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ORIGINAL PAPER

Quercetin enhances hypoxia-mediated apoptosis via direct inhibition of AMPK activity in HCT116 colon cancer

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Abstract Tumor hypoxia is considered the best validated target in clinical oncology because of its significant contribution to chemotherapy failure and drug resistance. As an approach to target hypoxia, we assessed the potential of quercetin, a flavonoid widely distributed in plants, as a anticancer agent under hypoxic conditions and examined its pharmacological mechanisms by primarily focusing on the role of AMP-activated protein kinase (AMPK). Quercetin significantly attenuated tumor growth in an HCT116 cancer xenograft in vivo model with a substantial reduction of AMPK activity. In a cell culture system, quercetin more dramatically induced apoptosis of HCT116 cancer cells under hypoxic conditions than normoxic conditions, and this was tightly associated with inhibition of hypoxia-induced AMPK activity. An in vitro kinase assay demonstrated that quercetin directly inhibits AMPK activity. Inhibition of AMPK by expressing a dominant-negative form resulted in an increase of apoptosis under hypoxia, and a constitutively active form of AMPK effectively blocked quercetin-induced apoptosis under hypoxia. Collectively, our data suggest that quercetin directly inhibits hypoxia-induced AMPK, which plays a protective role against hypoxia. Quercetin also reduced the activity of hypoxia-inducible factor-1 (HIF-1), a major transcription factor for adaptive cellular response to hypoxia. Moreover, quercetin sensitized HCT116 cancer cells to the anticancer drugs cisplatin and etoposide under hypoxic conditions. Our findings suggest that AMPK may serve as a novel target for overcoming tumor hypoxia-associated negative aspects.

Keywords Quercetin · AMPK · Hypoxia · HIF-1α · Apoptosis

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Introduction

Drug resistance exhibited by some tumors is one of the primary limitations to chemotherapy for the vast majority of cancer patients [1]. The microenvironment of growing tumors is highly associated with increased energy demand and oxygen deficits due to diminished vascular supply [2–4]. Hypoxia represents one of the most pervasive features associated with drug resistance in most solid tumors, and its negative impact on chemotherapy outcomes has been known for decades [3, 5]. Although the factors contributing to drug resistance are complex and multifactorial, hypoxia is considered a compelling therapeutic target for cancer therapy [2–5].

Recent epidemiological and dietary intervention studies have suggested that diet-derived flavonoids may have a beneficial role in cancer therapy because of their apoptotic or anti-angiogenic activities [6–8]. Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone) is one of the most ubiquitous flavonoids found in a wide range of plants, and its anti-tumor activity has been recognized in a number of different

cancer cells, including leukemia, lymphoma, colon, ovarian, cervical, prostate, and breast [9–12]. Various mechanisms have been suggested for quercetin's anti-tumor activity, including inhibition of glycolysis, up-regulation of cell cycle inhibitors such as $p21^{WAF1}$ and $p27^{KIP1}$, and down-regulation of oncogene expression [9, 13–16]. Nevertheless, the precise target for quercetin and its action mechanisms remain largely elusive.

AMPK is a heterotrimer which consists of a catalytic subunit (α) and two regulatory subunits (β and γ), and plays a central role in the regulation of energy homeostasis via the coordination of a number of adaptive responses under ATP-depleting metabolic stresses [17–19]. It is sensitively activated by AMP accumulation as a result of ATP depletion under a broad spectrum of cellular conditions, including hypoxia, nutritional deprivation, and oxidative stress. Phosphorylation at the Thr^{172} residue of the α subunit by the upstream kinase including LKB1 and calmodulin-dependent protein kinase kinase β (CaMKKβ) is also important for AMPK activation [20, 21]. We have recently demonstrated that the activity of AMPK is critical for HIF-1 transcriptional activity and vascular endothelial growth factor (VEGF) expression under oxygen- and glucose-deprived conditions in various cancer cells [22, 23], which suggests that the AMPK-mediated energy-sensing signal is crucial in the adaptive responses of cancer cells.

As an attempt to target hypoxia, we investigated the molecular mechanisms for quercetin-induced tumor apoptosis under hypoxic conditions in the present study. The anti-cancer effect of quercetin was tightly associated with a reduction of hypoxia-induced AMPK activity. This notion was supported by the HCT116 colon cancer xenograft model, cell culture, and in vitro kinase assay. Quercetin also decreased HIF-1 activity in several cancer cells and sensitized these cells to cisplatin and etoposide under hypoxic conditions.

Materials and methods

Materials

RPMI medium 1640 and fetal bovine serum were obtained from Invitrogen (Carlsbad CA). Quercetin, etoposide, cisplatin, Hoechst 33342, and propidium iodide were from Sigma–Aldrich (St. Louis, MO). Compound C was from Calbiochem (San Diego, CA). Antibodies recognizing phosphospecific forms of AMPKα-Thr^{172} and ACC-Ser^{79} were from Cell Signaling Technology (Boston, MA). Antibodies for PARP, α-actinin, AMPKα, and c-myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant AMPK enzyme was purchased from Upstate Biotechnology (Lake Placid, NY). Plasmid pEpoE-luc containing a HIF-1 binding site (5′-TACGTGCT-3′) was generously provided by Dr. Franklin Bunn (Hematology-Oncology Division, Brigham & Women's Hospital, Harvard Medical School, Boston, MA).

Cell culture and hypoxia

HCT116 ($p53^{+/+}$) colorectal cancer cells were maintained in RPMI medium supplemented with 10 % heat-inactivated fetal bovine serum and antibiotics at 37 °C with 95 % air and 5 % CO_2. Before exposure to hypoxia, culture medium was removed and replaced by a thin layer of fresh medium to decrease the diffusion distance of the ambient gas. Dishes were transferred to a Bactron Anaerobic/Environmental Chamber (Sheldon Manufacturing, Inc.), which was flushed with 0.1 % O_2, 5 % CO_2 and 95 % N_2 at 37 °C and incubated for the indicated time points.

Assessment of cell apoptosis and cell viability

Cell apoptosis was assessed via fluorescence-activated cell sorting (FACS) analysis, Hoechst 33342 staining, and internucleosomal DNA fragmentation analysis. Total cells were harvested by trypsinization, collected by centrifugation, and washed with PBS. After fixing with 70 % ethanol, cells were resuspended in PBS containing 10 μg/mL propidium iodide. After sorting out the viable cells, fluorescence intensity was measured by FACSCalibur flow cytometry and CellQuest software (Becton–Dickinson) using excitation and emission wavelengths of 488 and 525 nm, respectively. Chromatin was stained with Hoechst 33342 as described previously [24]. Cells evidencing condensed chromatin were considered apoptotic. Cell viability was measured by Vi-CELLTMXR Cell Viability Analyzer (Beckman Coulter) through the use of an automated trypan blue exclusion assay, which is based on uptake of trypan blue dye by dead cells due to loss of their membrane integrity. One milliliter of the cell suspension aliquot in a plastic cuvette was aspirated and mixed with trypan blue and then pumped into the flow cell for imaging. The instrument collected 50 images of cells to compute viability. The dead cells appear darker than the viable cells, allowing the contrast between live and dead cells to be used in determining cell viability.

