0026-895X/06/7005-1664–1671$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 70:1664–1671, 2006

Vol. 70, No. 5
25817/3144895
Printed in U.S.A.

Curcumin Inhibits Hypoxia-Inducible Factor-1 by Degrading Aryl Hydrocarbon Receptor Nuclear Translocator: A Mechanism of Tumor Growth Inhibition

Hyunsung Choi, Yang-Sook Chun, Seung-Won Kim, Myung-Suk Kim, and Jong-Wan Park

Department of Pharmacology (H.C., M.-S.K., J.-W.P.), Department of Physiology and Cancer Research Institute (Y.-S.C.), and Human Genome Research Institute (S.-W.K.), Seoul National University College of Medicine, Seoul, Korea

Received April 16, 2006; accepted July 31, 2006

ABSTRACT

Hypoxia-inducible factor-1 (HIF-1), a transcription factor composed of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), plays a key role in cell survival and angiogenesis in hypoxic tumors, and many efforts have been made to develop anticancer agents that target HIF-1α. However, although ARNT is also required for HIF-1 activity, ARNT has been disregarded as a therapeutic target. Curcumin is a commonly used spice and coloring agent with a variety of beneficial biological effects, which include tumor inhibition. In the present study, we tested the possibility that curcumin inhibits tumor growth by targeting HIF-1. The effects of curcumin on HIF-1 activity and expression were examined in cancer cell lines and in xenografted tumors. We found that curcumin inhibits HIF-1 activity and that this in turn down-regulates genes targeted by HIF-1. Moreover, of the two HIF-1 subunits, only ARNT was found to be destabilized by curcumin in several cancer cell types, and furthermore, ARNT expression rescued HIF-1 repression by curcumin. We also found that curcumin stimulated the proteasomal degradation of ARNT via oxidation and ubiquitination processes. In mice bearing Hep3B hepatoma, curcumin retarded tumor growth and suppressed ARNT, erythropoietin, and vascular endothelial growth factor in tumors. These results suggest that the anticancer activity of curcumin is attributable to HIF-1 inactivation by ARNT degradation.

Hypoxia commonly develops in solid tumors because the rate of tumor cell proliferation outpaces the rate of vessel formation. To survive under hypoxic conditions, tumor cells run numerous adaptive mechanisms, such as glycolysis, glucose uptake, and survival factor up-regulation (Hockel and Vaupel, 2001). Hypoxic adaptation involves gene induction via hypoxia-inducible factor-1 (HIF-1), which up-regulates ~60 genes by binding to 5′-RCGTG-3′ sequences in hypoxia response elements (Semenza, 2002). HIF-1 is a heterodimer composed of two basic-helix-loop-helix (bHLH) proteins of the PAS family, namely, HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT). Of these, HIF-1α is a primary transcription factor, which is tightly regulated by oxygen (Huang et al., 1998) and transactivates hypoxia-inducible genes (Jiang et al., 1997). Indeed, HIF-1α was found to be present at high levels in human tumor specimens, and its levels were found to be positively related to tumor progression, metastasis, and resistance to chemo/radiotherapy (Zhong et al., 1999; Birner et al., 2000). In addition, the effects of HIF-1α on tumor growth and angiogenesis have been demonstrated in xenograft tumor models (Ryan et al., 2000). Therefore, attention has been focused on HIF-1α alone, and as a result, several HIF-1α inhibitors are being developed for cancer chemotherapy (Giaccia et al., 2003; Yeo et al., 2004). In comparison, ARNT has been disregarded in the context of cancer therapy because it is regarded as a constitutively expressed partner of HIF-1α (Chilov et al., 1999), and thus, little effort has been made to uncover the identities of ARNT inhibitors.

ARNT belongs to class II of the bHLH PAS family and acts in partnership with several class I bHLH PAS family members, such as HIF-1α, HIF-2α, aryl hydrocarbon receptor, single-minded protein 1/2, and cardiovascular helix-loop-he-

This work was supported by research funds of Seoul National University College of Medicine (21-2005-014) and Seoul National University Cancer Research Institute (2005).

H.C. and Y.-S.C. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.025817.

ABBREVIATIONS: HIF, hypoxia-inducible factor; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix; PAS, Per-ARNT-Sim; EPO, erythropoietin; VEGF, vascular endothelial growth factor; RT-PCR, reverse-transcription polymerase chain reaction; AMC, 7-amino-4-methylcoumarin; TTBS, Tris-buffered saline/Tween 20; DMSO, dimethyl sulfoxide; NAC, *N*-acetyl cysteine; BSO, buthionine sulfoximine; β-gal, β-galactoside; HA, hemagglutinin; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; Z-VAD-FMK, Z-Val-Ala-Asp(OMe)-fluoromethyl ketone.

lix factor 1 (Gu et al., 2000). Although ARNT does not function as a main transcription factor, ARNT functions as a central axis in the gene expression networks of many essential physiological processes (e.g., in response to hypoxia and xenobiotics, and in neural/cardiovascular development) (Gu et al., 2000). In addition to its role as a partner, it has also been suggested to have a role as a primary transcription factor. We recently found that ARNT in concert with HIF-1α^{417} can play a key role in gene transcription via its C-terminal transactivation domain (Lee et al., 2004). In addition, ARNT-ARNT homodimer has been reported to have transcriptional activity (Sogawa et al., 1995). Therefore, the regulation of ARNT expression or activity may have a significant impact on cellular metabolisms, which provides a rationale for determining the identities of drugs that target ARNT.

Curcumin (diferuloylmethane) is a major component of the yellow spice turmeric derived from the rhizomes of *Curcuma longa* and is a commonly used flavoring and coloring agent. Curcumin has also been reported to show potential in terms of the prevention and treatment of cancer (Rao et al., 1995; Kawamori et al., 1999). In clinical pilot studies, curcumin has been associated with the regression of premalignant lesions (Cheng et al., 2001). However, its anticarcinogenic and anticancer actions are complicated (Aggarwal et al., 2003). Curcumin may block both tumor initiation and tumor promotion by up-regulating glutathione transferases (Piper et al., 1998), by inhibiting cytochrome P450 enzymes (Thapliyal and Maru, 2001), by reducing oxidative stress (Ruby et al., 1995) and inflammation (Plummer et al., 1999), by inhibiting tumor growth by inactivating pathways signaling oncogenes and growth factors (Han et al., 1999), by inducing cell cycle arrest and apoptosis (Moragoda et al., 2001), or by inhibiting angiogenesis (Arbiser et al., 1998). However, the mechanisms responsible for its anticancer effect remain obscure.

