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# Baicalein Induces Functional Hypoxia-Inducible Factor-1$\alpha$ and Angiogenesis[S]

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## ABSTRACT

Targeting the oxygen-sensing mechanisms of the hypoxia-inducible factor (HIF) pathway provides pharmacological ways of manipulating the HIF response. Because HIF-1$\alpha$-specific prolyl-4 hydroxylases (PHDs) prime degradation of HIF-1$\alpha$, we have made an effort to find a small molecule capable of up-regulating the HIF pathway by inhibiting prolyl hydroxylation. Through an in vitro high-throughput screen, we have discovered a PHD2 inhibitor baicalein, which is also found to abrogate asparaginyl hydroxylation of HIF-1$\alpha$. Such inhibitory effects are reversed by the addition of excess 2-oxoglutarate and iron(II), suggesting the involvement of baicalein's binding at the enzyme active sites, which has also been corroborated by spectroscopic binding assays between baicalein and enzyme. In addition, baicalein suppresses ubiquitination of HIF-1$\alpha$, which works in concert with the inhibition of the HIF-specific hydroxylases to increase the HIF-1$\alpha$ content, leading to induction of HIF-1-mediated reporter gene activity and target gene transcription in tissue culture cells, whereas it induces HIF-independent activation of other genes. Furthermore, in vivo organ models based on the chick chorioallantoic membrane assay demonstrate that baicalein promotes new blood vessel formation. Together, our results indicate that baicalein possesses a proangiogenic potential and thus might have the therapeutic utility in the treatment of ischemic diseases.

The hypoxic response in animals involves coordinated regulation of the expression of numerous genes that participate in development, physiology, and pathogenesis of cancer and ischemic disease (Pugh and Ratcliffe, 2003; Semenza, 2004). At the transcription level, hypoxia-inducible factor (HIF)-1, composed of an $\alpha/\beta$ heterodimer, mediates cellular adaptive processes to low oxygen availability as a pivotal transcription factor. Although both subunits are constitutively expressed, HIF-1$\alpha$ but not HIF-1$\beta$ is rapidly degraded during normoxia through the ubiquitin-proteasome system. HIF-1$\alpha$ proteolysis is controlled by hydroxylation of specific proline residues in HIF-1$\alpha$, which renders it able to recognize the von Hippel-Lindau tumor suppressor protein (VHL) in a protein complex of VHL-Elongin B-Elongin C (VBC) (Epstein et al., 2001; Ivan et al., 2001). Prolyl hydroxylation is catalyzed in an oxygen-sensitive manner by HIF-1$\alpha$-specific prolyl-4 hydroxylases (PHDs), which require oxygen and 2-oxoglutarate (2-OG) as cosubstrates, and iron(II) and ascorbic acid as cofactors (Bruick and McKnight, 2001). Among PHDs, PHD2 is known to serve as the critical oxygen sensor setting the low steady-state levels of HIF-1$\alpha$ in normoxia (Berra et al., 2003). HIF-1$\alpha$ also possesses a highly conserved site of asparaginyl hydroxylation within the C-terminal transactivation domain

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**ABBREVIATIONS:** HIF, hypoxia-inducible factor; VHL, von Hippel-Lindau tumor suppressor protein; VBC, von Hippel-Lindau protein-Elongin B-Elongin C; PHD, hypoxia-inducible factor-1$\alpha$-specific prolyl-4 hydroxylase; 2-OG, 2-oxoglutarate; FIH-1, factor-inhibiting hypoxia-inducible factor-1; HRE, hypoxia-response element; VEGF, vascular endothelial growth factor; FP, fluorescence polarization; GST, glutathione transferase; DMSO, dimethyl sulfoxide; B-P564, biotin-DLDLEALAPYIPADDDFQLR; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; RT, reverse transcriptase; PCR, polymerase chain reaction; CAM, chorioallantoic membrane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; CQ, clioquinol; shRNA, short hairpin RNA; bFGF, basic fibroblast growth factor; B, baicalein; H, hypoxia.

catalyzed by the additional iron(II)- and 2-OG-dependent enzyme factor-inhibiting HIF-1 (FIH-1) (Lando et al., 2002). This modification prevents recruitment of the transcriptional coactivator cAMP response element-binding protein-binding protein/p300. Thus, the presence of sufficient oxygen inactivates the HIF system by a dual process involving proteolytic destruction and inactivation of transcriptional activity. In contrast, the reduction in the activities of HIF-specific hydroxylases under hypoxia leads to stabilization of HIF-1$\alpha$, which forms a dimeric complex with HIF-1$\beta$. The complex then translocates to the nucleus where it binds to the hypoxia-response element (HRE) in the 5′-flanking regions of the target genes, including vascular endothelial growth factor (VEGF) and other cytoprotective proteins, thereby promoting hypoxic tolerance (Pugh and Ratcliffe, 2003; Semenza, 2004).

Efforts to target key components of signal transduction pathways have resulted in therapeutic successes, often through inhibition of upstream signal mediators. In signaling of tissue oxygen homeostasis, the master mediator HIF-1 regulates antiangiogenic and proangiogenic factors. Thus, considerable interest has been shown in the inhibition of angiogenesis for the treatment of cancer and conversely in the activation of angiogenesis for the treatment of ischemic disorders (Carmeliet, 2000; Maxwell and Ratcliffe, 2002; Brahimi-Horn and Pouysségur, 2007). Because of the primary role of the HIF-specific hydroxylases in regulation of HIF-1$\alpha$ protein levels, inhibition of the hydroxylases has been predicted to produce activation of downstream signals in the HIF system with enhanced transcription of the angiogenic factor VEGF, which might be beneficial in therapeutic angiogenesis.

We previously developed a convenient fluorescence polarization (FP)-based assay for quantitative measurements of prolyl hydroxylase activities (Cho et al., 2005). The present study uses this FP-based assay to screen a collection of 1040 biologically active compounds. Among the hit compounds as potential inhibitors for PHD2, we chose baicalein, which is a major component of the dried root of *Scutellaria baicalensis*, which is widely used in traditional Chinese medical applications. It has been found to exhibit antibacterial, free radical scavenging, antioxidant, anticancer, anti-inflammatory, and lipoxygenase-inhibitory activities (Ding et al., 1999; Gao et al., 1999; Shen et al., 2003; Zhang et al., 2003a), in addition to antiangiogenic activity shown in vivo and in vitro (Liu et al., 2003). Despite various interesting reports on the beneficial effects of baicalein, its proangiogenic potential has not yet been demonstrated. Accordingly, we explore the effects of baicalein on the HIF pathway in molecular, cellular, and organ levels, which unravel its unrecognized biological activity.

## Materials and Methods

**Cells, cDNAs, Proteins, and Reagents.** Human HepG2 hepatoma cells, human SH-SY5Y neuroblastoma cells, and mouse 3T3-L1 fibroblast cells were obtained from American Type Culture Collection (Manassas, VA) and maintained as recommended. The cells were incubated in a hypoxic incubator (Thermo Fisher Scientific, Waltham, MA) at 37°C to induce hypoxia. HIF-1$\alpha$ (GenBank accession number U22431), VHL (GenBank accession number NM000551), Elongin B (GenBank accession number NM007108), Elongin C (GenBank accession number NM005648), PHD2 (GenBank accession number AJ310543), and FIH-1 (GenBank accession number AF395830) human cDNAs were used as expression vectors. The p(HRE)$_4$-luc reporter plasmid (Hur et al., 2001) was used for reporter assays. Truncated GST-PHD2 and His-FIH-1 proteins for enzymatic reactions were expressed and purified as described previously (Cho et al., 2005, 2007). Baicalein, 2-OG, ascorbic acid, and iron(II) sulfate heptahydrate were purchased from Sigma-Aldrich (St. Louis, MO), cinnamyl-3,4-dihydroxy-$\alpha$-cyanocinnamate was from BIOMOL Research Laboratories (Plymouth Meeting, MA), and anti-human HIF-1$\alpha$ was from BD Biosciences Pharmingen (San Diego, CA). Culture media were obtained from Invitrogen (Carlsbad, CA), and fetal bovine serum was from Lonza Verviers SPRL (Verviers, Belgium). All other chemicals were of the highest grade of purity commercially available.

**Screening for PHD2 Inhibitors.** NINDS Custom Collection II (MicroSource Discovery Systems, Gaylordsville, CT) was used for screening potential inhibitors against PHD2 activities. An FP-based assay was performed using proteins and peptides prepared as described previously (Cho et al., 2005). A peptide substrate F-P564 (fluorescein isothiocyanate-aminocaproic acid-DLDLEALAPYIPADDDFQLR) at the final concentration of 1 $\mu$M was incubated with 0.2 $\mu$g/$\mu$l recombinant GST-PHD2 in NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) containing 200 $\mu$M ascorbic acid and 20 $\mu$M 2-OG in the presence of 20 $\mu$M compounds for 1 h at 25°C. The reaction mixtures containing DMSO alone with or without GST-PHD2 were included as positive or negative controls, respectively. The reactions were terminated by heating the reactants for 1 min at 95°C, followed by dilution to a final peptide concentration of 100 nM in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, and 0.25% Nonidet P-40) in the presence of 250 nM GST-VBC. The mixtures were gently mixed and transferred to 96-well plates, and their FP values were measured by a Wallac 1420 VICTOR$^2$ (PerkinElmer Life and Analytical Sciences, Boston, MA). After subtracting the FP value of the negative control, the percentage of inhibition was calculated based on the FP value of the positive control.

