Clioquinol, a Cu(II)/Zn(II) Chelator, Inhibits Both Ubiquitination and Asparagine Hydroxylation of Hypoxia-inducible Factor-1α, Leading to Expression of Vascular Endothelial Growth Factor and Erythropoietin in Normoxic Cells*

We found that the Cu(II) and Zn(II)-specific chelator Clioquinol (10–50 μM) increased functional hypoxia-inducible factor 1α (HIF-1α) protein, leading to increased expression of its target genes, vascular endothelial growth factors and erythropoietin, in SH-SY5Y cells and HepG2 cells. Clioquinol inhibited ubiquitination of HIF-1α in a Cu(II)- and Zn(II)-dependent manner. It prevents FIH-1 from hydroxylating the asparagine residue (803) of HIF-1α in a Cu(II)- and Zn(II)-independent fashion. Therefore, it leads to the accumulation of HIF-1α that is prolyl but not asparaginyl hydroxylated. Consistent with this, co-immunoprecipitation assays showed that Clioquinol-induced HIF-1α interacted with cAMP-responsive element-binding protein in normoxic cells, implying that Clioquinol stabilizes the trans-activating form of HIF-1α. Our results indicate that Clioquinol could be useful as an inducer of HIF-1α and its target genes in ischemic diseases.

Hypoxia-inducible factor 1 (HIF-1)³ is a key regulator of hypoxic adaptation that functions by activating the transcription of several genes involved in angiogenesis, erythropoiesis, and glycolysis (1, 2). It consists of two subunits; HIF-1α is rapidly degraded under normoxic condition by the ubiquitin-proteasome system, whereas HIF-1β is stable. Under normoxic conditions the proline 564 and/or 402 residues of HIF-1α are hydroxylated by HIF-1α-specific prolyl-4 hydroxylases (PHDs), which need O2, α-ketoglutarate, vitamin C, and Fe(II) (3–6). The hydroxylated prolines interact with von Hippel-Lindau (VHL) protein, a component of E3 ubiquitin ligase, and the HIF-1α is ubiquitinated by the VCB E3 ubiquitin-ligase complex, consisting of VHL protein, ElonginB, ElonginC, Cul2, and Rbx1 (7, 8). In hypoxic conditions, proline hydroxylation decreases and HIF-1α accumulates. Oxygen molecules inhibit not only the stabilization of HIF-1α but also its transactivation activity, because the protein, factor-inhibiting HIF-1α (FIH-1), catalyzes hydroxylation of its asparagine residue using O2, α-ketoglutarate, vitamin C, and Fe(II). Hydroxylation of this asparagine residue in the transactivation domain of HIF-1α prevents it from recruiting its coactivator, cAMP-response element-binding protein (CBP) (3, 9).

Thus, activation of HIF-1α is censored by two systems, proline hydroxylation and asparagine hydroxylation. HIF-1α and its targets, such as EPO and VEGF, are being evaluated as therapeutic agents for cerebral and myocardial infarctions, and a small lipophilic HIF-1α-activating compound is being sought as a treatment for these diseases. However, to generate fully functional HIF-1α, a putative HIF-1α activator should suppress both proline hydroxylation-dependent ubiquitination and asparagine hydroxylation.

We showed previously that the zinc chelator N,N,N’,N”-tetraakis (2-pyridylmethyl) ethylenediamine (TPEN) enhances the activity of PHD2 but that the level of HIF-1α protein does not fall because TPEN also inhibits its ubiquitination. Because TPEN does not prevent FIH-1 from hydroxylating the asparagine residue of HIF-1α, it leads the accumulation of nonfunctional HIF-1α (10, 11). Here, we report that another zinc chelator, Clioquinol, which has relatively low affinity but high selectivity for Zn(II) and Cu(II), has a different effect on the activity of HIF-1α. Both TPEN and Clioquinol inhibit ubiquitination of HIF-1α and cause its accumulation. However, in contrast to TPEN, Clioquinol prevents FIH-1 from hydroxylating HIF-1α. It therefore stabilizes functional HIF-1α, leading to expression of its target genes in normoxic cells.

