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Quercetin Suppresses Hypoxia-Induced Accumulation of Hypoxia-Inducible Factor-1α (HIF-1α) Through Inhibiting Protein Synthesis

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ABSTRACT

Quercetin, a ubiquitous bioactive plant flavonoid, has been shown to inhibit the proliferation of cancer cells and induce the accumulation of hypoxia-inducible factor-1α (HIF-1α) in normoxia. In this study, under hypoxic conditions (1% O_2), we examined the effect of quercetin on the intracellular level of HIF-1α and extracellular level of vascular endothelial growth factor (VEGF) in a variety of human cancer cell lines. Surprisingly, we observed that quercetin suppressed the HIF-1α accumulation during hypoxia in human prostate cancer LNCaP, colon cancer CX-1, and breast cancer SkBr3 cells. Quercetin treatment also significantly reduced hypoxia-induced secretion of VEGF. Suppression of HIF-1α accumulation during treatment with quercetin in hypoxia was not prevented by treatment with 26S proteasome inhibitor MG132 or PI3K inhibitor LY294002. Interestingly, hypoxia (1% O_2) in the presence of 100 μM quercetin inhibited protein synthesis by 94% during incubation for 8 h. Significant quercetin concentration-dependent inhibition of protein synthesis and suppression of HIF-1α accumulation were observed under hypoxic conditions. Treatment with 100 μM cycloheximide, a protein synthesis inhibitor, replicated the effect of quercetin by inhibiting HIF-1α accumulation during hypoxia. These results suggest that suppression of HIF-1α accumulation during treatment with quercetin under hypoxic conditions is due to inhibition of protein synthesis. J. Cell. Biochem. 105: 546–553, 2008.

KEY WORDS: QUERCETIN; HYPOXIA; HYPOXIA-INDUCIBLE FACTOR-1α; VEGF; PROTEIN SYNTHESIS; pVHL

Solid tumor cells survive and proliferate even in the severe hypoxia and nutrient deprivation resulting from the poor blood supply in a tumor [Dachs et al., 1997]. When the tumor cells are exposed to hypoxic stress, the transcriptional activator hypoxia-inducible factor-1 (HIF-1) increases and plays an important role in promoting cancer angiogenesis and anaerobic metabolism [Maxwell et al., 1997]. Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcriptional factor composed of α and β subunits. HIF-1 is overexpressed in many human cancers [Zhong et al., 1999], and the levels of its activity in cells correlate with tumorigenicity and angiogenesis [Maxwell et al., 1997]. HIF-1α is induced by hypoxia, growth factors, and oncogenes [Fukuda et al., 2002; Traxler et al., 2004]. Under normoxic conditions, the oxygen-dependent degradation domain of HIF-1α interacts with the von Hippel–Lindau protein (VHL), a recognition component of an E3 ubiquitin-protein ligase complex. Such HIF-1α/VHL protein interaction requires oxygen- and iron-dependent hydroxylation of proline residues (Pro402 and Pro564) in HIF-1α protein [Semenza, 2007] and results in the ubiquitination and subsequent degradation of HIF-1α protein via the 26S proteasome [Salceda and Caro, 1997; Kallio et al., 1999]. Under hypoxic conditions, the blockade of prolyl hydroxylation, ubiquitination and degradation lead to the remarkable accumulation and translocation of HIF-1α protein to the nucleus, where it forms an active complex with HIF-1β [Ivan et al., 2001; Jaakkola et al., 2001]. Vascular endothelial growth factor (VEGF) is mainly regulated by HIF-1α at a transcriptional level [Forsythe et al., 1996]. VEGF plays a critical role in tumor angiogenesis [Ferrara and Davis-Smyth, 1997]. Angiogenesis is the formation of new blood

Abbreviations used: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; pVHL, von Hippel–Lindau protein; PHD, proline hydroxylase; PI3-kinase, phosphatidylinositol 3′-kinase; CHX, cyclohexmide; EGF, epidermal growth factor.

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vessels from preexisting ones and is required for tumor growth and metastasis [Folkman, 2002]. Therefore, an antiangiogenic therapy that targets the HIF-1α/VEGF system is a promising strategy for the treatment of cancers.

