Glucose Deprivation Increases mRNA Stability of Vascular Endothelial Growth Factor through Activation of AMP-activated Protein Kinase in DU145 Prostate Carcinoma

Hee Yun, Minyoung Lee, Sung-Soo Kim, and Joohun Ha

From the Department of Biochemistry and Molecular Biology, Medical Research Center for Bioreaction to Reactive Oxygen Species, Kyung Hee University College of Medicine, Seoul 130-701, Korea

The induction of proangiogenic cytokines such as vascular endothelial growth factor (VEGF) is a critical feature of tumor angiogenesis. In the present study, we examined the mechanisms of VEGF gene expression induced by glucose deprivation in cancer cells, a role of AMP-activated protein kinase (AMPK) in the process, and the signal transduction pathway. AMPK functions as an energy sensor to provide metabolic adaptation under ATP-depleting conditions such as hypoxia and nutritional deprivation. Here, we show that glucose deprivation leads to a significant increase in the mRNA level of VEGF, GLUT1, and PFKFB3 genes in several cancer cells via a hypoxia-inducible factor-1-independent mechanism, and we demonstrate an essential role of AMPK in these gene expressions. Our data suggest that VEGF mRNA induction by glucose deprivation is due to an increase in mRNA stability, and the AMPK activity is necessary and sufficient to confer the stability to VEGF mRNA. We further show that reactive oxygen species is involved in glucose deprivation-induced AMPK activity in DU145 human prostate carcinomas, and c-Jun amino-terminal kinase acts as an upstream component in AMPK activation cascades under these conditions. LKB1, which was recently identified as a direct upstream kinase of AMPK, was not detected in DU145 cells. In conclusion, our results demonstrate a novel and major role of AMPK in the post-transcriptional regulation of VEGF, further implying its potential role in tumor angiogenesis.
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VEGF mRNA stability through ROS-mediated activation of AMPK in DU145 cancer cells under glucose deprivation. Moreover, we reveal for the first time that JNK acts as an upstream signal component of AMPK in the signal pathway.

EXPERIMENTAL PROCEDURES

Materials—RPMI medium 1640 and fetal bovine serum was from Invitrogen, PD908059, actinomycin D, and antibody for FLAG were from Sigma. SP600125, SB203580, and LY294002 were from TOCRIS. 2-Deoxy-o-[3H]glucose (6.0 Ci/mmol) was from PerkinElmer Life Sciences. The antibodies that recognize a phosphoactivated form of AMPKα Thr172, ERK1 and -2 (Thr202/Tyr204), stress-activated protein kinase/JNK (Thr183/Tyr185), p38 (Thr180/Tyr185), AKT Ser473 and acetyl-CoA carboxylase (ACC) Ser79, c-Jun Ser73, and LKB1 were from Cell Signaling Technology. Antibodies for HIF-1α, HIF-1β, and α-actinin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). AMPKα antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Plasmid pEpoE-luc (5′-TACGTGCT-3′) the cDNA construct of the dominant negative mutant of stress-activated protein kinase kinase 1, the FLAG-tagged dominant-negative mutant of JNK1 vector, and the FLAG-tagged wild-type LKB1 construct were provided by Dr. Franklin Bunn (Hematology-Oncology Division, Brigham & Women’s Hospital, Harvard Medical School, Boston, MA), Dr. J. Silva Gutkind (National Institutes of Health, Bethesda, MD), Dr. Roger J. Davis (Howard Hughes Medical Institute Research Laboratories, University of Massachusetts Medical Center, Worcester, MA), and Dr. Junying Yuan (Department of Cell Biology, Harvard Medical School, Boston, MA), respectively.

Cell Culture, Glucose Deprivation, and Hypoxia—DU145 (a human prostate carcinoma), HeLa S3 (a human cervix adenocarcinoma), HepG2 (a human hepatocellular carcinoma), AGS (a human gastric carcinoma), and HCT116 (a human colon carcinoma) were maintained in RPMI supplemented with 10% heat-inactivated FBS and antibiotics at 37 °C with 95% air and 5% CO2. Cells were rinsed three times with phosphate-buffered saline (PBS) and then exposed to glucose-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. Before exposure of hypoxia, culture medium was removed and replaced by a thin layer of fresh medium (0.15 ml/cm2) to decrease the diffusion distance of the ambient gas. Dishes were transferred to a Bactron anaerobic/environmental chamber (Sheldon Manufacturing, Inc.) using excitation and emission wavelengths of 488 and 525 nm.

