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Differential In Vitro and Cellular Effects of Iron Chelators for Hypoxia Inducible Factor Hydroxylases

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ABSTRACT

Hypoxia inducible factor 1α (HIF-1α), an essential transcriptional factor, is negatively regulated by two different types of oxygen and Fe^{2+}-dependent HIF hydroxylases, proline hydroxylase (PHD) and factor inhibiting HIF (FIH), under normoxia. Iron chelators have therefore been used for inducing HIF-1α expression by inhibiting the hydroxylases. In this study, the iron chelators displayed differential effects for PHD and FIH in cells depending on their iron specificity and membrane permeability rather than their in vitro potencies. The membrane permeability of the strict Fe^{2+}-chelator potentially inhibited both hydroxylases, whereas the membrane impermeable one showed no inhibitory effect in cells. In contrast, the depletion of the extracellular Fe^{3+} ion was mainly correlated to PHD inhibition, and the membrane permeable one elicited low efficacy for both enzymes in cells. The 3′-hydroxyl group of quercetin, a natural flavonoid, was critical for inhibition of intracellular hydroxylases. Since the 3′-methylation of quercetin is induced by catechol-o-methyl transferase, the enzyme may regulate the intracellular activity of quercetin. These data suggest that the multiple factors of iron-chelators may be responsible for regulating the intracellular activity HIF hydroxylases. J. Cell. Biochem. 114: 864–873, 2013.

KEY WORDS: HYPOXIA INDUCIBLE FACTOR; HIF HYDROXYLASES; IRON CHELATORS; HYDROXYLASE INHIBITION

Cells under hypoxia express a variety of genes involved in angiogensis and erythropoiesis, glycolytic energy metabolisms and so on [Semenza, 2003], and hypoxia-inducible factor 1 (HIF-1) is a key transcription factor regulating those gene expressions. HIF-1 is a α/β-heterodimeric transcription factor and plays a central role in oxygen homeostasis. The β subunit, known as aryl hydrocarbon receptor nuclear translocator, is constitutively expressed in cells, whereas HIF-1α is controlled by oxygen concentration. HIF-1α contains two oxygen-dependent regulating domains, an oxygen-dependent degradation domain (ODDD) containing two proline residues at positions 402 and 564 (Pro402 and Pro564, respectively) hydroxylated by prolyl hydroxylase domain enzymes (PHD1–3) and a carboxy-terminal transactivation domain (CTAD) containing an 803 asparagine residue (Asn803) hydroxylated by factor inhibiting HIF (FIH). Both PHDs and FIH require HIF-1α, oxygen and 2-oxoglutarate (2OG) as substrates and Fe^{2+} ion and ascorbate as cofactors. Under normoxic condition, the proline hydroxylation induces the HIF-1α degradation in proteosome via the von Hippel Lindau tumor suppressor (pVHL)-mediated polyubiquitinylation system [Jaakkola et al., 2001], and the asparagine hydroxylation abrogates the p300/CBP co-activator recruitment required for the HIF-1α-dependent transcription [Hewitson et al., 2002; Lando et al., 2002b]. Since those enzymes are inactivated under hypoxia, HIF-1α is stabilized and its transcriptional activity is activated.

PHD2 is considered to be the primary regulating enzyme that controls the HIF-1α function in cells, whereas FIH is considered a precise regulator of the HIF-1α function under hypoxia because FIH has higher binding affinity for oxygen than PHDs [Koivunen et al., 2004]. In fact, FIH has been proved to be more active than PHDs

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under hypoxia [Stolze et al., 2004; Dayan et al., 2006; Pouyssegur et al., 2006]. Recently the compounds, amphothericin B and bortezomib, have been reported to induced anemia and to inhibit tumor adaptation to hypoxia by enhancing FIH mediated Asn803 hydroxylation of HIF-1α regardless of PHDs, respectively [Yeo et al., 2006; Shin et al., 2008]. Therefore, selective inhibitors or stimulators of the HIF hydroxylases can be developed as anti-ischemia/anemia or anti-cancer drugs, respectively.

Since PHDs and FIH require Fe^{2+} for their function, iron-chelators have been studied as anti-ischemic compounds [Ivan et al., 2002; Tian et al., 2011]. However, studies of the relationships between the in vitro and cellular activities of the inhibitors are rare. In addition, their cellular effects are mainly focused on abilities of HIF-1α accumulation mediated by inhibition of PHDs, but there are very few studies on inhibition of the Asn803 hydroxylation of HIF-1α mediated by FIH because of lack of available antibody. Previously we developed a monoclonal antibody (Mab), SHN-HIF1α, specific to the Asn803 hydroxylated HIF-1α, and it has been successfully used for sensitive analysis of the Asn803 hydroxylation of intracellular HIF-1α [Lee et al., 2008].

The iron chelators, dipyridyl (DP) and desferoxamine (DFO), have been currently used for intracellular HIF-1α stabilization [Lando et al., 2002a; Yeo et al., 2006]. Our preliminary data showed that DFO had higher in vitro inhibitory activity than DP for both enzymes, but could not inhibit Asn803 hydroxylation even at 300 μM even under hypoxia unlike DP. DP is a strict Fe^{2+}-chelator with membrane permeability, whereas DFO is a Fe^{3+}-chelator without membrane permeability [Kicic et al., 2001]. We assumed that the differences may account for the discrepant results between their in vitro and cellular functions.

Quercetin (QUE) is the most abundant flavonoid in nature, and provides many beneficial effects protecting cancer, cardiovascular diseases, infections, aging and so on [Graf et al., 2005]. It has also been reported to induce the HIF-1α expression by its iron chelating property, but its cellular effects on the HIF-dependent transcription have been controversially reported [Jeon et al., 2007; Park et al., 2008; Triantafyllou et al., 2008].

It is important to understand the differential inhibition of HIF proline and asparagine hydroxylation by iron chelators. Recent studies have investigated the differential regulation of sites of HIF prolyl and asparaginyl hydroxylation in cells using hydroxyl residue-specific antibodies [Tian et al., 2011]. However, systemic studies for hydroxylase inhibitors according to their molecular natures have been rarely done. Here, we report the differential functions of the inhibitors in the regulation of HIF prolyl and asparaginyl hydroxylation in vitro and in cells. Our results reveal that the cellular function of iron chelators is regulated by multiple factors such as their iron selectivity, membrane permeability and intracellular modification rather than their in vitro potencies.