Flavonoids are present in fruits, vegetables, and beverages derived from plants and in many dietary supplements or herbal remedies [Moon et al., 2006]. Flavonoids have been described as health-promoting, disease-preventing dietary supplements and have activity as cancer-preventive agents [Moon et al., 2006]. Additionally, they are extremely safe and associated with low toxicity, making them excellent candidates for chemopreventive agents. Quercetin (3,5,7,3′,4′-pentahydroxyflavone), which is orally bioavailable, is a flavonoid, and its metabolites are potent antioxidants, having anti-tumor, anti-inflammatory, anti-allergic, and anti-viral activities [Middleton and Kandaswami, 1993; Kandaswami and Middleton, 1994; Wang, 2000; Vulcain et al., 2005]. Previous studies have shown that quercetin stabilizes HIF-1α protein and induces VEGF in endothelial cells in normoxic conditions [Wilson and Poellinger, 2002]. Quercetin-induced accumulation of HIF-1α/2α is mediated through chelation of iron [Park et al., 2008]. Quercetin inhibits the ubiquitination of HIF-1α/2α in normoxia by hindering proline hydroxylase (PHD) which requires cellular iron ions as cofactors; this may be a drawback for using quercetin as a cancer prevention agent, because it could promote tumor growth through increasing angiogenesis. Thus, in this study, we examined whether quercetin can affect HIF-1α accumulation and VEGF expression differently under hypoxic conditions. We observed that quercetin inhibits HIF-1α accumulation under hypoxic conditions in human prostate cancer LNCaP, colon cancer CX-1, and breast cancer SkBr3 cell lines. Our studies reveal that inhibition of HIF-1α accumulation is due to inhibition of protein synthesis by treatment with quercetin under hypoxic conditions.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Quercetin (>99% pure) was obtained from Sigma Chemical Co. (St. Louis, MO). Proteasome inhibitor MG-132 was obtained from Calbiochem (San Diego, CA). Monoclonal antibodies were purchased from the following companies: anti-ubiquitin from Santa Cruz (Santa Cruz, CA), anti-HIF-1α from BD Biosciences (San Jose, CA), anti-pVHL from PharMingen (San Diego, CA) and anti-actin antibody from ICN (Costa Mesa, CA). Anti-phospho-Ser^{473}-Akt and anti-Akt antibodies were from Cell Signaling (Beverly, MA).

CELL CULTURE AND HYPOXIA TREATMENT

Human prostate cancer LNCaP, colon cancer CX-1, and breast cancer SkBr3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). LNCaP and CX-1 or SkBr3 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) or DMEM medium (Gibco BRL, Gaithersburg, MD), respectively, with 10% fetal bovine serum (Hyclone, Logan, UT) and 26 μM sodium bicarbonate for monolayer cell culture. Cells were cultured at 37°C humidified atmosphere and 5% CO_2 in air. Petri dishes/flasks containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. Before the experiments, cells were grown to ~80% confluence in 60 or 100 mm tissue culture dishes. For hypoxia treatment, Petri dishes containing cells were incubated in a hypoxic chamber (Forma Scientific, Marietta, OH) with a 94:5:1 mixture of $N_2/CO_2/O_2$. It is difficult to accurately measure oxygen concentration in unstirred systems above monolayers. Thus, the culture mediums were replaced with deoxygenated mediums before hypoxia treatment as reported previously [Kwon et al., 2005]. Deoxygenated mediums were prepared prior to each experiment by equilibrating the medium with a hypoxic gas mixture containing 5% CO_2, 94% N_2, and 1% O_2 at 37°C.

DRUG TREATMENTS

Exponentially growing cells (70–80% confluence) in complete medium were co-treated with different concentrations of quercetin, followed by continual incubation in normal culturing conditions or exposure to hypoxia (1% O_2) for indicated time intervals according to the purpose of the experiment. To investigate the role of protein synthesis in the inhibition of hypoxia induced HIF-1α protein accumulation in the presence of quecetin, LNCaP cells were exposed to hypoxia for 8 h followed by treatment with 100 μM of cycloheximide to inhibit further protein synthesis in the presence or absence of 100 μM of quercetin for different time periods or various amounts of quercetin for 8 h. To study the effects of quercetin on the half-life or degradation of hypoxia induced HIF-1α protein accumulation, cells were pretreated with 10 μM of MG132 for 30 min and cultured in the presence of different concentrations of quercetin for 4 or 8 h under normoxic conditions or hypoxic conditions. HIF-1α protein levels were determined by Western blot analysis.

DETERMINATION OF CELL VIABILITY

One or 2 days prior to the experiment, cells were plated into 60-mm dishes at a density of 1×10^5 cells/plate in 5 ml tissue culture medium in triplicate. For trypan blue exclusion assay, trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min, and examined under a light microscope. At least 300 cells were counted for each survival determination.