Adenovirus infection

C-myc-tagged AMPK wild type α subunit (WT), a dominant-negative form (DN), and a constitutively active AMPK form (CA) were generated, prepared, and purified as described previously [23]. Infections with adenovirus expressing AMPK wild type (Ad-AMPK-WT), AMPK dominant-negative form (Ad-AMPK-DN) or the constitutively active

form (Ad-AMPK-CA) were conducted in phosphate-buffered saline for 30 min at 37 °C, after which fresh medium was added.

Animals and in vivo anti-tumor assay

Athymic BALB *nu/nu* mice (5–6 weeks old) were utilized in this study. All animal experiments were approved by the Ethics Committee for Animal Experimentation of Kyung Hee University. Human colon carcinoma cells (HCT116) were injected into the flanks of 5–6 weeks old nude mice. Five mice were assigned to each of the experimental groups. Intraperitonal injection of quercetin at 3-day intervals was initiated after the tumor achieved a minimal volume of 200 mm^3. Tumor volumes were monitored for 24 days and evaluated in accordance with the formula $(L \times l^2)/2$ via the measurement of tumor length (L) and width (l) with a set of calipers. In 3 h after the last treatment, the mice were sacrificed, and xenograft tissues were frozen or fixed in 4 % paraformaldehyde in PBS.

Immunofluorescence

Tumor xenograft tissues were fixed in 4 % paraformaldehyde in PBS, postfixed in 0.05 % glutaraldehyde and 4 % paraformaldehyde for 2 h at 4 °C, washed in PBS, and infiltrated with 2.3 M sucrose for 24 h at 4 °C. Tissues were mounted on microtomy pins and frozen in liquid nitrogen, and subsequently cryosections were prepared for immunofluorescence. The specimen was blocked for 1 h with normal goat serum in Triton X-100. After blocking, sections were incubated overnight with P-AMPK primary antibody at 4 °C. After rinsing in PBS, the specimen was stained with FITC-conjugated secondary antibody for 2 h at room temperature in the dark. Slides were then rinsed with PBS and stained with Hoechst 33342 for 30 min. Finally, the slides were rinsed and mounted with Vectashield® Mounting Medium (Vector Laboratories Inc.). Immunofluorescence images were captured using a LSM510 confocal laser microscope (Carl Zeiss).

Western blotting

For the preparation of whole-cell lysates, the homogenized tissues of the tumor xenograft and the treated cells were lysed on ice in the PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology) for 30 min. Supernatant fractions were recovered by centrifugation (14,000×g × 20 min, 4 °C), and the concentration of protein was determined using the Bradford protein assay. Samples were prepared with 2-mercaptoethanol and denatured by heating ant 95 °C for 3 min. The proteins were separated on 8–12 % polyacrylamide gels and were transferred to nitrocellulose membranes. The membranes were blocked and hybridized with the primary antibody. The protein bands were visualized using a chemiluminescence detection kit (Amersham Bio-sciences) after hybridization with the HRP-conjugated secondary antibody.

AMPK activity assay

AMPK activity was assessed by the phosphorylation of the SAMS peptide fused to glutathione *S*-transferase (GST). An enzyme assay was performed with or without a pharamacological inhibitor of AMPK (compound C) or quercetin at 30 °C in buffer solution containing 15 mM HEPES (pH 7.0), 200 μM 5′-AMP, 200 mM GST-SAMS, recombinant AMPK enzyme, 0.01 % Brij 35, 0.3 mM dithiothreitol (DTT), 15 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (10 μCi). After incubation, the fusion protein was purified with glutathione-Sepharose and run on 12 % acrylamide gels. After running, gels were dried and exposed to X-ray film.

ATP analysis

Intracellular ATP was extracted from cells and measured by the luciferin/luciferase method by using an ATP Determination Kit (Molecular Probes). The assay buffer (100 μl) containing 0.5 mM luciferin, 1.25 μg/ml luciferase, 25 mM Tris pH 7.8, 5 mM $MgSO_4$, 100 μM EDTA, and 1 mM DTT was mixed with 20 μl cell lysate. Luminescence was analyzed and normalized by using cellular proteins.

Reporter gene assay

Transfection of cells with pEpoE-luc reporter constructs was performed. Cells were seeded onto 24-well culture plates at a density of 4×10^4 cells/well and incubated for 24 h in medium. The plasmid was transfected into cells using Cytopure™ (Qbiogene) according to the manufacturer's instructions. After 24 h of transfection, cells were exposed to hypoxia with or without various concentrations of quercetin. Luciferase activity was determined by mixing 20 μl cell extract with 100 μl luciferase assay reagent (Promega) and subsequent measurement of relative light units for 10 s in a luminometer (TD-20/20 luminometer; Turner Designs).

Statistical analysis

Results are expressed as the means ± SE. We used 1-way ANOVA test followed by Newman-Keuls multiple comparison test or Student's t test. Differences were considered significant at a $P < 0.05$.

Results

Quercetin exhibits anti-tumor effects in the HCT116 human cancer xenograft model with a substantial reduction of AMPK activity

We first evaluated the anti-cancer effect of quercetin in nude mice harboring HCT116 tumor xenografts. A total of 2×10^6 HCT116 colon cancer cells were subcutaneously implanted on a flank in each of five nude mice in each treatment group. When the tumor masses each grew to a minimal volume of 200 mm^3, mice were intraperitoneally treated with 50 mg/kg quercetin every 3 days for 24 days. The tumor volumes were significantly reduced by quercetin treatments in comparison with the vehicle-treated control groups (Fig. 1a). Under these conditions, we also assessed AMPK activity via Western blot analysis (Fig. 1b) and immunofluorescence microscopic imaging (Fig. 1c). Quercetin significantly inhibited AMPK in HCT116 human cancer xenografts, as evidenced by the phosphorylation level at Thr^{172} in the active site of the AMPKα subunit and at Ser^{79} of acetyl-CoA carboxylase (ACC), a well characterized AMPK cellular substrate [25]. The results strongly suggest that the in vivo anti-cancer property of quercetin may be associated with reduction of AMPK activity.