**Mass Spectrometric Analyses of PHD2 and FIH-1 Activities.** B-P564 (biotin-DLDLEALAPYIPADDDFQLR) synthesized by Anygen (KwangJu, Korea) was used to analyze prolyl hydroxylation of HIF-1$\alpha$ by mass spectrometry. The B-P564 peptide at 3 $\mu$M was mixed with 0.20 $\mu$g/$\mu$l GST-PHD2 in NETN buffer containing 400 $\mu$M ascorbic acid and 100 $\mu$M 2-OG. For asparaginyl hydroxylation, 4 $\mu$M F-HIF-1$\alpha$-(788-822; fluorescein isothiocyanate-aminocaproic acid-DESGLPQLTSYDCEVNAPIQGSRNLLQGEELLRAL) was mixed with 0.55 $\mu$g/$\mu$l His-FIH-1 in hydroxylation buffer (20 mM Tris-HCl, pH 7.5, 5 mM KCl, and 1.5 mM $MgCl_2$) containing 400 $\mu$M ascorbic acid and 100 $\mu$M 2-OG. After incubation for 2 h at 25°C, the reactants were desalted with ZipTip$_{C18}$ (Millipore Corporation, Billerica, MA), and eluted from the tip by addition of $\alpha$-cyano-4-hydroxycinnamic acid in 50% acetonitrile/50% water containing 0.1% trifluoroacetic acid. The purified peptide solutions obtained after extensive washing with 0.1% trifluoroacetic acid in water were transferred to a MALDI sample plate, and MALDI-TOF measurements were performed with a Voyager analyzer (Applied Biosystems, Foster City, CA).

**UV-Visible Spectroscopic Measurements for Iron(II)-Baicalein Binding.** Oxygen was removed from ultrapure water by sonication for 30 min, which was used immediately for solution preparations to minimize the oxidation of baicalein. Baicalein at 24 $\mu$M was dissolved in 10 mM phosphate buffer, pH 7.4, and mixed with varying concentrations of iron(II) sulfate, followed by recording of absorption spectra from 230 to 500 nm using a Libra S22 UV-visible spectrometer (Biochrom, Berlin, Germany).

**Immunoblotting.** HepG2, SH-SY5Y, and 3T3L1 cells were grown to 80% confluence on 60-mm tissue culture plates, and whole cell extracts were prepared as described previously (Hur et al., 2001). For immunoblotting, whole cell lysates were resuspended in SDS sample buffer, boiled for 5 min, and run on SDS-polyacrylamide gel electrophoresis gels, followed by transfer of the proteins to nitrocellulose membranes by semidry transfer (Trans-Blot SD; Bio-Rad, Hercules, CA). Proteins were then reacted with anti-human HIF-1$\alpha$ antibody and/or with anti-Hsp70 or anti-14-3-3$\gamma$ antibody, and visualized by

enhanced chemiluminescence according to the manufacturer's instructions (Pierce Chemical, Rockford, IL), with anti-mouse Ig conjugated with horseradish peroxidase as a secondary antibody. Weak signals from protein bands on Western blots were visualized with a luminescence image analyzer (LAS-3000; Fuji, Tokyo, Japan).

**Reverse Transcriptase-PCR.** HepG2 and SH-SY5Y cells were treated with baicalein or clioquinol (CQ) at indicated concentrations in normoxic conditions for 16 h. One-microgram aliquots of isolated total RNA were used for reverse transcription. To amplify and visualize the cDNAs of VEGF and CA9 mRNA or 18S RNA, two sets of primers were used: for VEGF (AF022375), forward primer (ccatgaactttctgctgtctt) and reverse primer (atcgcatcaggggcacacag); for CA9, forward primer (ctgtcactgctgcttctgat) and reverse primer (tcctctccaggtagatcctc); for 18S rRNA (X03205), forward primer (accgcagctaggaataatggaata) and reverse primer (ctttcgctctggtccgtctt). After an initial melt at 95°C for 10 min, 27 cycles of amplification (95°C for 45 s, 56°C for 45 s, and 72°C for 60 s) were performed with 1 $\mu$M concentrations of each primer. For PCRs, a final 5-min extension at 72°C was performed, and the amplified products were analyzed on 1% ethidium bromide-stained agarose gels. The expression levels were measured with a luminescence image analyzer (LAS-3000; Fuji).

**Quantitative Real-Time RT-PCR.** Quantitative real-time RT-PCR was performed on the iQTM SYBR Green Supermix using MyiQ single color real-time PCR detection system (Bio-Rad). Oligonucleotide primers were designed as reported previously (Zhang et al., 2003b; Murakami et al., 2007) for VEGF, CA9, and BINP3 mRNA or 18S rRNA: for human VEGF, forward primer (aaccatgaactttctgctgtcttg) and reverse primer (ttcaccacttcgtgatgattctg); for mouse VEGF(120), forward primer (gccagcacatagagagaatgagc) and reverse primer (cggcttgtcacatttttctgg); for human CA9, forward primer (cagttgctgtctctcttgga) and reverse primer (tgaagtcagagggcaggagtg); and for mouse BINP3, forward primer (ttaaagggtgcgtgcgggttatct) and reverse primer (aaggcgagaatcctcatcctgcaa). All PCRs were performed in triplicate, and the expression levels normalized to that of 18s rRNA are presented as the averages of at least three experiments.

**Transient Transfection and Luciferase Assay.** Transfections were carried out using the Lipofectamine Plus reagent according to the manufacturer's instructions (Invitrogen). For luciferase assays, $5 \times 10^4$ HepG2 or 3T3-L1 cells in 24-well plates were transfected with 200 ng of hypoxia reporter plasmid $p(HRE)_4$-luc containing four copies of HRE and 50 ng of plasmid pCHO110 for $\beta$-galactosidase (Promega, Madison, WI). Cells were treated with baicalein and clioquinol at indicated concentrations or hypoxia 16 h before harvest. Cell extracts were prepared 48 h after transfection and analyzed with a luminometer (Turner Designs, Sunnyvale, CA) using the Luciferase Assay System (Promega). Measured luciferase activities were normalized for total protein concentrations, as determined by Bradford assay (Bio-Rad).

**Chorioallantoic Membrane Assay.** Fertilized chick eggs were maintained in a humidified incubator at 37°C with 55% humidity. After incubation for 10 days, a hypodermic needle was used to remove ~2 ml of egg albumin, and the chorioallantoic membrane (CAM) and yolk were allowed to drop away from the shell membrane. On day 10.5, the shell (1 $cm^2$) was punched out and the shell membrane was peeled away. For angiogenesis testing, sterile circular filter paper (0.5 cm in diameter; Whatman, Maidstone, UK) loaded with 10 $\mu$l of sample was air-dried and applied to the CAM surface on the Y-shaped vascular branch point. Ten percent fat emulsion (1–2 ml) was injected into the chorioallantois 3 days later, and it was observed under a microscope. When the CAM showed an avascular zone of >3 mm in diameter, the response was scored as positive.

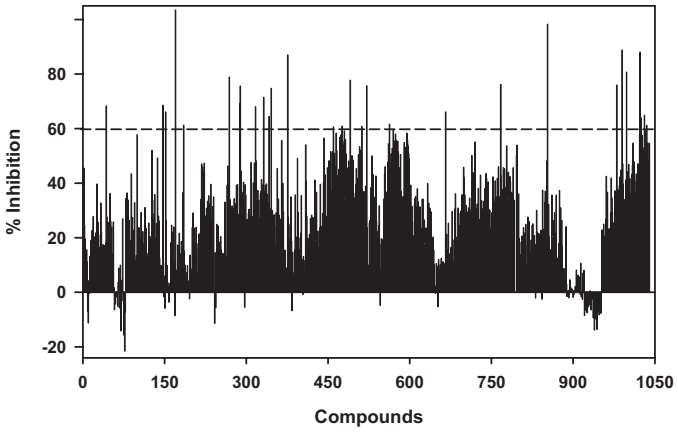


**Fig. 1.** Screen for PHD2 inhibitors. An FP-based assay for PHD2 activity was used for screening 1040 compounds in NINDS Custom Collection II. F-P564 peptide at 1 $\mu$M was incubated with 0.2 $\mu$g/$\mu$l GST-PHD2 in NETN buffer containing 200 $\mu$M ascorbic acid and 20 $\mu$M 2-OG in the presence of 20 $\mu$M chemicals for 1 h at 25°C. Then, 500 nM VBC was added to reactants diluted to a final peptide concentration of 100 nM in EBC buffer, followed by FP measurements. The percentage of inhibition was calculated and presented with a dotted line at 60% inhibition.

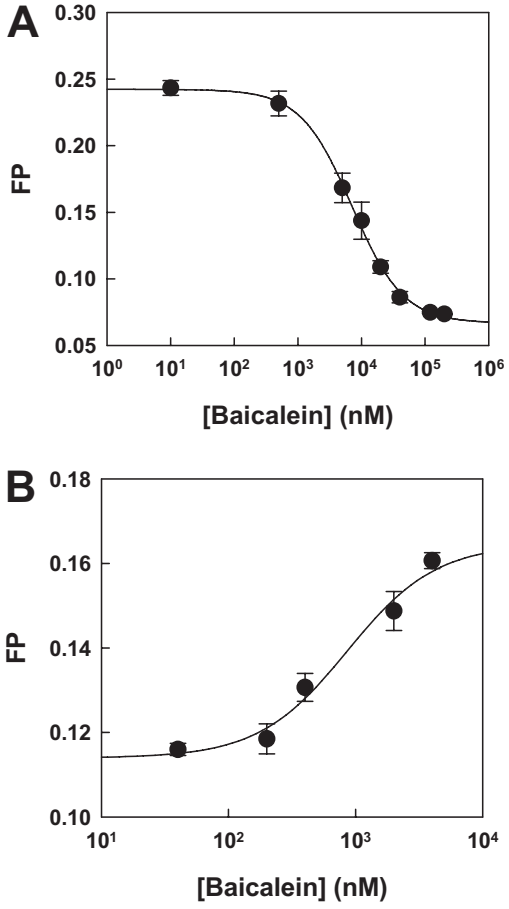


**Fig. 2.** Inhibitory effects of baicalein on hydroxylase activities. A, prolyl hydroxylation reactions were performed with varying concentrations of baicalein. F-P564 peptide at 1 $\mu$M was reacted with 0.05 $\mu$g/$\mu$l GST-PHD2 in NETN buffer containing 200 $\mu$M ascorbic acid and 20 $\mu$M 2-OG for 15 min at 25°C. The reactants were diluted in EBC buffer to yield a final peptide concentration of 100 nM in the presence of 500 nM VBC, followed by FP measurements. Each point represents the average of triplicate assays ± S.D. B, asparaginyl hydroxylation reactions by FIH were performed with varying concentrations of baicalein. F-HIF-1$\alpha$-(786-826) peptide at 1 $\mu$M was reacted with 0.55 $\mu$g/$\mu$l His-FIH in hydroxylation buffer containing 400 $\mu$M ascorbic acid and 100 $\mu$M 2-OG for 2 h at 25°C. The reactants were diluted in EBC buffer to yield a final peptide concentration of 100 nM in the presence of 400 nM GST-p300, followed by FP measurements. Each point represents the average of duplicate assays.