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF VEGF

LNCaP cells were plated in a 60 mm plate at a density of 1×10^5 cells/ml in RPMI medium and incubated overnight before the cells were subjected to treatment. After treatment, the cell culture media were removed for storage at −80°C. Levels of VEGF protein in the medium were determined by ELISA using a commercial kit (R&D Systems, Minneapolis, MN). Briefly, 200 μl of standard or cell culture supernatant were added to the wells of a microplate which was pre-coated with a monoclonal antibody specific for VEGF and incubated for 2 h at room temperature. After washing away any unbounded substances, an enzyme-linked polyclonal antibody against VEGF conjugated to horseradish peroxidase was added to

the wells and incubated for 2 h at room temperature. Following a wash, 200 μl of substrate solution was added to the wells and incubated for 30 min, and then 50 μl of stop solution was added to stop color development. The optical density of each well was determined using a microplate reader at 450 nm. The experiments were repeated three times with two replications of each experiment. Mean values from these samples were analyzed.

PROTEIN SYNTHESIS

The effect of quercetin on protein synthesis during normoxia or hypoxia was investigated by plating LNCaP cells (1×10^5) into 60 mm culture plates. The protein synthesis experimental procedure was as reported previously [Kwon et al., 2005]. In brief, after the cells were grown to ~80% confluence, the medium was replaced with complete medium containing 8 μCi/ml Tran ^{35}S-label, which contains mixtures of [^{35}S]L-methionine, [^{35}S]L-cysteine, [^{35}S]L-methionine sulfone, and [^{35}S]L-cysteic acid (MP Biomedicals, Irvine, CA). After the plates were incubated at 37°C for a period of time, the cells were washed thrice with cold PBS for 3 min and twice with cold 10% trichloroacetic acid for 5 min. Trichloroacetic acid-insoluble proteins were solubilized with 5 ml of 0.25 N NaOH; then, the solubilized sample (0.25 ml) was added to Packard Scint-A (10 ml). Radioactivity was determined with a Beckman LS-7500 scintillation counter. The protein content was estimated by the Lowry method [1951].

IMMUNOBLOT ANALYSIS

Cells were lysed with 1 × Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M sodium dodecyl sulfate (SDS), 0.3 mM bromophenol blue) and boiled for 7 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with 1 × lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–15% SDS–polyacrylamide gels. SDS–PAGE analysis was performed according to Laemmli [1970] using a Hoefer gel apparatus. Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS-Tween-20 (0.1%, v/v) for 1 h. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) at 4°C overnight. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL). To ensure equal protein loading, each membrane was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading.

STATISTICAL ANALYSIS

Statistical significance was examined using Student's *t*-test. The two-sample *t*-test was used for two-group comparisons. Values were reported as mean ± SD. $P < 0.05$ was considered significant and indicated by asterisks in the figures.

RESULTS

QUERCETIN INHIBITS THE ACCUMULATION OF HIF-1α IN HYPOXIC CONDITIONS

We previously observed that quercetin increases the intracellular level of HIF-1α by chelating iron in normoxia [Park et al., 2008]. In the present study, we investigated whether quercetin promotes hypoxia-induced accumulation of HIF-1α in LNCaP cells. HIF-1α accumulated during hypoxia or treatment with quercetin in normoxia (Fig. 1A,B). Surprisingly, we observed that reduction of HIF-1α accumulation occurred during treatment with quercetin in

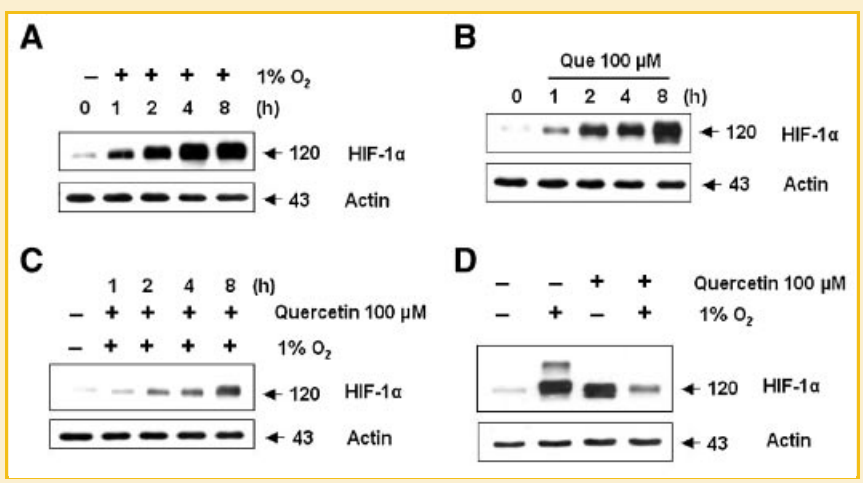

Fig. 1. Effect of hypoxia and quercetin on intracellular level of HIF-1α in LNCaP cells. Cells were exposed to 1% O_2 for various times (1–8 h) and/or treated with 100 μM quercetin (Que) for various times (1–8 h). A: Kinetics of HIF-1α accumulation in hypoxic conditions. B: Kinetics of HIF-1α accumulation during treatment with quercetin in normoxic conditions. C: Kinetics of HIF-1α accumulation during treatment with quercetin in hypoxic conditions. D: Cells were exposed to hypoxia alone, treated with quercetin alone, and hypoxia in combination with for 8 h then harvested. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-HIF-1α antibody. Actin was used as a loading control.

hypoxic conditions (Fig. 1C,D). The reduction of HIF-1α accumulation was dependent upon quercetin concentrations, but not treatment time, during hypoxia (Fig. 2). To examine whether our observations could be generalized, we tested with three different cell lines, LNCaP, SkBr3 and CX-1. Figure 3 shows that HIF-1α accumulation was suppressed by treatment with quercetin in hypoxic conditions in all three cell lines.