Quercetin more effectively induced apoptosis in HCT116 colon cancer cells under hypoxic conditions than normoxic conditions

We next compared the efficacy of quercetin under hypoxic and normoxic conditions. HCT116 cells were exposed to 100 μM quercetin during the indicated period of time or

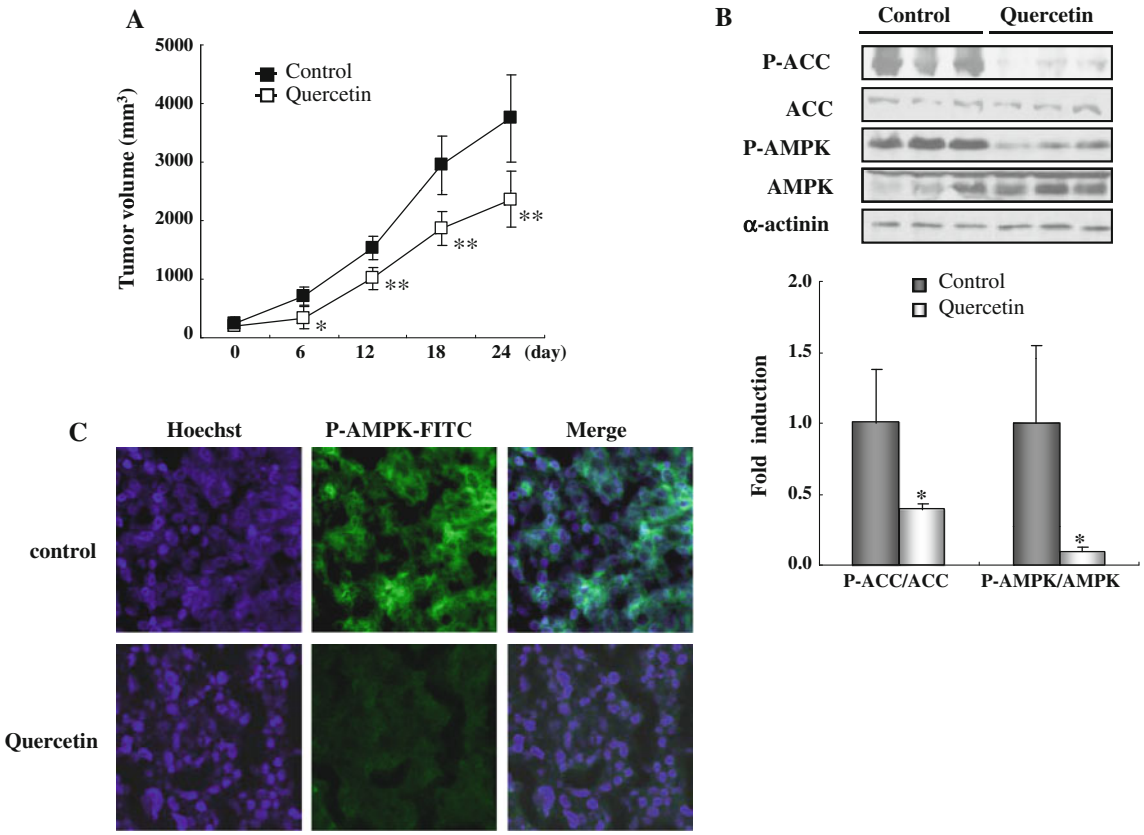

Fig. 1 Quercetin decreases in vivo tumor growth in nude mice. **a** HCT116 cells (2×10^6 cells) were subcutaneously injected into 5 week-old male nude mice of five per treatment group. After 1 week, mice were intraperitoneally treated with quercetin 50 mg/kg every 3 days for 24 days. The control group received vehicle only (30 % DMSO). Tumor volumes were calculated as described in "Materials and methods" (**$p < 0.01$ compared to control). **b** The last treatment of mice was performed 3 h prior to sacrificing the animal. Homogenized cell lysates were prepared from the control and quercetin-treated tumor tissue from three different sets of nude mice and subjected to Western blot assay using specific antibodies. The fold increases of the levels of P-ACC (ACC-Ser^{79})/ACC and P-AMPK (AMPKα-Thr^{172})/AMPK were presented after analysis using densitometry. **c** Immunofluorescence was obtained to confirm the results of Western blot analysis. In the confocal images, the *green* FITC fluorescence staining indicates the levels of AMPK phosphorylation (AMPKα-Thr^{172}) while chromatin was stained with Hoechst 33342 (Color figure online)

