0026-895X/06/7006-1856–1865$20.00
MOLECULAR PHARMACOLOGY
Vol. 70, No. 6
28076/3151282
Mol Pharmacol 70:1856–1865, 2006
Printed in U.S.A.

Bafilomycin Induces the p21-Mediated Growth Inhibition of Cancer Cells under Hypoxic Conditions by Expressing Hypoxia-Inducible Factor-1α[S]

Ji-Hong Lim, Jong-Wan Park, Myung-Suk Kim, Sang-Ki Park, Randall S. Johnson, and Yang-Sook Chun

Cancer Research Institute (J.-H.L., Y.-S.C.), Department of Physiology (Y.-S.C.), and Department of Pharmacology (J.-W.P., M.-S.K.), Seoul National University College of Medicine, Seoul, Korea; and Molecular Biology Section, Division of Biological Sciences, University of California San Diego, La Jolla, California (S.-K.P., R.S.J.)

Received June 19, 2006; accepted August 25, 2006

ABSTRACT

Bafilomycin A1, a macrolide antibiotic isolated from *Streptomyces* species, has been used as an inhibitor of vacuolar H^+ ATPase (V-ATPase). Bafilomycin has been also evaluated as a potential anticancer agent because it inhibits cell proliferation and tumor growth. Although these anticancer effects of bafilomycin are considered to be attributable to the intracellular acidosis by V-ATPase inhibition, the exact mechanism remains unclear. In the present study, we tested the possibility that bafilomycin targets a tumor-promoting factor, hypoxia-inducible factor-1α (HIF-1α). Bafilomycin A1 and its analog, concanamycin A, were found to up-regulate HIF-1α in eight human cancer cell-lines, and this effect is attributed to inhibited degradation of HIF-1α protein. Furthermore, the HIF-1α induction by bafilomycin was augmented by hypoxia, which caused a robust induction of p21 and cell cycle arrest in cancer cells. The cell cycle inhibition was shown only in cancer cells expressing both HIF-1α and p21. In HIF-1α(+/+) or HIF-1α(−/−) fibrosarcomas grafted in nude mice, bafilomycin showed the HIF-1α-dependent anticancer effect. Based on these results, the exorbitant expression of HIF-1α is likely to contribute to the anticancer action of bafilomycin.

Bafilomycin A1, a macrolide antibiotic isolated from *Streptomyces* species, has been used as an inhibitor of vacuolar H^+ ATPase (V-ATPase). It binds to a pocket formed by V0 sector subunit c (ATP6V0C) of the V-ATPase complex and inhibits H^+ translocation by preventing the rotation of the ATP6V0C multimer, which causes the accumulation of H^+ in the cytoplasm (Bowman et al., 2004). Biologically, it has been found that bafilomycin induces cell growth inhibition (Ohkuma et al., 1993) and apoptosis (Kinoshita et al., 1996, Nakashima et al., 2003). In vivo, bafilomycin also inhibits the growth of xenografted tumors (Ohta et al., 1998) and thus could be evaluated as a potential anticancer agent. These biological effects of bafilomycin are considered to be attributable to the intracellular acidosis by V-ATPase inhibition. There is the question of whether the acidosis is only one mechanism underlying the anticancer activity of bafilomycin; generally, the agents that have in vivo anticancer activities inhibit some cancer-specific events, but the acidosis by bafilomycin occurs in normal cells as well. Therefore, we hypothesized that bafilomycin targets a cancer-specific molecule.

Hypoxia-inducible factor-1α (HIF-1α) is a basic-helix-loop-helix protein of the PAS family (Wang and Semenza, 1995). It plays a key role in cellular adaptation to hypoxia by up-regulating 60 or more genes essential for angiogenesis and cell survival. To date, the roles of HIF-1α in tumor progression have been extensively investigated, and its overexpression is frequently found in various human tumors (Zhong et al., 1999). HIF-1α levels in tumors are positively related with tumor hypervascularity, aggressiveness, and poor prognosis (Birner et al., 2000, Zagzag et al., 2000). Moreover, xenograft studies have disclosed that HIF-1α is essential for tumor

This work was supported by a grant from the Korean Ministry of Health and Welfare Research Fund 2005 (A050479).
J.-H.L. and J.-W.P. contributed equally to this work.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.028076.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: V-ATPase, vacuolar H^+ ATPase; HIF-1α, hypoxia-inducible factor 1α; BM, bafilomycin A1; pVHL, Von Hippel-Lindau protein; FIH, factor-inhibiting hypoxia-inducible factor; VEGF, vascular endothelial factor; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; MEF, mouse embryonic fibroblast; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; β-gal, β-galactoside; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

growth and angiogenesis (Ryan et al., 2000). Therefore, HIF-1α is indicated as an aggravating factor in cancer diseases. However, in stark contrast with the above, HIF-1 α overexpression in hypoxic cancer cells has been suggested to inhibit cell division by inducing p21, p27, or p53 and to promote apoptosis by inducing p53, Nip3, Noxa, or HGTD-P (Goda et al., 2003; Bacon and Harris, 2004). Thus, HIF-1α seems to be a double-edged sword from the viewpoint of tumors subjected to hypoxia, and thus, its expression is probably controlled at optimal levels in growing tumors. In this respect, both HIF-1α suppression and overexpression could inhibit the growth of hypoxic tumors.

In the present study, we address the mechanism underlying the anticancer action of bafilomycin. Bafilomycin A1 and its analog, concanamycin A, were found to induce HIF-1α in eight human cancer cell-lines. The HIF-1α induction by bafilomycin was augmented by hypoxia, which caused a robust induction of p21 and cell cycle arrest in cancer cells. In fibrosarcoma xenografts, bafilomycin showed the HIF-1α-dependent anticancer effect. The exorbitant expression of HIF-1α is likely to contribute to the anticancer action of bafilomycin.

Materials and Methods

Materials. Bafilomycin A1 and MG132 were purchased from Alexis Biochemicals (Lausen, Switzerland), and concanamycin A, cycloheximide, propidium iodide, and other chemicals were from Sigma-Aldrich (St. Louis, MO). Culture media and fetal calf serum were purchased from GIBCO/BRL (Grand Island, NY). Anti-HIF-1α antiserum was generated in rabbits against a bacterially expressed fragment encompassing amino acids 418 to 698 of human HIF-1α, as described previously (Chun et al., 2001). p53, p27, mouse p21, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human p21, hemagglutinin, Von Hippel-Lindau protein (pVHL), and Flag antibodies were purchased from Cell Signaling (Beverly, MA), Roche (Basel, Switzerland), PharMingen (San Diego, CA), and Sigma-Aldrich, respectively.

Cell Culture. PC3 (prostate), Caki-1 (kidney), SiHa (uterine cervix), and Hep3B (liver) cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). Four lung cancer cell lines, HCC1171 (epithelial origin), HCC2108 (epithelial), HCC1195 (squamous), and HCC2279 (squamous), were obtained from the Korean Cell Line Bank (Seoul, Korea), and an HIF-1α-null mouse embryonic fibroblast (MEF) cell line was provided as described previously (Ryan et al., 2000). HCT116(p21−/−) and HCT116(p21+/+) were generous gifts from Dr. Deug Y Shin (Dankook University College of Medicine, Cheonan, Korea). Lung cancer cells were cultured in RPMI 1640 medium and other cells in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, in a 5% CO_2 humidified atmosphere at 37°C. O_2 levels in the chamber were either 20% (normoxic) or 1% (hypoxic). Bafilomycin A1 or concanamycin A were administered to medium 5 min before normoxic or hypoxic incubation for 4 h.