VGEF ELISA Assay—After stimuli, the medium was removed and stored at −80 °C until assayed. VGEF concentrations were determined using an ELISA kit (R & D Systems), following the manufacturer’s instructions. Samples from three different experiments were analyzed in duplicate.

Glucose Uptake—Cells cultured on 12-well cluster dishes were washed with Krebs-Ringer phosphate buffer (KRB) (136 mM NaCl, 4.7 mM KCl, 10 mM NaHCO3, 1.0 mM CaCl2, 1.0 mM MgCl2, and 11.1 mg/ml propidium iodide. After sorting out the viable cells, fluorescence intensity was measured by flow cytometry (BD Biosciences) using excitation and emission wavelengths of 488 and 525 nm, respectively.

RESULTS

AMPK Activity Is Required for Both Glucose Deprivation- and Hypoxia-induced VEGF, GLUT1, and PFKFB3 mRNA Expression in DU145 Cells—Initially, the examination of the effect of glucose deprivation and hypoxia on AMPK activity of DU145 human prostate carcinomas was carried out (Fig. 1A).
DU145 cells were exposed to a glucose-free medium for the indicated time at normoxic conditions. In case of hypoxia, cells were incubated in a regular medium containing 25 mM glucose and exposed to 1% O2. Both of the stimuli rapidly increased the phosphorylation level of Thr172 in the active site of the AMPK/H9251 catalytic subunit, which is essential for the enzyme activity (13). The phosphorylation level of serine 79 of ACC, which is the best characterized phosphorylation site by AMPK (14), was concomitantly increased. In the absence of any stimuli, the phosphorylation level of ACC Ser79 and AMPK/H9251 Thr172 remained at the basal level for at least 12 h. The total amount of ACC or AMPK subunit was essentially the same. Subsequently, we examined the sensitivity of AMPK activation to glucose concentrations (Fig. 1B). The phosphorylation level of ACC Ser79 and AMPKα Thr172 gradually increased as cells were exposed to decreasing concentrations of glucose, and a prominent activation of AMPK occurred at a glucose concentration lower than 1 mM (Fig. 1B). Collectively, these results indicate that AMPK is sensitively activated in response to both glucose and oxygen deprivations.

Hypoxia induces a number of genes that are involved in adaptive responses to low oxygen tension via a transcription factor HIF-1. The well known target genes of HIF-1 include VEGF, GLUT1, and PFKFB3, which facilitate angiogenesis, glucose uptake, and anaerobic glycolysis, respectively. We have previously demonstrated that AMPK is critical for HIF-1 activity and its target gene expression under hypoxic conditions (11). Here, we examined whether glucose deprivation could induce expression of the aforementioned genes and if AMPK would play any role under these conditions. RT-PCR analysis shows that mRNA levels of VEGF, GLUT1, PFKFB3, and β-actin genes significantly increased as cells were exposed to decreasing concentrations of glucose (Fig. 2A). In fact, glucose deprivation induced the mRNA levels of these genes as distinctively as hypoxia (Fig. 2B). Inhibition of endogenous AMPK activity via adenovirus-mediated expression of the dominant negative form of AMPK (DN) or by pretreatment with a specific inhibitor compound C (15) severely attenuated the expression of VEGF, GLUT1, PFKFB3 genes, under glucose- and oxygen-deprived conditions (Fig. 2B), thus implying a significant role of AMPK in these gene regulations. Throughout the subsequent experiments, glucose deprivation was performed by incubating cells in a glucose-free medium at normoxic conditions.