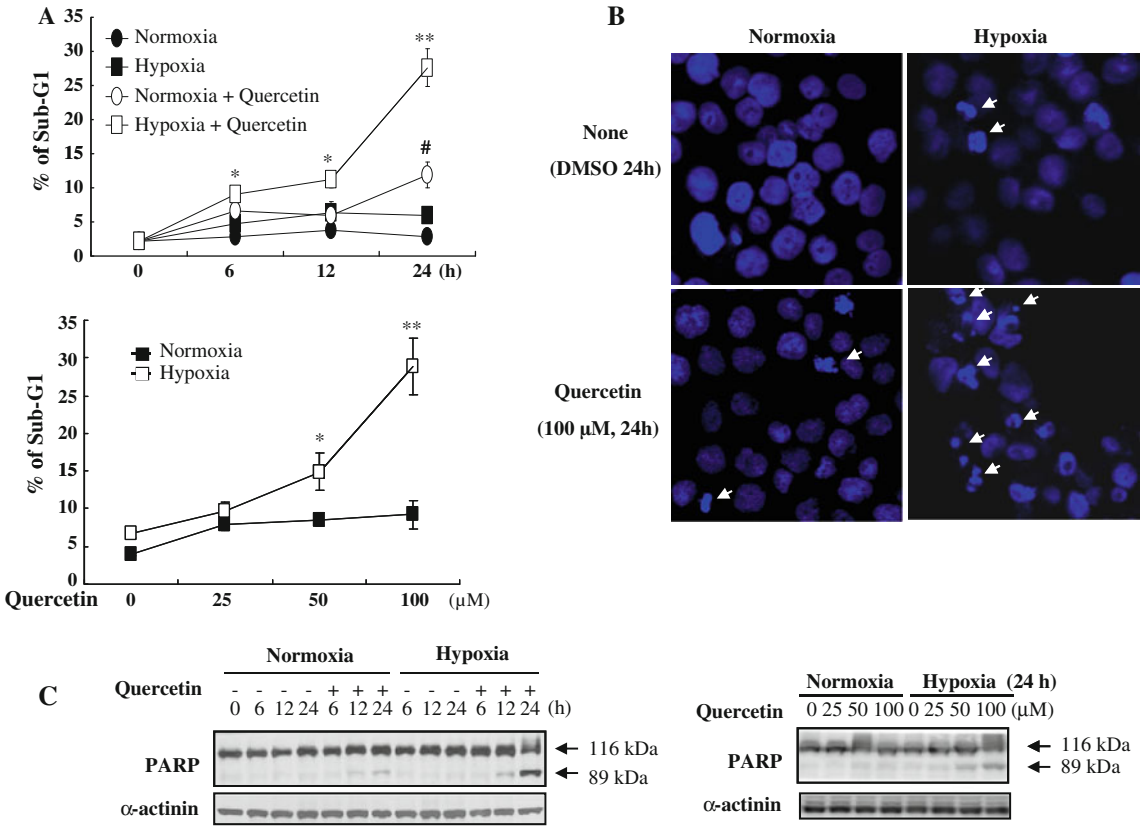

Fig. 2 Quercetin dramatically induces apoptosis of HCT116 colon cancer cells under hypoxic conditions. **a** HCT116 cells (2×10^5 cells) were incubated with 100 μM quercetin at indicated treatment durations (*upper panel*) or with indicated quercetin concentrations for 24 h (*lower panel*) under normoxic or hypoxic conditions. Cells were collected, fixed in 70 % ethanol, and stained by propidium iodide before FACS analysis. The percentages of sub-G1 DNA contents are indicated. The data are expressed as the means ± SE for three determinations in duplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with normoxia plus quercetin; $^{\#}p < 0.05$ compared with untreated cells. **b** The *arrow* indicates apoptotic cells as assessed by condensed chromatin after Hoechst 33342 staining in quercetin treated-HCT116 cells (100 μM, 24 h). **c** HCT116 cells were incubated with quercetin (100 μM) for the indicated time period or incubated with indicated concentrations for 24 h under normoxic or hypoxic conditions. Total cell extracts were prepared and subjected to Western blot analysis using anti-PARP and anti-α-actinin antibodies

the indicated concentrations of quercetin for 24 h under hypoxic and normoxic conditions (Fig. 2a). When cells were exposed to hypoxia (0.1 % O_2) alone for 24 h, no substantial increase in the degree of apoptosis was observed, as assessed by FACS analysis of sub-G1 DNA content. Quercetin increased apoptosis of HCT116 cells under both hypoxic and normoxic conditions in a time- and dose-dependent manner, but the efficacy as a putative anti-cancer agent was far higher under hypoxic conditions (Fig. 2a). The increase in quercetin-induced apoptosis under hypoxic conditions was also verified by Hoechest 33342 staining; distinctive nuclear condensation was observed (Fig. 2b). This result was further supported by Western blot analysis; the cleavage of poly (ADP-ribose) polymerase (PARP), which implies the initiation of apoptosis, was accelerated dramatically as the result of quercetin treatment under hypoxic conditions (Fig. 2c).

Quercetin inhibits hypoxia-induced AMPK activity in HCT116 colon cancer cells

We next addressed whether AMPK is associated with the anti-cancer effects of quercetin under hypoxic conditions. Hypoxia resulted in distinctive activation of AMPK (Fig. 3a, b). Under normoxic conditions, quercetin slightly activated AMPK until 6 h, and then AMPK activity was not detected until 24 h (Fig. 3a). In sharp contrast, AMPK activity markedly induced by hypoxia was dramatically inhibited by quercetin in both time- and dose-dependent manners (Fig. 3a, b), whereas AKT signaling pathway, which is known as a survival pathway under hypoxia, was not affected by hypoxia or quercetin (Fig. 3b). In addition to hypoxia, quercetin also effectively inhibited AMPK activity induced by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), ionomycin, glucose deprivation and