QUERCETIN INHIBITS PRODUCTION OF VEGF

It is well known that HIF-1α up-regulates the expression of VEGF [Towler, 2007]. We examined whether reduction of HIF-1α accumulation during treatment with quercetin in hypoxic conditions results in decrease in the extracellular level of VEGF. Figure 4A shows an increase in VEGF in time dependent manner (up to 16 h) during hypoxia in LNCaP cells. However, VEGF induction was decreased by treatment with quercetin during hypoxic conditions (Fig. 4B).

INHIBITION OF HIF-1α ACCUMULATION BY QUERCETIN DURING HYPOXIA IS PI3K–AKT SIGNALING PATHWAY-INDEPENDENT

Gort et al. [2006] reported that the oncogenic phosphatidylinositol 3′-kinase (PI3-kinase)/Akt signaling pathway regulates the expression of HIF-1α. To examine whether the inhibition of HIF-1α accumulation by treatment with quercetin during hypoxia occurs by inhibiting the PI3K signaling pathway, cells were treated with PI3K inhibitor LY294002 prior to hypoxia alone, quercetin alone, or combined hypoxia and quercetin treatment. Figure 5 shows that LY294002 caused dephosphorylation (inactivation) of Akt regardless of oxygen tensions. Interestingly, LY294002 inhibited accumulation of HIF-1α by quercetin, but not that by hypoxia. These results suggest that quercetin-induced inhibition of HIF-1α accumulation during hypoxia is not due to dephosphorylation of Akt.

QUERCETIN INHIBITS HYPOXIA-INDUCED ACCUMULATION OF HIF-1α IN A PROTEASOMAL-INDEPENDENT MANNER

Under normoxic conditions, HIF-1α protein is hydroxylated at Pro-402 and Pro-564 residues and is degraded rapidly and continuously by ubiquitination and the proteasomal system [Lisztwan et al., 1999; Ohh et al., 2000]. In contrast, under hypoxic conditions, HIF-1α protein is stabilized and escapes proteasomal degradation. To investigate whether inhibition of HIF-1α accumulation by quercetin under hypoxic conditions is mediated by promoting the activation of the proteasomal system, we performed an experiment in the presence of the proteasomal inhibitor MG-132. As shown in Figure 6, MG-132 induced accumulation of HIF-1α protein in normoxic conditions. MG-132 did not affect hypoxia-induced accumulation of HIF-1α. Interestingly, MG-132 did not affect the inhibition of HIF-1α accumulation by quercetin under hypoxic conditions. These results suggest that quercetin-induced inhibition of HIF-1α accumulation during hypoxia is not mediated through promoting HIF-1α degradation.

QUERCETIN DOES NOT AFFECT CELL VIABILITY UNDER HYPOXIC CONDITIONS

We examined whether quercetin induces cytotoxicity under hypoxic conditions and whether quercetin-induced cytotoxicity is responsible for suppression of HIF-1α accumulation. The effects of quercetin on cell viability and drug-induced PARP-1 cleavage, the hallmark feature of apoptosis, were determined by trypan blue exclusion dye assay and western blotting, respectively (Fig. 7). When LNCaP cell were treated with various concentrations of