MATERIALS AND METHODS

MATERIALS

The iron chelators, DP, 1,10-phenanthroline (PHT), DFO, quercetin (QUE), isorhamnetin (ISR), rhamnetin (RHA), and bathophenanthrolinedisulfonate disodium salt (BPS) were purchased from Sigma–Aldrich (USA). Pyridoxal isonicotinyl hydrazone (PIH) was purchased from Santa Cruz Biotechnology, Inc. (USA) and *N*-oxalylglycine (NOG) and dimethyl NOG (DMOG) were purchased from Frontier Scientific, Inc. (USA). *N*-oxalyl-D-phenylalanine (NOdF) was prepared as we described previously [McDonough et al., 2005]. Figure 1 shows the molecular structures of the inhibitors.

PEPTIDE SYNTHESIS

The peptides were synthesized by Fmoc solid-phase peptide synthesis (Fmoc-SPPS) using the standard dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) method. The crude peptides were purified by the reverse-phase HPLC using a C18-column, and evaluated by mass analyses using MALDI-TOF (Shimazu, Japan).

ENZYME PURIFICATION AND CHARATERIZATION

The expression and purification of FIH were performed according to a previously described method (REF). To prepare PHD2, the human PHD2 (179–426) gene was cloned into the *Bam*HI/*Hin*dIII site of the pET-28a vector. The expression and purification of PHD2 also followed the method for FIH purification, except for the use of the SP-sepharose chromatography instead of the Q-sepharose chromatography. The activities of the purified enzymes were initially analyzed by mass analyses using the substrate peptides, b803 (biotin-DESGLPQLTSYDCEVNAPIQGSRNLLQGEELLRAL) for FIH and b402 (biotin-KKPDKALTLLAPAAGDTIISLD) and b564 (biotin-DLDLEMLAPYIPMDDDFQL) for PHD2. The reaction was performed for 1 h at 37°C in 45 μl of reaction mixture with the enzyme (15 μg FIH or 30 μg PHD2), 15 μg substrate peptide, 200 μg catalase, 2 mM ascorbate, 2 mM dithiothreitol (DTT), and 0.1 mM ammonium ferrous sulfate (AFS) and 50 mM Tris pH 7.5 in the presence or absence of 0.6 mM 2OG, respectively. The masses of the resulting substrate peptides were measured by MALDI-TOF mass analyses. The hydroxylated forms of b803, b564, and b402 were named b803-OH, b564-OH, and b402-OH, respectively.

PRODUCTION OF MONOCLONAL ANTIBODY SPECIFIC TO B402-OH

We prepared a Mab to analyze the inhibitory functions of HIF hydroxylase inhibitors for PHD2. The peptides, 402i-OH (CGKKPDKALTLLAP$^{(OH)}$AAG), 402a-OH (CGKKPDKALTLLAP$^{(OH)}$AAGDTIISLD) and 402a (CGKKPDKALTLLAPAAGDTIISLD), were conjugated to carrier proteins, bovine serum albumin (BSA) or ovalbumin (OVA), using *N*-[γ-maleimidobutyryloxy] succinimde ester (GMBS) for sulfhydryl-amine conjugation according to the manufacturer's protocols (Pierce, USA). The peptide-protein conjugates were dialyzed in phosphate-buffered saline (PBS; 20 mM sodium phosphate and 150 mM sodium chloride, pH 7.4). To produce a Mab specific to 402a-OH, 30 μg of 402i-OH/BSA or 402i-OH/OVA was intraperitoneally immunized 4–5 times at 2 week intervals into Balb/c mice in Freund's adjuvant (Sigma, USA). Three days after the final intravenous boosting, the splenocytes derived from the immunized mice were fused with mouse P3/NS1/1-Ag4-1 (NS-1) myeloma cells. The hybridoma producing the Mab specific to 402a-OH were selected, and the Mab was named An402-OH.

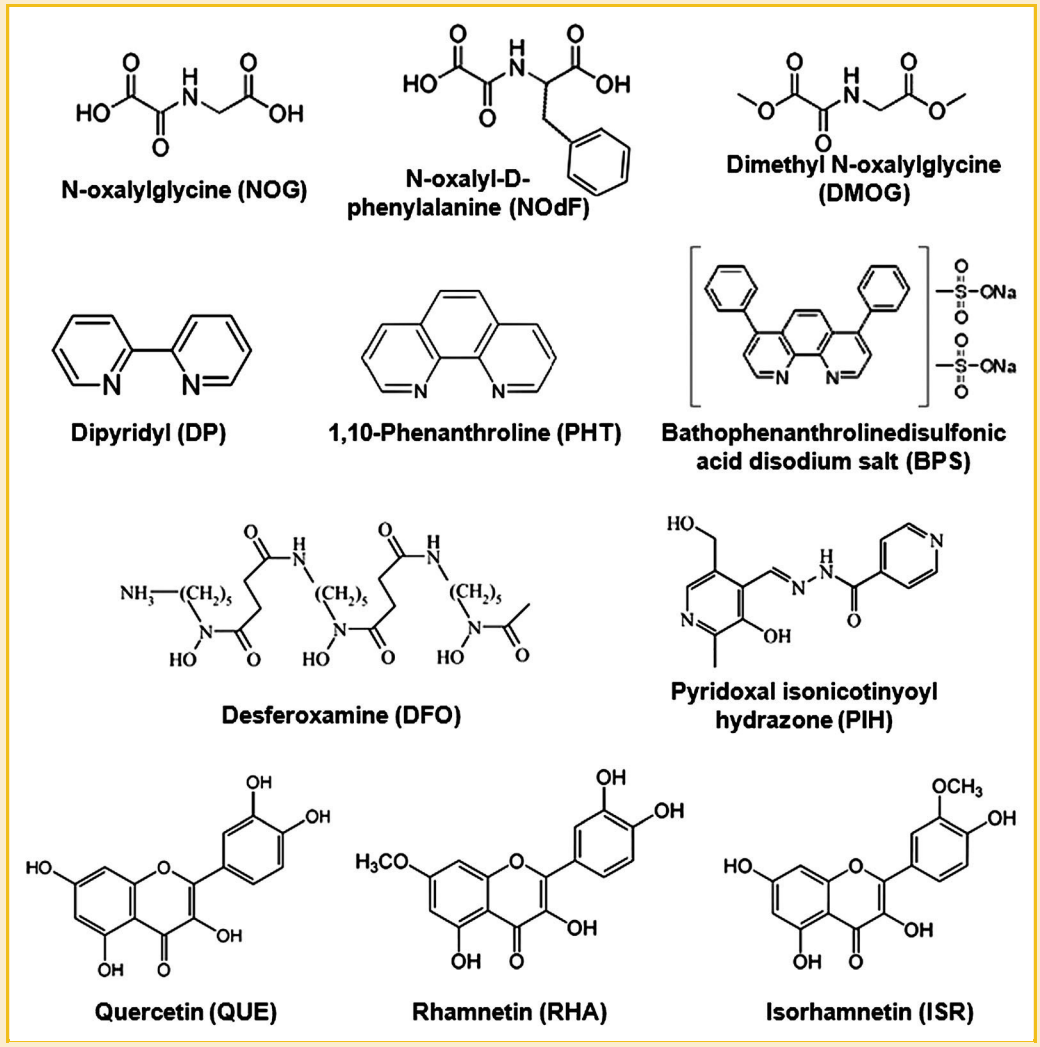

Fig. 1. The molecular structures of hydroxylase inhibitors used in this study.