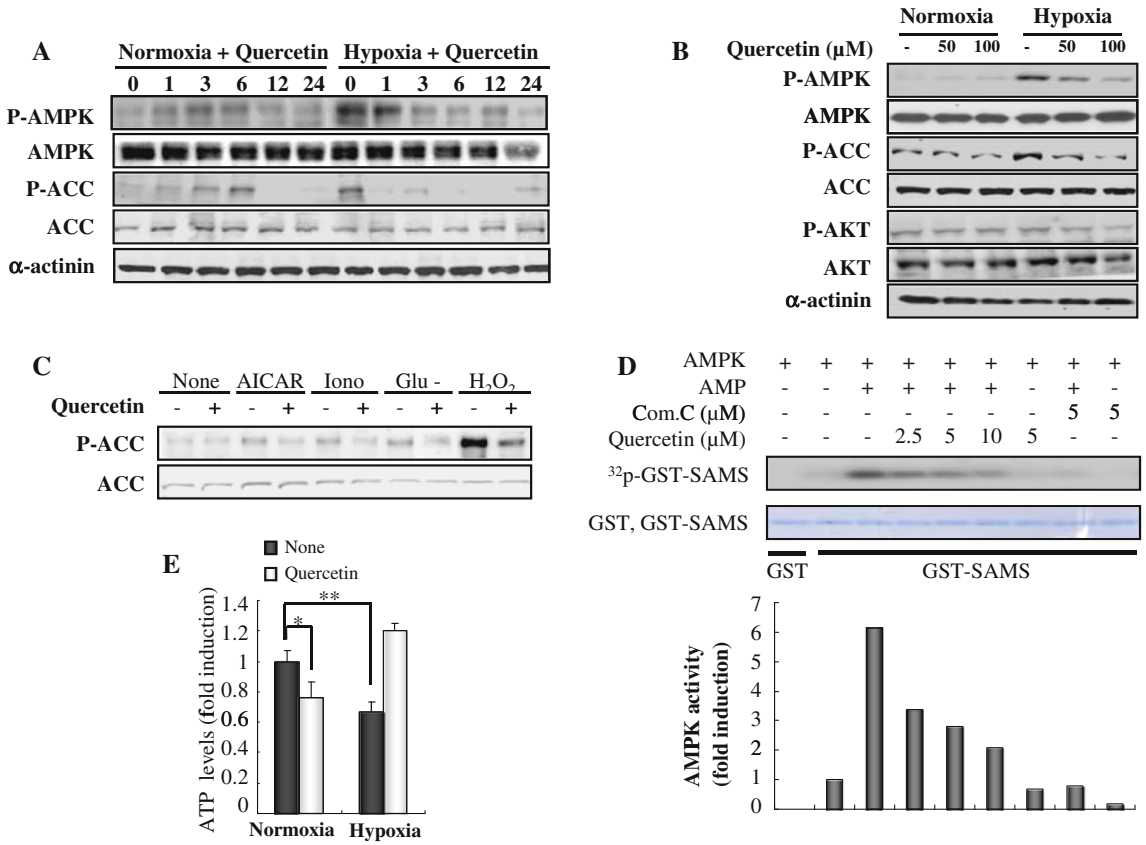

Fig. 3 Quercetin suppresses hypoxia-induced AMPK activity. **a** HCT116 cells were incubated with quercetin (100 μM) for the indicated time period under normoxic or hypoxic conditions. **b** HCT116 cells were incubated with the indicated quercetin concentrations for 24 h under normoxic or hypoxic conditions. Total cell extracts were prepared and subjected to Western blot analysis using specific antibodies. **c** HCT116 cells were pretreated with 100 μM quercetin for 30 min and then exposed to AICAR (1 mM, 1 h), ionomycine (1 mM, 30 min, Iono) glucose deprivation (1 h, Glu-), H_2O_2 (0.5 mM, 30 min). Total cell extracts were prepared and subjected to Western blot analysis using specific antibodies. **d** AMPK activity was assessed in vitro by the phosphorylation of the GST-SAMS peptide. An enzyme assay was performed in the presence of [γ-^{32}P] ATP with or without an AMPK pharmacological inhibitor in kinase assay buffer at 30 °C for 1 h, and then run on 12 % acrylamide gels. After running, gels were dried and exposed to X-ray film. The protein substrate is GST or GST-SAMS peptide and was stained with Coomassie blue. **e** HCT116 cells were incubated with 100 μM quercetin for 6 h under normoxia and hypoxia, and cellular ATP concentrations were determined as described in the "Materials and methods" section. The results are expressed as the means ± SE from at least three experiments

H_2O_2 (Fig. 3c). These conditions are known to activate AMPK via different mechanisms; AICAR allosterically activate AMPK, whereas ionomycin and glucose deprivation induces AMPK activation via its different upstream kinases, CaMKKβ and LKB1, respectively. The mechanism for AMPK activation via oxidative stress is not fully understood. Therefore, this result showing that quercetin is able to inhibit AMPK under various stimuli raises a possibility that quercetin may act as a direct inhibitor of AMPK. To address this possibility, we performed an in vitro kinase assay (Fig. 3d). Recombinant AMPK was activated in the presence of AMP, reflecting the nature of AMPK, and its activity was inhibited in the presence of the AMPK inhibitor Compound C. The results showed that quercetin directly inhibited AMPK activity in a dose-dependent manner.

As an attempt to understand the opposite effects of quercetin on AMPK under different oxygenic conditions, we next measured the effect of quercetin on intracellular level of ATP because AMPK is highly sensitive to this parameter (Fig. 3e). Under normoxic condition, quercetin decreased ATP level by 30 %, which may contribute to AMPK activation. Therefore, quercetin may exert two opposing effects on AMPK under normoxic condition: activation via ATP depletion and direct inhibition of the enzyme activity as demonstrated in in vitro kinase assay. As a result, a slight activation of AMPK by quercetin under normoxic condition may be a net result of two effects of quercetin.

Quercetin induces apoptosis of HCT116 colon cancer cells via inhibition of AMPK under hypoxic conditions

To determine the role of AMPK under hypoxic conditions, we took a molecular approach to inhibiting AMPK activity

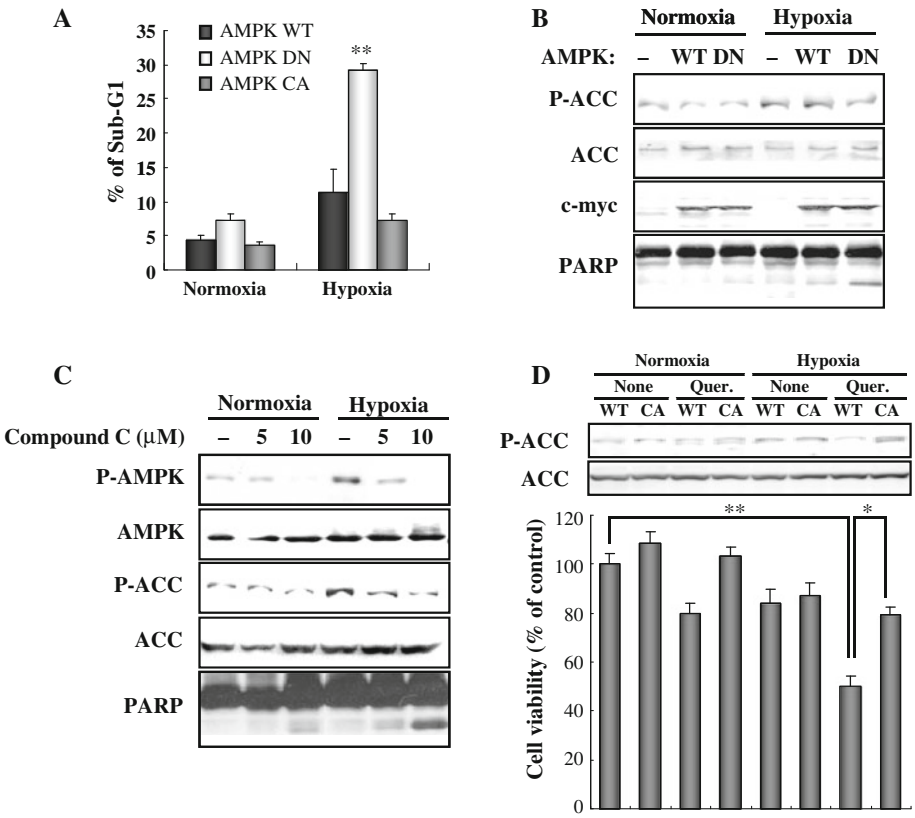

Fig. 4 Inhibition of AMPK activity decreases cell viability under hypoxic conditions. **a** HCT116 cells were infected with c-myc tagged adenovirus-AMPKα1 WT (wild-type), AMPKα1-DN (dominant-negative) or AMPKα1-CA (constitutively active form) at 100 plaque-forming units per cell and incubated for an additional 24 h. These cells were incubated for 24 h under normoxia or hypoxia and then analyzed for apoptosis via FACS analysis. The percentages of sub-G1 (apoptotic fraction) cells are shown; the data are expressed as the means ± SE for three determinations in duplicate ($^{**}p < 0.01$). **b** Under identical conditions, total cell extracts were prepared and subjected to Western blot analysis. **c** HCT116 cells were incubated with the indicated concentrations of compound C for 24 h under normoxic or hypoxic conditions, and then total cell extracts were prepared and subjected to Western blot analysis using specific antibodies. **d** After infection with adenovirus expressing AMPKα1-wild type (WT) or AMPKα1-constituitive active form (CA), HCT116 cells were exposed to hypoxia in the presence or absence of 100 μM quercetin. After 24 h, cell viability was examined by trypan blue exclusion assay or after 3 h, Western blot analysis was performed. The results are expressed as the means ± SE for four determinations in duplicate ($^{*}p < 0.05$ and $^{**}p < 0.01$)