Immunoblotting and Immunoprecipitation. To quantify protein levels, total cell lysates were prepared as described previously (Chun et al., 2001). Proteins were separated on SDS-polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk and incubated overnight at 4°C with a primary antibody diluted 1:1000. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (diluted 1:5000) for 2 h, and antigen-antibody complexes were visualized using an Enhanced Chemiluminescence Plus kit (Amersham Biosciences Corporation, Piscataway, NJ). β-Actin protein was used as an internal standard. For coimmunoprecipitation of HIF-1α and pVHL, cell lysates (150 μg of protein) were incubated with 10 μl of anti-HIF-1α antiserum or preimmune rabbit serum at 4°C for 4 h. The immune complex was further incubated with protein A/G-Sepharose beads (Amersham Biosciences) at 4°C for 4 h. The antigen-bead complexes obtained were

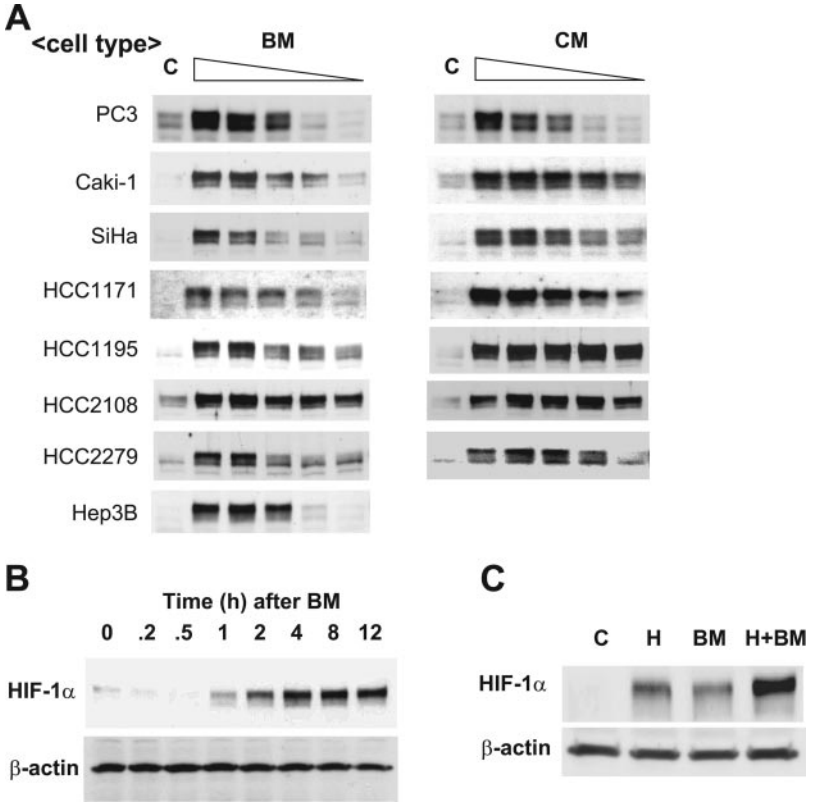

Fig. 1. Bafilomycin and concanamycin induce HIF-1α. A, dose-response of HIF-1α expression. Eight cancer cell lines were incubated with BM or concanamycin A (CM) for 4 h and harvested. The concentrations of BM and CM used were 10, 5, 2, 1, and 0.2 nM. Protein levels of HIF-1α were evaluated by immunoblotting. B, time course of HIF-1α expression. PC3 cells were incubated with 10 nM BM for the times indicated above the figure. C, synergistic induction of HIF-1α by bafilomycin and hypoxia. PC3 cells were incubated under normoxic or hypoxic conditions in the absence or in the presence of 10 nM BM for 4 h. HIF-1α levels were evaluated by immunoblotting.

washed extensively with lysis buffer. Immunocomplexes were eluted by boiling for 3 min in a sample buffer containing 2% SDS and 10 mM dithiothreitol, subjected to SDS-polyacrylamide gel electrophoresis, and then immunoblotted using anti-HIF-1α or anti-pVHL antibody.

Semiquantitative RT-PCR. To quantify mRNA levels, we used a highly sensitive, semiquantitative RT-PCR method, as described previously (Yeo et al., 2003). Total RNAs were isolated from cultured cells or rat kidney using Trizol (GIBCO/BRL). One microgram of RNA was reverse-transcribed, and the cDNA obtained was amplified over 18 PCR cycles in a reaction mixture containing 5 μCi [α-^{32}P]dCTP and 250 nM concentration of each primer set. PCR products were electrophoresed on a 4% polyacrylamide gel, and dried gels were autoradiographed. Primers for human HIF-1α, vascular endothelial growth factor (VEGF), phosphoglycerate kinase 1, enolase 1, and β-actin were constructed as described previously (Yeo et al., 2003).

Construction and Assay of a p21 Reporter Gene. To make a p21 promoter-driven luciferase reporter gene, the DNA coding human p21 promoter region (−966 to −2) containing a c-Myc binding site was cloned using PCR and then inserted into the KpnI and BglII sites of the pGL3 promoter plasmid (Promega, Madison, WI). PC3 cells were cotransfected with 0.5 μg each of reporter gene and plasmid cytomegalovirus-β-gal using Lipofectamine (Invitrogen, Carlsbad, CA). After being allowed to stabilize for 48 h, cells were 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.

Chromatin Immunoprecipitation. Cells were fixed with formaldehyde, and soluble chromatin samples were immunoprecipitated with anti-c-Myc or anti-HIF-1α at 4°C overnight (Jung et al., 2005). DNA isolated from immunoprecipitated material was amplified by semiquantitative PCR with [α-^{32}P]dCTP. The PCR primer sequences used were 5′-GATTTGTGGCTCACTTCGT-3′ and 5′-GCTCCACAAGGAACTGACT-3′, which produced a 320-base pair fragment, including the c-Myc binding site of the p21 gene. PCR products were electrophoresed in a 4% polyacrylamide gel, and dried gels were autoradiographed.

Cell Cycle Analysis. PC3 cells were plated in 10-cm culture dishes at concentrations determined to yield 70 to 80% confluence within 24 h. Cells were then incubated under normoxic or hypoxic