MONOCLONAL ANTIBODY-BASED ELISA

In vitro activity of the hydroxylase inhibitors was measured by the Mab-based ELISA method as described previously [Lee et al., 2008]. SHN-HIF1α and An402-OH were used for detection of the hydroxylated peptides. Briefly, one of the inhibitors was serially diluted in dimethyl sulfoxide (DMSO) to 0–5.5 mM. Two microliter of the diluted compounds were mixed with 10 μl of the enzyme solution (50 mM Tris pH 7.5, 2 mM DTT, 0.55 μM PHD2 or 0.22 μM FIH, 220 μg/μl catalase, and 11 μM AFS) and incubated for 10 min at 37°C. Ten microliter of the substrate solution (50 mM Tris pH 7.5, 2 mM DTT, 2.2 μM b402, 55 μM 2OG, and 1.1 mM ascorbate) was then added and incubated for an additional 1 h (PHD2) or 30 min (FIH) at 37°C. All reactions were stopped by adding 78 μl of 5 mM EDTA. The hydroxylation of b803 or b402 in the reactant was analyzed through indirect ELISA as described previously [Lee et al., 2008]. Briefly, 5 or 20 μl of each reactant for FIH or PHD2 was diluted in 95 or 80 μl 0.1% Tween 20-TBS, respectively, and the diluents were loaded on the 50 ng streptavidin coated microtiter plate. The plate was then incubated with the Mab, SHN-HIF1α or An402-OH, respectively, and the remaining steps were following the procedure [Lee et al., 2008; Shin et al., 2008]. The results were analyzed using GraphicPad Prism 3.0 (GraphPad Software, Inc., USA).

IMMUNOBLOTING

The cellular functions of the inhibitors were analyzed by immunobloting. Prior to experiments, cells were grown to about 70–80% confluence in 60 mm dishes. Cells were treated with one of the inhibitors dissolved in DMSO, and then incubated for 4 or 16 h under normoxia (21% oxygen) or hypoxia (1% oxygen). To test the iron-mediated recovery of the intracellular hydroxylase function, 100 μM Fe^{2+} or Fe^{3+} was added to the cells in the presence or absence of the inhibitor. The nuclear proteins of HeLa cells were extracted using a Nuclear Extraction Kit (Panomics, USA), and total extracts of Hep3B cells were prepared by treatment of SDS-buffer (1% SDS, 25 mM Tris–HCl (pH 6.8), 10% glycerol and 20 mM dithiotreitol and then sonication. The proteins in the extracts were separated by SDS–PAGE, and then immunoblots were performed with one of the antibodies, anti-HIF-1α 28b Mab, anti-P50, anti-

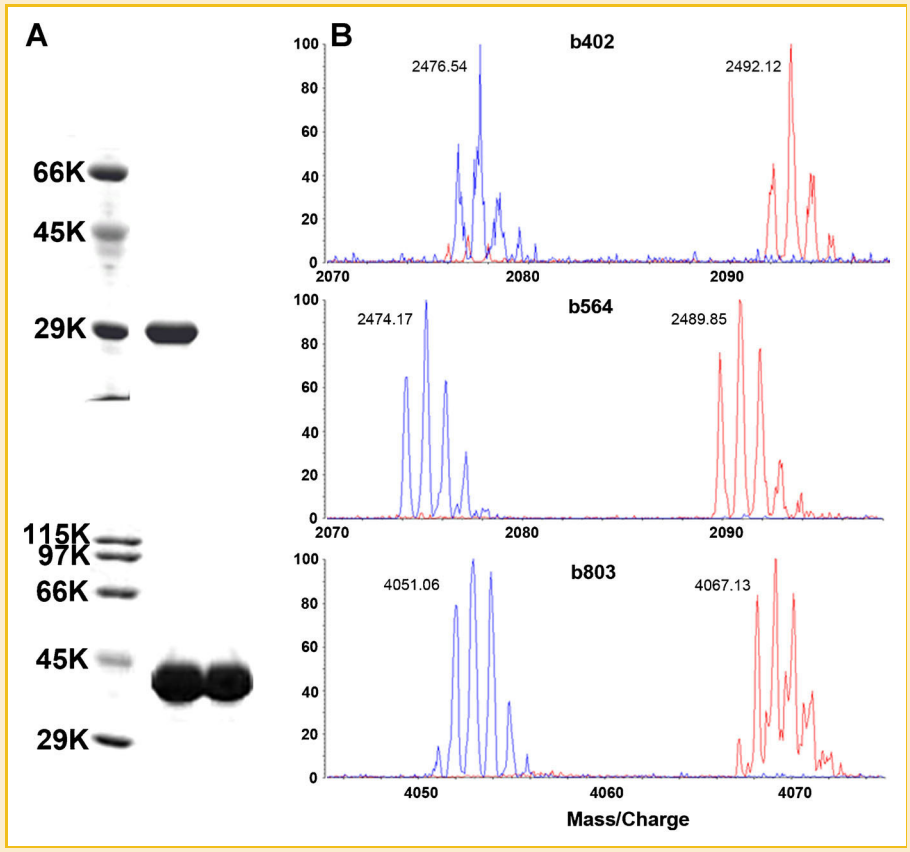

Fig. 2. SDS–polyacrylamide gel electrophoresis for the purified HIF hydroxylases, PHD2 (top) and FIH (bottom; A) and the molecular mass increases of the peptide substrates by PHD2 and FIH (B). b402 and b564 peptides were used as the PHD2 substrates and b803 was used as the FIH substrate. The hydroxylated and unhydroxylated peptides in the reaction mixtures were obtained in the presence (red) and absence (blue) of 2-oxoglutarate (2OG). The hydroxylated peptides by PHD2 or FIH show the 16 Da difference as compared with the unhydroxylated ones.

tubulin (Santa Cruz Biotechnology) and SHN-HIF1α [Lee et al., 2008]. The target proteins were detected by subsequent treatment of peroxidase conjugated anti-mouse or anti-rabbit IgG goat antibody (Santa Cruz Biotechnology) and Amersham ECL Western Blotting Detection Reagents (GE Healthcare, UK).