Since VEGF, GLUT1, and PFKFB3 are induced primarily via HIF-1 under hypoxia, we next tested whether HIF-1 is also involved in the regulation of these gene expressions under glucose-deprived conditions (Fig. 3). As expected, hypoxia dramatically induced HIF-1α expression and HIF-1 transcriptional activity, which is assessed by the reporter activity
(pEpoE-luc) driven by the human erythropoietin HIF-1-binding site (5'-TACGTGCT-3') and SV40 promoter. In contrast, glucose deprivation induced neither HIF-1α expression (Fig. 3A) nor the transcriptional activity of HIF-1 (Fig. 3B). This observation is indeed in good agreement with a previous report, which demonstrates that VEGF expression was still induced in Hif1α−/− embryonic stem cells by glucose deprivation (16). Consistent with our previous report (11), hypoxia-induced HIF-1 transcriptional activity was significantly blocked by AMPK inhibition (Fig. 3C). These findings first suggest that glucose deprivation can induce a subset of hypoxia-inducible genes via HIF-1-independent mechanisms. Second, it is likely that AMPK can regulate the expression of VEGF, GLUT1, and PFKFB3 genes via either an HIF-1-dependent or -independent mechanism, depending on the cellular microenvironments, thus highlighting the significant role of AMPK in these gene expressions.

**AMPK Activation Alone Is Sufficient to Induce VEGF, GLUT1, and PFKFB3 mRNA Expression via a HIF-1-independent Mechanism.** A, DU145 cells were identically treated as described in the legend to Fig. 2 either by compound C (Com.C) or by Ad-α1WT and Ad-α1DN infection. These cells were then exposed to AICAR (500 μM) for 12 h, and the mRNA level of VEGF, GLUT1, PFKFB3, and β-actin was analyzed by RT-PCR. B, DU145 cells were exposed to AICAR (500 μM) or hypoxia for 4 h. Total cell extracts were subjected to Western blot assay using anti-phosphospecific AMPK Thr172 (P-AMPK), anti-phosphospecific ACC Ser79 (P-ACC), anti-HIF-1α (HIF-1α), and anti-α-actinin (α-actinin) antibodies. C, after 24 h post-transfection with pEpoE-luc plasmid, DU145 cells were exposed to AICAR (500 μM) or hypoxia for an additional 12 h. Then cell lysates were subjected to the luciferase activity assay. The data represent means ± S.E. for six determinations.
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Fig. 5. AMPK inhibitor prevents glucose deprivation-induced VEGF secretion and glucose uptake. A, DU145 cells were pretreated with 10 μM compound C (Com.C) for 30 min and then exposed to glucose deprivation or hypoxia or AICAR (500 μM) for 24 h. Then culture media were collected, and the level of secreted VEGF at each condition was measured using a commercially available VEGF ELISA assay kit. B, under the identical condition, 0.5 μCi of 2-[3H]deoxy-D-glucose was added to culture media for 10 min at the end of the glucose deprivation or hypoxia or AICAR (500 μM) exposure period, and glucose uptake was measured. Results are the means ± S.E. of six determinations. DMSO, Me2SO.

Fig. 6. AMPK regulates the glucose deprivation-induced VEGF, GLUT1, and PFKFB3 gene expression in a broad range of cancer types. HeLa S3, AGS, and HCT116 cells were exposed to glucose deprivation for 12 h in the presence or absence of 20 μM compound C (Com.C). Then the mRNA level of VEGF, GLUT1, PFKFB3, and β-actin was analyzed by RT-PCR. Experiments were repeated three times with similar results, and a representative result is shown.

widely used to demonstrate a physiological role of AMPK (9). The mRNA levels of VEGF, GLUT1, and PFKFB3 genes were markedly increased by AICAR, and these effects were significantly abrogated by compound C or by Ad-αDN, indicating that AMPK itself, and not the subsidiary effects of AICAR, is sufficient to induce gene expression under normoxic conditions (Fig. 4A). Moreover, AICAR induced neither HIF-1α protein expression (Fig. 4B) nor HIF-1 transcriptional activity (Fig. 4C), reinforcing that AMPK can modulate the gene expression via a HIF-1-independent mechanism under normoxic conditions.