and examined the subsequent effect on cell apoptosis. The inhibition of endogenous AMPK activity by expression of a c-myc-tagged AMPK dominant-negative (DN) form resulted in a marked increase of apoptosis under hypoxic conditions, whereas its effect was marginal under normoxic conditions, and expression of the AMPK constitutively active (CA) form decreased hypoxia-induced apoptosis (Fig. 4a), indicating that AMPK protects cancer cells against hypoxic stress. This was further confirmed by Western blot analysis of poly (ADP-ribose) polymerase (PARP) cleavage (Fig. 4b). Pretreatment with Compound C also revealed identical results (Fig. 4c).

To examine the causal relationship between AMPK and quercetin-induced apoptosis, we expressed a constitutively active mutant of the AMPKα catalytic subunit (AMPK-CA) in HCT116 colorectal cells and assessed cell viability (Fig. 4d). As demonstrated via trypan blue exclusion assay, quercetin more effectively decreased cell viability under hypoxic conditions than under normoxic conditions, and expression of AMPK-CA significantly abrogated the inhibitory effect of quercetin on cell viability under hypoxia (Fig. 4d), indicating that quercetin inhibits AMPK activity, which plays a protective role against hypoxia and leads to induction of apoptosis.

Quercetin decreases hypoxia-induced transcriptional activity of HIF-1

To gain further insight into the mechanisms of quercetin's anti-cancer effects, we next examined the consequence of quercetin treatment on HIF-1 activity under normoxic and hypoxic conditions. HIF-1 is a heterodimeric transcription factor and plays a central role in the adaptive response to hypoxia via the coordinated upregulation of genes involved in angiogenesis, erythropoiesis, and glycolysis [26, 27]. The HIF-1α subunit is subjected to degradation under

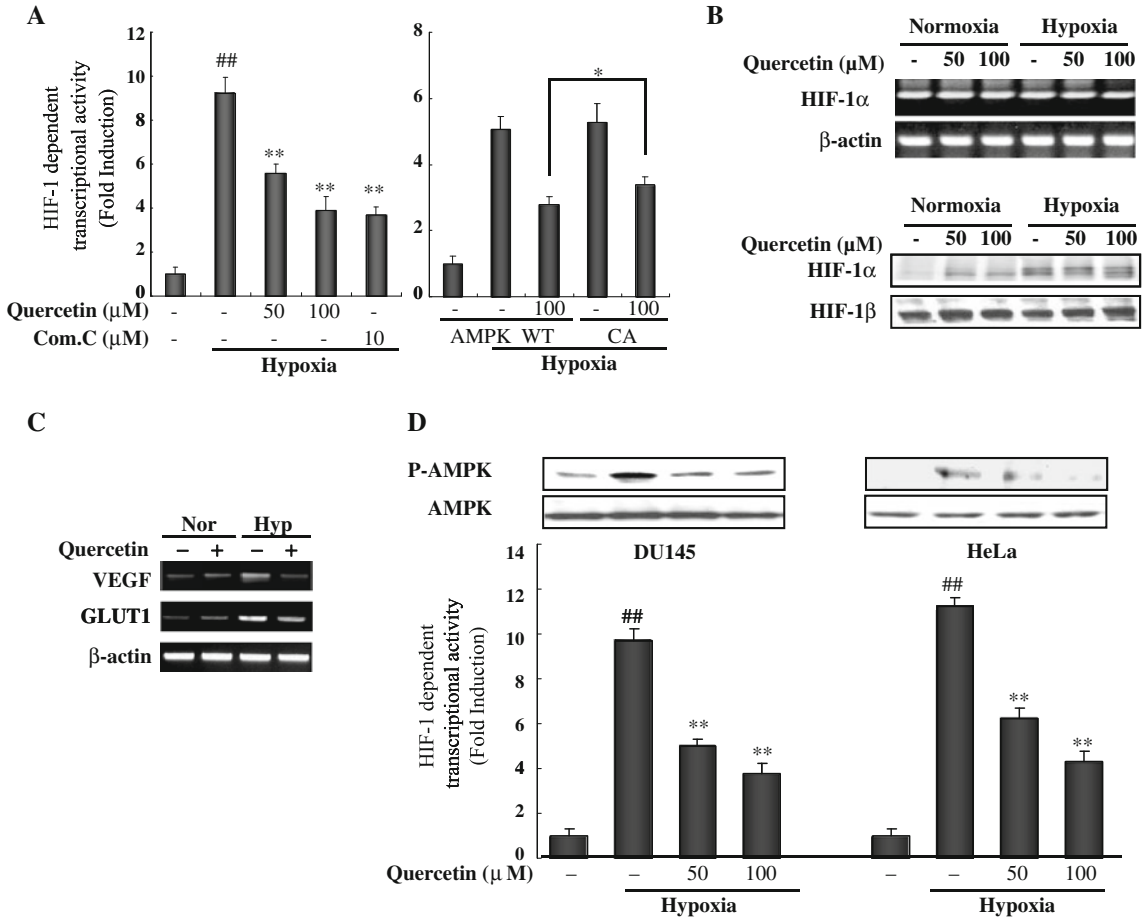

Fig. 5 Quercetin decreases hypoxia-induced activation of HIF-1. **a** HCT116 cells were transfected with pEpoE-luc reporter constructs. These cells were incubated with quercetin or Compound C for 12 h under hypoxia (*left panel*). HCT116 cells were infected with adenovirus-AMPKα1 WT or AMPKα1-CA and incubated with quercetin for 12 h (*right panel*). Luciferase activity was measured. Data was expressed as the means ± SE for three determinations in duplicate. $^{\#\#}p < 0.01$, compared with normoxia; $^{**}p < 0.01$, compared with hypoxia alone; $^{*}p < 0.05$. **b** HCT116 cells were incubated with the indicated quercetin concentrations for 12 h under normoxic or hypoxic conditions. The mRNA levels of HIF-1α and *β*-actin were determined via RT-PCR (*upper panel*). Under identical conditions, total cell extracts were prepared and subjected to Western blot assay using specific antibodies (*lower panel*). **c** HCT116 cells were incubated with 100 μM quercetin for 12 h under normoxic (*Nor*) or hypoxic conditions (*Hyp*). The mRNA levels of VEGF, GLUT1 and *β*-actin were determined via Reverse Transcriptase-PCR. **d** After transfection using pEpoE-luciferase reporter constructs, the indicated cell lines (DU145, HeLa S3) were treated with indicated quercetin concentrations in hypoxia, and luciferase activity was measured (12 h) or Western blot analysis was performed (3 h). Data was expressed as the means ± SE from for three determinations in duplicate. $^{\#\#}p < 0.01$, compared with normoxia, and $^{**}p < 0.01$, compared with hypoxia alone