LUCIFERASE REPORTER ASSAY

Hep3B cells containing the EPO/HRE-luciferase reporter gene were used for analyzing the inhibitor function. After treating one of the inhibitors to the cells for 16 h, the HIF-mediated transcriptional activity was analyzed by luciferase activity using Firefly Luciferase Assay System (Promega, USA). Luciferase activity was normalized to total cellular protein concentration measured by BCA protein assay kit (Thermo Scientific, USA).

RESULTS

CLONING OF PHD2 AND ENZYME PURIFICATION

The gene with the sequence 179–426 of PHD2 was cloned into pET-28a vector. The PHD2 protein contains the sequence 179–426 of PHD2 and N-terminal 18 additional sequence derived from the pET-28a vector after cleaving with thrombin, and the purified PHD2 mass agreed well with the calculated mass (29,435; Fig. 2A, top). The purified PHD2 and FIH showed above 90% purity (Fig. 2A, top and bottom, respectively). The activities of the purified PHD2 and FIH were initially measured by MALDI-TOF mass analyses as shown in Figure 2B. The 16 Da mass increases of both substrate peptides (b402 and b564) for PHD2 and b803 for FIH were observed in the presence of 2OG. These active enzymes were used for in vitro assay of hydroxylase inhibitors. The hydroxylated peptides were denoted b402-OH, b564-OH, and b803-OH.

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY AN402-OH

Previously, we proved that the SHN-HIF1α-based ELISA system is a powerful tool for sensitive analyses of FIH inhibitors [Lee et al., 2008]. In order to analyze PHD2 inhibitors, we produced a Mab, An402-OH, specific to the Pro402 hydoxylated HIF-1α peptide, and developed an An402-OH-based ELISA system. An402-OH elicited extreme specificity to the Pro402 hydroxylated HIF-1α peptides, 402a-OH and b402-OH, while had almost no affinity to 402a-WT and b402 without Pro402 hydroxylation (Fig. 3). Its isotype was identified to be IgG2b with the κ light chain.

IN VITRO INHIBITION OF HYDROXYLASES BY INHIBITORS

The SHN-HIF1α-based ELISA system was proven to be a powerful tool for analyzing FIH inhibitors [Lee et al., 2008]. The molecular

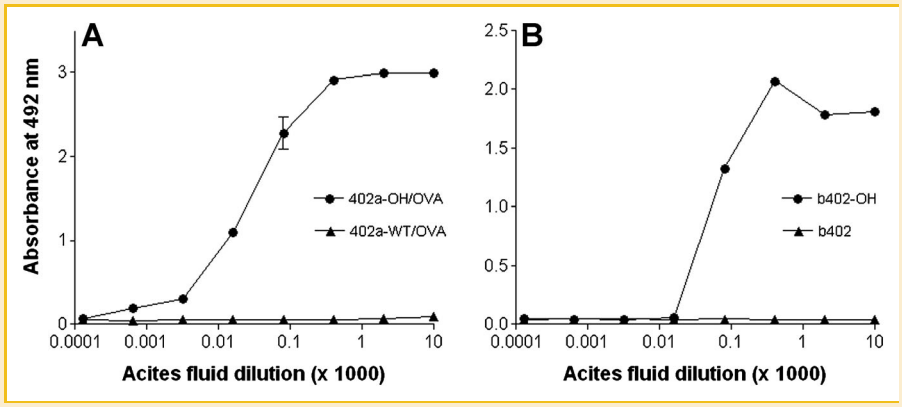

Fig. 3. Specific binding of An402-OH to the Pro402 hydroxylated HIF-1a peptides. The binding property of An402-OH to the immobilized peptides was tested by indirect ELISA. The symbols of panels A and B represented are the hydroxylated peptide (closed circle) and the unhydroxylated one (closed triangle). An402-OH only shows the specific bindings to the Pro402 hydroxylated peptides, 402a-OH (A) and b402-OH (B), but not to the unhydroxylated peptides, 402s-WT (A) and b402-OH (B).

structures of the inhibitors used in this study are shown in Figure 1. The An402-OH based ELISA system also proved to be useful for sensitive analyses of PHD2 inhibitors. The enzyme activity was reduced by the inhibitors in a dose-dependent manner, and the IC_{50} values of the inhibitors were calculated by the graphs after curve-fitting using the Graphpad Prizm 3.0 program.

The 2OG analogs show the differential inhibition pattern for PHD2 and FIH. NOG displayed a 35-times stronger inhibitory activity for FIH than for PHD2 ($IC_{50} = 1.10\ \mu M$ vs. 37.2 μM), and NOdF showed the strict and strong inhibition ($IC_{50} = 0.12\ \mu M$) for FIH only (Fig. 4A). These inhibitors showed the classical competitive inhibition pattern (Hill slope = 0.8–1.1).

In vitro activity of the iron chelator elicited relatively much less difference between PHD2 and FIH than the 2OG analog, and the Hill slope of each inhibition curve was above 2.5 (Fig. 4B,C, respectively). The results suggest that the iron chelation may be a key mechanism of hydroxylase inhibition rather than their direct enzyme binding. Although Fe^{2+} is essential for the hydroxylase activity, the strict Fe^{2+}-specific chelators, DP, PHT, and BSP, were less active to both enzymes than DFO and PIH known Fe^{3+} chelators. Since Fe^{2+} is the only iron ion in the reaction solution, the present data demonstrate that DFO and PIH have also high affinity to Fe^{2+}. DFO with the multiple iron chelating motifs in a molecule elicited higher inhibition for both enzymes (IC50 = 7.64 μM for PHD2 and 8.41 μM for FIH) than the other iron chelators.

QUE showed the strong inhibition for both PHD2 and FIH ($IC_{50} = 2.88$ and 6.94, respectively; Fig. 4D). QUE has three different iron-chelating motifs, 3′, 4′-dihydroxy, 3-hydroxy and 4-oxo, and 5-hydroxy and 4-oxo. Among them, the 3-hydroxy and 4-oxo motif and 5-hydroxy and 4-oxo motif have been reported to play an important role for the Fe^{2+} chelation [Leopoldini et al., 2006]. However, the present data show the importance of the 3′-hydroxy group for hydroxylase inhibition. ISR with a 3′-hydroxymethylation of QUE showed much less inhibitory activity for both enzymes than QUE, and moreover displayed less inhibition at 500 μM than at 100 μM unlike the other inhibitors (Fig. 4D). In contrast RHA with a 7-hydroxymethylation of QUE elicited the similar inhibition pattern as QUE.

The IC_{50} values of the inhibitors are summarized in Figure 4E.

CELLULAR INHIBITION OF INHIBITORS

To investigate cellular effects of the inhibitors, we performed Western blot analyses against nuclear extracts of HeLa cells and total extracts of Hep3B cells treated with the inhibitors. The intracellular HIF-1α accumulation induced by inhibition of PHDs have been extensively studied, but FIH-mediated Asn803 hydroxylation of HIF-1α has recently studied using our Mab, SHN-HIF1α [Lee et al., 2008, 2009; Tian et al., 2011].