In the present study, we examined the possibility that curcumin inhibits tumor growth by targeting HIF-1. Curcumin was found to inactivate HIF-1 by degrading ARNT and to down-regulate HIF-1-targeted genes. In xenografted hepatomas, curcumin also reduced the tissue levels of ARNT, erythropoietin, and vascular endothelial growth factor, and it retarded tumor growth. This is the first report to demonstrate that curcumin is a potential anti-HIF, anticancer agent.

Materials and Methods

Materials. Ammonium chloride, buthionine sulfoximine (BSO), curcumin, cycloheximide, hydrogen peroxide, *N*-acetyl cysteine (NAC), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Bafilomycin A1, MG132, and Z-VAD-FMK were purchased from Alexis Biochemicals (Lausen, Switzerland), and [α-^{32}P]CTP (500 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). Culture media and fetal calf serum were purchased from Invitrogen (Carlsbad, CA).

Cell Culture. Hep3B hepatoma, MKN28 gastric carcinoma, HT29 colon carcinoma, MCF7 mammary carcinoma, Caki-1 renal carcinoma, SiHa cervical carcinoma, PC3 prostate carcinoma, and H596 non–small-cell lung carcinoma cells were cultured in α-modified Eagle's medium, Dulbecco's modified Eagle's medium, or RPMI 1640 medium. All culture media were supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were grown in a humidified 5% CO_2 atmosphere at 37°C in an incubator in which oxygen tension was held at either 140 mm Hg [20% O_2 (v/v), normoxic conditions] or 7 mm Hg [1% O_2 (v/v), hypoxic conditions].

Xenografts of Hepatoma. Male nude (BALB/cAnNCrj-*nu*/*nu*) mice were purchased from Charles River Japan Inc. (Shin-Yokohama, Japan). Animals were housed in a specific pathogen-free room under controlled temperature and humidity, and all animal procedures were performed in accord with the procedures described in the Seoul National University Laboratory Animal Maintenance Manual. Mice were injected subcutaneously in the flank with 5×10^6 viable Hep3B cells. When Hep3B tumors measured 400 to 500 mm^3, the mice were randomly assigned to one of two groups. The first group ($n = 6$) were treated with DMSO vehicle, and the second group ($n = 6$) was intraperitoneally injected with curcumin (120 mg/kg once a day for 5 days). Tumors were measured daily using a caliper in two dimensions, and tumor volumes were calculated using the formula Volume = $a \times b^2/2$, where a is the width at the widest point of the tumor, and b is the maximal width perpendicular to a. Results were plotted as average tumor volume versus time.

Reporter Assays. Luciferase reporter genes containing the EPO enhancer region or the Gal4-binding motif and ARNT and Gal4-HIF-1α/CAD (amino acids 776–826) expression plasmids were generously donated by Dr. Eric Huang (National Cancer Institute, Bethesda, MD). Hep3B cells were cotransfected with 0.5 μg of each reporter gene and with cytomegalovirus-β-gal and/or ARNT or Gal4-CAD plasmids (Yeo et al., 2006) using the calcium phosphate method. pcDNA was added to ensure that final DNA concentrations in the control and experimental groups were at similar levels. After then allowing cells to stabilize for 48 h, they were incubated under either normoxic or hypoxic conditions in the absence or presence of curcumin for 16 h. They were then lysed to determine luciferase and β-gal activities. Luciferase activities were analyzed using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany), and β-gal assays were performed to normalize transfection efficiencies.

Immunoblotting and Immunoprecipitation. For immunoblotting, total proteins were separated on 6.5 or 10% SDS/polyacrylamide gel, and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were then blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS) at room temperature for 1 h and incubated overnight at 4°C with anti-HIF-1α (Chun et al., 2001), anti-ARNT (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HA (BIODESIGN International, Saco, ME), or anti-β-actin (Santa Cruz Biotechnology) diluted 1:5000 in 5% nonfat milk in TTBS. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antiserum was used as a secondary antibody (1:5000 dilution in 5% nonfat milk in TTBS, 1-h incubation) and antigen-antibody complexes were visualized using an Enhanced Chemiluminescence Plus kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). For immunoprecipitation, cell lysates were incubated with anti-HIF-1α or anti-ARNT antibody and then with protein A/G-Sepharose beads (GE Healthcare). After washing, immunocomplexes were eluted using SDS sample buffer containing 10 mM dithiothreitol and then were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using anti-ARNT, anti-HIF-1α, or anti-HA antibody.

Semiquantitative RT-PCR for ARNT and HIF-1-Induced mRNAs. To quantify mRNA levels, we used a highly sensitive, semiquantitative RT-PCR, as described previously (Chun et al., 2001). Total RNAs were isolated from cultured cells using TRIzol (Invitrogen). After verifying their qualities on a 1% denaturing agarose gel, 1 μg of the total RNAs was reverse-transcribed at 48°C for 1 h, and the cDNAs so obtained were amplified over 18 polymerase chain reaction cycles (94°C for 30 s, 53°C for 30 s, and 68°C for 30 s) in a 20-μl reaction mixture containing 5 μCi [α-^{32}P]dCTP and 250 nM concentration of each primer set. Polymerase chain reaction products (5 μl) were electrophoresed in a 4% polyacrylamide gel, and dried gels were autoradiographed. Primers for human ARNT, EPO, VEGF, phosphoglycerate kinase 1, enolase 1, aldolase A, and β-actin

were constructed as described previously (Chun et al., 2001; Lee et al., 2004).

Proteasome Activity Assay. Proteasome activity was analyzed using a proteasome substrate, LLVY, conjugated with the fluorophore 7-amino-4-methylcoumarin (AMC), provided by Chemicon Inc. (Temecula, CA). Frozen MKN28 cells were quickly thawed and vigorously vortexed in a lysis/assay buffer containing 25 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.1% Nonidet P-40, and 0.002% SDS. The cell lysate (10 μl) was incubated with 50 μM LLVY-AMC, and the free AMC released by the proteasome was quantified using a 380/460 nm filter set in a fluorometer (Cytofluor 2350 plate reader; Millipore).

Statistical Analysis. All data were analyzed using Microsoft Excel 2000 (Microsoft, Redmond, WA), and results are expressed as means and standard deviations. The unpaired Student's *t* test (SPSS 10.0 for Windows; SPSS Inc., Chicago, IL) was used to compare data between the control and curcumin-treated groups. Differences were considered significant for P values of <0.05, and all statistical tests were two-sided.