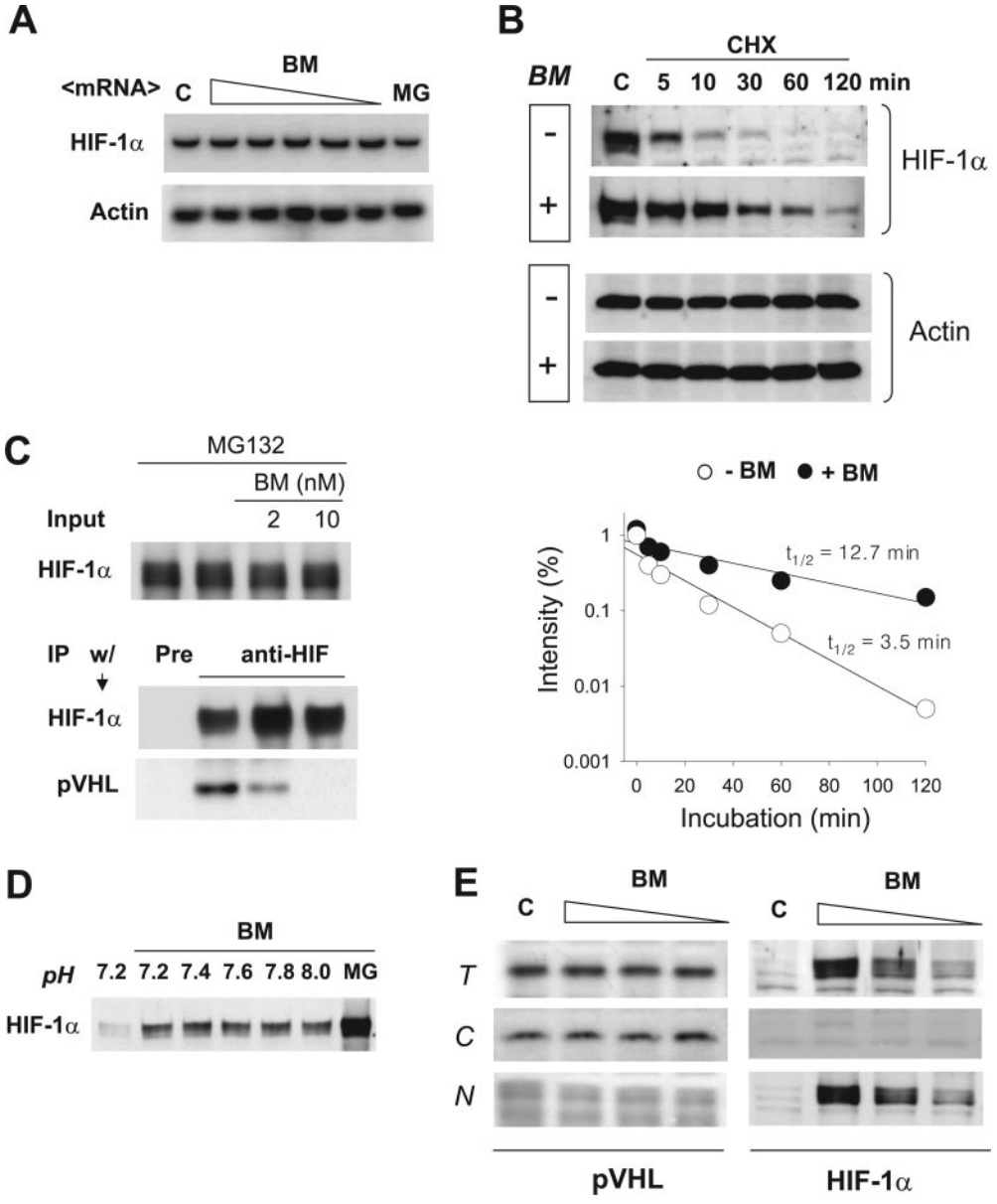

Fig. 2. Bafilomycin stabilizes HIF-1α protein. A, HIF-1α mRNA levels. Total RNAs were extracted from PC3 cells treated with 10, 5, 2, 1, or 0.2 nM BM, and HIF-1α and β-actin mRNAs were analyzed by semiquantitative RT-PCR. B, HIF-1α stability. PC3 cells were incubated in the absence (−BM) or presence (+BM) of 10 nM bafilomycin A1 for 4 h and then treated with 60 μg/ml cycloheximide (CHX). After 5, 10, 30, 60, or 120 min, the cell lysates were analyzed by immunoblotting using anti-HIF-1α and anti-β-actin antibodies (top). Band intensities were quantified using a Microcomputer Imaging Device model 4 (MCID-M4) image analysis system and are plotted at the bottom. Half-lives ($t_{1/2}$) were calculated from the slopes of first-order decay curves. Each point represents the mean value of three separate experiments. C, bafilomycin dissociates pVHL from HIF-1α. PC3 cells were treated with BM and MG132 (10 μM) for 6 h. HIF-1α was immunoprecipitated using anti-HIF-1α antibody or nonimmunized rabbit serum (Pre), and the coprecipitation of pVHL with HIF-1α was analyzed with anti-pVHL antibody. D, HIF-1α expression under alkaline conditions. A HEPES buffer (50 mM, pH 7.2–8.0) was added to buffer-free Dulbecco's modified Eagle's medium supplemented with 5 mM $NaHCO_3$ and 10% fetal calf serum. Cells were incubated in the alkaline media for 30 min and treated with 10 nM BM for 4 h. MG, treatment of 10 μM MG132 proteasome inhibitor. E, sublocalization of pVHL and HIF-1α. After PC3 cells were treated with BM (10, 2, and 1 nM) for 4 h, cells were harvested and fractionated to total (*T*), cytosolic (*C*), and nuclear (*N*) fractions. HIF-1α and pVHL proteins were analyzed in each fraction using anti-HIF-1α and anti-pVHL antibodies.

conditions in the absence or presence of bafilomycin A1 for 16 h. Cells were then harvested and resuspended in 200 μl of phosphate-buffered saline and fixed in 75% ethanol for 30 min on ice. After washing with phosphate-buffered saline, cells were labeled with propidium iodide (0.05 mg/ml) in the presence of RNase A (0.5 mg/ml) and then incubated in the dark for 30 min. DNA contents were analyzed using a Becton Dickinson FACStar flow cytometer (BD Biosciences, San Jose, CA). Propidium iodide was excited using an argon laser at 488 nm and detected at 630 nm.

Animals and Tumor Grafts. 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. All animal procedures were performed according to the procedures described in the Seoul National University Laboratory Animal Maintenance Manual. Mice aged 7 to 8 weeks were injected subcutaneously in the flank with 5×10^6 viable cells of HIF-1α(+/+) or HIF-1α(−/−) MEF. Tumor volumes were measured with a caliper and calculated using the formula Volume $= a \times b^2/2$, where a was the width at the widest point of the tumor, and b was the maximal width perpendicular to a.

Tumor Histology and TUNEL Staining. Tumors were removed 4 days after injecting bafilomycin A1, fixed with formalin, and embedded in paraffin. Serial sections (6 μm thick) were cut from each paraffin block and stained with hematoxylin and eosin (H&E). Necrosis was identified at a magnification of 40× and examined using a Sony XC-77 CCD camera and a Microcomputer Imaging Device