DMOG, a membrane-permeable prodrug form of NOG, was less efficient to induce HIF-1α expression under normoxia (no HIF-1α band at 200 μM and slight accumulation of HIF-1α at 500 μM), but strongly inhibited the Asn803 hydroxylation for the hypoxia-induced HIF-1α (approximate 50% inhibition between 1.6 and 8 μM) under hypoxia in HeLa cells (Fig. 5A). This result is correlated to the in vitro data.

In contrast, the iron-chelators displayed almost no correlation between in vitro and cellular data. Recently, the iron chelators, DFO and DP, manifested different dose-dependent effects. DP but not DFO restricts Asn803 hydroxylation [Tian et al., 2011]. We hypothesized that the results may be caused by the properties of iron chelators. The membrane permeable Fe^{2+}-specific chelators, DP and PHT, could enhance HIF-1α accumulation by PHD2 inhibition as well as prevent Asn803 hydroxylation by FIH inhibition (Fig. 5B,C), even though they have less in vitro potency. PHT and DP induced HIF-1α accumulation at 50 μM under normoxia, but DP showed almost no inhibition on Asn803 hydroxylation unlike PHT (Fig. 5D). BSP, a membrane-impermeable Fe^{2+} specific chelator, showed no cellular effect even at 300 μM, however, even though it has almost the same in vitro inhibitory potency as PHT.

The Fe^{3+}-chelators displayed the different results as compared with strict Fe^{2+} chelators. The membrane-impermeable DFO effectively enhanced the HIF-1α accumulation at 150 μM under normoxia, while failed to prevent Asn803 hydroxylation even under hypoxia (Fig. 5B,C). The membrane-permeable Fe^{3+}-chelator PIH was less effective to induce the HIF-1α accumulation than DFO, but was able to partially inhibit Asn803 hydroxylation at 300 μM

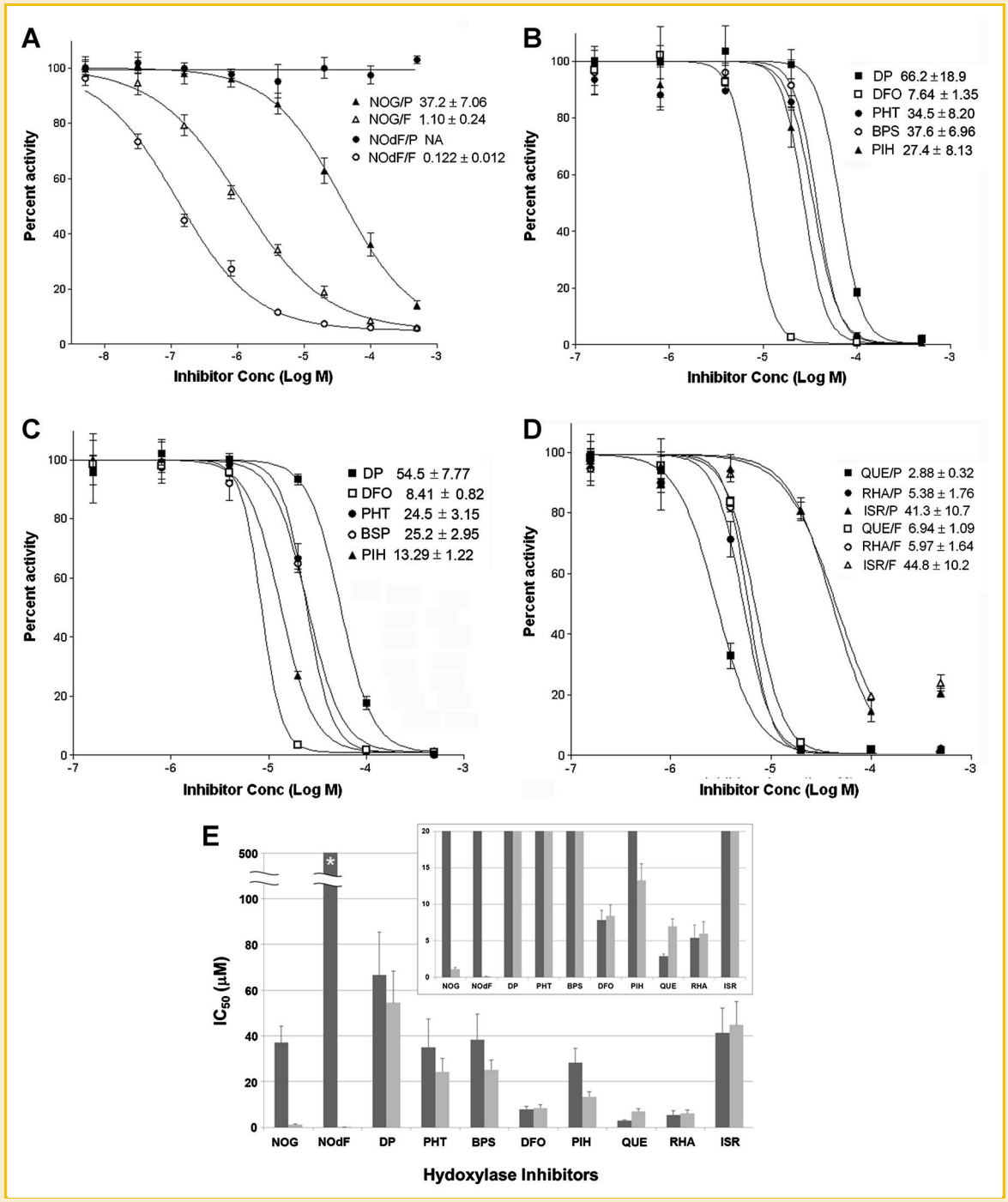

Fig. 4. In vitro inhibitory activities of hydroxylase inhibitors against PHD2 and FIH. (A) shows the inhibition patterns of the serially diluted 2OG analogs to PHD2 and FIH. (B,C) show the inhibitory patterns of the serially diluted iron specific chelators for PHD2 and FIH, respectively. (D) shows the serially diluted QUE and its methylated analogs to PHD2 and FIH. The hydroxylases, PHD2 and FIH, are represented to/P and/F, respectively, in (A) and (D). The numbers in (A–D) indicate the IC_{50} values and standard deviations, and the results are also summarized in (E). The dark and light gray bars represent the IC_{50} values for PHD2 and FIH, respectively. NOdF (*) shows no activity even at the maximum concentration (500 μM) tested. The IC_{50} values of ISR are calculated using the plots ranged from 0.16 to 100 μM because of unusual increase at 500 μM.