**MTT Assay.** HepG2 and SH-SY5Y cells were cultured on 96-well culture plates at $1.5 \times 10^5$ cells/ml for 24 h, and they were treated with varying concentrations of baicalein for 24 and 48 h. After the culture medium was replaced to prevent any possible background absorbance arising from baicalein itself, the assay was performed using TACS MTT Assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. In brief, 10 $\mu$l of MTT reagent was added to each well, followed by incubation for 4 h at 37°C. Then, 100 $\mu$l of detergent reagent was added to the wells, the plate was left in the dark for 16 h at room temperature, and the absorbance of each well was measured at 570 nm with a reference wavelength of 650 nm.

## Results

**Screening Inhibitory Compounds against PHD2 Activity.** We have previously developed an FP-based assay for the specific interaction between the prolyl hydroxylated HIF-1$\alpha$ peptide and recombinant VBC (Cho et al., 2005), which was successfully used for quantitative measurements of the activity of prolyl hydroxylase. To identify compounds capable of inhibiting the activity of GST-PHD2, we optimized this FP-based assay on 96-well plates and screened a structurally diverse library of 1040 compounds compiled by the National Institute of Neurological Disorders and Stroke in a blinded manner. When the threshold value was set at 60% inhibition, 26 compounds were identified from the primary inhibitory assessment conducted at 20 $\mu$M compounds in duplicates (Fig. 1). These selected compounds were then subjected to secondary cell-based assays by detecting HIF-1$\alpha$ upon treatment of human HepG2 cells with the compounds in normoxic conditions. From the secondary analyses, two prominent compounds that increased functional HIF-1$\alpha$ were found to be cyclopirox olamine and baicalein. The former was studied previously as a bidentate iron chelator capable of stabilizing HIF-1$\alpha$ (Linden et al., 2003), but the latter has never been investigated as a HIF-1$\alpha$ stabilizer. Therefore, we chose baicalein for investigation of its effects and mechanisms.

The inhibitory potency of baicalein was further evaluated quantitatively using the FP-based interaction assay. As shown in Fig. 2A, FP values decreased gradually with increasing baicalein concentrations, finally reaching to a similar FP value as that of the negative control without GST-PHD2, which indicated full abrogation of the activity of GST-PHD2 at 200 $\mu$M baicalein. The $IC_{50}$ value was determined to be 7.01 $\pm$ 0.31 $\mu$M, indicating a high inhibitory effect of baicalein on the PHD2 activity. Alternatively, baicalein did not interfere with the binding of the proline-564 hydroxylated peptide with VBC protein up to 500 $\mu$M baicalein (data not shown). Therefore, baicalein had inhibitory effects only on the prolyl hydroxylase activities, but not on the HIF-VBC interactions. In addition, a similar FP-based assay developed previously for the interaction of F-HIF-1$\alpha$-(786-826) peptide with GST-p300 (Cho et al., 2007) was used to determine the inhibitory effects of baicalein on the FIH-1 activity. FP values increased with increasing baicalein concentrations (Fig. 2B), indicating that baicalein-inhibited FIH-1 could not hydroxylate the asparagine residue of the HIF-1 peptide, and the unhydroxylated peptide was captured by GST-p300. Because the $IC_{50}$ value was determined to be 860 $\pm$ 50 nM, baicalein was found to exert a more potent inhibitory effect on the asparaginyl hydroxylation of HIF-1 than on its prolyl hydroxylation. Furthermore, higher concentrations of baicalein interfered with the binding of HIF-1$\alpha$ to p300, with an $IC_{50}$ of ~6.0 $\mu$M (see Supplemental Data).

**Inhibition of HIF Hydroxylation Examined by MALDI-TOF.** To directly examine the PHD2 activity, another HIF-1$\alpha$-derived peptide B-P564 was incubated with GST-PHD2, and

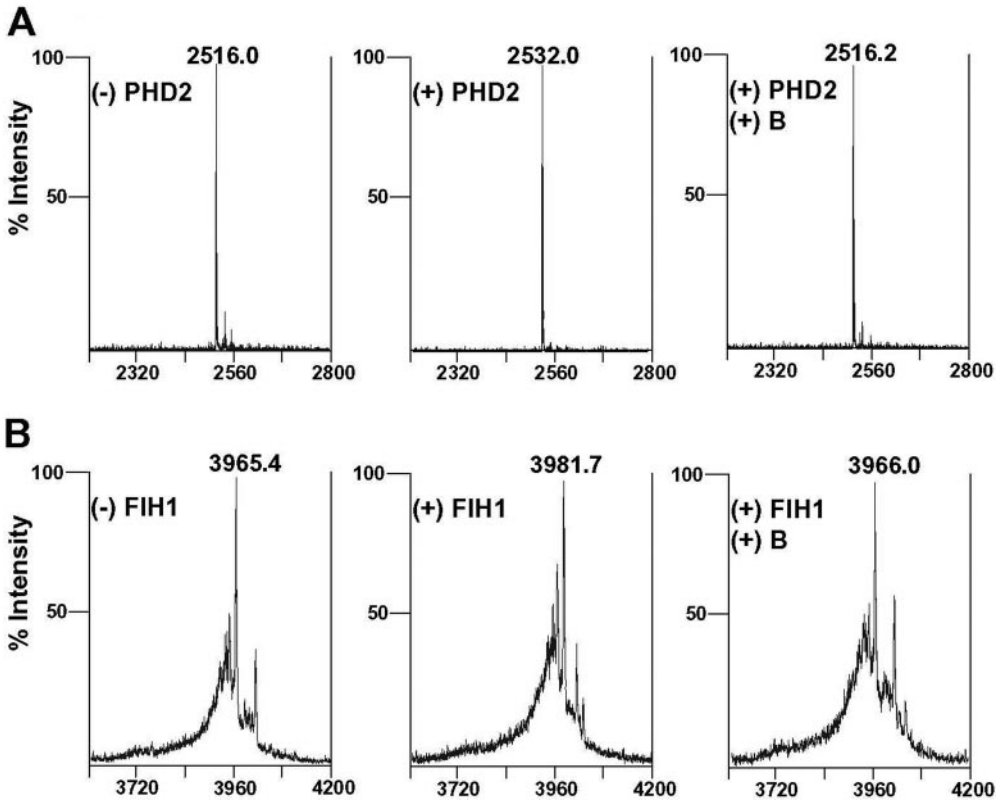


**Fig. 3.** Inhibitory effects of baicalein on hydroxylations of HIF-1$\alpha$. A, 3 $\mu$M B-P564 peptide was mixed without or with 0.20 $\mu$g/$\mu$l GST-PHD2 in NETN buffer containing 400 $\mu$M ascorbic acid and 100 $\mu$M 2-OG. To investigate the effect of baicalein in PHD2 activity, the prolyl hydroxylation was performed in the presence or absence of 400 $\mu$M baicalein (denoted as B) for 2 h at 25°C. MALDI-TOF spectra were obtained after desalting the reactants with $ZipTip_{C18}$. B, 4 $\mu$M F-HIF-1$\alpha$-(788-822) peptide was incubated without or with 0.55 $\mu$g/$\mu$l His-FIH-1 in hydroxylation buffer containing 400 $\mu$M ascorbic acid and 100 $\mu$M 2-OG. The asparaginyl hydroxylation was performed in the presence or absence of 200 $\mu$M baicalein for 2 h at 25°C, and MALDI-TOF spectra were obtained after purifying the reactants with $ZipTip_{C18}$. Note that the indicated molecular weights correspond to the peptides with detached fluorescein isothiocyanate (mol. wt. 389.0) in a sodium (mol. wt. 23.0)-added form during the MALDI-TOF measurements.

the change in molecular mass of the peptide was determined by MALDI-TOF analyses. Although B-P564 treated with GST-PHD2 had an increase in molecular mass of ~16 Da, yielding a peak at 2532.0 Da, the peptide incubated with the enzyme in the presence of 400 $\mu$M baicalein showed a peak at 2516.2 Da similar to the untreated peptide control (Fig. 3A). This result is consistent with the finding that baicalein inhibits the PHD2 activity. We then tested whether baicalein exerts an inhibitory effect on the FIH-1 activity because FIH-1 also belongs to the family of iron(II)- and 2-OG-dependent dioxygenases. As shown in Fig. 3B, treatment of F-HIF-1$\alpha$-(788-822) peptide with His-FIH-1 resulted in a new peak at 3981.7 Da corresponding to a mass increase of ~16 Da, demonstrating asparaginyl hydroxylation of the peptide. In contrast, when the peptide was incubated with His-FIH-1 in the presence of 200 $\mu$M baicalein, there was no mass increase from the enzyme-untreated peptide (Fig. 3B), indicating that baicalein also effectively inhibits the asparaginyl-hydroxylating activity of FIH-1.