normoxia and is stabilized under hypoxia. HCT116 cells were transiently transfected with a plasmid (pEpoE-luc) containing a luciferase reporter driven by the human erythropoietin HIF-1 binding site (5′-TACGTGCT-3′) and the SV40 promoter (Fig. 5a, left panel). Hypoxia markedly induced HIF-1-dependent luciferase activity, and the induced HIF-1 activity was inhibited by quercetin in a dose-dependent manner. In accordance with our previous report showing that AMPK is critical for HIF-1 activity [23], Compound C also effectively inhibited HIF-1 activity (Fig. 5a, left panel). In addition, the expression of AMPK-CA slightly abrogated the inhibitory effect of quercetin on hypoxia-induced HIF-1-dependent luciferase activity (Fig. 5a, right panel). Quercetin did not affect the mRNA level of HIF-1α under either normoxic or hypoxic conditions (Fig. 5b, upper panel). At the HIF-1α protein level, the differential effect of quercetin was observed at normoxic and hypoxic conditions. Quercetin slightly increased HIF-1α under normoxic conditions, whereas it had little effect on the hypoxia-induced HIF-1α protein level (Fig. 5b, lower panel). To further confirm the inhibitory effect of quercetin on hypoxia-induced HIF-1 activity, we investigated the mRNA level of HIF-1 target genes including VEGF and GLUT1. As a result, quercetin markedly reduced hypoxia-induced mRNA levels of VEGF and GLUT1 (Fig. 5c). In addition to HCT116 cells, quercetin also inhibited hypoxia-induced AMPK and HIF-1 activities in other cancer cell lines, including DU145

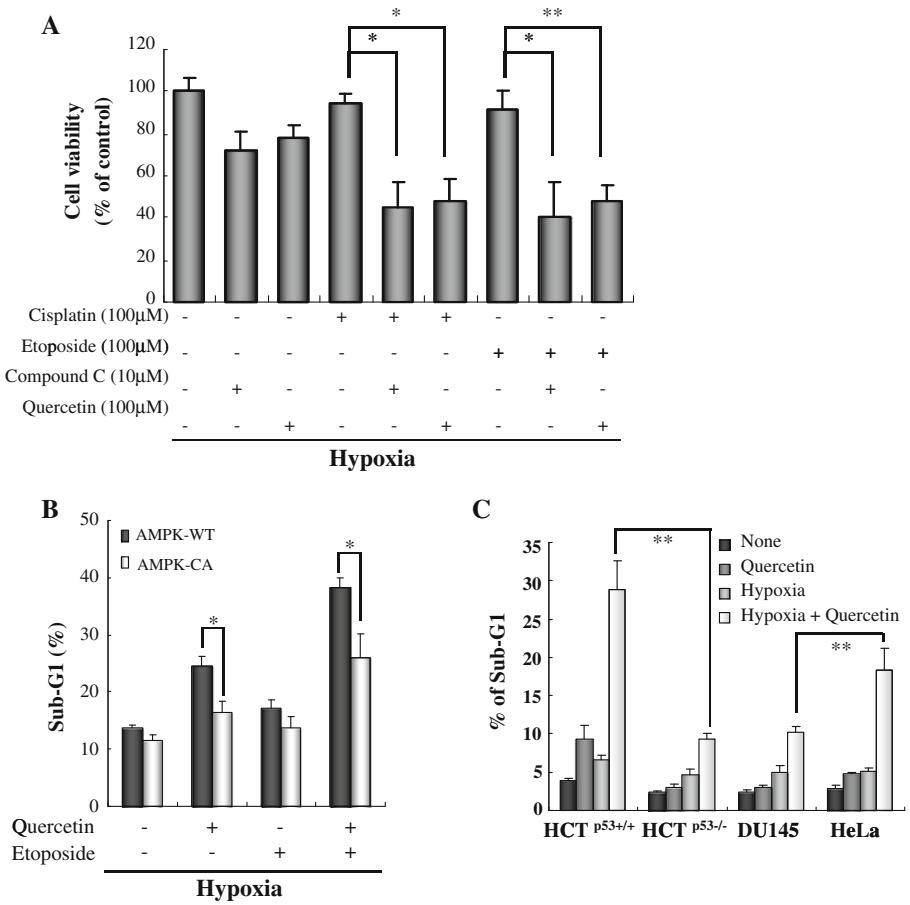

Fig. 6 Quercetin sensitizes HCT116 cells to cisplatin or etoposide-induced apoptosis under hypoxic conditions. **a** After pretreatment of quercetin or compound C for 30 min, HCT116 cells were incubated with cisplatin or etoposide under hypoxia for 24 h. Cell viability was measured by trypan blue exclusion assay. Data was expressed as the means ± SE from three individual experiments ($*p < 0.05$ and $**p < 0.01$). B. After infection with adenovirus-AMPKα1 WT or AMPKα1-CA, HCT116 cells were pretreated with 100 μM quercetin for 30 min and incubated with 100 μM etoposide under hypoxia for 24 h. Then, cells were analyzed for apoptosis via FACS analysis. The percentages of sub-G1 (apoptotic fraction) cells are shown; the data represent the means ± SE in triplicate ($*p < 0.05$). C. HCT116 $p53^{+/+}$ cells, HCT116 $p53^{-/-}$ cells, DU145 cells (prostate cancer, p53 mutant), HeLa S3 cells (cervical adenocarcinoma, p53 wild type) were incubated with 100 μM quercetin for 24 h under normoxic or hypoxic conditions and analyzed for apoptosis via FACS analysis. The percentages of sub-G1 (apoptotic fraction) cells are shown; the data represent the means ± SE for three determinations in duplicate ($**p < 0.01$)

prostate and HeLa S3 cervical cancer cells (Fig. 5d). Based on these results and our previous report showing that AMPK is critical for HIF-1 activity [23], we speculate that quercetin provokes the dominant apoptotic effect by inhibiting AMPK activity under hypoxic conditions, leading to reduction of HIF-1 activity.