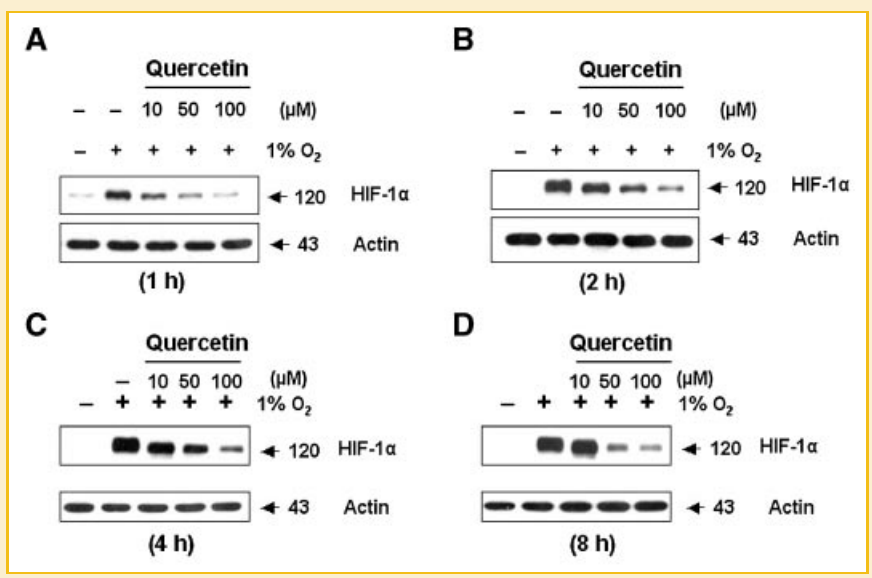

Fig. 2. Quercetin inhibits hypoxia-induced HIF-1α accumulation in LNCaP cells. Cells were exposed to 1% O_2 in combination with various concentrations (10–100 μM) of quercetin for (A) 1 h, (B) 2 h, (C) 4 h, or (D) 8 h. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-HIF-1α antibody. Actin was used as a loading control.

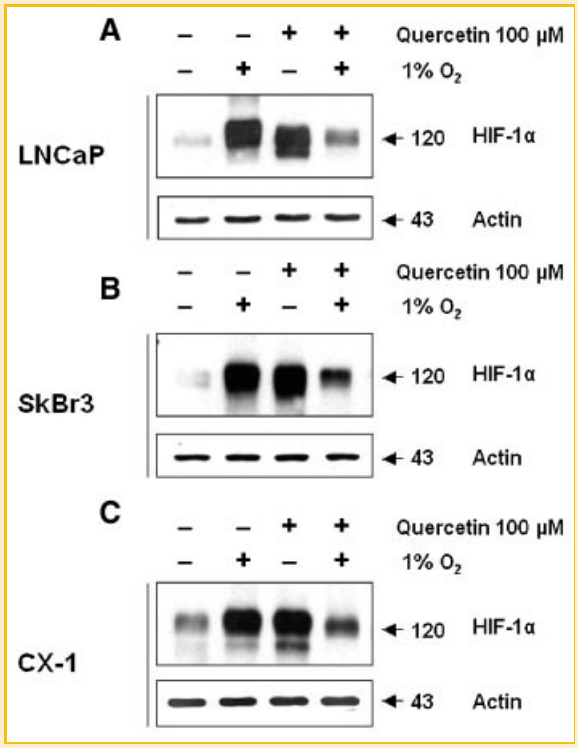

Fig. 3. Effect of quercetin on HIF-1α accumulation during hypoxia in various cancer cell lines. LNCaP, SkBr3, and CX-1 cells were exposed to 1% O_2 alone, treated with 100 μM quercetin alone, or 100 μM quercetin in combination with 1% O_2 for 8 h. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-HIF-1α antibody. Actin was used as a loading control.

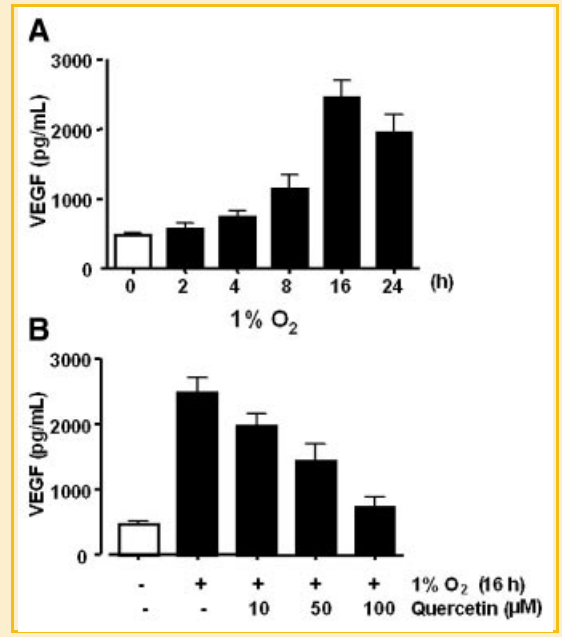

Fig. 4. Production of VEGF during hypoxia was decreased by quercetin. A: LNCaP cells were exposed to 1% O_2 for various times (2–24 h). B: LNCaP cells were treated with various concentrations of quercetin (10–100 μM) for 16 h during hypoxia. The concentration of VEGF protein in the culture media was determined by ELISA. The assays were performed with triplicate experimental samples. The results represent the mean values of VEGF concentrations and error bars represent standard error of the mean from triplicate samples.

quercetin (10–100 μM) for 8 h in hypoxia, no significant concentration-dependent reduction of the viability was observed (Fig. 7A). In addition, PARP cleavage and caspase activation were not observed during treatment with 100 μM quercetin under hypoxia for 8 h (Fig. 7B).