(Fig. 5B,C). Taken together, these results suggest that the cellular functions of iron-chelators may be determined by their iron-specificity and membrane permeability.

QUE is also reported to induce the HIF-1α accumulation in cells by their iron chelating property [Park et al., 2008]. QUE has the chelating ability with the iron ions (Fe^{2+} and Fe^{3+}) and copper ions (Cu^{+} and Cu^{2+}) with different stoichiometry, respectively [Leopoldini et al., 2006]. Since QUE and its methylated analogs used in this study showed slight cellular toxicity to the Hep3B cells at 100 μM such as vacuole formation, 50 μM or less concentration of the

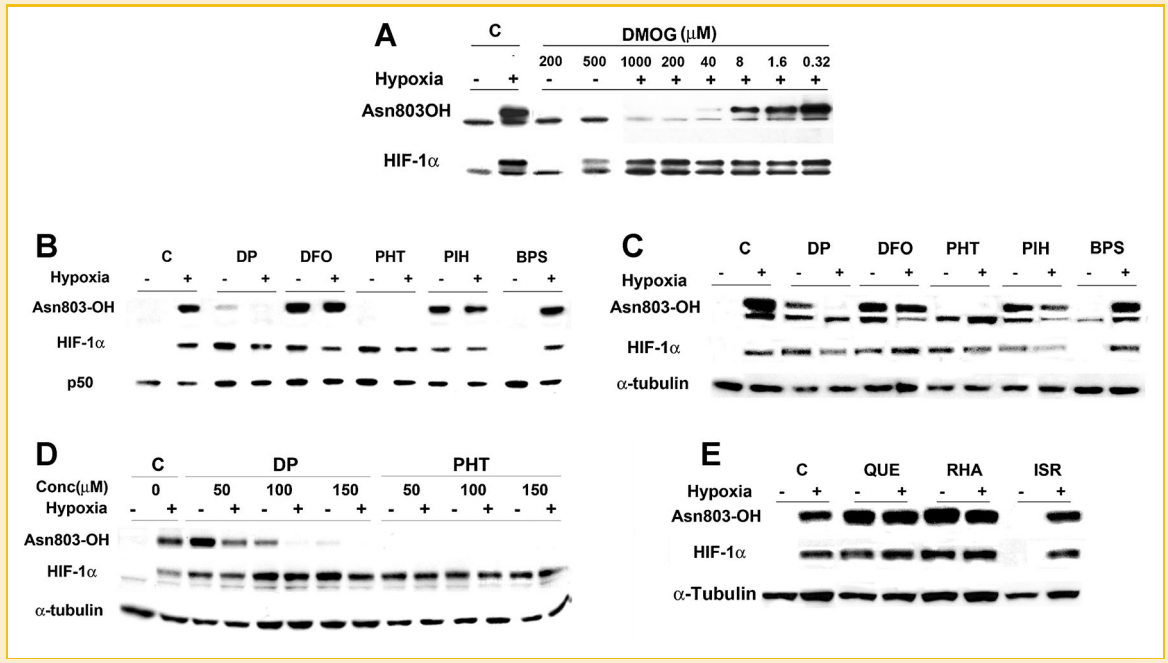

Fig. 5. The cellular function of the inhibitors in HeLa and Hep3B cells under normoxia and hypoxia. The HeLa and Hep3B cells were treated with various inhibitors for 16 h under normoxia and hypoxia. The nuclear extracts of HeLa cells (A,B) and the total extract of Hep3B cells (C–E) were separated by SDS–PAGE and immunoblotted by SHN–HIF1α and the anti HIF-1α antibody for detecting the Asn803 hydroxylated HIF-1α and total HIF-1α, respectively. The anti-p50 or anti-α-tubulin antibody was used as a control antibody for the nuclear or total cell extracts, respectively. The HIF-1α stabilized under hypoxic condition (1% oxygen concentration) still contains the Asn803 hydroxylated form. The inhibitor concentrations used in (B,C,E) are 100 μM (DP and PHT), 150 μM (DFO), 300 μM (PIH and BPS), and 50 μM (QUE, RHA, and ISR).

compound was used to treat cells. QUE can penetrate the cell membrane [Triantafyllou et al., 2008], and therefore the methylated compounds, ISR and RHA, are also considered to penetrate the cell membrane. QUE induced HIF-1α accumulation, but failed to reduce Asn803 hydroxylation (Fig. 5E). RHA showed similar effectiveness in HIF-1α accumulation to QUE, while ISR showed no inhibitory functions for both PHDs and FIH in cells (Fig. 5E).

The iron chelator-dependent HIF-1α accumulation has been reported to be reversed by adding iron ions. Fe^{2+} and Fe^{3+} were found to reverse the iron chelator-mediated HIF-1α accumulation in Hela cells under normoxia. Fe^{2+}, but not Fe^{3+}, effectively inhibited HIF-1α accumulation mediated by the strict Fe^{2+} chelators, DP and PHT, whereas the DFO-mediated HIF-1α accumulation was prevented by treatments of both Fe^{2+} and Fe^{3+} (Fig. 6). These results also support that DP and PHT have strict Fe^{2+}-specificity but DFO nonspecifically chelates both Fe^{2+} and Fe^{3+} ions as we expected.

EFFECTS OF INHIBITORS ON HIF-MEDIATED TRANSCRIPTIONAL ACTIVITY

To test whether the inhibitors enhance the HIF transcriptional activity, we used Hep3B cells containing the stable EPO/HRE-dependent GL3-luciferase reporter gene. Simultaneous treatment of siRNAs corresponding to PHDs and FIH has been known to synergistically enhance the HIF transcription [Dayan et al., 2006]. In this concept, we considered that PHT and DP enhance the HIF-transcriptional activity than the other inhibitors. In fact, it has been reported that the cells treated with 100 μM DP show higher HIF-1α-dependent transcription than those under hypoxia or those treated with 1 mM DMOG [Lando et al., 2002b]. As we expected, DP and PHT elicited more significant enhancement of the luciferase activity than the other inhibitors (Fig. 7). Especially, the most effective PHT displayed twice higher activity even at 1.56 μM than no treatment, and had 16 times higher activity than DP. Interestingly, the luciferase activities enhanced by PHT and DP were more efficient than their in vitro hydroxylase inhibitions unlike the other inhibitors. These results suggest that the synergistic inhibitions of both PHDs and FIH are important for effective increase of HIF transcription in cells [Dayan et al., 2006]. Although QUE and RHA stabilized intracellular HIF-1α (Fig. 5E), they displayed less than twice enhancement of HIF transcription at 50 μM as the previous report [Triantafyllou et al., 2008]. We assumed that the