Clioquinol has been used in Alzheimer, Parkinson, and Huntington diseases as a Cu(II)- and Zn(II) chelator that reverses Zn(II)- or Cu(II)-induced metalloprotein precipitation (12).
Measurement of PHD Activity by a VHL Pulldown Assay—The human PHD2 gene (identical to AJ310543) was cloned into the pET21b His2 (+) vector and overexpressed in Escherichia coli as histidine-tagged fused proteins and purified by nickel-affinity chromatography (11). The in vitro VHL pulldown assay was performed as described by Jaakkola et al. (6). Briefly, [35S]methionine-labeled VHL protein was synthesized by in vitro transcription and translation using the pcDNA3.1/hygro-VHL plasmid, according to the instruction manual (catalogue number L1170; Promega). GST-ODD (amino acids 401–603 of human HIF-1α) was expressed in E. coli and purified with glutathione-unionflow resin according to the instruction manual (catalogue number 8912-1; BD Biosciences Clontech). Resin-bound GST-ODD (200 μg of protein/∼80 μl of resin volume) was incubated in the presence of 2 mM ascorbic acid, 100 μM FeCl2, and 5 mM α-ketoglutarate with 1.5–3 μM of PHD2 in 200 μl of NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) with mild agitation for 90 min at 30 °C. The reaction mixture was centrifuged and washed three times with 10 volumes of NETN buffer. Resin-bound GST-ODD was mixed with 10 μl of 35S-labeled VHL in 500 μl of EBC buffer (120 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% (v/v) Nonidet P-40). After mild agitation at 4 °C for 2 h, the resin was washed three times with 1 ml of NETN buffer, and proteins were eluted in 3X SDS sample buffer, fractionated by 12% SDS-PAGE, and detected by autoradiography. The amount of each sample loaded was monitored by staining the GST-ODD with Coomassie Blue.

In Vitro Ubiquitination—HeLa cells were washed twice with cold hypotonic extraction buffer (20 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride). After removing the buffer, the cells were disrupted in a Dounce homogenizer, and the crude extract was centrifuged at 10,000 × g for 10 min at 4 °C to remove cell debris and nuclei. Aliquots of the supernatant (S-10 fraction) were stored at −70 °C. Ubiquitination assays were carried out at 30 °C for 270 min in a total volume of 40 μl containing 2 μl of 35S-labeled human HIF-1α-programmed reticulocyte lysate, 27 μl of S-10 extract (50 μg of protein), 4 μl of 10X ATP-regenerating system (20 mM Tris, pH 7.5, 10 mM ATP, 10 mM magnesium acetate, 300 mM creatine phosphate, 0.5 mg/ml creatine phos-

**FIGURE 1. Effect of Clioquinol on stabilization of HIF-1α.** A, effect of TPEN and Clioquinol on stabilization of HIF-1α. HepG2 and SH-SY5Y cells were treated with TPEN (5 μM) and Clioquinol (indicated doses) in normoxic or hypoxic conditions (1% O2). For the indicated hours. HIF-1α was detected by Western analysis with anti-human HIF-1α antibody (1:800 dilution; BD Biosciences). B, effect of the Zn(II) and Cu(II) on HIF-1α stabilization by Clioquinol. SH-SY5Y cells were treated with Clioquinol (50 μM) for 6 h. HIF-1α was detected by Western analysis with antibody (1:800 dilution; BD Biosciences).
Clioquinol-induced HIF-1α and Its Targets

**RESULTS**

**Clioquinol Stabilizes Functional HIF-1α Protein**—Human hepatoma HepG2 cells and neuroblastoma SH-SY5Y cells were exposed to the cation chelators TPEN and Clioquinol in both normoxic and hypoxic (1% \(O_2\)) conditions, and levels of HIF-1α protein were measured by Western blotting. Both TPEN and Clioquinol dramatically increased the amount of HIF-1α in normoxic cells (Fig. 1A). The data in Fig. 1B show that addition of Zn(II) and Cu(II) together reversed the effect of Clioquinol, indicating that it stabilizes HIF-1α by depleting Zn(II), and temperature, excess salts were removed with ZipTip C18 (Millipore). The peptide was eluted from the tip with \(\alpha\)-cyano-4-hydroxycinnamic acid in acetonitrile/water containing 0.1% trifluoroacetic acid (50:50, v/v) followed by extensive washing with 0.1% trifluoroacetic acid in water. The eluted peptide solution was transferred to a MALDI sample plate and MALDI-TOF measurements were performed with a Voyager analyzer (Applied Biosystems).