**Mechanistic Examination of the Inhibitory Effects of Baicalein on HIF-Specific Hydroxylases.** HIF-specific hydroxylases contain 2-OG that binds to iron(II) at the active site via bidentate coordination (Elkins et al., 2003; McNeill et al., 2005), which is mimicked in the binding of 2-OG analogs to the enzymes (McDonough et al., 2006; Hewitson et al., 2007). We therefore tested whether the inhibitory effects of baicalein on the hydroxylation reactions involve its coordination with iron(II) by performing FP-based competitive assays with varying concentrations of 2-OG. Although baicalein at 200 $\mu$M inhibited the prolyl hydroxylation activity of GST-PHD2 in the presence of 100 $\mu$M 2-OG shown by a low FP value, FP values increased with increasing concentrations of 2-OG, finally reaching to the level of the reaction mixture incubated without baicalein (Fig. 4A). Such reversion of the inhibition suggests that baicalein works as a 2-OG-competitive inhibitor of PHD2. Conversely, 2-OG in the same concentration range did not exhibit any effect on the prolyl

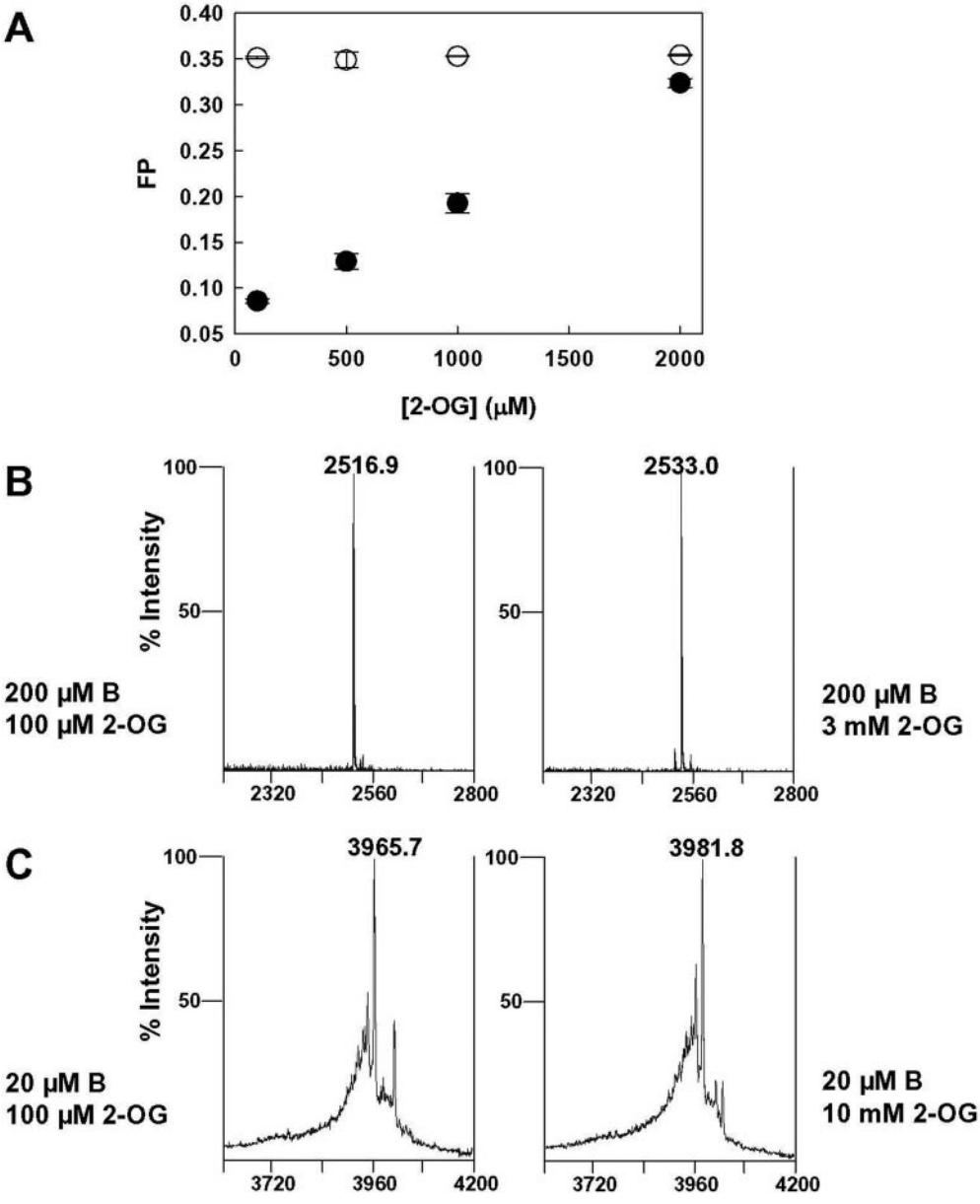


**Fig. 4.** Reversal of baicalein inhibited-hydroxylation activities by 2-OG. A, 1 $\mu$M F-P564 peptide was reacted with 0.05 $\mu$g/$\mu$l recombinant GST-PHD2 in NETN buffer containing 200 $\mu$M ascorbic acid in the presence (●) or absence (○) of 200 $\mu$M baicalein, with increasing concentrations of 2-OG. After incubation for 2 h at 25°C, 500 nM VBC was added to reactants diluted to a final peptide concentration of 100 nM in EBC buffer, followed by FP measurements. Each point represents the average of triplicate assays ± S.D. B, 1 $\mu$M B-P564 peptide was incubated with 0.05 $\mu$g/$\mu$l recombinant GST-PHD2 in the NETN buffer containing 400 $\mu$M ascorbic acid in the presence of 200 $\mu$M baicalein (denoted as B). To examine the effect of 2-OG, hydroxylation reactions were performed with 100 $\mu$M or 3 mM 2-OG. C, 3 $\mu$M F-HIF-1$\alpha$-(788-822) peptide was reacted with 0.51 $\mu$g/$\mu$l His-FIH-1 in hydroxylation buffer containing 400 $\mu$M ascorbic acid in the presence of 20 $\mu$M baicalein. To examine the effect of 2-OG, hydroxylation reactions were performed with 100 $\mu$M or 3 mM 2-OG. After incubation for 2 h at 25°C, MALDI-TOF spectra were obtained for reactants desalted with ZipTip$_{C18}$.

hydroxylation in the absence of baicalein or the binding of the peptide to VBC (Fig. 4A). To confirm this reversal effect of 2-OG by mass analyses, prolyl hydroxylation reactions were carried out with 100 $\mu$M or 3 mM 2-OG in the presence of 200 $\mu$M baicalein. As shown in Fig. 4B, the inclusion of 3 mM 2-OG allowed the hydroxylation reaction to occur as revealed by the mass increase of the peptide peak, whereas the B-564 peptide reacted with 100 $\mu$M 2-OG showed no hydroxylation reaction at all. In addition, we investigated whether excess 2-OG could reverse the inhibitory effect of baicalein on the FIH-1 activity. When the reactions with His-FIH-1 were performed in the presence of 100 $\mu$M or 10 mM 2-OG, excess 2-OG was found to completely reverse the activity inhibition of baicalein as demonstrated by appearance of the peak of the hydroxylated peptide in the MALDI-TOF spectrum (Fig. 4C). From these results, we could draw a conclusion that baicalein is probably capable of competing with 2-OG for the active sites of PHD2 and FIH-1, leading to the inhibition of their activities.

We then tested the effects of iron(II) similarly to the competitive experiments performed with 2-OG. When GST-PHD2 and His-FIH-1 were expressed in bacterial cultures, iron(II) ions were automatically packed into the enzymes because the media contain many minerals (Cho et al., 2005, 2007). We confirmed that the bacterially expressed GST-PHD2 and His-FIH-1 proteins are capable of catalyzing prolyl and asparaginyl hydroxylation reactions at the same rate regardless of the iron(II) inclusion in assays (data not shown). As shown in Fig. 5A, in the absence of baicalein, there was no change in FP with the exogenous addition of iron(II), albeit an insignificantly small decrease at 2 mM iron(II). In contrast, FP values in the presence of 200 $\mu$M baicalein increased with increasing iron(II) concentrations from 0.5 to 1.5 mM (Fig. 5A). This deinhibition of the PHD2 activity implies that the added iron(II) ions presumably form a complex with baicalein, thus limiting the availability of baicalein, leading to recovery of the enzyme activity. However, the FP value decreased again at 2 mM iron(II). Because