In accordance with the transcript level (Figs. 2 and 4), the amount of secreted VEGF protein, as measured by a commercial ELISA kit, increased ~1.5–3-fold in culture media of DU145 cells that were exposed to glucose deprivation, hypoxia, or AICAR for 24 h (Fig. 5A). Under these conditions, the rate of glucose uptake increased to a similar extent (Fig. 5B). The pretreatment of DU145 cells with 10 μM compound C significantly abrogated the VEGF secretion and glucose uptake. We further examined the role of AMPK in several different human cancer cell lines, including HeLa S3 cervix adenocarcinoma, AGS gastric adenocarcinoma, and HCT116 colon carcinoma (Fig. 6). Glucose deprivation induced the mRNA level of VEGF, GLUT1, and PFKFB3 in the above mentioned cells, and the inductions were significantly attenuated by compound C. Therefore, AMPK is likely to play a significant role in the regulation of these gene expressions in a broad range of cancer types.

Glucose Deprivation Increases the Stability of VEGF mRNA via Activation of AMPK—As an initial attempt to understand the underlying mechanisms for glucose-deprived induction of VEGF mRNA as well as a role of AMPK, subsequently, the effects of glucose deprivation, hypoxia, and AICAR on the VEGF promoter using the luciferase reporter system were analyzed and compared. As schematically presented in Fig. 7A, we used a plasmid construct containing a 2.2-kb VEGF promoter region (pVEGF-luc), in which the hypoxia-responsive element was located at 900 base pairs upstream of the transcription initiation site (17–19). After 24 h post-transfection with pVEGF-luc, DU145 cells were exposed to glucose deprivation, AICAR, and hypoxia for 8 h (Fig. 7B). As expected, hypoxia induced a luciferase activity ~3-fold. In contrast, this construct showed no response to both glucose deprivation and AICAR, suggesting that VEGF mRNA induction by these two stimuli may not be a result of transcriptional activation. It is known that hypoxia also enhances the stability of VEGF mRNA (5), and several RNA-binding proteins, such as HuR (20) and heterogeneous nuclear ribonucleoprotein L (21), which recognize specific sequences in the 3′-UTR of the mRNA, have been implicated to play a role in post-transcriptional regulation. Since glucose deprivation has no effect on pVEGF-luc, we suspected that it could increase VEGF mRNA stability via 3′-UTR. To test this possibility, we designed another VEGF promoter construct, in which a 0.6-kb cDNA fragment corresponding to VEGF 3′-UTR nucleotides 1319–1910 was inserted between the luciferase gene and the polyadenylation site. This region of 3′-UTR is known to confer instability to VEGF mRNA (20, 22). This construct was designated as pVEGF-luc-3′-UTR (Fig. 7A), and its basal luciferase activity was ~40% lower than that of pVEGF-luc. In sharp contrast to pVEGF-luc, glucose deprivation and AICAR distinctively increased the luciferase activity of pVEGF-luc-3′-UTR, showing ~2–3-fold induction, and hypoxia also induced the luciferase activity ~4-fold (Fig. 7B). Pretreatment of AMPK inhibitor almost completely blocked the luciferase activity of pVEGF-luc-3′-UTR induced by the stimuli. Considering the known roles of VEGF mRNA 3′-UTR, the present findings raise a possibility that AMPK may regulate VEGF mRNA stability in response to glucose deprivation.

To test such a possibility, our next step was to directly determine the rate of decay of the VEGF mRNA after treat-
ment of cells with the transcription inhibitor, actinomycin D (Fig. 8). DU145 cells were exposed to glucose deprivation for 8 h, and then the cells were further incubated in the presence of actinomycin D for an indicated time period. Northern blot analysis shows that glucose deprivation indeed increased the half-life of VEGF mRNA from <1 to 3 h (Fig. 8A). Subsequently, to examine the role of AMPK in the regulation of VEGF mRNA stability, compound C was added to the cells that had been deprived of glucose for 8 h together with actinomycin D. This treatment almost completely abolished the effect of glucose deprivation, implying a critical role of AMPK in the regulation of VEGF mRNA stability. To confirm the role of AMPK, further examination of the effect of AICAR on VEGF mRNA stability was carried out (Fig. 8B). AICAR treatment for 8 h increased VEGF mRNA level to a similar degree as glucose deprivation, and its half-life also increased to >4 h in the presence of actinomycin D (Fig. 8B). Removal of AICAR resulted in a rapid decay of the mRNA, indicating that AMPK activation was autonomously sufficient to confer the stability to VEGF mRNA. These results thus provide strong evidence that glucose deprivation stabilizes VEGF mRNA through activation of AMPK. When these experiments (Fig. 8) were reanalyzed by RT-PCR, almost identical results were obtained (data not shown).

