a luciferase reporter (pEpoE-luc) driven by the human erythropoietin HIF-1-binding site (5′-TACGTGCT-3′) and SV40 promoter (35), and we investigated the effect of AMPK inhibition on HIF-1-dependent luciferase expression (Fig. 5). Hypoxia or CoCl2 induced a HIF-1-dependent luciferase activity ∼7–11-fold, whereas the cells transfected with pEpoEm-luc with a mutated site (5′-TAAAAGCT-3′) showed no response to these stimuli (Fig. 5B). Hypoxia- or CoCl2-induced luciferase activity was significantly diminished by pretreatment of compound C (Fig. 5A) or by cotransfection of pcDNA3 expression vector containing AMPK-α1DN cDNA (Fig. 5B), indicating that AMPK activity is indeed required for the HIF-1 transcriptional activity and thereby expression of its target genes. We further examined the role of AMPK in several different human cancer cell lines including HepG2 hepatocellular carcinoma, HeLa cervix carcinoma, and MCF7 breast adenocarcinoma (Fig. 5C). Hypoxia rapidly activated AMPK as well in these cells (data not shown), and the HIF-1-dependent luciferase expressions induced by hypoxia were also significantly attenuated by cotransfection of AMPK-α1DN expression vector in these cells (Fig. 5C). Therefore, AMPK activity is likely to be necessary for the HIF-1 transcriptional activity in a broad range of cancer types. Although we have used the adenovirus-mediated gene transfer throughout the present study, we performed the cotransfection assay in this particular experiment (Fig. 5, B and C) because infection of DU145 cells with a null adenovirus containing no exogenous gene caused an aberrantly high expression of the luciferase gene even in the absence of any stimuli.

AMPK Is Not Involved in Modulation of HIF-1α Protein Expression, Stabilization, or Nuclear Translocation—As an initial attempt to understand the underlying mechanisms how AMPK regulates the HIF-1 transcriptional activity and its target gene expression, we examined the effects of AMPK inhibition on HIF-1α protein level because the functional activity of HIF-1 is primarily regulated by accumulation of HIF-1α protein (16–18). DU145 cells were infected with Ad-α1WT or Ad-α1DN and exposed to hypoxia or CoCl2 for 4 h, and then total cellular protein extracts were subjected to Western blot analysis to determine HIF-1α protein level. The results showed that AMPK inhibition did not affect total protein level of HIF-1α (Fig. 6A, 1st 7 lanes). Under these conditions, the mRNA level of HIF-1α or the protein level of HIF-1β was not affected by AMPK inhibition either (data not shown). Moreover, pharmacological activation of AMPK under normoxic conditions by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), which becomes a potent AMPK activator after its intracellular phosphorylation to AMP-mimetic AICA-ribotide (1–3), did not induce HIF-1α protein expression, either
The changes in the level of a phosphoactivated form of each kinase in DU145 cells that were exposed to hypoxia for the indicated times (B). Then protein extracts were prepared and subjected to the luciferase activity assay. The data represents means ± S.E. for six determinations.

AMPK activation alone is not sufficient to stimulate HIF-1 transcriptional activity under normoxic conditions. DU145 cells were infected with adenovirus with no exogenous gene (Null), Ad-α1WT (WT), or Ad-α1DN (DN). After 24 h of infection, cells were exposed to hypoxia (1% O2) or CoCl2 (100 µM) for 4 h. Total, nuclear, and cytosolic fractions of protein extracts were isolated and subjected to Western blot analyses to determine the protein level of HIF-1α. β-Tubulin and U1–70K were used as specific markers of cytoplasmic and nuclear proteins, respectively. Adenovirus-mediated expression of AMPK α subunit was detected with c-Myc antibody. B, DU145 cells were treated with different concentrations of AICAR for the indicated times under normoxic conditions. Then total cellular protein extracts were prepared and subjected to the Western blot analyses to determine the protein level of HIF-1α (HIF-1α), the phosphorylation level of ACC-α Ser79, and the protein level of ACC (ACC). The band intensities of P-ACC were measured, normalized to those of ACC, and expressed as a fold induction of the basal activity. Protein extracts of DU145 cells exposed to hypoxia for 4 h were used as a positive control.