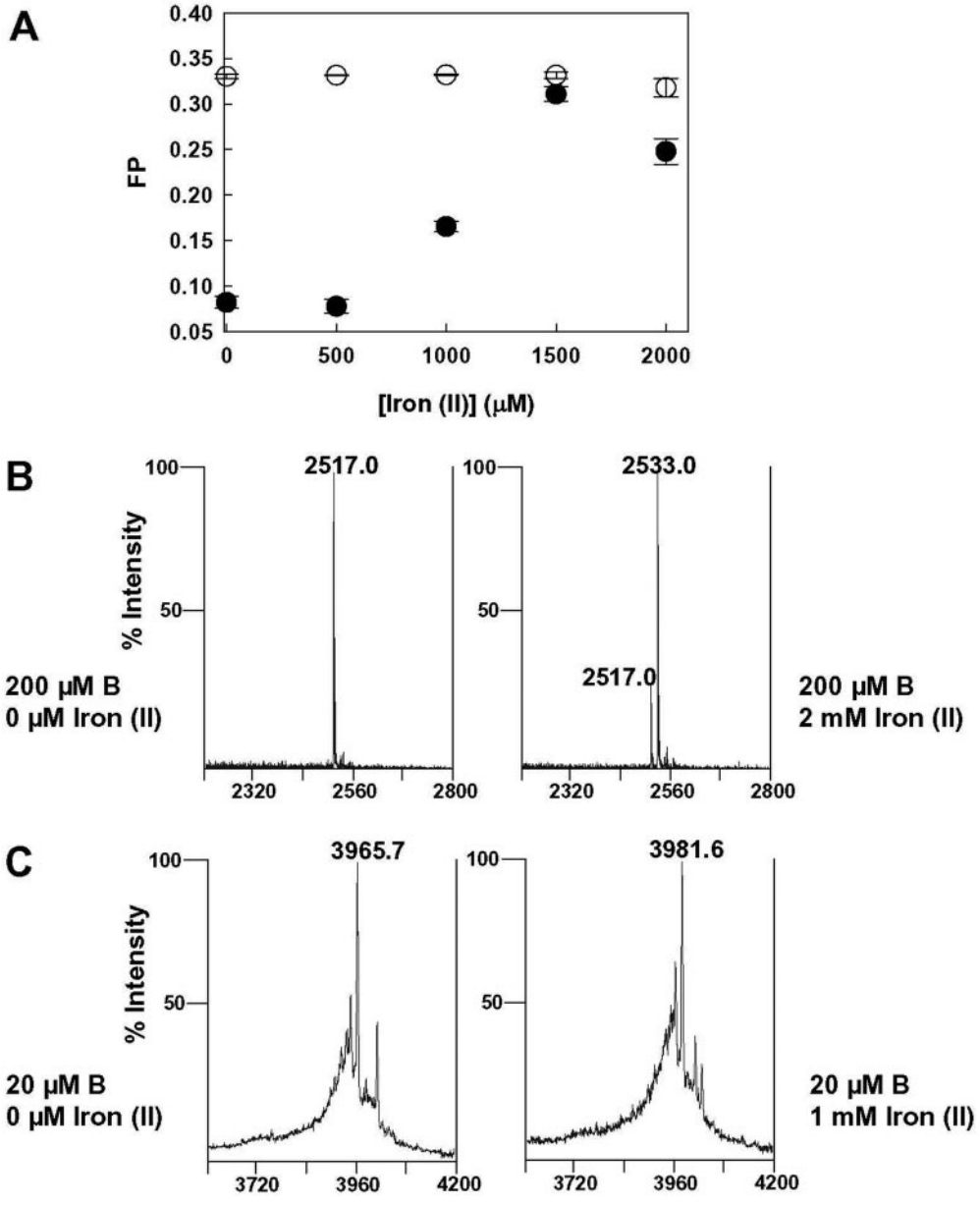


**Fig. 5.** Reversal of baicalein-inhibited hydroxylation activities by iron(II). A, 1 $\mu$M F-P564 peptide was reacted with 0.05 $\mu$g/$\mu$l GST-PHD2 in NETN buffer containing 200 $\mu$M ascorbic acid and 100 $\mu$M 2-OG in the presence (●) or absence (○) of 200 $\mu$M baicalein, with increasing concentrations of iron(II). After incubation for 2 h at 25°C, 500 nM VBC was added to reactants diluted to a final peptide concentration of 100 nM in EBC buffer, followed by FP measurements. Each point represents the average of triplicate assays ± S.D. B, 1 $\mu$M B-P564 peptide was incubated with 0.05 $\mu$g/$\mu$l recombinant GST-PHD2 in NETN buffer containing 400 $\mu$M ascorbic acid and 100 $\mu$M 2-OG in the presence of 200 $\mu$M baicalein (denoted as B). To examine the effect of iron(II), hydroxylation reactions were performed with or without 2 mM $FeSO_4$. C, 3 $\mu$M F-HIF-1$\alpha$-(788-822) peptide was reacted with 0.51 $\mu$g/$\mu$l His-FIH-1 in hydroxylation buffer containing 400 $\mu$M ascorbic acid and 100 $\mu$M 2-OG in the presence of 20 $\mu$M baicalein. To investigate the effect of iron(II), hydroxylation reactions were performed without or with 1 mM $FeSO_4$. After incubation for 2 h at 25°C, MALDI-TOF spectra were obtained for reactants desalted with $ZipTip_{C18}$.

ferrous ions can bind both baicalein and 2-OG, 2-OG needed for the prolyl hydroxylation activity might be partially removed excess iron(II), thereby exhibiting lower activity at 2 mM iron(II). We also confirmed these effects of iron(II) on the PHD2 activity by MALDI-TOF analyses. Consistent with the FP-based competitive assay data, the presence of 1.5 mM iron(II) permitted the full prolyl hydroxylation reaction (data not shown), but a residual unhydroxylated peak at 2517.0 Da was observed with 2 mM iron(II) (Fig. 5B). For asparaginyl hydroxylation, the mass analysis data showed complete restoration of the activity in the presence 1 mM iron(II) (Fig. 5C).

To obtain a direct evidence of the complexation between baicalein and ferrous ions, we performed absorbance measurements for baicalein as titrated with iron(II). Whereas baicalein had a strong absorbance peak in the UV region at 269 nm and a minor peak at 359 nm, absorbance changes were observed with increasing concentrations of ferrous sulfate added to the baicalein solution, as indicated by arrows (Fig. 6A). Iron(II) also induced a small but measurable concentration-dependent shift toward the blue from 359 to 353 nm. When the difference of absorbance at 269 nm was plotted as a function of the iron(II) concentration, the curve exhibited a plateau when the molar ratio of baicalein to iron(II) is ~3:2.

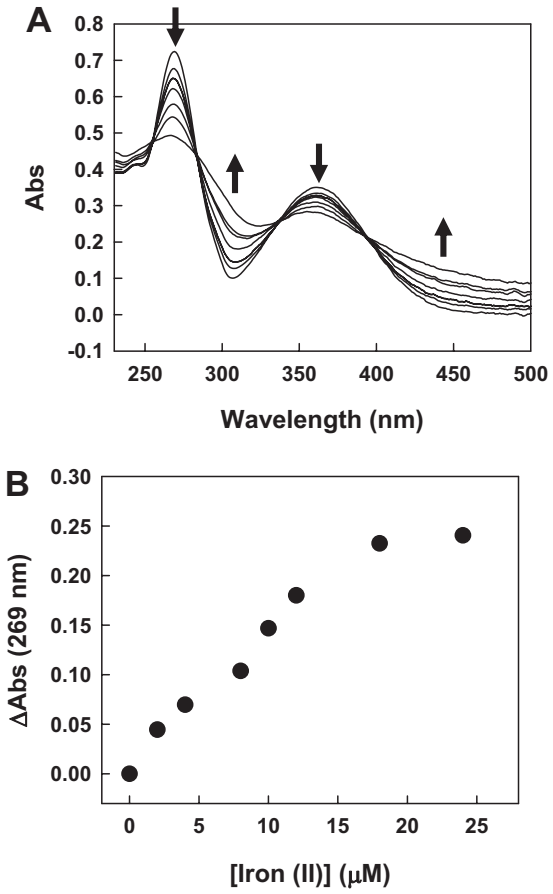


**Fig. 6.** Baicaline-iron(II) binding. A, 24 $\mu$M baicalein in 10 mM phosphate buffer, pH 7.4, was titrated with increasing concentrations of $FeSO_4$ (0, 2, 4, 8, 10, 12, 18, and 24 $\mu$M). After incubation for 2 min, the absorption spectra of baicalein were obtained from 230 to 500 nm. The direction of arrows indicates the increase of ferrous ion concentration. B, changes of absorbance at 269 nm in Fig. 7A were determined and plotted as a function of the iron(II) concentration.

We also performed spectroscopic scanning measurements of the baicalein-PHD2 solutions to directly demonstrate baicalein binding to PHD2. When baicalein at a fixed concentration was mixed with PHD2, absorbance of baicalein at 360 nm decreased with increasing concentrations of PHD2, and the difference of absorbance at 360 nm plotted as a function of the enzyme concentration yielded a saturated curve (Fig. 7A). In contrast, a PHD2 mutant containing mutations of two acidic amino acid residues participating in iron coordination at the active site of the enzyme (McDonough et al., 2006) did not show significant absorbance changes with increasing baicalein concentrations as shown in Fig. 7B. Therefore, these results corroborated the direct interaction of baicalein with PHD2. In addition, baicalein was found to bind FIH-1 as determined by spectroscopic scanning measurements (Fig. 7C).

**Stabilizing Effects of Baicalein on Functional HIF-1$\alpha$ Protein.** After we confirmed that baicalein inhibits the PHD2 and FIH-1 activities in in vitro assays, we investigated whether baicalein is able to stabilize functional HIF-1$\alpha$ in human hepatoma HepG2 and neuroblastoma SH-SY5Y cells. Baicalein has been reported to induce apoptosis possibly mediated by mitochondrial dysfunction and Bcl-2 regulation in HepG2 cells (Chang et al., 2002), whereas no cytotoxicity was demonstrated in SH-SY5Y cells treated with baicalein at lower than 125 $\mu$M (Gao et al., 2001). Therefore, before examination of HIF-1$\alpha$ stabilization, we performed MTT assays for HepG2 and SH-SY5Y cells, by which no detectable cytotoxic activity of baicalein was observed for HepG2 cells within the tested concentration range up to 1 mM for 48 h, whereas lower concentrations of baicalein induced apoptosis in SH-SY5Y cells even after 24-h treatment (see Supplemental Data). These results indicate that baicalein seems to exhibit cell type-dependent harmful effects on the cell viability.

We then used Western blotting to measure levels of HIF-1$\alpha$ protein after the normoxic HepG2 and SH-SY5Y cells were exposed to baicalein. The results in Fig. 8A show that baicalein induced HIF-1$\alpha$ in both cells in a dose-dependent manner. Compared with clioquinol-treated cells as a positive control as its stabilization effect on the functional HIF-1$\alpha$ was established previously (Choi et al., 2006), baicalein seemed less potent. We also examined the ubiquitination state of stabilized HIF-1$\alpha$. When normoxic HepG2 cells were exposed to MG132, which is known to specifically inhibit the 26S proteasome, the high-molecular-mass HIF-1$\alpha$ protein was recognized by anti-HIF-1$\alpha$ antibody (Fig. 8B), indicating blockage of the degradation of ubiquitin-conjugated HIF-1$\alpha$ even in normoxic conditions. In contrast, the cells treated with baicalein exhibited much less high molecular mass HIF-1$\alpha$, regardless of the presence or absence of MG132 (Fig. 8B). These results demonstrated that the inhibitory effect on the HIF-specific hydroxylases is likely to result in a reduction in the ubiquitination of HIF-1$\alpha$.

Because baicalein is known as a selective 12-lipoxygenase inhibitor, we tested the possibility of involvement of 12-lipoxygenase in the drug action by using another 12-lipoxygenase inhibitor with a similar potency, cinnamyl-3,4-dihydroxy-$\alpha$-cyanocinnamate. As shown in Fig. 8C, HepG2 cells treated with cinnamyl-3,4-dihydroxy-$\alpha$-cyanocinnamate up to 100 $\mu$M did not induce stabilization of HIF-1$\alpha$.