**JNK Acts as an Upstream Component in AMPK Activation Cascades under Glucose Deprivation Conditions**—Like many protein kinases, AMPK lies within phosphorylation cascades, and it is activated by phosphorylation at the Thr172 residue in α catalytic subunit via an upstream AMPK kinase. Recent work performed by several research groups has identified LKB1 as a major AMPK kinase (23–25). LKB1 is a tumor suppressor kinase, whose gene mutation leads to a dominantly inherited cancer termed Peutz-Jeghers syndrome (26). Hence, it was suggested that LKB1/AMPK may represent a link between metabolism and cancer cell proliferation. However, the precise role of LKB1 on AMPK activation still remains largely unknown. Thus, to understand the role of LKB1 and AMPK in cancer cell adaptation to metabolic stress, we initially measured the expression level of LKB1 in DU145 cells and compared its level with that of HeLa S3 cells (Fig. 9). It has been reported that HeLa S3 cells do not express LKB1 (27). The Western blot analysis and RT-PCR revealed that LKB1 was not expressed in DU145 cells and HeLa S3 cells. When these two cell lines were transfected with a FLAG-tagged LKB1 expression vector, its mRNA and protein were detected. These findings indicate that AMPK gets phosphorylated and activated by a mechanism that does not involve LKB1 in DU145 cells.

Several MAP kinases or lipid kinases have been implicated in the regulation of VEGF gene expression under hypoxic conditions (28). Hence, our next attempt was to elucidate the AMPK upstream signal pathway by checking a cross-talk with ERK, JNK, p38 MAP kinase, and phosphatidylinositol 3-kinase (PI3K/AKT under glucose-deprived conditions (Fig. 10). The activation kinetics of these kinases was initially determined under glucose deprivation by examining the level of the phosphorylated form of each kinase. ERK and JNK were rapidly activated within 1 h, which is a comparable rate of AMPK activation (Fig. 1), whereas activation of AKT and p38 MAP kinase was detected in 8–12 h (Fig. 10A). Total amount of each kinase remained constant (data not shown). To seek an upstream component in the AMPK cascade, cells were pre-treated with a specific inhibitor for each kinase (PD98059 for ERK, SP600125 for JNK, SB202190 for p38 MAP kinase, LY294002 for phosphatidylinositol 3-kinase) and exposed to glucose deprivation for 12 h. Among these, a JNK inhibitor, SP600125, led to a significant reduction in the phosphorylation state of AMPKα Thr172 and ACC Ser27 (Fig. 10B). A dose-dependent effect of SP600125 was also observed (data not shown), suggesting that JNK may act as an upstream component for AMPK activation under glucose-deprived conditions. To confirm this possibility, we have taken a molecular approach to inhibit the JNK1 activity. Expression of a dominant negative mutant form of stress-activated protein kinase kinase 1, which is an upstream kinase of JNK, (Fig. 10C), or a dominant negative form of JNK1 (Fig. 10D) distinctively blocked AMPK

![Diagram](http://www.jbc.org/)