**Co-immunoprecipitation**—HepG2 cells were grown to 80% confluence on 100-mm tissue culture plates and treated with drugs as indicated for 6 h in normoxic or hypoxic conditions. Whole cell extracts were prepared as previously described (6). For immunoprecipitation, 200-μg samples of whole cell lysates were preclarified by incubating with 1 μg of anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and 20 μl of 0.5% ImmunoPure immobilized protein A/G gel (Pierce) for 30 min at 4°C. The cleared extracts were mixed with 1 μg of anti-CBP antibody (Santa Cruz Biotechnology) and 1% Triton X-100. After addition of 15 μl of 0.5% ImmunoPure immobilized protein A/G gel, they were rotated overnight at 4°C. The immunoprecipitates were pelleted, washed four times with phosphate-buffered saline, and resuspended in SDS sample buffer. They were then boiled for 5 min and run on 8% SDS-polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes by semi-dry transfer (Trans-Blot; Bio-Rad). Co-immunoprecipitated proteins were reacted with anti-human HIF-1α antibody (BD Biosciences) and/or anti-CBP antibody and visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce) with anti-mouse or rabbit Ig conjugated with horseradish peroxidase as secondary antibody. Weak signals from protein bands on Western blots were visualized with a luminence image analyzer (Model LAS-3000; Fuji).

**Measurement of FIH-1 Activity**—The human FIH-1 gene (AF395830) was cloned into pET28a vector (Novagen), and FIH-1 was overexpressed in E. coli as a histidine-tagged fusion protein and purified by nickel-affinity chromatography. The fusion protein was further purified by gel filtration chromatography (Hi-Load Superdex 200) and concentrated by ultrafiltration. FIH-1 activity was measured by GST-CBP pulldown assays as described previously (10, 18). Alternatively FIH-1 activity was measured by Asn hydroxylation of F-HIF-1α peptide. Hydroxylation of peptide was measured by mass spectrophotometric analysis. F-HIF-1α (amino acids 788–822) peptide (fluorescein isothiocyanate-aca-DESGLPQ1T5SYDCEV/NAPIQGSRNLLQGEELHALR) containing fluorescein conjugated with an N-terminal-inserted aminocaproic acid (aca) linker (AnyGen, Kwangju, Korea) developed for another assay was used for the FIH-1 reaction. The peptide was incubated at a final concentration of 4 μM with 2.8 μg of recombinant FIH-1 in 20 mm Tris buffer, pH 7.5, 5 mm KCl, and 1.5 mm MgCl₂ containing 100 μm 2-ketoglutarate and 400 μM ascorbic acid in a total volume of 50 μl. After incubation for 2 h at room temperature, excess salts were removed with ZipTip C18 (Millipore). The peptide was eluted from the tip with 0.1% trifluoroacetic acid (50:50, v/v) followed by extensive washing with 0.1% trifluoroacetic acid in water. The eluted peptide solution was transferred to a MALDI sample plate and MALDI-TOF measurements were performed with a Voyager analyzer (Applied Biosystems).