AMPK activation alone is not sufficient to stimulate HIF-1 transcriptional activity under normoxic conditions. DU145 cells were cotransfected with pEPoE-luc and pcDNA3 expression vector containing HIF-1α cDNA, and then the effect of AMPK activation by AICAR on HIF-1-dependent luciferase expression was examined under normoxic conditions (Fig. 7). Introduction of exogenous HIF-1α resulted in 11-fold induction of HIF-1-dependent luciferase activity under normoxic conditions. AICAR treatment of these cotransfected cells stimulated AMPK activity in a dose- and time-dependent manner as demonstrated by the phosphorylation level of ACC-α Ser79, and a 4-fold induction of AMPK activity, which is a similar degree of activation observed under hypoxic conditions, was achieved by 0.25 mM AICAR treatment for 1 h (Fig. 6B, upper panel). During 12 h of exposure, 0.25 mM AICAR did not further stimulate HIF-1-dependent luciferase activity under normoxic conditions (Fig. 7A), suggesting that AMPK activation alone is not sufficient to stimulate HIF-1 activity. Exposure of these cells to 0.25 mM AICAR (Fig. 7A) or 0.5 mM AICAR for longer than 8 h (Fig. 7B) led to a slight decrease in HIF-1-dependent luciferase activity, and this may be due to the nonspecific effect of AICAR because a prolonged exposure to AICAR was reported to lead to an AMPK-independent cell death (41).
(Fig. 8A). Immunoblot analyses with phosphospecific antibodies against ERK, p38, JNK, and AKT kinase revealed that only ERK1 and ERK2 were mildly and progressively activated in response to hypoxia. Compared with the kinetics of AMPK activity, ERK activation was quite slow, being detected in 2–4 h after hypoxic exposure. A phosphoactivated form of p38, JNK, and AKT was not detected at all during 24 h of exposure to hypoxia. As a positive control for potency of each antibody, protein extracts of DU145 cells exposed to 1 mM H2O2 for 30 min were used, and a distinctively phosphoactivated form of each kinase was detected. To evaluate further the functional role of ERK for HIF-1 regulation, DU145 cells were transiently transfected with pEpoE-luc plasmid (MEK1 inhibitor), and exposed to hypoxia for 24 h. However, the hypoxia-induced luciferase expression was not attenuated by PD98059 but rather slightly increased (Fig. 8B). However, this increase was not statistically significant, indicating that the ERK activity induced by hypoxia is not involved in the HIF-1 regulation in DU145 cells. Moreover, inhibition of ERK activity by PD98059 also did not affect the phosphorylation state of ACC-α Ser79, either (Fig. 8C). Therefore, AMPK is likely to modulate the HIF-1 transcriptional activity through its own signaling pathway that is independent of PI3-kinase/AKT and these MAP kinases.

**Previous Section Next Section**

**DISCUSSION**

Cellular oxygen concentration in all higher organisms is precisely regulated because it serves as a substrate for oxidative phosphorylation, a key step in the electron transport chain. Moreover, oxygen circulation is critical not only for maintaining ATP generation but also for regulation of metabolism. Even a slight decrease in normal oxygen concentration can impair ATP generation, hence affecting cell viability, and disruption of oxygen homeostasis is implicated in the etiology of many disease processes including cancer, heart disease, cerebrovascular disease, and chronic lung disease (42). For this reason, cells possess highly sophisticated mechanisms in response to hypoxia. In the present study, we explored a couple of such protective mechanisms, mainly focusing on the relationship between AMPK and HIF-1, which represent a cellular energy sensor/effecter (1–3) and oxygen sensor (4–7), respectively. In cancer, the cell adaptation to hypoxic stress involves an enhancement of the expression of angiogenic growth factor, growth of new blood vessels. VEGF is a specific mitogen for vascular endothelial cells, and the binding on its receptor on these cells promotes their proliferation, leading to vessel formation. The effective vascular remodeling after ischemic injury in heart or brain is positively affected in HIF-1-dependent mechanisms (42). In the case of myocardial ischemia, the role of AMPK as a critical mediator in controlling fatty acid and glucose metabolism has been demonstrated by several research groups (3, 15, 43). Therefore, in addition to the well-known fact that AMPK is to be involved in the regulation of glucose metabolism and lipid metabolism, the functional role of ERK for HIF-1 regulation, DU145 cells were transiently transfected with pEpoE-luc plasmid, pretreated with PD98059 (MEK1 inhibitor), and exposed to hypoxia for 24 h. However, the hypoxia-induced luciferase expression was not attenuated by inhibition of AMPK activity via pharmacological or molecular approach. Identical results were also obtained from cells that were exposed to catabolic ions, which mimicked hypoxia. These results thus indicate that a part of energy-sensing signals and oxygen-sensing signals are tightly linked at the molecular level, converging into HIF-1 modulation of both hypoxic and normoxic stress. As a result, an energy-sensing signal appears to be one of the critical components for the oxygen-regulated gene expression.