QUERCETIN EFFECTIVELY INHIBITS PROTEIN SYNTHESIS UNDER HYPOXIC CONDITIONS AND INHIBITION OF PROTEIN SYNTHESIS IS RESPONSIBLE FOR INHIBITION OF HIF-1α ACCUMULATION

Our data clearly demonstrate that quercetin induces HIF-1α accumulation under normoxia and inhibits accumulation of HIF-1α under hypoxia. A fundamental question is how quercetin has such contradictory effects. One possibility is that quercetin inhibits the translational process. To investigate this possibility, protein synthesis was measured when cells were treated with quercetin at various times during normoxia/hypoxia. As shown in Figure 8A, protein synthesis was inhibited by 48% during incubation for 8 h under hypoxic conditions. Quercetin inhibited protein synthesis by 71% or 94% during incubation for 8 h under normoxia or hypoxia, respectively. Significantly increased quercetin concentration-dependent inhibition of protein synthesis was observed under hypoxic conditions (Fig. 8B). To confirm whether increased inhibition of protein synthesis is responsible for inhibition of HIF-1α accumulation during hypoxia, cells were

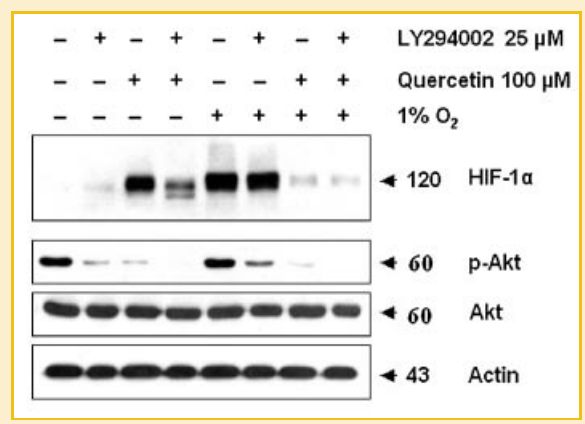

Fig. 5. Role of Akt in the accumulation of HIF-1α in LNCaP cells. Cells were exposed to 1% O_2, treated with 100 μM quercetin, or treated with 100 μM quercetin in combination with 1% O_2 in the presence or absence of 25 μM LY294002 for 8 h. For treatment with LY294002, a PI3K inhibitor, cells were pretreated with LY294002 (25 μM) for 30 min followed by treatment with quercetin and/or hypoxia for 8 h in the presence of LY294002. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-HIF-1α, anti-phospho-Akt (S473), or anti-Akt antibody. Actin was used as a loading control.

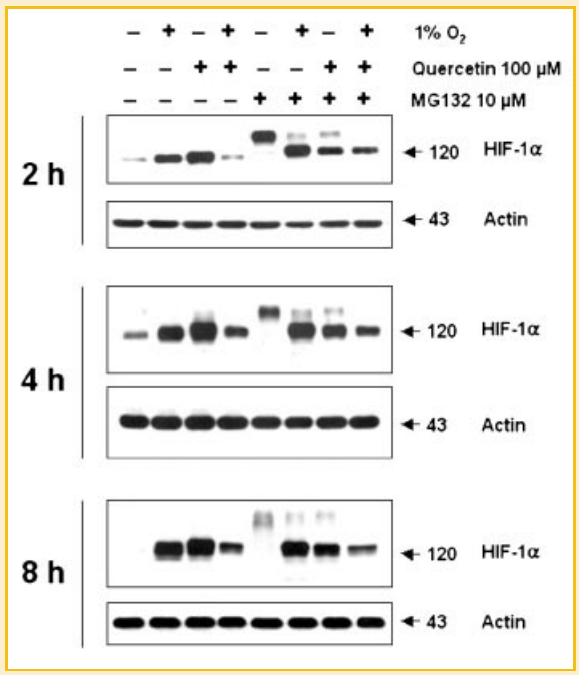

Fig. 6. Effect of MG132 on the alteration of intracellular level of HIF-1α by hypoxia, quercetin, or quercetin in combination with hypoxia in LNCaP cells. Cells were exposed to 1% O_2, treated with 100 μM quercetin, or treated with 100 μM quercetin in combination with 1% O_2 for various times (2–8 h) in the presence or absence of 10 μM MG132. Equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-HIF-1α antibody. Actin was shown as an internal standard.