**Fig. 7. The potential role of VEGF 3′-UTR in its mRNA induction by glucose deprivation.** A, a schematic diagram of luciferase reporter gene constructs used in the analysis of VEGF expression. The construct pVEGF-luc contains 2.2 kilobase pairs of the VEGF promoter region, in which a hypoxia response element (HRE) is located at –900 bp. Transcription initiation site is numbered as +1. pVEGF-luc-3′-UTR was constructed by subcloning a 0.6-kb fragment of VEGF 3′-UTR downstream of the luciferase gene. B, DU145 cells were transiently transfected with pVEGF-luc or pVEGF-luc-3′-UTR. After 24 h of post-transfection, cells were exposed to glucose deprivation, AICAR (500 μM), and hypoxia for an additional 8 h in the presence or absence of 20 μM compound C (Com.C). Then cell lysates were subjected to luciferase activity assay. The data represent means ± S.E. for six determinations.
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Glucose deprivation increases VEGF mRNA stability via AMPK activity. A, DU145 cells were exposed to glucose deprivation for 8 h. Then actinomycin D (Act.D; 2 µg/ml) was added at time 0, and the cells were further incubated for an additional 6 h under glucose deprivation. Compound C (Com.C; 20 µM) was added at time 0 together with actinomycin D. B, after treatment with AICAR (500 µM) for 8 h, DU145 cells were washed and incubated with media containing actinomycin D for 6 h in the presence and absence of AICAR. At the indicated time point, VEGF mRNA level was determined by Northern blot analysis and quantified by phosphorimaging. Experiments were repeated twice with similar results, and a representative result is shown.

LKB1 is not expressed in DU145 cells. DU145 and HeLa S3 cells were transiently transfected with a FLAG-tagged LKB1 expression vector. After 24 h, post-transfection, total cell extracts were subjected to a Western blot assay using anti-LKB1 (LKB1), anti-FLAG (Flag), and anti-α-actinin (α-actinin) antibodies. The arrow indicates LKB1. Total RNA extract was prepared under identical conditions and subjected to RT-PCR using specific primers for LKB1 and β-actin genes. WB, Western blot. Experiments were repeated four times with similar results, and a representative result is shown.

Activation induced by glucose deprivation. The potency of each dominant negative form was confirmed by examining the phosphorylation level of its substrate, JNK and c-Jun. These findings strongly suggest that JNK is involved in AMPK activation cascades. Moreover, a JNK inhibitor also abrogated the glucose deprivation-induced VEGF mRNA level (Fig. 10E). This result is in good agreement with a previous report demonstrating that JNK is essential for VEGF mRNA stability (29). Inhibitors for p38 MAP kinase and AKT, but not for ERK, also effectively blocked VEGF mRNA induction (Fig. 10E), and therefore phosphatidylinositol 3-kinase/AKT and p38 are likely to modulate the VEGF gene expression without involving the AMPK signal pathway.

Glucose Deprivation Induces VEGF Expression as Well as Activation of Both AMPK and JNK via ROS Generation—It has been reported that glucose deprivation led to accumulation of ROS, presumably superoxide and hydrogen peroxide, as a result of down-regulation of the mitochondrial free radical scavenger system (30, 31). Because AMPK and JNK are highly sensitive to oxidative stress (32, 33), we hypothesized that the glucose deprivation-induced AMPK activation cascade could
FIG. 10. JNK acts as an upstream component in AMPK activation cascades under glucose deprivation conditions. A, DU145 cells were exposed to glucose deprivation for the indicated times, and the phosphoactivated form of each kinase (P-ERK, P-AKT, P-JNK, and P-p38) was examined using specific antibodies. Protein extracts of DU145 cells exposed to 1 mM H2O2 for 30 min were used as a control to support the potency of each antibody (H2O2). B, DU145 cells were exposed to glucose deprivation for 12 h in the presence of compound C (Com.C; 20 µM), PD98059 (PD; 25 µM), SP600125 (SP; 25 µM), SB202190 (SB; 25 µM), or LY294002 (LY; 25 µM). Then the phosphorylation level of AMPK-αThr172 (P-AMPK) and ACC Ser79 (P-ACC) was examined by Western blot analysis. C, after 72 h of transfection with stress-activated protein kinase kinase 1 wild type (WT) or dominant negative (DN) plasmid, DU145 cells were exposed to glucose deprivation for 4 h, and then total cell extracts were subjected to Western blot assay using anti-phospho-JNK (P-JNK), anti-phospho-AMPKα Thr172 (P-AMPK), and anti-AMPK α (AMPK-α) antibodies. D, DU145 cells were stably transfected with JNK1-WT or JNK1-DN plasmid. These cells were exposed to glucose deprivation for 4 h, and then total cell extracts were subjected to a Western blot assay using anti-phospho-c-Jun (P-cJun), anti-phospho-AMPKα Thr172 (P-AMPK), and anti-AMPK α (AMPK-α) antibodies. E, cells were exposed to glucose deprivation for 12 h in the presence of the indicated kinase inhibitor, and mRNA levels of VEGF were measured by RT-PCR. Experiments were repeated 2–4 times with similar results, and a representative result is shown.