Results

Curcumin Blunts the Hypoxic Expression of HIF-1-Dependent Genes. We first examined the effect of curcumin on cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assays. The viabilities and shapes of Hep3B cells were not altered 16 h after 1 to 20 μM curcumin treatment (data not shown). Thus, we applied curcumin with this range in all experiments. EPO enhancer reporter activities were markedly enhanced in hypoxic cells versus those in normoxic cells, and curcumin significantly inhibited this hypoxic activation at concentrations as low as 2 μM in a dose-dependent manner (Fig. 1a). Moreover, curcumin suppressed the hypoxic expression of HIF-1 target genes, such as EPO, VEGF, aldolase, enolase, and phosphoglycerate kinase (Fig. 1b). These results suggest that curcumin reduces the expressions of hypoxia-induced genes by inhibiting HIF-1 activity.

HIF-1 Inactivation by Curcumin Is Due to ARNT Down-Regulation. HIF-1 can be inactivated in two ways: 1) by repressing its transcriptional activity; and 2) by reducing the availabilities of its subunits, HIF-1α and/or ARNT. To examine the effect of curcumin on the transcriptional activity of HIF-1, we coexpressed the DNA binding domain of Gal4 fused with the C-terminal transactivation domain of HIF-1α and the Gal4 reporter. Reporter activities were found to be greatly enhanced by hypoxia but were wholly unaffected by curcumin (Fig. 2a), indicating that curcumin does not affect the transcriptional activity of HIF-1α. Next, we checked the effects of curcumin on HIF-1α and ARNT levels. Curcumin reduced ARNT levels in both normoxic and hypoxic cells dose-dependently (Fig. 2b) and time-dependently (Fig. 2c), whereas it did not affect HIF-1α expression. To examine whether ARNT down-regulation causes curcumin-induced HIF-1 inhibition, cells were transfected with HIF-1α or ARNT. Expression of HIF-1α alone failed to recover EPO reporter activity loss by curcumin. However, ARNT up-regulation effectively recovered EPO reporter activity in a gene dose-dependent manner (Fig. 2d). These results suggest that ARNT down-regulation by curcumin is responsible for the suppression of HIF-1-dependent gene induction under hypoxia. To examine the effects of curcumin on ARNT expression in other cancer cell types, seven cancer cell lines were treated with 10 μM curcumin for 16 h. Curcumin reduced ARNT levels generally in these cell lines, and in particular, ARNT levels were markedly reduced by 10 μM curcumin in MKN28 (-77%) and H596 cells (-64%), to the extent observed in Hep3B cells, although they were moderately reduced in HT29 (-35%), MCF7 (-49%), SiHa (-41%), and PC3 (-27%), with the exception of Caki1 cells (Fig. 2e). At a higher concentration of curcumin (20 μM), ARNT levels were also reduced by -55% in Caki1 cells (Fig. 2e). These results show that ARNT inhibition by curcumin is not limited to the Hep3B cell line.

Curcumin Stimulates the Degradation of ARNT. ARNT can be regulated in three ways; 1) by de novo synthesis; 2) by enhancing its stability; and 3) by dimerizing with HIF-1α. To investigate the dimerization of ARNT and HIF-1α, we coimmunoprecipitated ARNT with HIF-1α, as shown in Fig. 3a. The amount of ARNT bound to HIF-1α was found to be reduced as much as that in the input sample was reduced by curcumin, suggesting that HIF-1α binding to ARNT was not affected by curcumin and that ARNT levels were reduced before the dimerization process. Next, we investigated ARNT mRNA levels and found that it was constitutively expressed in curcumin-treated cells (Fig. 3b). To examine the degradation rate of ARNT, we measured ARNT levels after blocking de novo protein synthesis with cycloheximide. ARNT levels in untreated cells were not altered sig-

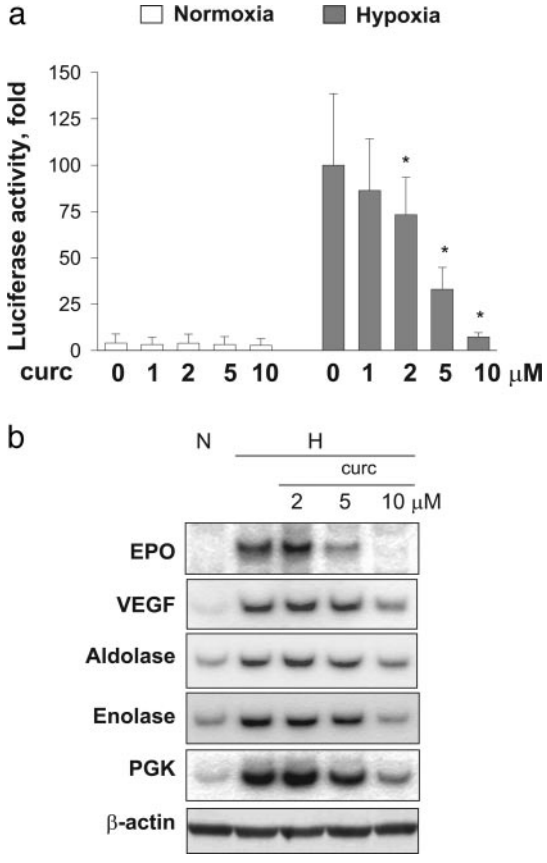
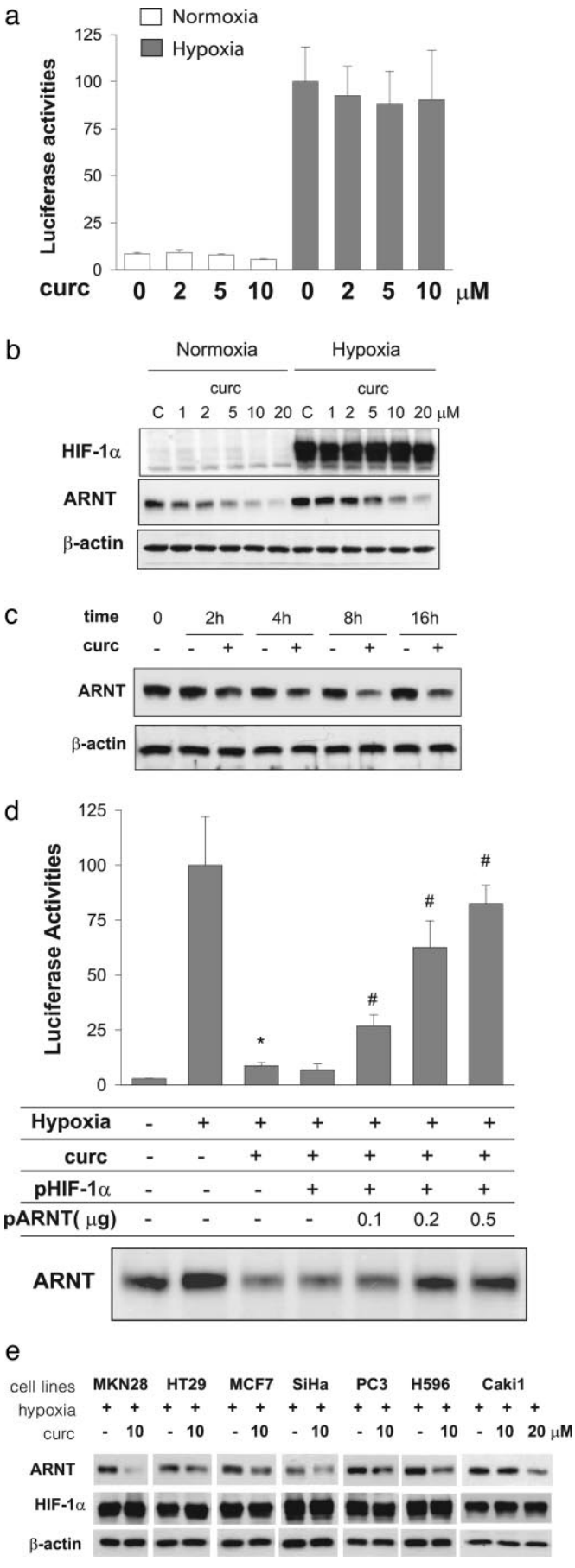