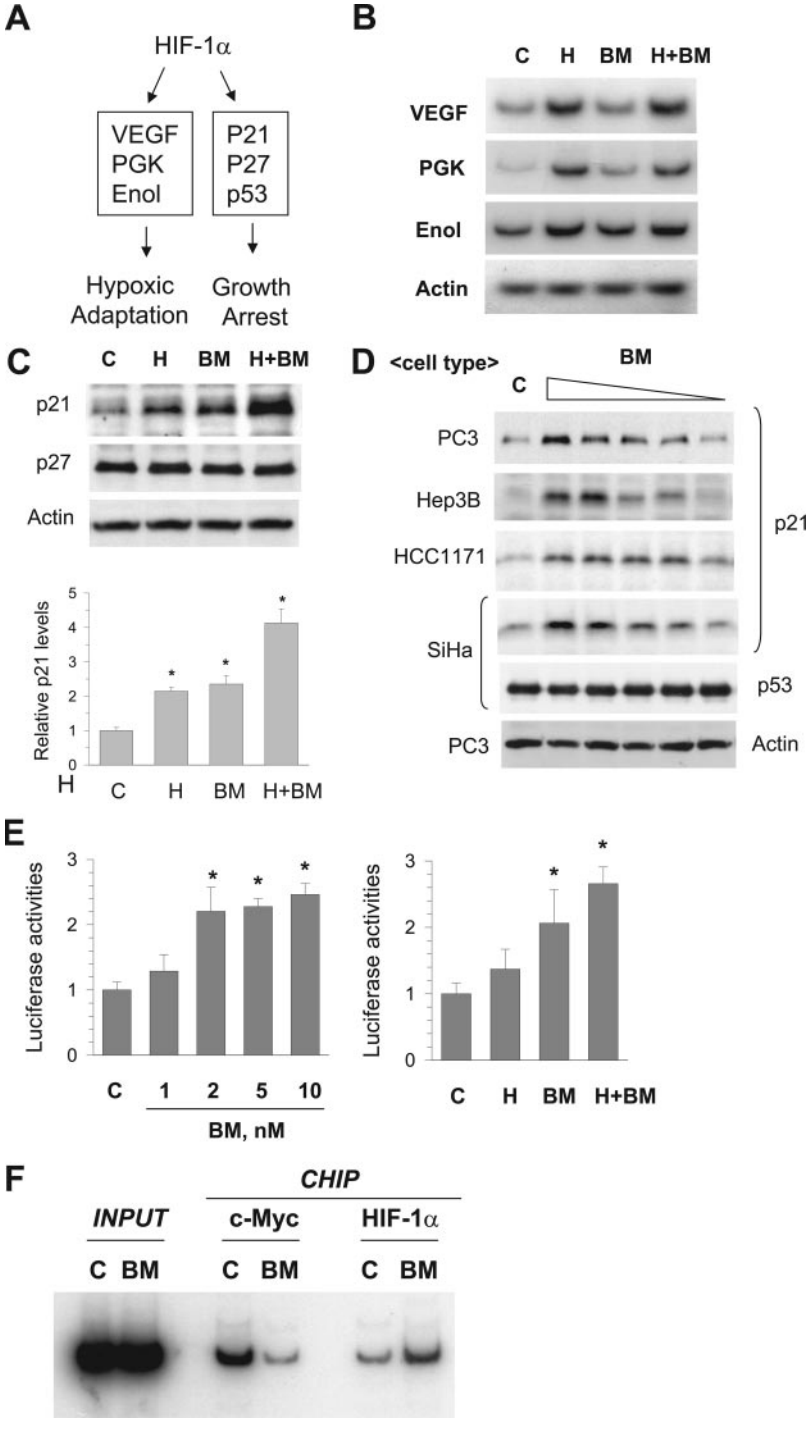

Fig. 3. Bafilomycin induces p21 expression. A, two different roles of HIF-1α. B, PC3 cells were incubated under normoxic or hypoxic conditions for 16 h in the absence or in the presence of 10 nM of BM. Expression of genes related to angiogenesis and glycolysis. mRNA levels of VEGF, phosphoglycerate kinase 1 (PGK), enolase 1 (Eno), and β-actin were determined by semiquantitative RT-PCR. C, normoxic control; H, hypoxia; BM, BM treatment; H+BM, BM treatment under hypoxic conditions. C, synergistic induction of p21 by BM and hypoxia. In PC3 cells, p27 and β-actin levels were analyzed by immunoblotting. Band intensities of p21 were quantified using the MCID-M4 image analysis system and are plotted on the bottom. Each point represents the mean and standard deviation of four separate experiments.*, $P < 0.05$ versus the normoxic control. D, bafilomycin-induced p21 expression in various cell lines. Four cancer cell lines were incubated with various concentrations (10, 5, 2, 1, or 0.2 nM) of BM for 4 h and then harvested for p21, p53, and β-actin immunoblotting. E, bafilomycin activates the p21 promoter. PC3 cells were transfected with a p21 promoter reporter plasmid, and treated with BM for 16 h. Luciferase activities are quoted as relative values versus the control value and are plotted as the means ± S.D. of four experiments. *, $P < 0.05$ versus the control. F, bafilomycin-induced HIF-1α dissociates c-Myc from p21 promoter. After PC3 cells were treated with 10 nM bafilomycin for 16 h, c-Myc or HIF-1α binding to p21 promoter was analyzed using chromatin immunoprecipitation. Proximal promoter DNAs of p21 were amplified by 32 cycles of PCR, electrophoresed, and autoradiographed.

model 4 (MCID-M4) image analysis system (Sony, Tokyo, Japan). The extent of necrosis was determined in four different cross-sections per tumor by dividing the total cross-sectional necrotic area by the total cross-sectional area. An ApopTag in situ apoptosis detection kit of Oncor (Gaithersburg, MD) was used to evaluate apoptotic death. Serial sections were dewaxed, treated with proteinase K, incubated with equilibration buffer for 10 min, and then incubated with working-strength TdT enzyme solution at 37°C for 2 h. The reaction was terminated by incubation in working-strength stop/wash buffer for 30 min at 37°C. Sections were then incubated with antidigoxigenin peroxidase and then with diaminobenzidine and 0.01% H_2O_2 for 5 min at room temperature. Finally, they were lightly counterstained with H&E and examined under an optical microscope.

Statistical Analysis. All data were analyzed using Microsoft Excel 2000 (Microsoft, Redmond, WA), and results are expressed as means and S.D. The Mann-Whitney *U* test (SPSS 10.0 for Windows; SPSS Inc., Chicago, IL) was used to compare reporter activities or protein levels in cultured cells. Tumor volumes of the control and the bafilomycin-treated groups were compared using analysis of variance followed by Duncan's multiple range test. Differences were considered statistically significant at the $P < 0.05$ level. All statistical tests were two-sided.

Results

HIF-1α Is Induced by Bafilomycin A1 or Concanamycin A. In eight cancer cell-lines, HIF-1α expression was increased in a dose-dependent manner by bafilomycin A1 (Fig. 1A, left column), whereas β-actin expression was not (data not shown). We examined the effect of another V-ATPase inhibitor, concanamycin A (Forgac, 1989), on HIF-1α expression, and found that it also induced HIF-1α (Fig. 1A, right column). HIF-1α expression in PC3 cells was increased in a time-dependent manner by bafilomycin A1 (Fig. 1B). When PC3 cells were incubated under hypoxic conditions, HIF-1α levels were synergistically increased by bafilomycin (Fig. 1C). To clarify the mechanism of HIF-1α induction by bafilomycin, we examined HIF-1α expression at the mRNA and protein levels. Bafilomycin A1 (BM) did not affect HIF-1α mRNA levels (Fig. 2A) but inhibited HIF-1α degradation to extend protein half-life by 4-fold (Fig. 2B). Proteasomal degradation of HIF-1α is preceded by the binding of pVHL and HIF-1α. We thus examined the effect of bafilomycin on the pVHL-HIF-1α interaction using immunoprecipitation and found that bafilomycin strongly inhibited the pVHL binding to HIF-1α. Bafilomycin seems to deregulate the pVHL-dependent HIF-1α degradation process. Recently, it was reported that HIF-1α can be stabilized under acidic conditions by nuclear sequestration of pVHL (Mekhail et al., 2004). Because the inhibition of V-ATPase could acidify the cytoplasm, we tested the possibility that this pH-dependent regulation of HIF-1α is responsible for the bafilomycin effect. However, the bafilomycin effect on HIF-1α was not attenuated in alkaline media (Fig. 2D). After treatment of bafilomycin, pVHL was not sequestered to the nucleus, whereas HIF-1α was present specifically in the nucleus (Fig. 2E). Moreover, an acridine orange staining showed that endosomal pH was not changed by 4-h treatment with 10 nM BM (Supplemental Result S1). These results suggest that the HIF-1α stabilization by bafilomycin is irrelevant to the pH-dependent regulation.

Bafilomycin Induces p21 Expression. HIF-1α is known to have two different functions: hypoxic adaptation and growth arrest (Fig. 3A). To determine the significance of bafilomycin-induced HIF-1α expression, we first analyzed mRNA levels of VEGF, phosphoglycerokinase 1, and enolase 1. However, these mRNAs were not increased significantly by bafilomycin, whereas they were up-regulated under hypoxic conditions (Fig. 3B). Moreover, bafilomycin did not enhance the transcriptional activity of HIF-1α in an erythropoietin enhancer-luciferase reporter system (data not shown). This suggests that HIF-1α induced by bafilomycin is less active than that by hypoxia. We next examined the expressions of cell cycle inhibitors p21, p27, and p53. In PC3 cells, p21(cip1/waf1) expression was increased synergistically by bafilomy-