So far, more than 40 HIF-1 target genes have been reported, and their protein products play important roles in angiogenesis, vascular reactivity and remodeling, energy metabolism, erythropoiesis, cell proliferation, and survival (16–18). Although it is unknown at this point whether AMPK activity is required for every HIF-1 target gene expression, the current implication of AMPK in the modulation of HIF-1 action seems to extend to the mechanisms of angiogenesis. AMPK may generate an energy stress, which in turn may affect the expression of target genes and the growth of new blood vessels. VEGF is a specific mitogen for vascular endothelial cells, and the binding on its receptor on these cells promotes their proliferation, leading to vessel formation. The effective vascular remodeling after ischemic injury in heart or brain is positively affected in HIF-1-dependent mechanisms (42). In the case of myocardial ischemia, the role of AMPK as a critical mediator in controlling fatty acid and glucose metabolism has been demonstrated by several research groups (3, 15, 43). Therefore, in addition to the well-known fact that AMPK is to be involved in the regulation of glucose metabolism and lipid metabolism, the functional role of ERK for HIF-1 regulation, DU145 cells were transiently transfected with pEpoE-luc plasmid, pretreated with PD98059 (MEK1 inhibitor), and exposed to hypoxia for 24 h. However, the hypoxia-induced luciferase expression was not attenuated by inhibition of AMPK activity via pharmacological or molecular approach. Identical results were also obtained from cells that were exposed to catabolic ions, which mimicked hypoxia. These results thus indicate that a part of energy-sensing signals and oxygen-sensing signals are tightly linked at the molecular level, converging into HIF-1 modulation of both hypoxic and normoxic stress. As a result, an energy-sensing signal appears to be one of the critical components for the oxygen-regulated gene expression.

More relevant to our study, HIF-1 and VEGF also contribute to tumor pathogenesis by facilitating angiogenesis, by which tumors contain a unique blood supply during development (16). 1) A HIF-1 gene is critical for cancer growth and progression (44). In the case of HIF-1 and VEGF, both are expressed in a variety of primary malignant tumors, and the overexpression of either type of cancer correlated well with treatment failure and mortality (46). In addition to secretion of proangiogenic factors such as VEGF, an increased rate of anaerobic glycolysis is another hallmark feature of hypoxic adaptation of cancer cells. In fact, we demonstrated that hypoxia-induced VEGF secretion or glucose uptake was significantly blocked by AMPK inhibition (Fig. 4). Considering the functional role of AMPK in the regulation of cell adaption to hypoxic stress, we are tempted to speculate that AMPK may play an important role in cancer pathogenesis under hypoxic conditions. Moreover, our study may have clinical implications regarding an anti-cancer therapy. By taking advantage of the hypothesis environments of tumors, a selective anti-cancer therapy such as development of drugs that are toxic only under hypoxic condition has been attempted (47). In these strategies, HIF-1 has been considered as a putative anti-cancer target, and such an anti-cancer drug targeting HIF-1 is indeed under development (47, 48). Similarly, inhibition of AMPK in hypoxic tumor cells may fit into such a selective anti-cancer therapy because its activities were not only selectively induced under hypoxia but also required for the putative anti-cancer target HIF-1, as demonstrated in the current study. In general, the AMPK system is considered as a protective mechanism under stress conditions in normal cells, and we believe that its role in cancer cell is not an exception either.