**Effects of Baicalein on Hypoxia-Induced Transactivation by HIF-1$\alpha$.** Because HIF-1$\alpha$ is critical for the

expression of various genes, and inhibition of asparaginyl hydroxylation of HIF-1$\alpha$ by baicalein would enhance the transactivation of HIF-1$\alpha$ through its interaction with cAMP response element-binding protein-binding protein/p300, we next investigated whether baicalein induces endogenous HIF-1 target genes, including VEGF. When the relative amounts of VEGF mRNA were determined by Northern analysis, RT-PCR results in Fig. 9A revealed that VEGF expression is up-regulated by baicalein in normoxic HepG2 cells, although much lesser than by clioquinol. In addition, the level of CA9 mRNA, which is known to be induced under hypoxia via the transcription factor HIF-1 (Wykoff et al., 2000), increased in baicalein-treated HepG2 cells. In contrast, no significant difference of the level of VEGF mRNA was found in baicalein-treated SH-SY5Y cells (Fig. 9A). Because rather less dramatic effects were observed in transcription of the target genes, the transactivation activity of HIF-1$\alpha$ was further assessed in cell cultures, using a Gal4-driven reporter plasmid encoding the luciferase gene under the control of the Gal4 binding site. When HepG2 cells were cotransfected with plasmid pGal4/HRE, which exhibits specific HIF-1-dependent gene induction (Choi et al., 2006), baicalein increased the transactivation activity of HIF-1$\alpha$ in a concentration-dependent manner (Fig. 9B). Furthermore, quantitative analyses using real-time RT PCR were performed to examine the effects of baicalein on gene induction. In HepG2 cells, VEGF (Fig. 9C) and CA9 (Fig. 9D) were up-regulated in the presence of baicalein, although their induction levels were much lower than for CQ-treated cells. In addition, baicalein was found to increase the levels of VEGF (Fig. 9E) and CA9 mRNAs (Fig. 9F) slightly but significantly in SH-SY5Y cells. Therefore, our results demonstrated that baicalein probably up-regulates HIF-1$\alpha$-mediated transactivation in normoxic HepG2 cells.

To confirm the effect of baicalein on the transactivation activity of HIF-1$\alpha$, HIF-1$\alpha$ knock-down 3T3-L1 cells prepared with short hairpin RNA (shRNA) were used in hypoxia-driven reporter assays. As expected, the cells treated with a retroviral vector system containing shHIF-1$\alpha$ did not



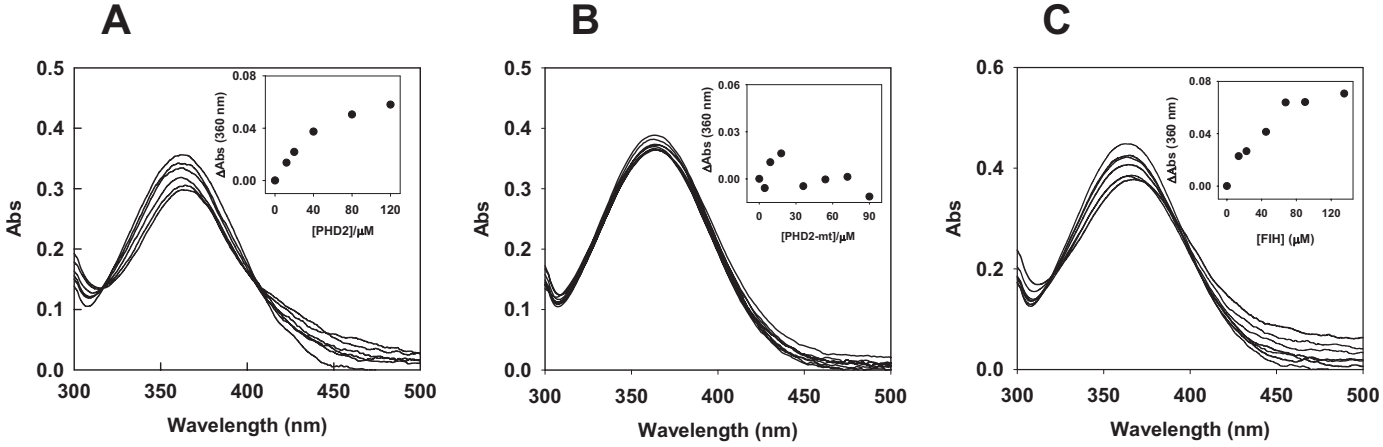


**Fig. 7.** Baicalein-PHD2 binding. Baicalein at 25 $\mu$M in EBC buffer was mixed with increasing concentrations of PHD2 (A), PHD2 mutant containing H313A and D315A (B), or FIH-1 (C), and their absorption spectra were obtained from 300 to 500 nm. The inset graph is for changes of absorbance at 360 nm determined and plotted as a function of the enzyme concentration.

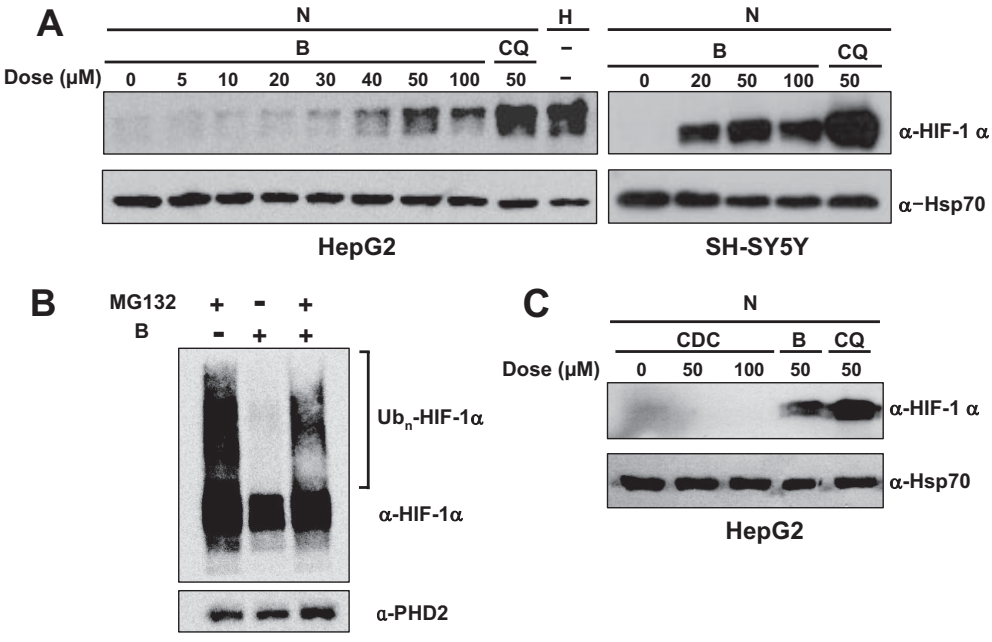


**Fig. 8.** Effect of baicalein on stabilization of HIF-1$\alpha$. A, HepG2 and SH-SY5Y cells were treated with baicalein (denoted as B) at the indicated doses and 50 $\mu$M CQ in normoxic or hypoxic (1% $O_2$) conditions for 6 h. HIF-1$\alpha$ and Hsp70 were detected by Western analysis. N and H represent normoxia and hypoxia, respectively. B, HepG2 cells were treated with or without 50 $\mu$M baicalein in the presence or absence of 10 $\mu$M MG132 in normoxic conditions for 6 h. High-molecular-mass ubiquitinated HIF-1$\alpha$ (denoted as $Ub_n$-HIF-1$\alpha$) and nonubiquitinated HIF-1$\alpha$ were determined by Western analysis. C, HepG2 cells were treated with or without cinnamyl-3,4-dihydroxy-$\alpha$-cyanocinnamate (denoted as CDC) at the indicated doses in normoxic conditions for 6 h, and their HIF-1$\alpha$ and 70-kDa heat shock proteins (Hsp70) were detected by Western analysis along with the samples treated with 50 $\mu$M baicalein and 50 $\mu$M clioquinol.

showed any significant induction of HIF-1$\alpha$ under hypoxic conditions, whereas the control shRNA-treated cells did (Fig. 10A). When these cells were assayed for luciferase expression in the presence of baicalein, they exhibited no transactivation activity (Fig. 10B). We also examined the expression of genes in HIF-1$\alpha$ knockdown 3T3-L1 cells in which baicalein and hypoxic insult did not exert stabilization of HIF-1$\alpha$ (Fig. 11A). As shown in Fig. 11B, the levels of VEGF increased with increasing concentrations of baicalein similarly as in control cells. It is noteworthy that HIF-1$\alpha$ knockdown 3T3-L1 cells in hypoxic conditions showed a significant increase of VEGF mRNA, albeit less than control cells (Fig. 11B). In contrast, BINP3 was not induced by baicalein or hypoxia treatment (Fig. 11C). Therefore, the induction of HIF-1$\alpha$ is