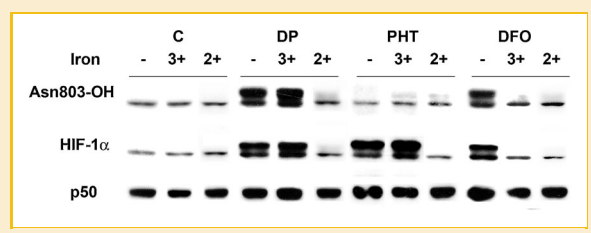

Fig. 6. Effects of iron ions on the iron-specific chelator-induced HIF-1α accumulation and Asn803 hydroxylation under hypoxia. The HeLa cells treated for 16 h with one of the inhibitors, 50 μM DP, 50 μM PHT, and 100 μM DFO, in the presence or absence of 100 μM Fe^{2+} or Fe^{3+} as indicated. The nuclear extracts of HeLa cells were separated by SDS–PAGE and immunoblotted with SHN-HIF1α, the anti HIF-1α or anti p50 antibody.

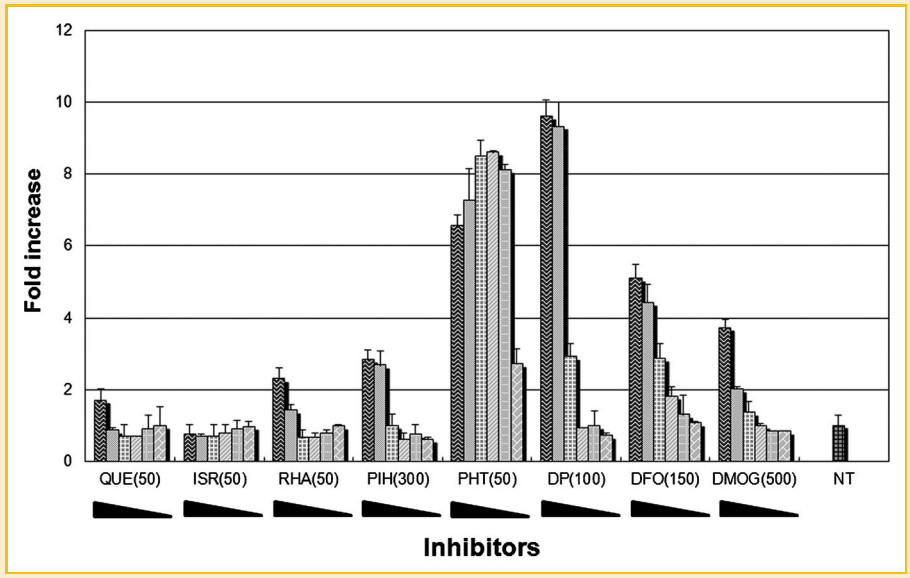

Fig. 7. Effects of the inhibitors on the HIF-dependent transcription. The Hep3B cells with the EPO/HRE-luciferase gene were treated with the inhibitors. Each inhibitor was 1/2 serially diluted from the concentration (μM) in the parentheses, and then treated cells for 16 h. The luciferase activities in the cells were analyzed using Firefly Luciferase Assay System (Promega, USA). Luciferase activity was normalized to total cellular protein concentration measured using BCA protein assay kit (Thermo Scientific, USA). The results are expressed as fold increase in the normalized luciferase activity in relation to the control (NT).

COMT-mediated 3′-o-methylation of QUE or RHA may be one of the reasons reducing its potency in cells.

DISCUSSION

The in vitro analyses of small molecule libraries are the first step to screen drug candidates. Previously, we have developed the SHN-HIF1α-based ELISA system for screening FIH inhibitors, and proved that our method is at least 30 times or more sensitive than the previous methods [Lee et al., 2008]. In this study, we also developed a Mab, An402-OH, extreme specificity to Pro402 hydroxylated peptides, and the An402-OH-based ELISA system was also proven to be a powerful tool for sensitive assays of PHD2 inhibitors. Since the Mab-based ELISA system is not impeded by the substrate types, it is considered to be powerful tools for screening the HIF hydroxylase inhibitors.

The structural difference of the 2OG binding domains between PHD2 and FIH has been reported to determine the 2OG analog selectivity. The D-enantiomer of N-oxalyl amino acids are weaker PHD inhibitors because of structural instability [Chowdhury et al., 2009], and the lack of activity of NOdF is explained in this aspect. However, the lower activity of NOG to PHD2 than to FIH cannot be explained using this concept. We think that the narrower opening to the 2OG binding site of PHD2 [McDonough et al., 2006] may be related to the differential activity.

In contrast to the 2OG analog, the inhibition mechanism of the iron chelator for the hydroxylases is depriving the available iron ions in solution. However, the cellular effects of iron chelators were quite different from their in vitro activities. Although some similar results were also reported [Tian et al., 2011], the detail mechanisms have not been proposed. We focused on the asymmetric distribution of iron ions between the extracellular and intracellular compartments. The Fe^{3+} ions are the major form in the extracellular fluid, while the Fe^{2+} ions are abundant in cytoplasm localizing the hydroxylases. The uptake of extracellular Fe^{3+} is mediated by transferrin (TF)/Tf receptor (TfR) system, the endocytic Fe^{3+} convert to Fe^{2+} in endosomes, and then transports to cytoplasm via divelent metal transporter 1 (DMT1) [MacKenzie et al., 2008]. Therefore, the iron specificity and membrane permeability of a chelator are considered to contribute primarily its cellular function. Based on the asymmetric iron distributions and the properties of the iron specific chelators, here we propose a model for possible action mechanisms of the iron chelators (Fig. 8). In this aspect, the membrane-permeable strict Fe^{2+} chelators, PHT and DP, are considered to directly access and deplete the intracellular Fe^{2+} without obstacle of the extracellular Fe^{3+}, and this may contribute efficient inhibitions of the intracellular PHDs and FIH. The fact that the Fe^{2+}-specific chelator function is reversed by the co-treatment of Fe^{2+}, but not Fe^{3+}, also supports our assumption. Previously, the simultaneous treatment of PHD2 siRNA and FIH siRNA has been reported to synergistically enhance the HIF transcriptional activity as compared with the treatment of PHD2 siRNA only [Dayan et al., 2006]. In this aspect, PHT and DP providing the similar effects are more powerful for enhancing the HIF-dependent transcription as shown in Figure 7. DFO has been reported not to directly penetrate into cells by passive diffusion, but can enter into cells by pinocytosis [Lloyd et al., 1991]. However, the internalized DFO remains in lysosomes after pinocytosis. The presence of DFO in the lysosomal lumen has been reported to reduce also the concentrations of ferritin, the major iron (Fe^{3+}) storage protein, and iron in cytoplasm by induction of autophagy-mediated ferritin degradation in lysosomes [De Dome-