exposed to hypoxia for 8 h and subsequently treated with 100 μM cyclohexmide (CHX), a protein synthesis inhibitor, under hypoxic conditions (Fig. 9A). We previously reported that protein synthesis is inhibited within 10 min by 90% or 99% with 10 or 100 μM CHX, respectively, in normoxia [Lee and Dewey, 1986; data not shown]. Figure 9A shows that hypoxia-induced accumulated HIF-1α level was rapidly reduced by treatment with CHX. In addition, combined CHX and quercetin, in comparison to CHX alone or quercetin alone, more effectively decreased the intracellular level of HIF-1α even under hypoxic conditions (Fig. 9B,C). Our data also show that treatment with quercetin alone for 15 min after hypoxia for 8 h did not significantly reduce the intracellular HIF-1α. This is probably because inhibition of protein synthesis by quercetin is not as effective as that by CHX. Our data suggest that inhibition of protein synthesis is mainly responsible for suppression of HIF-1α accumulation by quercetin under hypoxic conditions.

DISCUSSION

In this study, we observe that quercetin inhibits hypoxia-induced HIF-α accumulation in a dose-dependent manner in several human cancer cell lines. Inhibition of HIF-α accumulation by quercetin is mediated through inhibition of protein synthesis under hypoxic conditions.

The intracellular level of HIF-α is regulated by protein translation and proteasome-dependent degradation. In general, up-regulation of HIF-α in tumor cells is mediated by enhanced HIF-α protein synthesis via a variety of growth factors such as epidermal growth factor (EGF), whereas hypoxia-induced HIF-α accumulation occurs because of the shutdown of the proteasomal degradation system in the absence of oxygen [Tanimoto et al., 2000; Phillips et al., 2005; Liu et al., 2006; Peng et al., 2006; Tanaka et al., 2006]. In normoxia, epidermal growth factor receptor (EGFR) signaling activates the PI3K-Akt pathway, subsequently increasing the level of HIF-1α [Peng et al., 2006; Tanaka et al., 2006]. Reactive oxygen species (ROS) regulate EGF-induced HIF-1α expression through activation of Akt [Liu et al., 2006]. Although Tanaka et al. [2006]

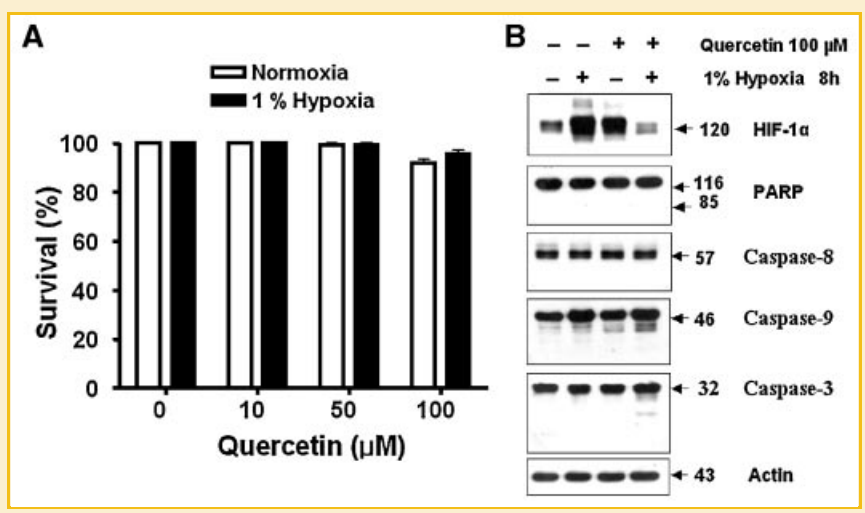

Fig. 7. Effect of quercetin on the cell viability of LNCaP cells. Cells were treated with quercetin for 8 h under normoxia or hypoxia. A: Cell viability was determined by trypan blue dye exclusion assay. Error bars represent the mean ± SE from three separate experiments. B: Cells were harvested and samples were prepared for analysis of the intracellular level of HIF-1α, cleavage of PARP, caspase-8, caspase-9, and caspase-3. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted. Actin was used to confirm the equal amount of proteins loaded in each lane.