Fig. 1. The effects of curcumin on HIF-1 activity and on the expressions of hypoxia-induced genes in Hep3B cells. a, HIF-1 inhibition by curcumin. A luciferase reporter plasmid containing EPO enhancer was transfected into Hep3B cells. Luciferase activities were measured after incubation under normoxic or hypoxic conditions for 16 h with various concentrations of curcumin (curc). Results are quoted as relative values versus the normoxic control and are plotted as means $\pm$ S.D. of 12 experiments. *, $P < 0.05$ versus the hypoxic control. b, hypoxia-inducible gene inhibition by curcumin. The mRNAs of EPO, VEGF, aldolase A, enolase 1, phosphoglycerokinase 1, and β-actin were analyzed by semiquantitative RT-PCR. Results are representative of three separate experiments.



nificantly for up to 2 h after cycloheximide treatment and then gradually reduced. However, in curcumin-treated cells, ARNT levels were significantly reduced 2 h after cycloheximide treatment, and ARNT almost disappeared after 8 h of incubation (Fig. 3c, left). To calculate the half-life of ARNT, its band intensities were quantified using an image analysis system; results are plotted in the right in Fig. 3c. The half-life of ARNT was found to be shortened by more than 50% in curcumin-treated cells, which suggests that ARNT is destabilized by curcumin.

Curcumin Destabilizes ARNT Via an Oxidative Process. The effect of curcumin on the intracellular redox state is disputed. Because it contains an unsaturated aliphatic chain and two aromatic rings, the electrons in curcumin are highly conjugated. Such structures generally act as antioxidants by forming stable radicals after receiving electron(s) (Priyadarsini et al., 2003). Conversely, they can act as prooxidants by transferring electron(s) to molecular oxygen (Bhaumik et al., 1999). To examine whether the intracellular redox state is associated with attenuated ARNT levels, cells were treated with the antioxidant NAC, which was found to effectively block ARNT degradation by curcumin (Fig. 4a). Conversely, H_2O_2 reduced ARNT expression (Fig. 4b). In addition, BSO, which induces oxidative stress by depleting glutathione, reduced ARNT expression (Fig. 4c).

Curcumin Degrades ARNT Via the Ubiquitin-Proteasome System. To further examine the ARNT degradation process, curcumin was coadministered into media with protease inhibitors (i.e., the proteasome inhibitor MG132, the caspase inhibitor Z-VAD-FMK, or the lysosome inhibitors NH_4Cl or bafilomycin A1). We first checked the viability of Hep3B in the presence of curcumin and these inhibitors. Because Hep3B cells were injured by coadministration of MG132 and curcumin (data not shown), we could not examine the effect of MG132 on the curcumin-induced degradation of ARNT in Hep3B. Instead, we used MKN28 and H596, which also showed good responses to curcumin in Fig. 2e. MG132 effectively prevented the ARNT degradation in both cell lines (Fig. 5a). To confirm the proteasome-dependent degradation of ARNT, the effect of MG132 on the degradation rate of ARNT was examined. The half-life of ARNT was noticeably prolonged by MG132 in curcumin-treated cells (Fig. 5b). However, other protease inhibitors, such as Z-VAD-FMK, NH_4Cl, and bafilomycin A1, failed to recover ARNT

Fig. 2. Effects of curcumin on expression of HIF-1 subunits. a, no effect on transcriptional activity. A Gal4-CAD plasmid was cotransfected with a Gal4-luc reporter plasmid into Hep3B cells. Luciferase activities are quoted as relative values versus the normoxic control and are plotted as means ± S.D. of 12 experiments. b, ARNT suppression by curcumin. After 16-h incubation, HIF-1α and ARNT protein levels in Hep3B cells were analyzed by Western blotting. c, time course of ARNT suppression. Hep3B cells were incubated under hypoxic conditions for the indicated times with 10 μM curcumin and prepared for Western blotting of ARNT. d, recovery of HIF-1 activity by ARNT expression. Hep3B cells were cotransfected with EPO reporter plasmid and HIF-1α or ARNT plasmid and were incubated under hypoxic conditions in the presence of 10 μM curcumin for 16 h. Results are plotted as means ± S.D. of 12 experiments. *, $P < 0.05$ versus the hypoxic control; #, $P < 0.05$ versus the hypoxic curcumin-treated group. Bottom, ARNT levels in cell lysates prepared for the reporter assay. e, effect of curcumin on ARNT expression in various cell lines. The indicated cell lines were treated with DMSO (−) or 10 μM curcumin (+) and were incubated under hypoxic conditions for 16 h. Protein levels were analyzed by Western blotting. Band intensities were quantified using a Microcomputer Imaging Device model 4 (MCID-M4) image analysis system.