DISCUSSION

VEGF is a key regulator of tumor angiogenesis, and low oxygen tension in poorly vascularized tumors has been considered as a major inducer of VEGF expression (1, 2). However, hypoxic response mechanisms do not seem to be completely responsible for the increased VEGF expression in tissues facing insufficient vascularization. Some regions of rapidly growing solid tumors are frequently subjected to a differential degree of deficits in the levels of glucose and oxygen. Thus, in order to elicit an efficient angiogenic feedback response, it is necessary that cancer cells also up-regulate VEGF expression in response to glucose deprivation, and our findings in the present study support this notion. Here, we showed that VEGF, GLUT1, and PFKFB3 genes, which are well known targets of HIF-1 and are hypoxia-inducible, were induced in glucose-deprived cancer cells via a HIF-1-independent mechanism (Figs. 2, 3, and 6). These results thus suggest that the reduced glucose availability in cancer cells can initiate a series of adaptive responses such as angiogenesis, glucose uptake, and glycolysis by up-regulating identical genes that can also be induced by hypoxia, but via a different mechanism. We further demonstrated an essential role of AMPK in the gene inductions and observed that AMPK activity was necessary and sufficient to induce the genes in normoxic conditions (Figs. 2 and 4). Furthermore, as demonstrated in the present case (Figs. 2 and 3) or in our...
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previous study (11), AMPK can positively regulate the gene expression by modulating HIF-1 activity under hypoxic conditions. Therefore, our findings collectively suggest that AMPK can regulate the gene expressions in response to a shortage of either oxygen or glucose via multiple mechanisms, a HIF-1-dependent or HIF-1-independent manner, thus highlighting its significance in cancer cell adaptive responses. Both hypoxia and glucose deprivation represent metabolic stresses accompanying the intracellular ATP depletion. Thus, the energy-sensing signal mediated by AMPK appears to be one of the critical components for cancer cell adaptation and survival.

We next focused our attention on the mechanism of VEGF mRNA induction by glucose deprivation and a role of AMPK in this process. In accordance with previous reports (6, 7), our data show that the increased steady-state level of VEGF mRNA by glucose deprivation is not caused by transcriptional activation but is due to an increase in VEGF mRNA stability via its 3′-UTR. This suggestion was based on the following observations. First, the VEGF promoter containing a hypoxia response element showed no response to glucose deprivation, whereas its activity was induced –3-fold by hypoxia (Fig. 7). Second, the reporter plasmid containing 3′-UTR of VEGF mRNA, however, responded to glucose deprivation, showing –3-fold induced luciferase activity (Fig. 7). Third, glucose deprivation significantly increased the half-life of VEGF mRNA in the presence of actinomycin D (Fig. 8). Taken together, our data support a possibility that post-transcriptional regulation is critical for VEGF induction under glucose-deprived conditions. However, we cannot completely rule out a possibility that transcriptional activation would be partially responsible through an unknown glucose-responsive element located outside the VEGF promoter sequences tested in this study. In the present study, we have identified AMPK as an essential regulatory component for the regulation of VEGF mRNA stability. AMPK activity was not only required for hypoglycemia-induced VEGF induction, but also it was sufficient to confer stability to VEGF mRNA under normoxic conditions (Fig. 8). At this point, we do not understand the precise mechanisms by which VEGF mRNA stability is modulated by AMPK. Although regulation of mRNA turnover is now considered as a critical step of gene regulation, the mechanisms are poorly understood. Adenylate- and uridylate-rich elements present in the 3′-UTR of many short lived mRNAs, such as those of growth factors, cytokines, proto-oncogenes, and cell cycle regulatory genes, have been linked to the stability of these mRNAs, and a variety of RNA-binding proteins that selectively bind to adenylate- and uridylate-rich elements of these mRNAs have been identified (34). Although several RNA-binding proteins, such as HuR (20), heterogeneous nucleo ribonucleoprotein L (21), and poly(A)-binding protein-interacting protein 2 (35), have been demonstrated to bind to the 3′-UTR of VEGF mRNA and to participate in mRNA stabilization under hypoxic conditions, it is unknown whether or not hypoglycemia-induced VEGF mRNA stability is mediated by the similar proteins and mechanisms. We are currently investigating such a possibility and attempting to identify a component(s) involved in VEGF mRNA stabilization, which is directly regulated by AMPK.