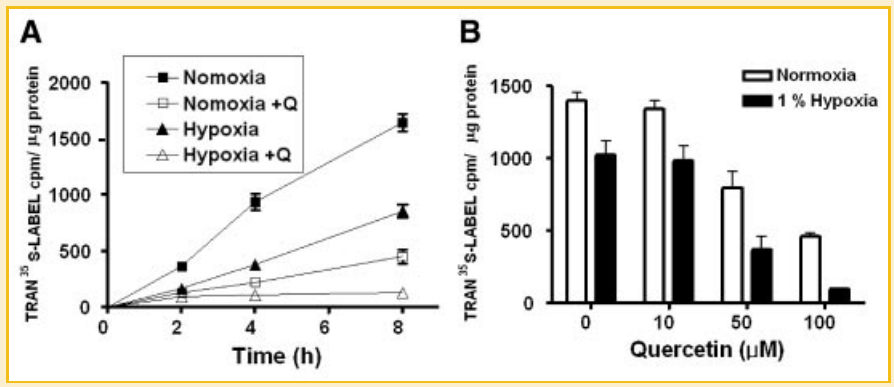

Fig. 8. Effect of quercetin on the incorporation of Tran ^{35}S-label into protein during normoxia or hypoxia in LNCaP cells. A: Cells were incubated in complete medium containing 8 µCi/ml Tran ^{35}S-label for various times (2–8 h) during hypoxia (1% O_2) or normoxia in the presence or absence of 100 µM quercetin. B: Cells were incubated in complete medium containing 8 µCi/ml Tran ^{35}S-label for 8 h during hypoxia (1% O_2) or normoxia in the presence or absence of various concentrations of quercetin (10–100 µM). Error bars represent the mean ± SE from triplicate samples.

reported that HIF-1α is markedly down-regulated after treatment with a PI3K inhibitor, LY294002, our data clearly reveal that HIF-1α can accumulate in the presence of quercetin which can dephosphorylate (inhibit) Akt or treatment with LY294002 under hypoxic conditions (Fig. 5). These results suggest that inhibition of HIF-1α accumulation during treatment with quercetin and hypoxia is not related to inhibition of Akt activity.

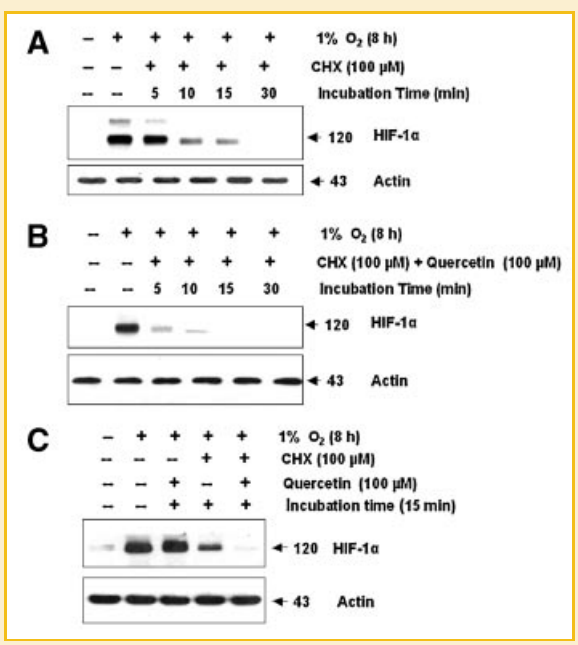

Fig. 9. Effect of cycloheximide/quercetin on hypoxia-induced accumulated HIF-1α in LNCaP cells. A: Cells were exposed to 1% O_2 for 8 h and then treated with 100 µM cycloheximide (CHX) for various times (5–30 min). B: Cells were exposed to 1% O_2 for 8 h and then treated with combined 100 µM cycloheximide (CHX) and 100 µM quercetin for various times (5–30 min). C: Cells were exposed to 1% O_2 for 8 h and then treated for 15 min with 100 µM CHX, 100 µM quercetin, or 100 µM CHX in combination with 100 µM quercetin. Cell lysates containing equal amounts of protein (20 µg) were separated by SDS–PAGE and immunoblotted with anti-HIF-1α antibody. Actin was used as a loading control.

In this study, we observed that quercetin inhibits protein synthesis by 71% during incubation for 8 h under normoxia (Fig. 8). These results were consistent with previous observations [Ito et al., 1999]. Quercetin-induced protein synthesis inhibition is associated with phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) [Ito et al., 1999]. These reports seem to contradict the observations which reveal an accumulation of HIF-1α during treatment with quercetin under normoxic conditions. However, these are not contradictory observations. Inhibition/promotion of HIF-1α accumulation is probably due to shifting the balance between HIF-1α translation and degradation. Although quercetin inhibits protein synthesis, it also causes iron chelation to inhibit HIF-1α degradation by chelating iron [Leopoldini et al., 2006; Park et al., 2008]. In this case, inhibition of degradation overrides inhibition of translation and leads to the accumulation of HIF-1α. However, inhibition of HIF-α translation overrides inhibition of HIF-1α degradation when cells are treated with quercetin under hypoxic conditions. Consistent with our observations, ARC-111 and topotecan have been shown to inhibit hypoxia-mediated HIF-1α accumulation by decreasing HIF-1α translation [Rapisarda et al., 2004; Meng et al., 2007]. In contrast, geldanamycin decreases hypoxia-induced HIF-α accumulation via the enhancement of HIF-α degradation through the proteasome pathway [Mabjeesh et al., 2002].

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