**Co-immunoprecipitation**—HepG2 cells were grown to 80% confluence on 100-mm tissue culture plates and treated with drugs as indicated for 6 h in normoxic or hypoxic conditions. Whole cell extracts were prepared as previously described (6). For immunoprecipitation, 200-μg samples of whole cell lysates were preclarified by incubating with 1 μg of anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and 20 μl of 0.5% ImmunoPure immobilized protein A/G gel (Pierce) for 30 min at 4°C. The cleared extracts were mixed with 1 μg of anti-CBP antibody (Santa Cruz Biotechnology) and 1% Triton X-100. After addition of 15 μl of 0.5% ImmunoPure immobilized protein A/G gel, they were rotated overnight at 4°C. The immunoprecipitates were pelleted, washed four times with phosphate-buffered saline, and resuspended in SDS sample buffer. They were then boiled for 5 min and run on 8% SDS-polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes by semi-dry transfer (Trans-Blot; Bio-Rad). Co-immunoprecipitated proteins were reacted with anti-human HIF-1α antibody (BD Biosciences) and/or anti-CBP antibody and visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce) with anti-mouse or rabbit Ig conjugated with horseradish peroxidase as secondary antibody. Weak signals from protein bands on Western blots were visualized with a luminence image analyzer (Model LAS-3000; Fuji).

**RESULTS**

**Clioquinol Stabilizes Functional HIF-1α Protein**—Human hepatoma HepG2 cells and neuroblastoma SH-SY5Y cells were exposed to the cation chelators TPEN and Clioquinol in both normoxic and hypoxic (1% \(O_2\)) conditions, and levels of HIF-1α protein were measured by Western blotting. Both TPEN and Clioquinol dramatically increased the amount of HIF-1α in normoxic cells (Fig. 1A). The data in Fig. 1B show that addition of Zn(II) and Cu(II) together reversed the effect of Clioquinol, indicating that it stabilizes HIF-1α by depleting Zn(II), and

**Measurement of FIH-1 Activity**—The human FIH-1 gene (AF395830) was cloned into pET28a vector (Novagen), and FIH-1 was overexpressed in E. coli as a histidine-tagged fusion protein and purified by nickel-affinity chromatography. The fusion protein was further purified by gel filtration chromatography (Hi-Load Superdex 200) and concentrated by ultrafiltration. FIH-1 activity was measured by GST-CBP pulldown assays as described previously (10, 18). Alternatively FIH-1 activity was measured by Asn hydroxylation of F-HIF-1α peptide. Hydroxylation of peptide was measured by mass spectrophotometric analysis. F-HIF-1α (amino acids 788–822) peptide (fluorescein isothiocyanate-aca-DESGLPQ1T5SYDCEV/NAPIQGSRNLLQGEELHALR) containing fluorescein conjugated with an N-terminal-inserted aminocaproic acid (aca) linker (AnyGen, Kwangju, Korea) developed for another assay was used for the FIH-1 reaction. The peptide was incubated at a final concentration of 4 μM with 2.8 μg of recombinant FIH-1 in 20 mm Tris buffer, pH 7.5, 5 mm KCl, and 1.5 mm MgCl₂ containing 100 μm 2-ketoglutarate and 400 μM ascorbic acid in a total volume of 50 μl. After incubation for 2 h at room temperature, excess salts were removed with ZipTip C18 (Millipore). The peptide was eluted from the tip with 0.1% trifluoroacetic acid (50:50, v/v) followed by extensive washing with 0.1% trifluoroacetic acid in water. The eluted peptide solution was transferred to a MALDI sample plate and MALDI-TOF measurements were performed with a Voyager analyzer (Applied Biosystems).

**Co-immunoprecipitation**—HepG2 cells were grown to 80% confluence on 100-mm tissue culture plates and treated with drugs as indicated for 6 h in normoxic or hypoxic conditions. Whole cell extracts were prepared as previously described (6). For immunoprecipitation, 200-μg samples of whole cell lysates were preclarified by incubating with 1 μg of anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and 20 μl of 0.5% ImmunoPure immobilized protein A/G gel (Pierce) for 30 min at 4°C. The cleared extracts were mixed with 1 μg of anti-CBP antibody (Santa Cruz Biotechnology) and 1% Triton X-100. After addition of 15 μl of 0.5% ImmunoPure immobilized protein A/G gel, they were rotated overnight at 4°C. The immunoprecipitates were pelleted, washed four times with phosphate-buffered saline, and resuspended in SDS sample buffer. They were then boiled for 5 min and run on 8% SDS-polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes by semi-dry transfer (Trans-Blot; Bio-Rad). Co-immunoprecipitated proteins were reacted with anti-human HIF-1α antibody (BD Biosciences) and/or anti-CBP antibody and visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce) with anti-mouse or rabbit Ig conjugated with horseradish peroxidase as secondary antibody. Weak signals from protein bands on Western blots were visualized with a luminence image analyzer (Model LAS-3000; Fuji).
Cu(II) (19, 20). Addition of Fe(II) failed to reverse the effect of Clioquinol, indicating that Fe(II) is not involved in stabilization.