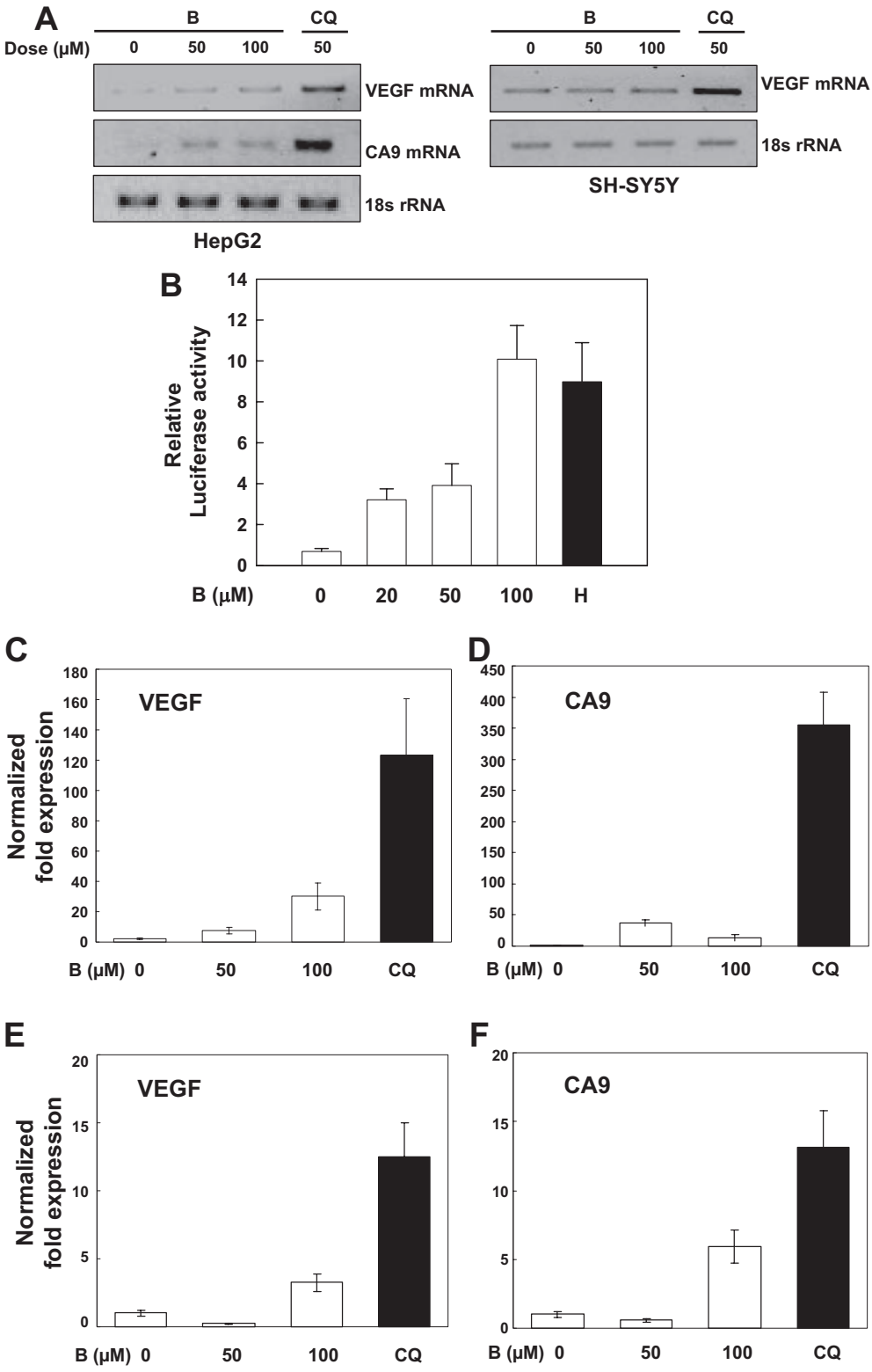


**Fig. 9.** Effect of baicalein on the transactivation activity of HIF-1$\alpha$. A, HepG2 and SH-SY5Y cells were treated without or with baicalein (denoted as B) at the indicated doses and 50 $\mu$M CQ in normoxic conditions for 16 h. Amplification of VEGF for 27 cycles was carried out by RT-PCR using 1-$\mu$g aliquots of isolated total RNA to determine the linear portion of the application reaction. The amplified products were analyzed on 1% agarose gels. B, HepG2 cells were transfected with both $p(HRE)_4$-luc and pCHO110, followed by culturing in the presence of varying concentrations of baicalein under normoxic conditions or in the absence of baicalein under hypoxic conditions for 16 h. Each bar represents the average of triplicate assays ± S.D. mRNA levels of VEGF (C) and CA9 (D) were quantitated by real-time RT-PCR in HepG2 cells that were treated without or with baicalein (denoted as B) at the indicated doses and 50 $\mu$M CQ in normoxic conditions for 16 h. mRNA levels of VEGF (E) and CA9 (F) were quantitated by real-time RT-PCR in SH-SY5Y cells that were treated without or with baicalein (denoted as B) at the indicated doses and 50 $\mu$M CQ in normoxic conditions for 16 h. Data were normalized by the level of 18s rRNA, and each bar represents the average of triplicate assays ± S.D.

critical for the effects of baicalein on up-regulation of some genes but not of VEGF, suggesting the possibility that baicalein might exert its effects via HIF-independent regulation of VEGF.

**Induction of Angiogenesis in the CAM by Baicalein.** To determine whether baicalein exerts proangiogenic activity, we carried out the CAM assay. At 13.5 days, baicalein strongly elicited an angiogenic response, forming new capillaries from the exiting vascular network similarly to the VEGF-treated positive control, whereas the eggs treated with DMSO alone exhibited no significant growth of new blood vessels (Fig. 12A). Applications of baicalein at 27 and 135 ng showed 82 and 75% of positive responses, respectively, whereas the control vehicle DMSO showed low angiogenic activity of 18% (Fig. 12B). As expected, VEGF was active in 82% of the eggs, exhibiting high angiogenic activity. These results support that baicalein acts as an inducer of angiogenesis in vivo.

## Discussion

Angiogenesis is a dynamic process in which vasculogenic and angiogenic signals are controlled by local release of vascular growth factors. Because VEGF is a principal endogenous growth factor implicated in early vasculogenesis, its administration by gene or protein therapy has been extensively attempted in therapeutic angiogenesis for the treatment of ischemic disorders (Ferrara and Kerbel, 2005). However, the use of VEGF therapies in patients with myocardial or limb ischemia yielded largely disappointing results, partly due to inadequate delivery strategies (Shah and Losordo, 2005). Alternatively, targeting of the HIF pathway has been suggested to provide advantages over the therapies using single angiogenic factors (Brahimi-Horn and Pouysségur, 2007). Although HIF-1 gene therapy might serve as a means to increase functional HIF-1, a pharmaceutical approach has

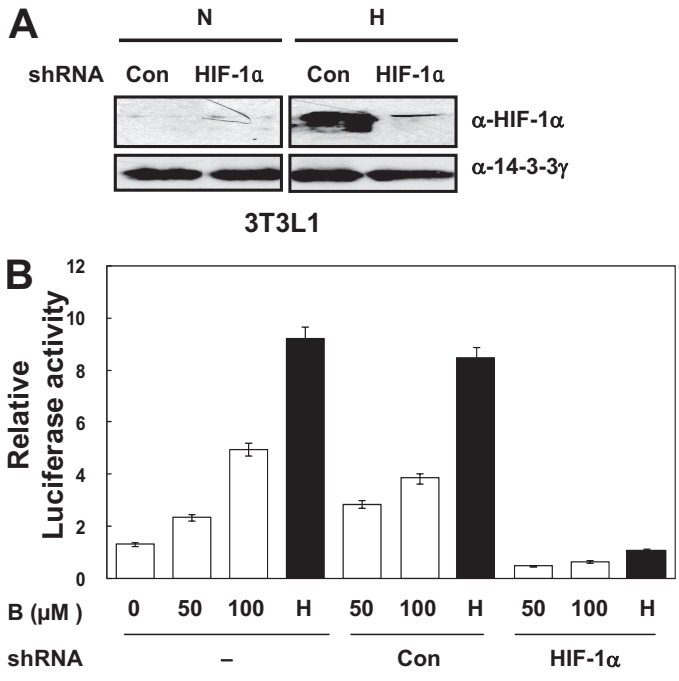


**Fig. 10.** Baicalein-induced transactivation in HIF-1$\alpha$ knockdown cells. A, HIF-1$\alpha$ knockdown 3T3-L1 cells were generated using a retroviral vector system. shRNA oligonucleotides against mouse HIF-1$\alpha$ (5′-GATCCGTGTGAGCTCACATCTTGATTTCAAGAGAATCAAGATGTGAGCTCACATTTTTTTAGATCTG-3′) were ligated with pSIREN-RetroQ vector (BD Biosciences, San Jose, CA) to produce pSIREN-RetroQ-shHIF-1$\alpha$, according to the instructions of the manufacturer. 3T3-L1 cells were then infected with the retrovirus encoding either shHIF-1$\alpha$ or control shRNA. The infected 3T3-L1 cells were selected by using 2 $\mu$g/ml puromycin, treated under normoxic or hypoxic (1% $O_2$) conditions for 12 h and analyzed for the protein levels of HIF-1$\alpha$ and 14-3-3$\gamma$ by Western analysis. N and H indicate normoxia and hypoxia, respectively. B, 3T3-L1 cells were transfected with both p(HRE)$_4$-luc and pCHO110, followed by culturing in the presence of varying concentrations of baicalein under normoxic conditions or in the absence of baicalein under hypoxic conditions for 16 h. Each bar represents the average of triplicate assays ± S.D.