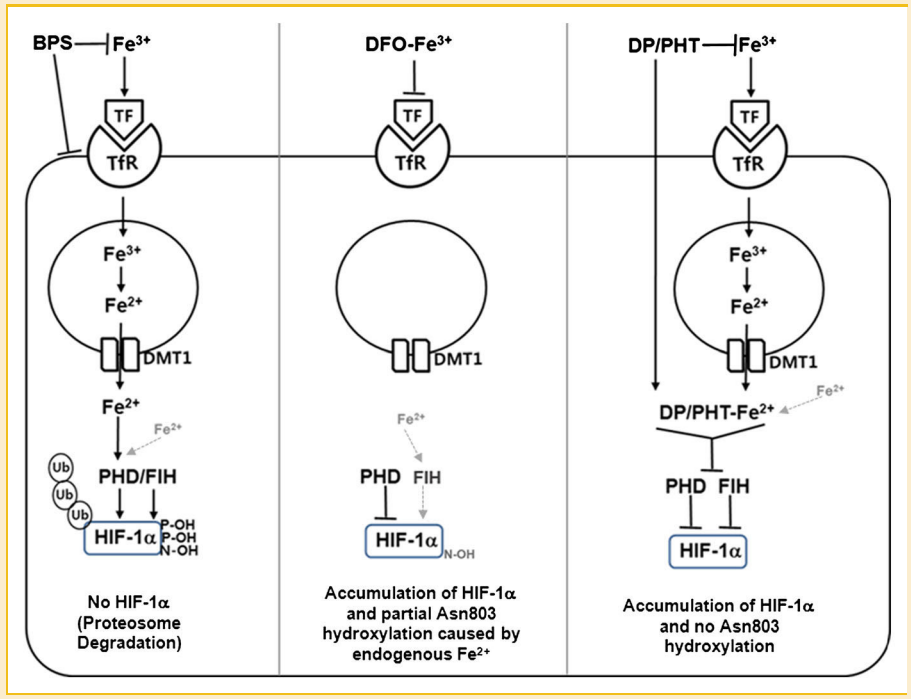

Fig. 8. A model for possible inhibition mechanisms of iron specific chelators according to their iron specificity and membrane permeability. The external Fe^{3+} is transported into endosome by transferrin (TF)/transferrin receptor (TfR)-mediated endocytosis. In endosome, Fe^{3+} is dissociated with TF and converted to Fe^{2+}. Fe^{2+} is then transported to cytoplasm via divalent metal transporter 1 (DMT1). The membrane impermeable and strict Fe^{2+} chelator BPS cannot chelate the external Fe^{3+} and the cytoplasmic Fe^{2+}, and this may be related to no inhibitory activity of BPS. DFO prevents the intracellular transport of the external Fe^{3+}, but may not block the function of the pre-existing Fe^{2+} in cytoplasm (gray). This may be sufficient to inhibit the PHD activity, but may not be enough to fully inhibit the FIH activity. In contrast, DP and PHT, which can be internalized into cells by diffusion, may directly chelate the cytoplasmic Fe^{2+}, and this induces the simultaneous abrogation of the PHD and FIH activity.

nico et al., 2009]. In addition, DFO can prevent the intracellular uptake of the extracellular Fe^{3+}, and can reduce the intracellular iron concentration as we suggested in Figure 8. These processes may efficiently reduce the iron concentration in cytoplasm by DFO, even though it cannot be accessible to the cytoplasmic iron. These processes are sufficient for stabilization of HIF-1α by inhibition of PHDs, while FIH is still active in this condition because the pre-existing Fe^{2+} in cytoplasm may be enough to maintain the FIH function as we suggested in Figure 8. The results also suggest that FIH may have higher affinity to Fe^{2+} than PHDs. No cellular function of BPS can be inferred from the fact that it cannot deplete the extracellular Fe^{3+} as well as intracellular Fe^{2+}. The membrane-permeable Fe^{3+}-chelator PIH may provide more complicated effects in cells because it may bind and directly transport the external Fe^{3+} ions in cytoplasm. The increase of Fe^{3+} in cytoplasm may provide negative effects for hydroxylase inhibition.

The membrane permeable QUE has the 3-different iron chelating motifs belonging to the 3′-hydroxy/4′-hydroxy, 3-hydroxy/4-oxo, and 5-hydroxy/4-oxo ones. It has been reported that the 3-hydroxy/4-oxo and to 5-hydroxy/4-oxo groups are the potential iron-chelation sites of QUE [Leopoldini et al., 2006]. However, the present data show that the 3′-hydroxy/4′-hydroxy motif at the catechol moiety of QUE plays a more important role in the inhibition of the HIF hydroxylases (Fig. 6). It has been suggested a possibility of the specific interaction between QUE and FIH [Welford et al., 2003]. Since ISR still has the metal chelating moieties, the unusual inhibition patterns and less inhibitory activity of ISR for HIF hydroxylases are also proposed the possibility. QUE can be converted to ISR by COMT [Cornish et al., 2002]. No intracellular hydroxylase inhibition of ISR suggests that COMT may regulate the QUE functions in cells. COMT is also considered to be a negative regulating factor for hydroxylase inhibitors acting by the catechol moiety. The QUE-induced HIF-1α transcription is not effective as reported [Triantafyllou et al., 2008]. It has been suggested that QUE and its analogs prevent HIF-1α transport into nucleus in the report. It is difficult to obtain HIF-1α in the nuclear extract, and therefore the total extracts were used for analyzing QUE and its analogs. However, we believe that COMT may play an important role in the intracellular QUE function, because ISR with a 3′-methylation of QUE shows no function in cells.

In conclusion, we systemically investigated the in vitro and cellular functions of iron chelators for HIF hydroxylases, PHD2 and FIH. The iron specificity and membrane permeability of iron chelators were more critical for their cellular function rather than their in vitro inhibitory potencies. The direct depletion of the cytoplasmic Fe^{2+} by the membrane-permeable and strict Fe^{2+}-specific chelator proved to be more effective for abrogating both enzyme activities in cells, while the membrane-impermeable one showed no effect in cells. In contrast, the depletion of extracellular Fe^{3+} by the membrane-impermeable Fe^{3+} chelator was enough to inhibit the PHDs but not FIH. Taken together, we propose a model for possible inhibition mechanisms of iron specific

chelators (Fig. 8). The 3′-hydroxyl group of QUE is critical for in vitro and cellular inhibition of hydroxylases, implicating that its methylation by COMT is considered one of the controlling methods of QUE as well as the other hydroxylase inhibitors acting by the catechol moiety. These results offer important factors for developing ant-ischemic drugs.

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