To see whether this stabilization led to expression of HIF-1α target genes we measured the expression of VEGF by reverse transcriptase PCR. In the event, Clioquinol but not TPEN increased expression of VEGF in normoxic cells (Fig. 2A), whereas TPEN reduced the hypoxia-induced expression of VEGF in SH-SY5Y cells. Similarly, Clioquinol, but not TPEN, induced expression of luciferase under the control of hypoxia-responsive elements (HRE) in normoxic cells whereas TPEN decreased expression in hypoxic cells (Fig. 2B). Ultimately, the activated HIF-1α binds to the HRE located in the enhancer region and thus increases transcription of target genes such as VEGF and EPO. Thus, the two chelators both stabilized HIF-1α protein but differed in their effects on transactivation of HIF-1α.

Effects of Clioquinol on the Activity of PHD2 and the Ubiquitination of HIF-1α—To account for this effect of Clioquinol we tested whether it changed the activity of PHD2. We examined the hydroxylated activity of PHD2 by measuring capture of 35S-labeled VHL by the ODD domain (amino acids 401–603) of human HIF-1α protein, because the interaction of VHL with HIF-1α depends on hydroxylation of the proline-402/564 residues. Human PHD2 was expressed with a histidine tag in E. coli and purified as described previously (11). We treated the purified full-length PHD2 with either Clioquinol or TPEN and measured its activity by VHL pulldown assays. TPEN increased the activity of PHD2 (10), whereas Clioquinol did not. This result demonstrates that the two zinc chelators differ in their effect on the activity of PHD2 (Fig. 3A).

We also looked for any change in the ubiquitination of HIF-1α in the presence of Clioquinol. We synthesized 35S-labeled HIF-1α and subjected it to in vitro ubiquitination in the presence of ubiquitin, ubiquitin aldehyde, and the S-10 fraction of cells. The ladder-like appearance of high molecular weight HIF-1α in Fig. 3B confirms that the 35S-labeled HIF-1α was ubiquitinated in vitro. Interestingly, Clioquinol repressed this ubiquitination.

Anti-HIF-1α antibody recognizes high molecular mass HIF-1α protein in cell lysates exposed to the proteasome inhibitor MG132, which specifically inhibits the 26S proteasome, thereby reducing the degradation of ubiquitin-conjugated HIF-1α even in normoxic conditions. We detected much less high molecular mass HIF-1α in cells exposed to either Clioquinol or TPEN (Fig. 3C). This observation shows that Clioquinol blocks the ubiquitination of HIF-1α, thereby causing it to accumulate in normoxic cells in agreement with the results of the in vitro ubiquitination assay.

Effect of Clioquinol on the Activity of FIH-1—Transactivation of HIF-1α is inhibited by hydroxylase of its asparagine 803 residue catalyzed by an asparagine hydroxylase named FIH-1 (9, 21, 22). The fact that the HIF-1α accumulated in the presence of Clioquinol is able to induce expression of its target genes implies that Clioquinol inhibits this hydroxylation. Because Asn-803 hydroxylation inhibits the interaction of HIF-1α with its coactivator, CBP, HIF-1α is activated when FIH-1 activity decreases (23). Radiolabeled HIF-1α was incubated with purified recombinant FIH-1 (10). Untreated 35S-labeled HIF-1α interacted with bead-bound GST-CBP protein, whereas FIH-1-treated HIF-1α failed to do so (Fig. 4A, lane 1 versus 4 and Fig. 4B, lane 1 versus 4). Because FIH-1 hydroxylates the asparagine 803 residue of HIF-1α, reducing recruitment of CBP to the transactivation domain of HIF-1α, the activity of FIH-1 is inversely related to the interaction between HIF-1α and CBP. We first tested the effect of Clioquinol on FIH-1 activity. In the presence of Clioquinol (Fig. 4A, 10 and 50 μM, lanes 5 and 6; Fig. 4B, 50 μM, lane 5; Fig. 4, C and D, lanes 4), the activity of FIH-1 was reduced so that the interaction between HIF-1α and CBP increased, whereas TPEN failed to reduce FIH-1 activity (Fig. 4B, lane 4 versus 6) (10). The difference between Clioquinol and TPEN reflects the fact that Clioquinol, but not TPEN, activates the expression of HIF-1α target genes (Fig. 2A). Although the zinc finger motif CH1 domain is located in the HIF-1α-interacting region of CBP, neither Clio-
Clioquinol-induced HIF-1α and Its Targets