levels (Fig. 5c). These results suggest that curcumin degrades ARNT via the proteasome. Because the proteasome-dependent degradation is preceded by ubiquitination, we next examined whether ARNT is ubiquitinated by curcumin. Figure 5d showed that ARNT immunoprecipitated from curcumin-treated cells was highly ubiquitinated. This supports the possibility that curcumin promotes ARNT ubiquitination and thereby stimulates the proteasome-dependent proteolysis. In contrast to these findings, curcumin (Jana et al., 2004) and oxidative stress (Ding and Keller, 2001) were reported to decrease the proteasome activity. To know how much the proteasome activity decreased in our experimental settings, we measured the activity 8 h after treatment with various concentrations of curcumin or 1 mM H_2O_2. Here, 10 μM curcumin or 1 mM H_2O_2 inhibited the proteasome activity by 18 or 31%, respectively, whereas 2 and 5 μM curcumin did not affect the activity (Fig. 5e, left). We also examined the time course of the curcumin effect. The proteasome activity decreased 8 h after curcumin treatment but did not 4 or 6 h (Fig. 5e, right). As reported previously, curcumin and H_2O_2 seem to inhibit protein degradation via the proteasome. However, curcumin failed to stabilize HIF-1α, which is quickly degraded by the ubiquitin-proteasome system under normoxic conditions (Fig. 2b). This suggests that such an inhibition of the proteasome is not enough to block the proteolysis of all the proteins. Despite the reduction in the proteasome activity, curcumin can destabilize ARNT specifically by ubiquitinating it. In the same aspect, we can explain the oxidative stress-induced degradation of ARNT. Indeed, Grune et al. (1995) demonstrated that oxidative stress increases proteasomal proteolysis by modifying cellular proteins even though it decreases the proteasome activity. This suggests that the protein modification, rather than total proteasome activity, is the rate-limiting step in proteolysis.

The Anticancer Effect of Curcumin Is Associated with ARNT Reduction. HIF-1 plays a critical role in tumor promotion. We here found that curcumin had a novel effect: HIF-1 inhibition as a result of ARNT degradation. Thus, we examined the possibility that curcumin inhibits tumor growth in vivo by inhibiting HIF-1. Figure 6a shows the growth rates of Hep3B hepatoma grafted into nude mice, plotted as average tumor volume versus time. Curcumin was found to significantly halt tumor growth. Furthermore, ARNT levels were markedly reduced in curcumin-treated tumors, whereas HIF-1α levels were not changed (Fig. 6b). Typical HIF-1-dependent proteins EPO and VEGF were also suppressed. Therefore, HIF-1 inhibition seems to contribute to the anticancer effect of curcumin in vivo.

Discussion

In this study, we demonstrate that curcumin is a potential anticancer agent and that it achieves this by targeting HIF-1. In Hep3B hepatoma cells, curcumin inhibited the activity of the EPO reporter to reflect HIF-1 activity and blocked the hypoxic induction of HIF-1-transcribed mRNAs. From a mechanistic perspective, curcumin suppressed ARNT expression without altering the expression and the transcriptional activity of HIF-1α. This ARNT-suppressing effect of cur-

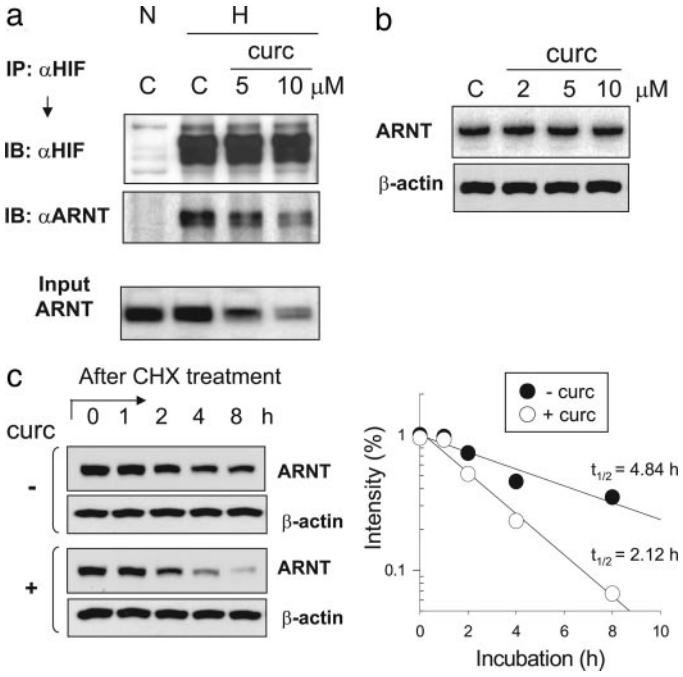


Fig. 3. Effects of curcumin on ARNT binding with HIF-1α and on ARNT mRNA expression and protein stability. a, no interference with HIF-1α binding. Hep3B cells were incubated under normoxic or hypoxic conditions with curcumin for 16 h and homogenized. HIF-1α was then immunoprecipitated, and the coprecipitated ARNT was detected by Western blotting. b, no change in ARNT mRNA expression. ARNT and β-actin mRNAs of Hep3B cells were analyzed by semiquantitative RT-PCR. c, ARNT protein degradation by curcumin. After 4-h incubation with 10 μM curcumin, Hep3B cells were treated with 60 μg/ml cycloheximide (CHX). After further incubation for 0, 1, 2, 4, and 8 h, the cell lysates (10 μg of protein) were analyzed by Western blotting (left). The band intensities were quantified using the MCID-M4 image analysis system, and ARNT half-life ($t_{1/2}$) was calculated from the slope of the first-order decay curve (right). Points represent the mean values of three separate experiments.

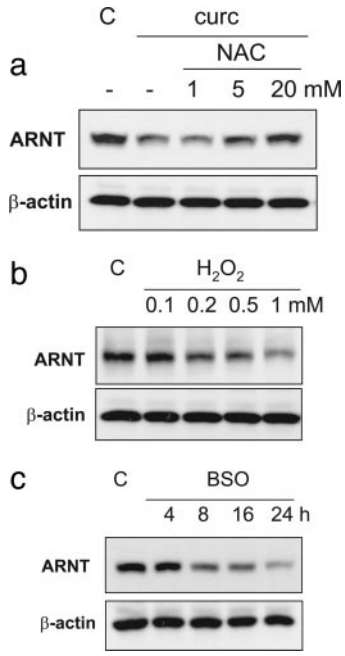


Fig. 4. Involvement of oxidative stress in curcumin-induced ARNT degradation. a, recovery of ARNT by NAC. Hep3B cells were cotreated with 10 μM curcumin and NAC for 8 h, and ARNT was analyzed by Western blotting. b, ARNT down-regulation by H_2O_2. Cells were treated with H_2O_2 for 8 h and were prepared for ARNT Western blotting. c, ARNT down-regulation by glutathione depletion. Cells were treated with 1 mM BSO for the indicated times and were prepared for ARNT Western blotting.