HIF-1 regulation in mammalian cells is extremely complex. At this point, we do not understand the precise mechanism by which HIF-1 activity is modulated by AMPK. However, its activity is not likely to be required for the HIF-1 protein induction or stabilization under hypoxic conditions, and moreover, nuclear translocation of HIF-1α seems to be independent of AMPK activity (Fig. 6), implying that AMPK-mediated HIF-1 regulation occurs at some other post-translational level of HIF-1. Because AMPK activation alone was not able to stimulate HIF-1 activity (Fig. 7), we further examined a cross-talk with PI3-kinase/AKT, ERK, JNK, or p38 MAP kinase to elucidate the AMPK signaling pathway. However, we were not able to demonstrate any significant functional role of these kinases for HIF-1 activity in DU145 cells (Fig. 8). Identified substrates of PI3-kinase/AKT or MAP kinases in the regulation of HIF-1 activity may be distinct cell types as well as hypoxic stimuli-dependent. For example, the PI 3-kinase/AKT pathway has been known to be required for growth factor-dependent HIF-1 induction (49–51). However, hypoxic induction of this pathway is not only cell type-dependent, but its requirement for the hypoxia-induced HIF-1 activity was also recently challenged (52, 53). Consistent with our result (Fig. 8), a previous report showed that hypoxia did not maintain a high AKT pathway activity in prostate cancer cells (54). Similarly, hypoxia is able to increase ERK activity in some cell lines, but their functional requirement for HIF-1 activity depends on cell type as well (39). Likewise, ERK was activated in DU145 cells, but its activity seems to be dispensable for the HIF-1 activity (Fig. 8). In contrast to these protein or lipid kinases, AMPK activity may be more generally required for HIF-1 activity under hypoxic stress in a variety of cancer cells because the nature of AMPK is to be activated by the reduced cellular energy level, which is an inevitably occurring phenomena under hypoxic stress. Our demonstration of the significance of AMPK for HIF-1 activity may support our data in last four activities/stressors (46, 49–51).

Consequently, independent of PI3-kinase/AKT and MAPK pathway, AMPK is likely to possess its own signaling pathway leading to the post-translational modification of HIF-1α, and we assume that there are at least three different scenarios for this event. First, it is conceivable that AMPK activation could lead to indirect phosphorylation of HIF-1α via unknown protein kinase cascades because AMPK activation alone did not stimulate the HIF-1 activity induced by introduction of exogenous HIF-1α under normoxic conditions (Fig. 7). However, HIF-1α phosphorylation may exclude a possibility that AMPK activity may be involved in this pathway. Second, AMPK activation could be due to another post-translational modification. Second, nitric oxide (NO) may mediate the effect of AMPK activity on the transcriptional activity of HIF-1. NO has been shown to increase HIF-1 transcriptional or DNA binding activity (55, 56), and AMPK was known to phosphorylate endothelial nitric oxide synthase at Ser1177, which activates the enzyme (57, 58). Third, AMPK may regulate the transcriptional activity of HIF-1α by modulating the activity of associating cofactors such as p300. HIF-1α contains two transcription activation domains, which confer transcriptional activation of the target genes by anchoring with general transcriptional factors such as CBP/p300, TFI2, or SRC-1. It was recently reported that AMPK could phosphorylate p300, thus modulating a subset of p300 activities, although a physiological role of this phosphorylation still remains to be elucidated (59). Thus, p300 could modulate HIF-1 transcriptional activity in an AMPK-dependent manner. We are currently investigating these possibilities to further reveal a detailed mechanism of how AMPK activity transmits the positive signal for HIF-1 activity.

Most of the currently identified substrates of AMPK are metabolic enzymes, and its role thus has been focused on the regulation of metabolic pathways, which leads to maintenance of ATP homeostasis. However, our data presented here imply that AMPK may play
critical role(s) in far more various cellular physiologies than ever speculated because HIF-1 is essential for embryonic vascularization and development, tumor angiogenesis, and tissue ischemia.

- The abbreviations used are: AMPK, AMP-activated protein kinase; HIF-1, hypoxia-inducible factor 1; VEGF, vascular endothelial growth factor; GLUT1, glucose transporter 1; ERK, extracellular signal-regulated kinase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; PI, phosphatidylinositol; JNK, c-Jun amino-terminal kinase; MEK, MAPK/ERK kinase; ACC, acetyl-CoA carboxylase; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild type; DN, dominant negative; ELISA, enzyme-linked immunosorbent assay; MAP, mitogen-activated protein; MAPK, MAP kinase.

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