Quercetin sensitizes HCT116 cells to cisplatin and etoposide under hypoxic conditions

We next examined whether quercetin can modulate cellular response to anti-cancer drugs, such as cisplatin or etoposide, under hypoxic conditions. HCT116 cells were exposed to hypoxia for 24 h in the presence/absence of quercetin, Compound C, cisplatin, or etoposide, and then cell viability was measured (Fig. 6a). Under hypoxic conditions, cisplatin or etoposide alone was not able to decrease cell viability to a significant degree. However, these drugs in combination with either quercetin or compound C resulted in a synergistic and dramatic reduction of cell viability (Fig. 6a). Moreover, expression of AMPK-CA significantly abrogated the apoptosis induced by etoposide in combination with quercetin under hypoxic conditions (Fig. 6b). Collectively, our results show that AMPK inhibition by quercetin or compound C sensitizes cancer cells to anti-cancer drugs.

Since p53 plays a major role in the AMPK-mediated apoptosis, we next compared the sensitivity of several cancer cells harboring wild type p53 or mutant p53 to quercetin-induced apoptosis under hypoxic condition. HCT116 $p53^{+/+}$ cells, HCT116 $p53^{-/-}$ cells, DU145 cells (prostate cancer, $p53^{mutant}$), HeLa S3 cells (cervical adenocarcinoma, $p53^{wild\ type}$) were incubated with 100 μM

quercetin for 24 h under normoxic or hypoxic conditions and analyzed for apoptosis via FACS analysis (Fig. 6c). The result revealed that p53 wild type cells are much more sensitive to quercetin-induced apoptosis under hypoxic condition (Fig. 6c). These results suggested that quercetin exert the apoptotic effect via p53-dependent signaling pathway.

Discussion

In the present study, we observed the differential effects of quercetin in induction of cancer apoptosis, which depends on oxygen concentration; quercetin more effectively induced apoptosis of cancer cells under hypoxic conditions than normoxic conditions. This is highly relevant to clinical oncology because hypoxia represents a compelling therapeutic target due to its major impact on tumorigenesis and resistance to therapy [2, 5]. Based on the following observations, we speculated that AMPK is a critical factor that determines the differential effect of quercetin. First, hypoxia resulted in ATP depletion in HCT116 cancer cells (Fig. 3e), which led to activation of AMPK (Fig. 3a, b). Second, AMPK plays a critical role in the protection of cells against hypoxia (Fig. 4). Third, quercetin effectively inhibited AMPK activity (Fig. 3a, b), thereby leading to dramatic induction of apoptosis under hypoxic conditions (Figs. 1, 2). Fourth, the result of an in vitro kinase assay suggests that AMPK is a direct molecular target of quercetin (Fig. 3d). Quercetin, which functions as AMPK inhibitor, also sensitized HCT116 cells to the anti-cancer drugs cisplatin and etoposide under hypoxia (Fig. 6). This observation precisely matched our previous report that inhibition of AMPK sensitized cancer cells to cisplatin [28]. Therefore, our data suggest that AMPK, which is sensitively activated by hypoxia and protects tumor cells against ATP depletion, may serve as a molecular target for cancer therapy.

In contrast to our present findings, several recent papers demonstrated that quercetin exerts anti-cancer activity via activation of AMPK [29–31]. Numerous reports support the role of AMPK as a survival factor in cancer cells as well as in other tissues, and such a role is tightly associated with AMPK's central function in the regulation of energy homeostasis [28, 32–35]. However, at the same time, AMPK has been implicated in the induction of cell cycle arrest or apoptosis via p53 pathway activation or by suppressing the mTOR pathway [36, 37]. It is reasonable to speculate that AMPK may promote the conservation of the remaining energy via induction of cell cycle arrest under ATP-depleting stresses, and if the stress exceeds a certain threshold, AMPK seems to be able to induce apoptosis. Indeed, several tumor suppressor proteins, including LKB1, TSC2, and p53, are associated with the AMPK signaling network [38]. Therefore, AMPK activation by quercetin may contribute to cell cycle arrest or apoptosis of cancer cells, as asserted in these reports [29–31], but it is noteworthy that such assertions were driven by experiments performed under normoxic conditions. In fact, we also observed that quercetin slightly activated AMPK under normoxic conditions for a short period of time (Fig. 3a). However, our data clearly indicate that quercetin can inhibit AMPK in vitro and in vivo, and the major anti-cancer effect of quercetin is tightly associated with its ability to inhibit hypoxia-activated AMPK. Collectively, quercetin seems to be able to activate AMPK under normoxic conditions, whereas it inhibits AMPK under hypoxic conditions. To explain why opposite effects occur under different oxygenic conditions, we measured the intracellular ATP (Fig. 3e). Quercetin reduced the intracellular ATP level approximately 30 % under normoxic conditions, which may contribute to AMPK activation. Therefore, under normoxic condition, quercetin may exert two opposing effect on AMPK; AMPK activation via ATP reduction and AMPK inhibition via direct binding. Thus, the slight activation of AMPK under normoxic condition may be a net result of these two effects. A recent report suggested that reduction of PP2C expression by quercetin be a factor for AMPK activation [34]. However, at this point, the mechanisms for the differential effect of quercetin on AMPK under different oxygen concentrations remain enigmatic. Further investigation of this issue will enhance our understanding of the anti-cancer properties of quercetin.

HIF-1 has been intensively characterized due to its central role in the adaptation of cancer cells to hypoxia [3, 5]. It is overexpressed in the majority of human cancers and metastases and has been known to contribute to tumor growth as well as drug resistance [2–5]. We previously reported that AMPK activity is critical for HIF-1 activity as well as HIF-1α protein stability, revealing a tight link between energy sensing and oxygen sensing [23, 39]. Here, we demonstrated that quercetin significantly inhibited HIF-1 transcriptional activity without altering the level of mRNA and protein of HIF-1α in several cancer cells (Fig. 5), and we speculate that AMPK inhibition by quercetin significantly contributes to suppression of HIF-1 activity although we cannot rule out the possibilities that other molecules also mediate quercetin's effect on HIF-1.

In conclusion, we demonstrated that quercetin exerts a more successful anti-cancer effect under tumor hypoxia and revealed AMPK to be a molecular target. The current study, together with previous results, suggests that inhibition of AMPK activity is an effective strategy to overcome drug resistance associated with hypoxia.

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