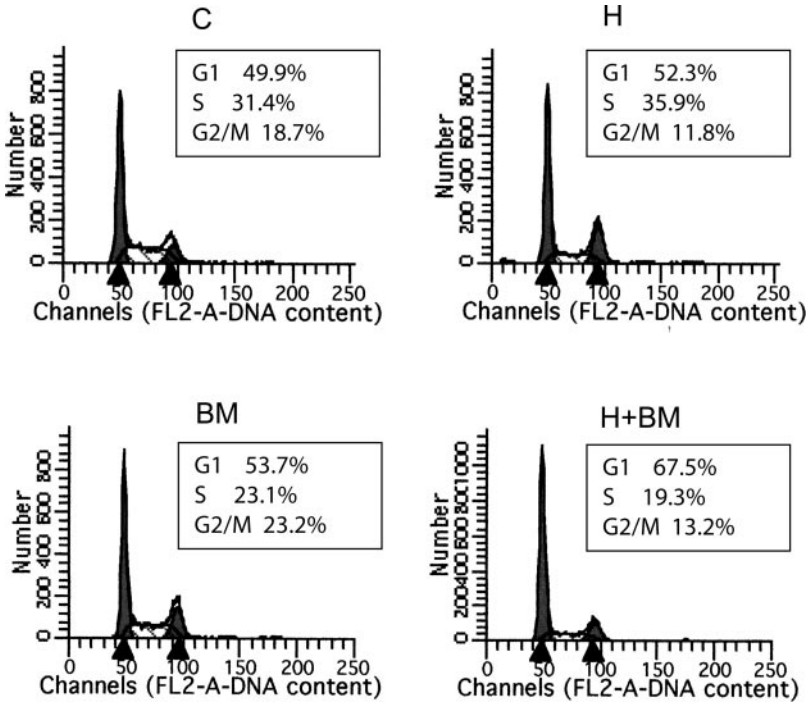

Fig. 4. Bafilomycin induces G_1 arrest under hypoxic conditions. PC3 cells were incubated under normoxic or hypoxic conditions for 16 h in the absence or presence of 10 nM BM. Cell cycle analysis was performed as described under *Materials and Methods*. C, normoxic control; H, hypoxia; BM, BM treatment; H+BM, BM treatment under hypoxic conditions.

cin and hypoxia in combination (Fig. 3C). However, p27 was not induced by bafilomycin, and p53 was not expressed in PC3 cells. Moreover, in four cancer cell lines, bafilomycin induced p21 in a dose-dependent manner but did not affect p53 expression (Fig. 3D). To examine whether bafilomycin stimulates p21 promoter activity, we made a luciferase reporter plasmid containing the p21 promoter. Figure 3E shows that bafilomycin enhanced p21 promoter activity in a dose-dependent manner (left) and further increased it under hypoxic conditions (right). Regarding the mechanism of p21 induction by HIF-1α, Koshiji et al. (2004) has demonstrated that HIF-1α dissociates c-Myc (a repressor) from p21 promoter and thereby derepresses p21. Therefore, we examined whether bafilomycin inhibits the c-Myc binding to p21 promoter via HIF-1α and found that in bafilomycin-treated cells, the c-Myc binding noticeably decreased, but the HIF-1α binding increased (Fig. 3F). This suggests that bafilomycin-induced HIF-1α counteracts c-Myc-mediated repression of p21 and expresses p21.

HIF-1α Is Essential for Bafilomycin-Induced p21 Expression and Cell Cycle Arrest. Because p21 functions as a cell cycle inhibitor, we examined whether bafilomycin induces cell cycle arrest via p21. As expected, the G_1 population of PC3 cells was increased synergistically by bafilomycin and hypoxia in combination (Fig. 4). To confirm that HIF-1α mediates bafilomycin-induced p21 expression and cell cycle arrest, we examined the effects of bafilomycin in HIF-1α(+/+) and HIF-1α(−/−) MEF cells. HIF-1α(+/+) cells showed a good response to bafilomycin in HIF-1α expression, but HIF-1α(−/−) cells failed to express HIF-1α (Fig. 5A). It is interesting that p21 induction and G_1 arrest did not occur in HIF-1α(−/−) cells, whereas both events were well-observed in HIF-1α(+/+) cells (Fig. 5, B and C). To examine whether p21 mediates the bafilomycin-induced cell cycle arrest, HCT116 cells were treated with bafilomycin. HIF-1α levels in both p21(+/+) and p21(−/−) cells were synergistically enhanced by bafilomycin and hypoxia, but p21 induction was observed only in p21(+/+) cells (Fig. 6A). Although HIF-1α was induced in both cell lines, bafilomycin did not arrest the cell cycle in p21(−/−) cells under hypoxic conditions, whereas it did in p21(+/+) cells (Fig. 6B).

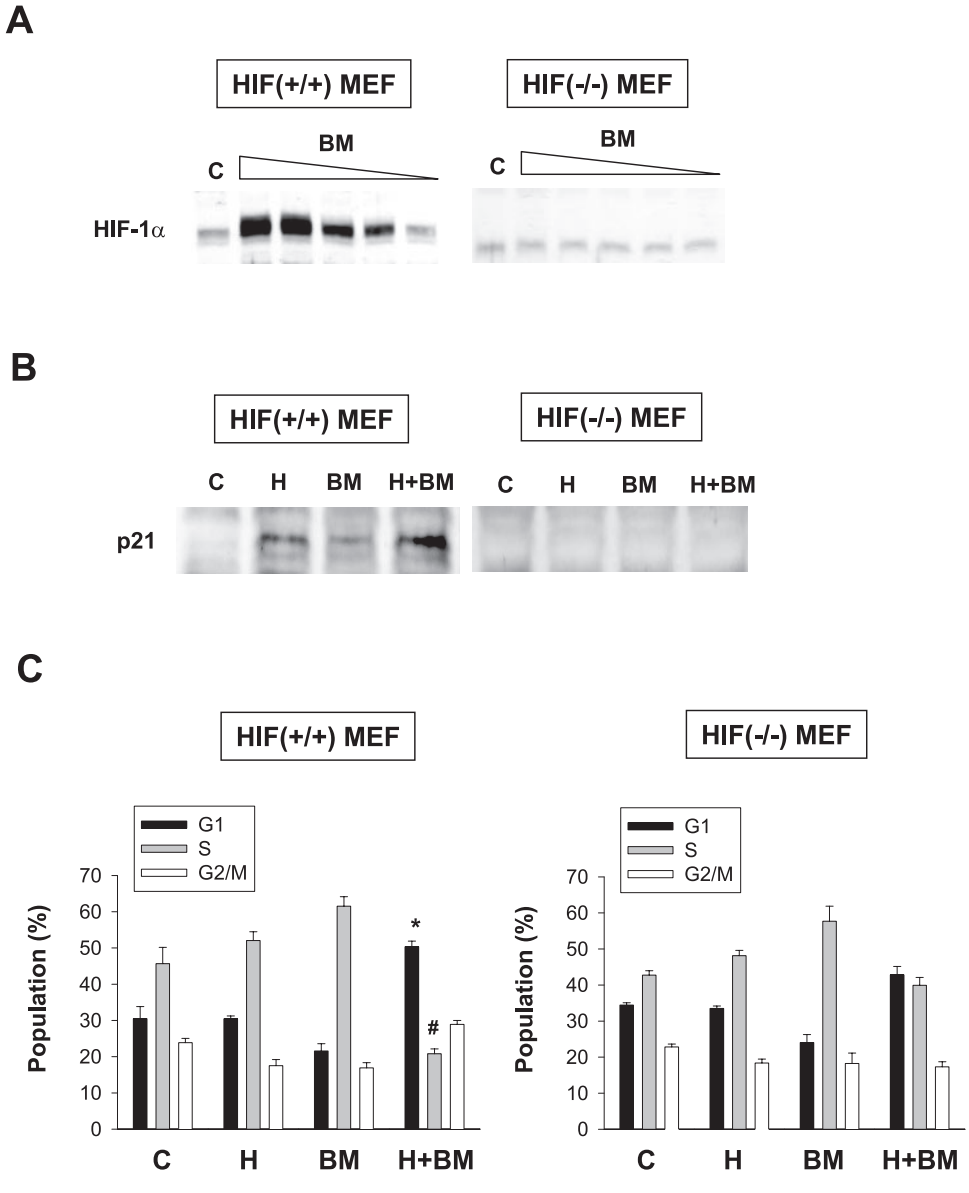

Fig. 5. HIF-1α is required for bafilomycin-induced p21 expression and cell cycle arrest. A, bafilomycin-induced HIF-1α expression in MEF cells. Wild-type (HIF-1α+/+) and HIF-1α null (HIF-1α−/−) MEF cells were treated with various concentrations (10, 5, 2, 1, or 0.2 nM) of BM for 4 h and then harvested for HIF-1α immunoblotting. B, bafilomycin-induced p21 expression. MEF cells were incubated under normoxic or hypoxic conditions for 16 h in the absence or presence of 10 nM BM and then harvested for p21 immunoblotting. C, HIF-1α-dependent cell cycle arrest. After 16-h incubation, the cell cycle was analyzed in HIF-1α(+/+) (left) or HIF-1α(−/−) (right) MEF cells. Each bar represents the mean and standard deviation of four experiments. C, normoxic control; H, hypoxia; BM, BM treatment; H+BM, BM treatment under hypoxic conditions. *, $P < 0.05$ versus G_1-phase population of the C group; #, $P < 0.05$ versus S-phase population of the C group.