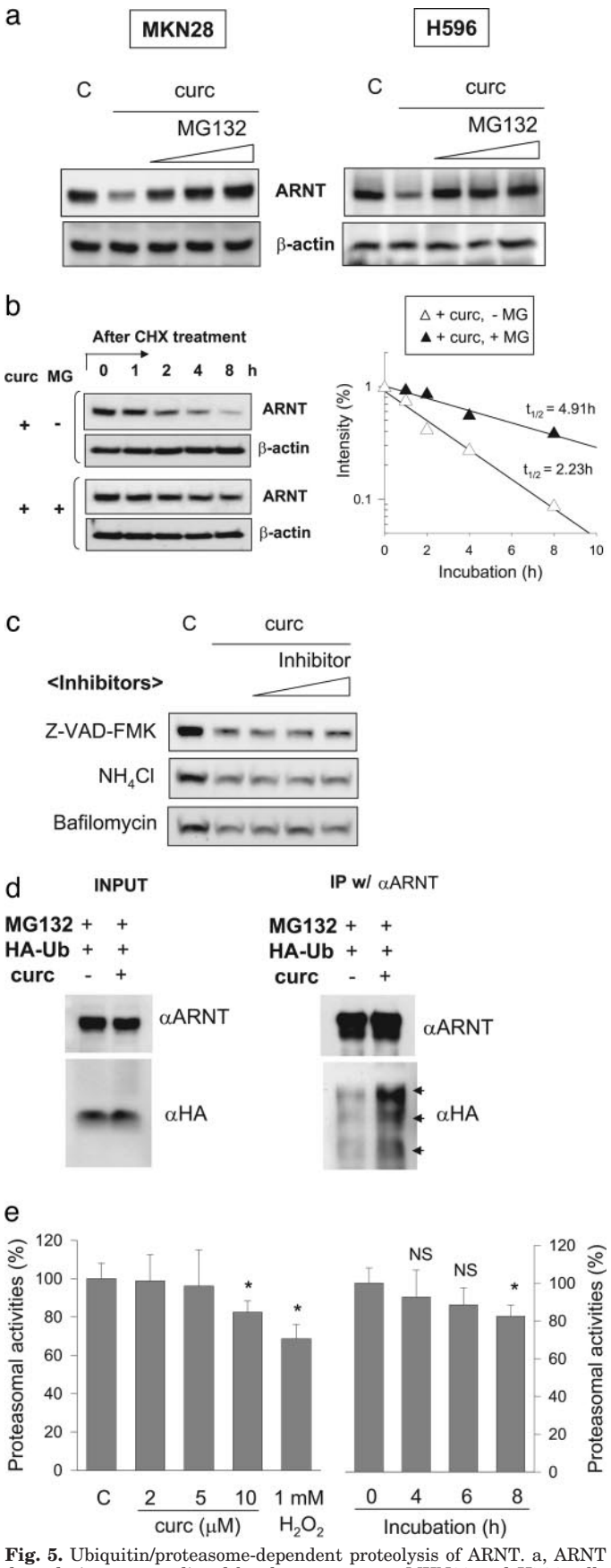


Fig. 5. Ubiquitin/proteasome-dependent proteolysis of ARNT. a, ARNT degradation was mediated by the proteasome. MKN28 and H596 cells were treated with 10 μM curcumin for 8 h in the presence of MG132 proteasome inhibitor (1, 5, and 10 μM) and were prepared for ARNT Western blotting. b, proteasome inhibition rescues ARNT degradation by curcumin. After pretreating MKN28 cells with 10 μM curcumin in the presence of DMSO (−MG) or 10 μM MG132 (+MG) for 4 h, they were treated with 60 μg/ml cycloheximide (CHX). After further incubation for 0, 1, 2, 4, and 8 h, ARNT levels were measured by Western blotting (left) and quantified using the MCID-M4 system. The protein half-life ($t_{1/2}$) was calculated from the slope of the first-order decay curve (right). Points represent the mean values of three separate experiments. c, ARNT degradation was not mediated by caspases and lysosomes. Hep3B cells were treated with 10 μM curcumin for 8 h in the presence of Z-VAD-FMK caspase inhibitor (20, 50, and 100 μM), NH_4Cl lysosome inhibitor (0.5, 2, and 10 mM), or bafilomycin A1 lysosome inhibitor (5, 20, and 100 nM). d, curcumin stimulates the ubiquitination of ARNT. MKN23 cells were transfected with 4 μg of the plasmid HA-tagged ubiquitin (HA-Ub) using Lipofectamine. After 8-h treatment with 10 μM curcumin and 10 μM MG132, immunoprecipitated ARNT or HA-Ub-conjugated ARNT was detected using anti-ARNT or anti-HA antibody, respectively. Arrows indicate the protein bands of ubiquitinated ARNT. e, curcumin or oxidative stress inhibits the proteasome activity. MKN28 cells were treated with various concentrations of curcumin or 1 mM H_2O_2 for 8 h (left) or were treated with 10 μM curcumin for the indicated incubation time (right). The proteasome activity was analyzed using the Chemicon assay kit. The AMC fluorescence was excited at 380 nm and measured at 460 nm. The proteasome activity was calculated using authentic AMC standards in the assay kit. Results represent means (n = 4) and standard deviations. *, $P < 0.05$ versus the control (C) or the zero time control (0). NS, no significant difference.

cumin was also demonstrated in other cancer cell lines. HIF-1 activity inhibited by curcumin was rescued by ARNT expression. We also found that curcumin stimulated proteasomal degradation of ARNT via oxidation and ubiquitination processes. In mice bearing Hep3B hepatomas, curcumin retarded tumor growth and suppressed ARNT, EPO, and VEGF expression in tumor tissue. Taken together, these results suggest that the anticancer effect of curcumin is due to HIF-1 inhibition by ARNT degradation.

Because HIF-1α determines the transcriptional activity of the HIF-1 complex, it is not surprising that HIF-1α is viewed as a better target for inhibiting HIF-1 than ARNT; considerable effort has been made to develop anticancer drugs using HIF-1α inhibitors. However, in addition to HIF-1α, many cancer cells have also HIF-2α (alternatively named EPAS-1 or MOP2) (Peng et al., 2000). HIF-2α is similar to HIF-1α in terms of its protein structure and regulation (Wiesener et al., 1998), and it binds with ARNT to form the HIF-2 transcription complex, which also participates in hypoxic gene regulation. Rankin et al. (2005) reported that the deletion of *HIF1A* is insufficient to prevent vascular-tumor development in von Hippel-Lindau-deficient mice, which may be because *HIF2A* compensates for the loss of *HIF1A* in terms of hypoxic gene regulation. On the other hand, the deletion of ARNT completely inhibited tumor formation because both HIF-1 and HIF-2 cannot be formed in its absence. Thus, ARNT represents a common target for the inhibition of both HIF-1 and HIF-2 in tumors. In this aspect, curcumin, which inhibits ARNT expression both in vitro and in vivo, may be a good lead compound in developing novel anticancer agents to target HIFs.