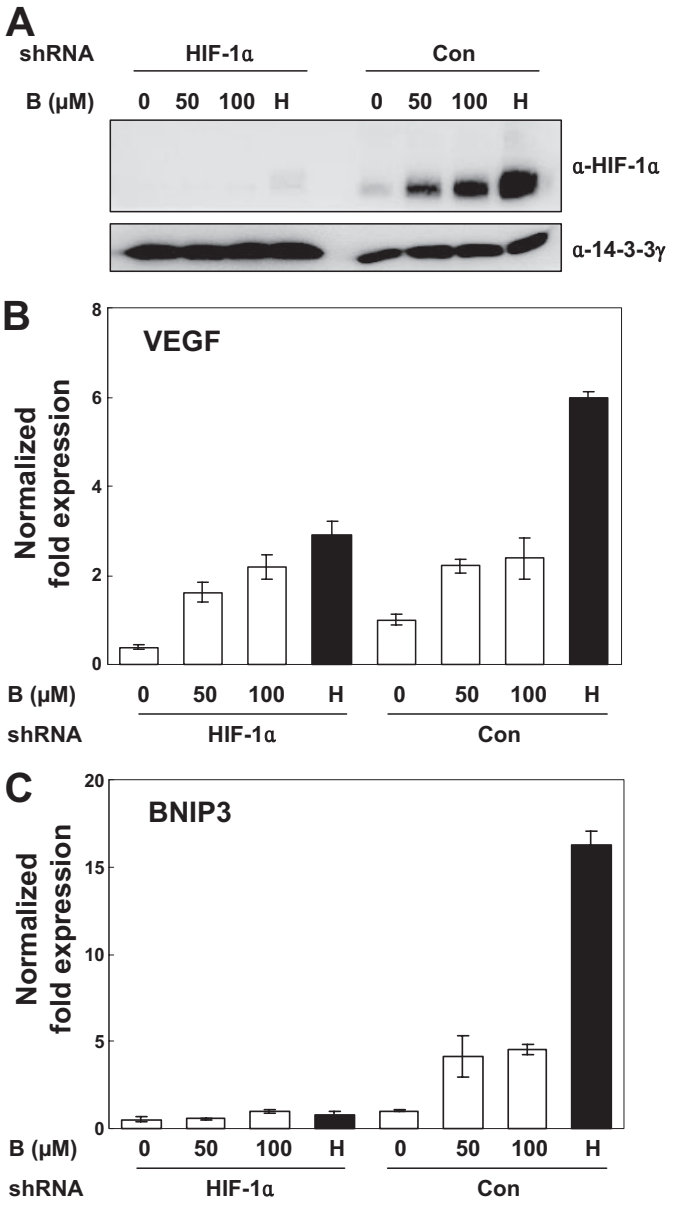


**Fig. 11.** Baicalein-induced transcription of genes in HIF-1$\alpha$ knockdown cells. A, 3T3-L1-sh-control, 3T3-L1-sh-HIF-1$\alpha$ cells were treated with baicalein in normoxic conditions for 6 h at the indicated doses or without baicalein in hypoxic conditions for 6 h and analyzed for the protein levels of HIF-1$\alpha$ and 14-3-3$\gamma$ by Western analysis. H and Con indicate hypoxia and control cells, respectively. mRNA levels of VEGF (B) or BCL2/adenovirus E1B interacting protein 1, NIP3 (BNIP3) (C) were quantitated by real-time RT-PCR in 3T3-L1 cells treated with baicalein at the indicated doses or without baicalein in hypoxic conditions for 16 h. Data were normalized by the level of 18s rRNA, and each bar represents the average of triplicate assays ± S.D.

been taken to stabilize HIF-1 through the inhibition of prolyl hydroxylase activity (Ivan et al., 2002; Hirsilä et al., 2003; Linden et al., 2003; Siddiq et al., 2005), which would be beneficial particularly regarding ease of application.

In this context, the initial aim of the present study was to find inhibitors of PHD2 because of its principal role in the stabilization of HIF-1. As a result of exploiting the previously developed FP-based assay in the discovery of small-molecule inhibitors, baicalein was selected and further shown to inhibit the activity of FIH-1 with higher potency. As expected, the synergistic hypoxia-mimic effects exerted by baicalein-mediated inhibitions of prolyl and asparaginyl hydroxylation and of the ubiquitination of HIF-1$\alpha$ led to stabilized functional HIF-1$\alpha$. Although induction of a proangiogenic factor VEGF was only marginal, we demonstrated that baicalein has the ability to elicit a proangiogenic response in the CAM assay. Furthermore, baicalein also seemed to up-regulate VEGF in an HIF-independent manner, which may possibly involve other transcriptional activation pathways such as peroxisome proliferator-activated receptor-$\gamma$ coactivator-1$\alpha$ (Arany et al., 2008). However, elucidation of the exact pathway by which baicalein controls angiogenesis needs further investigation.

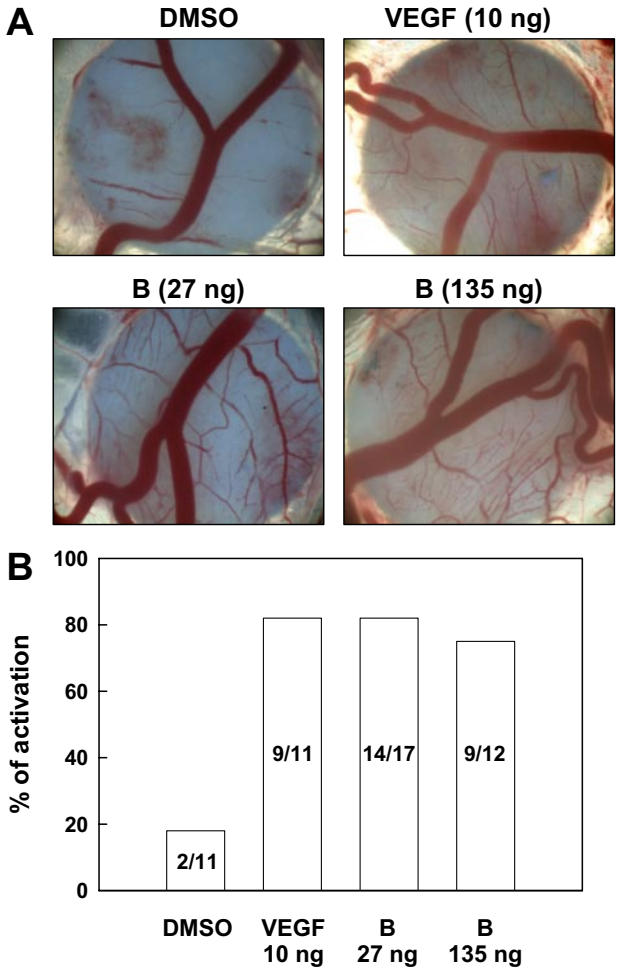


**Fig. 12.** Induction of angiogenesis in the CAM by baicalein. Ten microliters of baicalein (27 and 135 ng) in DMSO was added to the CAM surface of 10.5-day-old chick embryos. DMSO alone and 10 ng of VEGF were used as negative and positive controls, respectively. After 3 days of incubation, the eggs were observed under microscope. A, representative images. B, angiogenic activation is expressed as the percentage of positive CAMs, and the numbers inside each bar indicate the number of positive CAMs per the number of surviving eggs.

Baicalein is a small, lipophilic flavonoid that readily crosses the biological membranes, which would facilitate effective delivery for therapeutic applications. So far, baicalein has never been presented as an activator of the HIF pathway despite its wide ranges of effects on various enzymes and signaling pathways. Rather, baicalein has been reported to exhibit an anticancer activity as an antiangiogenic agent demonstrated by inhibition of endothelial cell proliferation, migration, and differentiation (Liu et al., 2003). In their CAM assays, baicalein at fairly high doses (50–200 nmol) blocked angiogenesis only in the presence of bFGF, but the effects of the compound at lower concentrations or in the absence of bFGF have not been examined. On the contrary to these results, our CAM assays demonstrated strong proangiogenic effects with much lower amounts of baicalein (0.1–0.5 nmol) in the absence of bFGF. Another observation revealed that baicalein as a selective 12-lipoxygenase inhibitor reduces VEGF expression in human prostate cancer PC-3 cells because 12-lipoxygenase mediates an increase in VEGF promoter activity in a phosphatidylinositol 3-kinase-dependent manner (Nie et al., 2006). Although the exact mechanism by which baicalein modulates angiogenesis in vivo remains to be explored in more detail, it could be postulated that baicalein might exert different effects depending on the doses and the cell types, especially considering that we observed different responses in VEGF transcription and apoptotic induction between baicalein-treated HepG2 and SH-SY5Y cells. From the fact that the complex process of angiogenesis requires the coordinated, sequential involvement of several cellular events, we could also speculate that multiple control systems might be switched on and off within a short period, and thus any modulating molecule that affects one or several steps of this process may exert an effect on angiogenesis. In addition, it is worth noting that some flavonoids such as quercetin have been reported to exert antiangiogenic cytotoxic effects by blocking cell proliferation signals of phosphatidylinositol 3-kinase (Igura et al., 2001) and VEGF-inducing effects by inhibiting PHD2 (Jeon et al., 2007).

The HIF-specific hydroxylases bear a conserved 2-histidine-1-carboxylate iron coordination motif at the catalytic site, as revealed by their crystal structures (Dann et al., 2002; McDonough et al., 2006). Iron(II) in the catalytic site is transiently associated with 2-OG for the hydroxylation reaction, although a recombinant PHD2 seems to contain iron(II) and 2-OG tightly bound at the active site (McNeill et al., 2005). Because the requirement of iron(II) and 2-OG renders the enzymes susceptible to pharmacological attack, efforts have been made to inhibit the HIF-specific hydroxylases by removal of iron from the enzyme active sites with iron chelators or by competing for iron with 2-OG analogs (Dann and Bruick, 2005). Among 2-OG mimetic compounds, *N*-oxalylglycine and its derivatives have been extensively applied for inhibiting the HIF-specific hydroxylases (Epstein et al., 2001; Elkins et al., 2003). Furthermore, some selective inhibitors complexed with the enzymes have been demonstrated in modeled or crystallized structures (Dann et al., 2002; McDonough et al., 2005; Warshakoon et al., 2006).

With regard to the action of baicalein, it was confirmed to be a strong chelator for iron(II) ions with a stoichiometry of ~3:2, deduced from the experiments on the spectral titration

of baicalein with iron(II). This binding stoichiometry is apparently consistent with the structural feature of baicalein containing a trihydroxy pyrogallol moiety in the A ring and the $C_4$ = O in the C ring of the flavonoid. In addition to the demonstration of the direct binding of baicalein to iron, we observed that the inhibitory effects of baicalein on HIF-specific hydroxylases could be reversed competitively by excess iron(II) and 2-OG. More directly, the binding between baicalein and PHD2 was also demonstrated. Therefore, from the mechanistic perspective, the inhibition of PHD2 and FIH-1 activities by baicalein may well be ascribed to its direct complexation with the active site iron of the enzymes. Likewise, a very recent report on a flavonoid quercetin has revealed the iron chelation as its inhibitory mechanism for PHD2 (Jeon et al., 2007). Elucidation of the exact chelating moiety of baicalein that associates preferentially with the active site iron, however, requires further investigation.

In summary, our studies demonstrate induction of some genes by baicalein, whose action is initiated at the level of HIF-specific hydroxylases, and its proangiogenic effect. Although these two effects seem to be independent and the angiogenic pathway that is controlled by baicalein is unclear, these results confirm the feasibility of a novel approach for therapeutic angiogenesis in which neovascularization is achieved by use of a small molecule. Because baicalein may inhibit other 2-OG-dependent dioxygenases, its structural modifications and mechanistic studies will aid in the design of more specific inhibitors for therapeutic development.

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