HIF-1α Is Required for the Anticancer Action of Bafilomycin. The HIF-1α-dependent inhibition of cell proliferation could be a good strategy for solid tumor treatment because HIF-1α overexpression is a typical feature of tumors subjected to hypoxia or with oncogene or tumor suppressor gene mutations (Semenza, 2003). Thus, we examined in vivo the relation between the anticancer activity of bafilomycin and HIF-1α expression. Mice grafted with HIF-1α(+/+) or HIF-1α(−/−) fibrosarcomas were treated with a single peritoneal injection of bafilomycin. The top of Fig. 7, A and B, illustrates the gross morphologies of the tumors that were excised from the flanks of mice 4 days after vehicle or bafilomycin treatment. HIF-1α(+/+) tumors in mice treated with bafilomycin were much smaller than those in animals treated with vehicle only. Figure 7A shows the growth rate of the HIF-1α(+/+) tumor, plotted as average tumor volume versus time. Bafilomycin inhibited tumor growth significantly. However, bafilomycin failed to inhibit the growth of HIF-1α (−/−) tumor (Fig. 7B). These results indicate that HIF-1α expression is responsible for the anticancer effect of bafilomycin. In tumor histology, bafilomycin-treated HIF-1α(+/+) tumors showed disorganized cell layers and condensed chromatin (Fig. 7C) and frequent TUNEL-positive nuclei (Fig. 7D). Necrotic areas were markedly enhanced in bafilomycin-treated HIF-1α(+/+) tumors (Fig. 7E). We also confirmed that bafilomycin induced the expressions of HIF-1α and p21 in the tumors (Fig. 7F). However, none of these bafilomycin effects were observed in HIF-1α(−/−) tumors.

Discussion

In this study, we found that bafilomycin increased HIF-1α. HIF-1α induced by bafilomycin arrested the cell cycle in the G_1 phase by inducing p21, which is believed to be responsible for the anticancer effect of bafilomycin in vivo. In this work, we emphasize that the anticancer effect of bafilomycin in vivo seems likely to depend on exorbitant expression of HIF-1α in hypoxia, and this effect could provide a potential therapeutic strategy for solid tumors having a hypoxic region.

HIF-1α has been suggested to act as both a positive and a negative regulator of tumor growth. Many xenograft experiments using HIF-1α null tumors (Ryan et al., 2000) and HIF-1α inhibitors (Yeo et al., 2003) have demonstrated that HIF-1α is associated with tumor growth. On the contrary, Carmeliet et al. (1998) reported that HIF-1α null tumors grew unexpectedly rapidly even though tumor vessel formation was impaired. They also found that cancer cells in HIF-1α(−/−) tumors were more proliferative and less apoptotic than those in HIF-1α(+/+) tumors. Mack et al. (2003) also demonstrated that HIF-1α stabilization by VHL gene knockout decreased rather than increased tumor growth, despite the induction of HIF-1 target genes. Regarding this controversy surrounding the role of HIF-1α in tumor growth, a recent report may provide an answer (Blouw et al., 2003). HIF-1α(−/−) astrocytoma was found to grow slower in vessel-poor subcutaneous spaces with poor vascularity than in vessel-rich brain parenchyma. Thus, the differential roles of HIF-1α on tumor growth seem to depend on the extant microenvironment of the tumor. If so, under some circumstances, HIF-1α overexpression might be a better strategy for cancer therapy than its inhibition, and hence, the attractiveness of bafilomycin as an HIF-1α targeting anticancer drug is revealed. Moreover, "tumor-promoting effect" of HIF-1α predominantly depends on its transcriptional activity, whereas "tumor-inhibiting effect" of HIF-1α does not. Because bafilomycin treatment only induced the expression of HIF-1α with low transcriptional activity, it could specifically enhance the

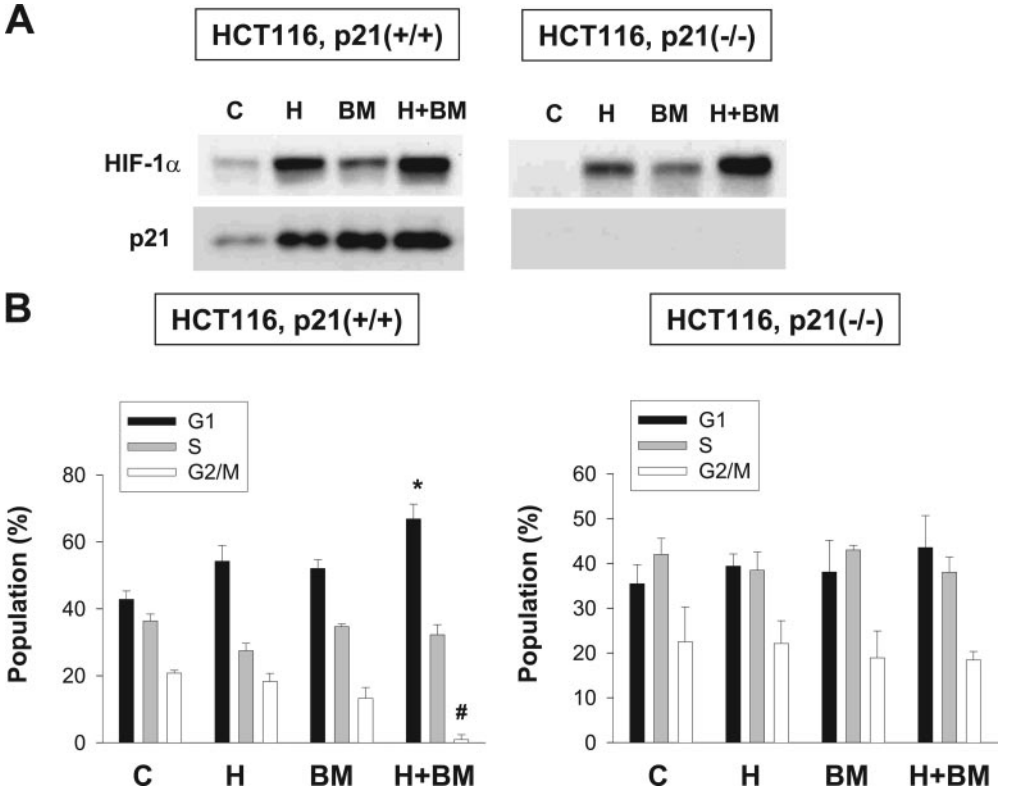

Fig. 6. p21 is required for cell cycle arrest by bafilomycin. A, bafilomycin-induced p21 expression in HCT116 cells. p21(+/+) and p21(−/−) HCT116 cells were incubated under normoxic or hypoxic conditions for 16 h in the absence or presence of 10 nM BM. HIF-1α and p21 levels were analyzed by immunoblotting method. B, BM-induced cell cycle arrest in HCT116 cells. After 16-h incubation, the cell cycle was analyzed in p21(+/+) (left) or p21(−/−) (right) HCT116 cells. Each bar represents the mean and standard deviation of four experiments. C, normoxic control; H, hypoxia; BM, BM treatment; H+BM, BM treatment under hypoxic conditions. *, $P < 0.05$ versus G_1-phase population of the C group; #, $P < 0.05$ versus G_2/M-phase population of the C group.