Curcumin has been reported to have various biological actions. Such a multiplicity of activities may be the result of its reactive α,β-unsaturated β-diketone moiety, which is able to covalently bind proteins (Aggarwal et al., 2003). Moreover, the curcumin radical, produced by reactive oxygen species, could be more reactive than curcumin at binding proteins (Bhaumik et al., 1999). Indeed, such protein binding by curcumin has been reported to induce the degradation of p50 in

the nuclear factor-κB complex (Brennan and O'Neill, 1998). In addition, curcumin has been shown to degrade many proteins, such as p53 (Tsvetkov et al., 2005), cyclin D1 (Kwon et al., 2005), $p185^{ErbB2}$ (Hong et al., 1999), c-Jun (Uhle et al., 2003), and CCAAT/enhancer-binding protein (Balasubramanian and Eckert, 2004). In the present study, curcumin was found to induce ARNT degradation, and this was found to depend on oxidative stress. Therefore, we speculate that the reactive oxygen species-derived curcumin radical, rather that unchanged curcumin, directly binds or oxidizes ARNT, and that this modified ARNT is targeted by the E3/ubiquitin ligase complex and is proteolysed. However, this binding of curcumin to ARNT remains to be proven.

Little is known of the mechanism of ARNT regulation. Although ARNT is present at consistent levels, it has a relatively short half-life (4.84 h) even in resting cells (Lee et al., 2004), which suggests that ARNT expression is finely regulated. Thus, if the rates of its synthesis and degradation are perturbed, ARNT levels might be altered. The present study demonstrates that ARNT is rapidly degraded by curcumin and that its levels are reduced as early as 4 h after curcumin treatment. Moreover, proteasomes are likely to mediate the ARNT degradation. However, MG132 did not enhance the ARNT levels in the absence of curcumin (data not shown). This suggests that the proteasomal degradation of ARNT does not participate in the normal turnover of ARNT, but it may be a stress response.

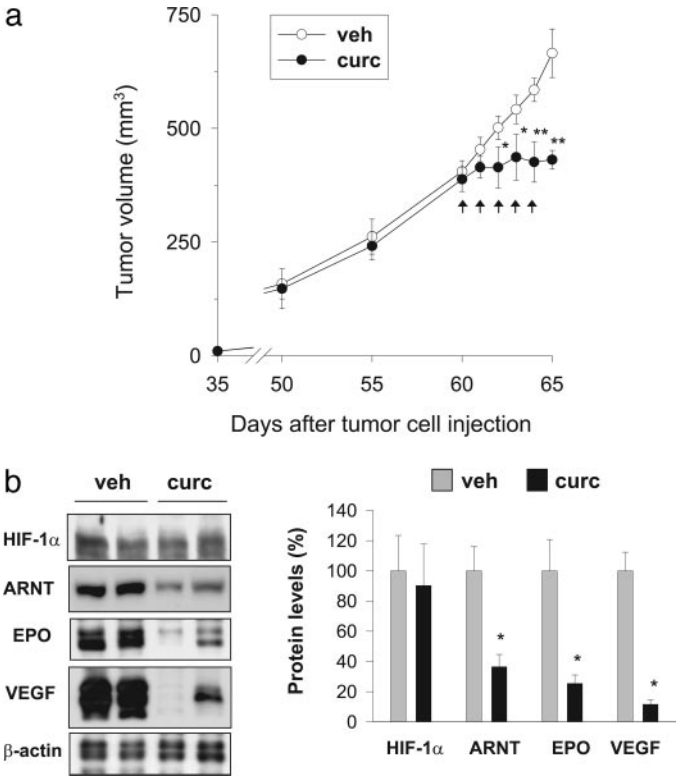


Fig. 6. Effects of curcumin on tumor growth and hypoxic gene expression. a, tumor growth inhibition by curcumin. Tumor-bearing mice were intraperitoneally treated with DMSO vehicle (veh) or 120 mg/kg curcumin (curc) once a day for 5 days (arrows). Results are plotted as means ± S.D. of tumor volumes from six mice. *, $P < 0.05$; **, $p < 0.01$ versus the vehicle-treated group. b, protein levels in tumors. Tumor tissues were homogenized in a 1% SDS lysis buffer and were analyzed by Western blotting. Band intensities were quantified using the MCID-M4 system and are plotted as means ± S.D. *, $P < 0.01$ versus the vehicle-treated group.

Curcumin is considered to be a safe material because curry has been used as an everyday food in India for more than 3000 years. In a recent phase I clinical trial (Cheng et al., 2001), curcumin showed no toxicity at up to 8 g/day (approximately 115 mg/kg/day). In addition, the pharmacokinetics and the biologically effective dose of curcumin have been investigated in patients with cancer (Sharma et al., 2004). However, the clinical indications for curcumin treatment and its anticancer effectiveness have not been determined. Nevertheless, the improved availability of agents targeting different molecules increases the possibility of achieving better combinations for cancer therapy. In this respect, the anti-HIF activity of curcumin may be useful in combination with conventional anticancer agents.

Acknowledgments

We thank Dr. Eric Huang (National Cancer Institute, Bethesda, MD) for generously donating essential plasmids and for advice.

References

Aggarwal BB, Kumar A, and Bharti AC (2003) Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res* **23:**363–398.

Arbiser JL, Klauber N, Rohan R, van Leeuwen R, Huang MT, Fisher C, Flynn E, and Byers HR (1998) Curcumin is an in vivo inhibitor of angiogenesis. *Mol Med* **4:**376–383.

Balasubramanian S and Eckert RL (2004) Green tea polyphenol and curcumin inversely regulate human involucrin promoter activity via opposing effects on CCAAT/enhancer-binding protein function. *J Biol Chem* **279:**24007–24014.

Bhaumik S, Anjum R, Rangaraj N, Pardhasaradhi BV, and Khar A (1999) Curcumin mediated apoptosis in AK-5 tumor cells involves the production of reactive oxygen intermediates. *FEBS Lett* **456:**311–314.

Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, and Oberhuber G (2000) Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. *Cancer Res* **60:**4693–4696.

Brennan P and O'Neill LA (1998) Inhibition of nuclear factor kappaB by direct modification in whole cells—mechanism of action of nordihydroguaiaritic acid, curcumin and thiol modifiers. *Biochem Pharmacol* **55:**965–973.

Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, et al. (2001) Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* **21:** 2895–2900.