One of the intriguing findings in the present study is that AMPKα Thr172 can be phosphorylated in response to ATP-depleting stimuli in DU145 cells, which do not express LKB1 (Figs. 1 and 9). Phosphorylation of Thr172 in the active site of AMPKα catalytic subunit is essential for the enzyme activity (13), and LKB1 has been identified as a major AMPK kinase (23–25). Its ability to phosphorylate AMPKα Thr172 as well as a similar residue of 13 human AMPK-related kinases was recently demonstrated (36). Nevertheless, the role of LKB1 as

FIG. 11. ROS generated by glucose deprivation induces VEGF mRNA expression and activation of AMPK and JNK. A, DU145 cells were exposed to glucose deprivation for the indicated time period, and then 2′,7′-dichlorofluorescein diacetate (10 μM) was added for 30 min. The changes in fluorescence intensity were measured by fluorescence-activated cell scanning analysis. B, after exposure to glucose deprivation for 2 h in the presence or absence of catalase (5,000 units/ml), the phosphorylation level of AMPKα Thr172 (P-AMPK), ACC Ser79 (P-ACC), and JNK (P-JNK) and the protein level of AMPK-α and JNK were examined. Total RNA was extracted under identical conditions and subjected to RT-PCR using specific primers for VEGF, and β-actin genes. WB, Western blot. Experiments were repeated twice with similar results, and a representative result is shown.
an AMPK kinase remains obscure because LKB1 activity does not change in response to physiological conditions that phosphorylate AMPKα Thr172 and stimulate AMPK activity in vitro (24) and in vivo (37). One of the potential hypotheses to explain this phenomenon is that binding of AMP to AMPK renders it a better substrate for AMPK kinase (38), so it seems possible that AMP-generating metabolic stresses increase the phosphorylation level of AMPKα Thr172 without altering LKB1 kinase activity. However, our results raise another possibility that other AMPK kinases that might be sensitive to metabolic stresses may also exist. As we measured the expression level of LKB1 in DU145 cells by RT-PCR and by Western blot analysis, its level was not detected at all (Fig. 9). Nonetheless, the phosphorylation level of AMPKα Thr172 in DU145 cells was significantly elevated by glucose starvation, hypoxia, and AICAR (Figs. 1 and 4). In fact, Altarejos et al. (39) recently reported that ischemia stimulates the activity of AMPK kinase in rat heart, which is distinct from LKB1, although the identity of this kinase is not determined. In accordance with such a study, our results suggest that multiple AMPK kinases may exist to phosphorylate and regulate AMPK, thus increasing the complexity of understanding the regulatory mechanisms for AMPK cascade.

In the present study, we have identified JNK as an upstream component for AMPK cascade in glucose-deprived conditions using a pharmacological and molecular approach (Fig. 10), but JNK is not likely to directly phosphorylate AMPK, because anisomycin, a strong activator of JNK, stimulated JNK but not AMPK in DU145 cells (data not shown). It was demonstrated that JNK is essential for VEGF mRNA stability and that such an AMPK kinase in rat heart, which is distinct from LKB1, although the identity of this kinase is not determined. In accordance with such a study, our results suggest that multiple AMPK kinases may exist to phosphorylate and regulate AMPK, thus increasing the complexity of understanding the regulatory mechanisms for AMPK cascade.

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