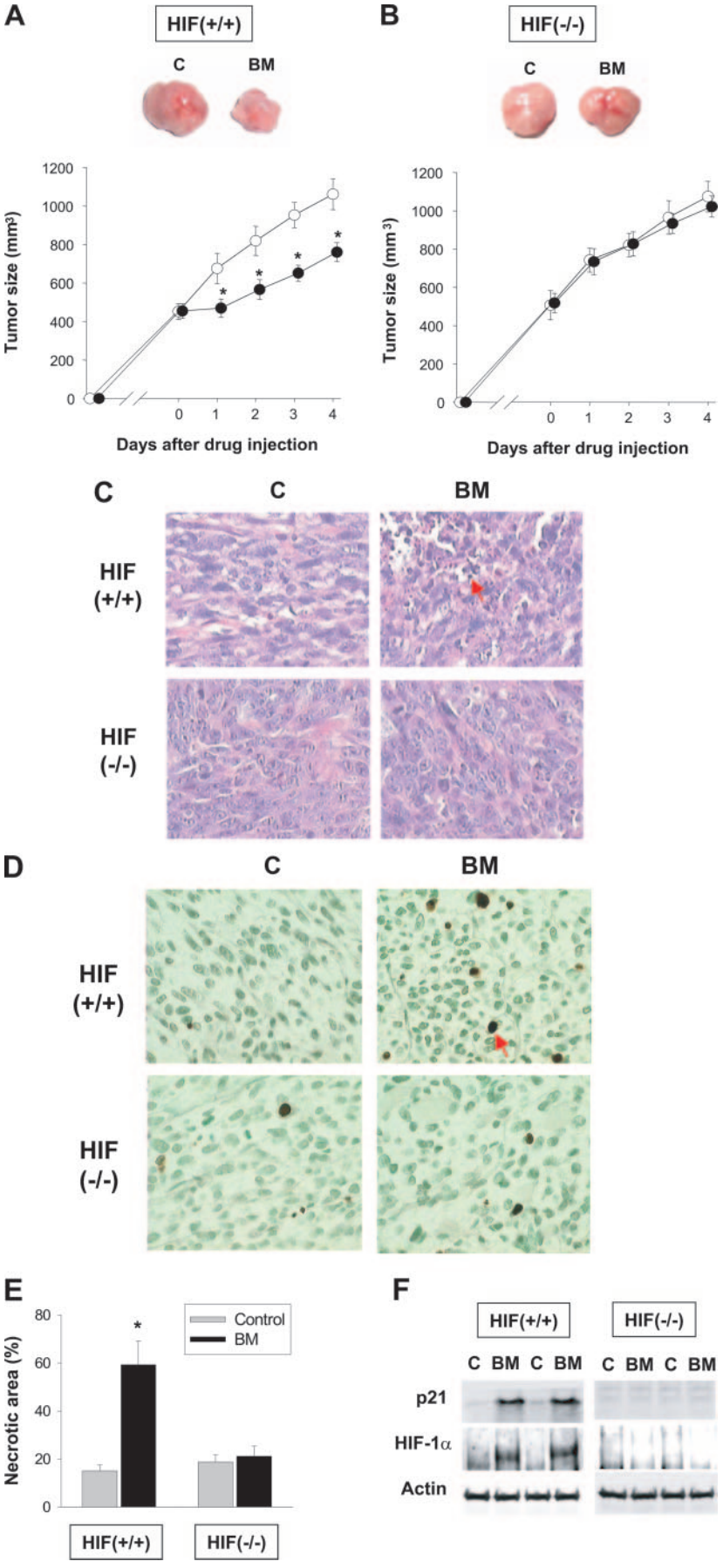

Fig. 7. HIF-1α is required for the anticancer activity of bafilomycin. Nude mice were injected subcutaneously in the flanks with HIF-1α(+/+) or HIF-1α(−/−) MEF cells. After tumors had reached 400 to 500 mm^3 in size, the mice were administered an intraperitoneal injection of BM (1 mg/kg) or vehicle (50 μl of dimethyl sulfoxide). Tumor sizes were subsequently measured daily for 4 days and then excised. The growth curves of HIF-1α(+/+) and HIF-1α(−/−) fibrosarcomas are plotted in A and B, respectively. Each data point represents the mean and standard deviation [$n = 9$ for HIF-1α(+/+) control; $n = 9$ for HIF-1α(+/+) BM-treated; $n = 9$ for HIF-1α(−/−) control; $n = 7$ for HIF-1α(−/−) BM-treated]. ∗, $P < 0.05$ versus the vehicle group. Illustrations at the top are tumors excised from mice 4 days after BM injection. C, tumor histology. Tumor sections were cut from paraffin blocks and stained with H&E. Cells showing chromatin condensation (arrow) were frequently found in BM-treated HIF-1α(+/+) tumors. Magnification, ×200. D, detection of apoptotic cells in tumors. Apoptotic cells were detected by TUNEL staining. TUNEL-positive nuclei (arrows) were frequently found in BM-treated HIF-1α(+/+) tumors. E, necrosis in tumors. Massive necrosis was found in BM-treated HIF-1α(+/+) tumors, whereas small, scattered areas of necrosis were present in BM-treated HIF-1α(−/−) tumors. The necrotic areas were evaluated in H&E-stained slides and are presented as percentages of total area. Each bar represents the mean and standard deviation of 24 or more specimens. ∗, $P < 0.05$ versus the vehicle group. F, p21 is induced only in BM-treated HIF-1α(+/+) tumors. Tumors were excised the day after BM injection and were frozen quickly in liquid nitrogen. Tumor lysates were assessed by immunoblotting p21, HIF-1α, and β-actin.

tumor-inhibiting effect. Therefore, bafilomycin may be a candidate for anticancer agent by exorbitantly inducing HIF-1α in hypoxic tumors.

Although bafilomycin induced HIF-1α levels under normoxic conditions, it has little effect on the expressions of HIF-1 targeted genes. This effect of bafilomycin on HIF-1α could be suspected of the factor-inhibiting HIF (FIH). In the absence of a hypoxic signal, HIF-1α can be inactivated by FIH. FIH hydroxylates an asparagine residue (Asn803) within the transactivation domain of HIF-1α, which blocks its recruitment of p300 coactivator (Lando et al., 2002). Moreover, under hypoxic conditions, asparagine hydroxylation is inhibited due to limited oxygen, and HIF-1α remains unmodified and activated. This seems to be one of the reasons why HIF-1α induced by bafilomycin has little transcriptional activity; similarly, HIF-1α induced by heat shock was found to have no transcriptional activity (Katschinski et al., 2002). However, HIF-1α induced by bafilomycin induced p21 transcription. Recently, Koshiji et al. (2004) demonstrated the mechanism of p21 induction by HIF-1α (i.e., c-Myc represses p21 transcription): HIF-1α displaces c-Myc binding from p21 promoter and thereby derepresses p21. They also demonstrated that the N terminus of HIF-1α without the transactivation domain is sufficient to antagonize c-Myc. Therefore, HIF-1α induced by bafilomycin can induce p21 expression despite a low transcriptional activity.