Chilov D, Camenisch G, Kvietikova I, Ziegler U, Gassmann M, and Wenger RH (1999) Induction and nuclear translocation of hypoxia-inducible factor-1 (HIF-1): heterodimerization with ARNT is not necessary for nuclear accumulation of HIF-1alpha. *J Cell Sci* **112:**1203–1212.

Chun YS, Choi E, Yeo EJ, Lee JH, Kim MS, and Park JW (2001) A new HIF-1 alpha variant induced by zinc ion suppresses HIF-1-mediated hypoxic responses. *J Cell Sci* **114:**4051–4061.

Ding Q and Keller JN (2001) Proteasome inhibition in oxidative stress neurotoxicity: implications for heat shock proteins. *J Neurochem* **77:**1010–1017.

Giaccia A, Siim BG, and Johnson RS (2003) HIF-1 as a target for drug development. *Nat Rev Drug Discov* **2:**803–811.

Grune T, Reinheckel T, Joshi M, and Davies KJ (1995) Proteolysis in cultured liver epithelial cells during oxidative stress. Role of the multicatalytic proteinase complex, proteasome. *J Biol Chem* **270:**2344–2351.

Gu YZ, Hogenesch JB, and Bradfield CA (2000) The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* **40:**519–561.

Han SS, Chung S, Robertson DA, Ranjan D, and Bondada S (1999) Curcumin causes the growth arrest and apoptosis of B cell lymphoma by downregulation of egr-1, c-myc, bcl-XL, NF-kappa B, and p53. *Clin Immunol* **93:**152–161.

Hockel M and Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* **93:**266–276.

Hong RL, Spohn WH, and Hung MC (1999) Curcumin inhibits tyrosine kinase activity of p185neu and also depletes p185neu. *Clin Cancer Res* **5:**1884–1891.

Huang LE, Gu J, Schau M, and Bunn HF (1998) Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* **95:**7987–7992.

Jana NR, Dikshit P, Goswami A, and Nukina N (2004) Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway. *J Biol Chem* **279:**11680–11685.

Jiang BH, Zheng JZ, Leung SW, Roe R, and Semenza GL (1997) Transactivation and inhibitory domains of hypoxia-inducible factor 1α. Modulation of transcriptional activity by oxygen tension. *J Biol Chem* **272:**19253–19260.

Kawamori T, Lubet R, Steele VE, Kelloff GJ, Kaskey RB, Rao CV, and Reddy BS (1999) Chemopreventative effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Res* **59:**597–601.

Kwon YK, Jun JM, Shin SW, Cho JW, and Suh SI (2005) Curcumin decreases cell proliferation rates through BTG2-mediated cyclin D1 down-regulation in U937 cells. *Int J Oncol* **26:**1597–1603.

Lee KH, Park JW, and Chun YS (2004) Non-hypoxic transcriptional activation of the aryl hydrocarbon receptor nuclear translocator in concert with a novel hypoxia-inducible factor-1alpha isoform. *Nucleic Acids Res* **32:**5499–5511.

Moragoda L, Jaszewski R, and Majumdar AP (2001) Curcumin induced modulation of cell cycle and apoptosis in gastric and colon cancer cells. *Anticancer Res* **21:**873–878.

Peng J, Zhang L, and Drysdale L (2000) The transcription factor EPAS-1/hypoxia-inducible factor 2α plays an important role in vascular remodeling. *Proc Natl Acad Sci USA* **97:**8386–8391.

Piper JT, Singhal SS, Salameh M, Torman RT, Awasthi YC, and Awasthi S (1998) Mechanisms of anticarcinogenic properties of curcumin: the effect of curcumin on glutathione linked detoxification enzymes in rat liver. *Int J Biochem Cell Biol* **30:**445–456.

Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S, and Howells L (1999) Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signalling complex. *Oncogene* **18:**6013–6020.

Priyadarsini KI, Maity DK, Naik GH, Kumar G, and Mohan H (2003) Role of phenolic O-H and methylene hydrogen on the free radical reactions and antioxidant activity of curcumin. *Free Radic Biol Med* **35:**475–484.

Rankin EB, Higgins DF, Walisser JA, Johnson RS, Bradfield CA, and Haase VH (2005) Inactivation of the arylhydrocarbon receptor nuclear translocator (Arnt) suppresses von Hippel-Lindau disease-associated vascular tumors in mice. *Mol Cell Biol* **25:**3163–3172.

Rao CV, Rivenson A, Simi B, and Reddy BS (1995) Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res* **55:**259–266.

Ruby AJ, Kuttan G, Babu KD, Rajasekharan KN, and Kuttan R (1995) Anti-tumour and antioxidant activity of natural curcuminoids. *Cancer Lett* **94:**79–83.

Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, and Johnson RS (2000) Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. *Cancer Res* **60:**4010–4015.

Semenza GL (2002) HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med* **8:**S62–S67.

Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, et al. (2004) Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. *Clin Cancer Res* **10:**6847–6854.

Sogawa K, Nakano R, Kobayashi A, Kikuchi Y, Ohe N, Matsushita N, and Fujii-Kuriyama Y (1995) Possible function of Ah receptor nuclear translocator (Arnt) homodimer in transcriptional regulation. *Proc Natl Acad Sci USA* **92:**1936–1940.

Thapliyal R and Maru GB (2001) Inhibition of cytochrome P450 isozymes by curcumins in vitro and in vivo. *Food Chem Toxicol* **39:**541–547.

Tsvetkov P, Asher G, Reiss V, Shaul Y, Sachs L, and Lotem J (2005) Inhibition of NAD(P)H:quinone oxidoreductase 1 activity and induction of p53 degradation by the natural phenolic compound curcumin. *Proc Natl Acad Sci USA* **102:**5535–5540.

Uhle S, Medalia O, Waldron R, Dumdey R, Henklein P, Bech-Otschir D, Huang X, Berse M, Sperling J, Schade R, et al. (2003) Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome. *EMBO (Eur Mol Biol Organ) J* **22:**1302–1312.

Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, et al. (1998) Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* **92:**2260–2268.

Yeo EJ, Chun YS, and Park JW (2004) New anticancer strategies targeting HIF-1. *Biochem Pharmacol* **68:**1061–1069.

Yeo EJ, Ryu JH, Cho YS, Chun YS, Huang LE, Kim MS, and Park JW (2006) Amphotericin B blunts erythropoietin response to hypoxia by reinforcing FIH-mediated repression of HIF-1. *Blood* **107:**916–923.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* **59:**5830–5835.

Address correspondence to: Dr. Jong-Wan Park, Department of Pharmacology, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea. E-mail: parkjw@snu.ac.kr