Because c-Myc also represses p27 transcription by direct binding of Myc/Max complexes in p27 promoter, HIF-1α can derepress p27 by counteracting c-Myc, as it does p21 (Gartel and Shchors, 2003). Indeed, several reports demonstrated that HIF-1α mediates the hypoxic induction of p27 (Goda et al., 2003; Mack et al., 2005). If so, p27 could be induced by bafilomycin treatment, but it was not induced in our experimental settings. We think this discrepancy could result from differences in p21 and p27 responses to HIF-1α; p27 responds to HIF-1α less sensitively than p21, which can be supported by the following reports. After 48-h hypoxia, p21 mRNA levels increased by 4-fold, whereas p27 mRNA levels increased by approximately 90% in HIF-1α(+/+) B cells (Goda et al., 2003). Likewise, Mack et al. (2005) also demonstrated that p21 transcription was induced by 9-fold in hypoxia, but p27 transcription was induced by 1-fold. Moreover, p21 mRNA levels of VHL(−/−) MEF (overexpressing HIF-1α) markedly increased 140-fold versus those of VHL(+/+) MEF, whereas p27 mRNA levels increased just 9-fold. In the present study, we also found that p27 was induced by expressed HIF-1α less sensitively than p21 (Supplemental Result S2). Based on these results, it is speculated that the amount of bafilomycin-induced HIF-1α is enough for p21 induction but not for p27 induction.

The in vivo effect of bafilomycin on tumor growth has been evaluated previously in xenografted pancreatic tumors (Ohta et al., 1998). Bafilomycin A1 (1 mg/kg) was injected once daily into nude mice from when tumors reached approximately 50 mm^3. However, they did not observe a significant retardation in tumor growth until tumors reached 300 mm^3, which required 21 injections. However, when we injected mice having fibrosarcoma with 1 mg/kg bafilomycin A1, we observed its inhibitory effect on tumor growth the following day. In view of the fact that the cell proliferation inhibition by bafilomycin depends on HIF-1α levels and that this is synergistically enhanced by hypoxia, it is believed that bafilomycin can inhibit the growth of large tumors subjected to hypoxia more effectively than that of small tumors, which explains its dramatic effect on 400- to 500-mm^3 tumors. If so, bafilomycin may be a useful therapeutic agent for large solid tumors.

HIF-1α is tightly regulated by oxygen tension. Under aerobic conditions, HIF-1-prolyl hydroxylases hydroxylate Pro402 and Pro564 of HIF-1α using molecular oxygen, and then the pVHL-E3 ubiquitin ligase complex binds to the hydroxylated HIF-1α, which results in ubiquitination and proteasomal degradation of HIF-1α (Epstein et al., 2001; Jaakkola et al., 2001). Under hypoxic conditions, however, HIF-1α hydroxylation is inhibited due to the limitation of oxygen, thereby precluding the binding of pVHL and stabilizing HIF-1α. In addition, a recent report demonstrated that pVHL was localized to the nucleoli under acidic (~pH 6.0) conditions, whereas it was present diffusely in the nucleus and the cytoplasm under neutral pH conditions (Mekhail et al., 2004). They also found that the nucleolar localization of pVHL restricted the pVHL binding to HIF-1α and caused the stabilization of HIF-1α. Considering the acidifying effect of bafilomycin, it is plausible that HIF-1α stabilization by bafilomycin is attributable to intracellular acidosis and nucleolar sequestration of pVHL. However, the HIF-1α stabilization by bafilomycin occurred even under alkaline pH conditions, and pVHL was not redistributed by bafilomycin. Furthermore, we found that the endosomal pH was not noticeably changed by 10 nM bafilomycin, whereas it was by 100 nM bafilomycin. Intracellular pH is unlikely to be changed by treating cells with bafilomycin at a low concentration (10 nM) and for a short time (4 h). Therefore, these results suggest that bafilomycin stabilizes HIF-1α via other mechanisms rather than via acidification. The mechanism underlying bafilomycin-induced HIF-1α stabilization remains to be investigated in the next study.

Acknowledgments

We thank Dr. Deug Y Shin (Dankook University College of Medicine) for the HCT116 cell lines.

References

Bacon AL and Harris AL (2004) Hypoxia-inducible factors and hypoxic cell death in tumour physiology. *Ann Med* **36:**530–539.

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.

Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, and Bergers G (2003) The hypoxic response of tumors is dependent on their microenvironment. *Cancer Cell* **4:**133–146.

Bowman EJ, Graham LA, Stevens TH, and Bowman BJ (2004) The bafilomycin/concanamycin binding site in subunit c of the V-ATPases from *Neurospora crassa* and *Saccharomyces cerevisiae*. *J Biol Chem* **279:**33131–33138.

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, et al. (1998) Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature (Lond)* **394:**485–490.

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.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, et al. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107:**43–54.

Forgac M (1989) Structure and function of vacuolar class of ATP-driven proton pumps. *Physiol Rev* **69:**765–796.

Gartel AL and Shchors K (2003) Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. *Exp Cell Res* **283:**17–21.

Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, and Johnson RS (2003) Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. *Mol Cell Biol* **23:**359–369.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim AV, Hebestreit HF, Mukherji M, Schofield CJ, et al. (2001) Targeting of HIF-alpha to

the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science (Wash DC)* **292:**468–472.
Jung JE, Lee HG, Cho IH, Chung DH, Yoon SH, Yang YM, Lee JW, Choi S, Park JW, Ye SK, et al. (2005) STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells. *FASEB J* **19:**1296–1298.
Katschinski DM, Le L, Heinrich D, Wagner KF, Hofer T, Schindler SG, and Wenger RH (2002) Heat induction of the unphosphorylated form of hypoxia-inducible factor-1α is dependent on heat shock protein-90 activity. *J Biol Chem* **277:**9262–9267.
Kinoshita K, Waritani T, Noto M, Takizawa K, Minemoto Y, Nishikawa A, and Ohkuma S (1996) Bafilomycin A1 induces apoptosis in PC12 cells independently of intracellular pH. *FEBS Lett* **398:**61–66.
Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, and Huang LE (2004) HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *EMBO (Eur Mol Biol Organ) J* **23:**1949–1956.
Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, and Bruick RK (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* **16:**1466–1471.
Mack FA, Patel JH, Biju MP, Haase VH, and Simon MC (2005) Decreased growth of Vhl−/− fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. *Mol Cell Biol* **25:**4565–4578.
Mack FA, Rathmell WK, Arsham AM, Gnarra J, Keith B, and Simon MC (2003) Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. *Cancer Cell* **3:**75–88.
Mekhail K, Gunaratnam L, Bonicalzi ME, and Lee S (2004) HIF activation by pH-dependent nucleolar sequestration of VHL. *Nat Cell Biol* **6:**642–647.
Nakashima S, Hiraku Y, Tada-Oikawa S, Hishita T, Gabazza EC, Tamaki S, Imoto I, Adachi Y, and Kawanishi S (2003) Vacuolar H+-ATPase inhibitor induces apoptosis via lysosomal dysfunction in the human gastric cancer cell line MKN-1. *J Biochem (Tokyo)* **134:**359–364.
Ohkuma S, Shimizu S, Noto M, Sai Y, Kinoshita K, and Tamura H (1993) Inhibition of cell growth by bafilomycin A1, a selective inhibitor of vacuolar H^+-ATPase. *In Vitro Cell Dev Biol Anim* **29A:**862–866.
Ohta T, Arakawa H, Futagami F, Fushida S, Kitagawa H, Kayahara M, Nagakawa T, Miwa K, Kurashima K, Numata M, et al. (1998) Bafilomycin A1 induces apoptosis in the human pancreatic cancer cell line Capan-1. *J Pathol* **185:**324–330.
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 (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3:**721–732.
Wang GL and Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* **270:**1230–1237.
Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS, and Park JW (2003) YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. *J Natl Cancer Inst* **95:**516–525.
Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, and Semenza GL (2000) Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. *Cancer* **88:**2606–2618.
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. Y. S. Chun, Department of Physiology, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea. E-mail: chunys@snu.ac.kr