A

B

C

D

E

FIGURE 4. Effect of Clioquinol on the activity of FIH-1. FIH-1 was expressed and purified as described previously (10). [35S]-labeled HIF-1α was hydroxylated for 1 h at 30°C with 1.5 μg of purified FIH-1 protein in the presence or absence of 10 and 50 μM Clioquinol (A) or 10 μM TPEN (B), with or without Zn(II) (50 and 100 μM) (C), or Cu(II) (50 and 100 μM) (D). Resin-bound GST-CBP-N (amino acids 1–450 of CBP) (18) was then added to each mixture, incubation continued for 1 h at 4°C, and the HIF-1α captured by CBP visualized by SDS-PAGE and autoradiography. Sample loading was monitored by measuring GST-CBP (CH1) stained with Coomassie Blue. E, the asparagine-specific effect of Clioquinol. [35S]-labeled HIF-1α-C(N803A) (amino acids 400–826, asparagine-803 is substituted with alanine) and [35S]-labeled HIF-1α-C(N803A) were synthesized by in vitro transcription and translation and hydroxylated with FIH-1 in the presence of Clioquinol. Resin-bound GST-CBP-N (CBP amino acids 1–450) was added to each mixture. HIF-1α-C(N803A) captured by CBP were visualized by SDS-PAGE and autoradiography. Sample loading was monitored by measuring GST-CBP-N (CH1) stained with Coomassie Blue.

To determine the activity of the FIH-1 by measuring hydroxylation of Asn-803 rather than by assessing its interaction with CBP, we incubated HIF-1α peptide (amino acids 788–822) with purified recombinant FIH-1 and determined the change in molecular mass of the peptide MALDI-TOF analysis. After treatment with FIH-1 the peptide gave a new MALDI-TOF peak corresponding to an increase of molecular weight of 16 (Fig. 5, A and B). This confirms that the recombinant FIH-1 hydroxylates Asn-803 of HIF-1α. In contrast, when the peptide was incubated with FIH-1 and Clioquinol its molecular weight did not increase (Fig. 5, B and D). This observation confirms that Clioquinol inhibits the Asn-hydroxylating activity of FIH-1.

Effects of Clioquinol on Hypoxia-induced Transactivation by HIF-1α—The fact that Clioquinol inhibits asparagine hydroxylation of HIF-1α implies that it increases the transactivation activity of HIF-1α, defined as its interaction with its coactivator CBP. Co-immunoprecipitation assays showed that HIF-1α interacts with CBP in both Clioquinol-treated cells and hypoxic cells, but not in MG132- or TPEN-treated cells, indicating that the HIF-1α accumulated upon exposure to MG132 or TPEN differs from that in Clioquinol-treated cells in terms of its ability to interact with CBP (Fig. 6A). Interestingly, neither TPEN nor Clioquinol interferes with the interaction between HIF-1α and CBP in vitro (Fig. 6B), although CBP has the zinc finger CH1 domain required for interaction with HIF-1α. This suggests that the CH1 domain is not affected by the two chelators at the concentrations used.

To confirm that Clioquinol increases the transactivation activity of HIF-1α in vivo, we used a Gal4-driven reporter plas-
mid encoding the firefly luciferase gene under the control of the Gal4 binding site. We transfected a Gal4-driven reporter plasmid into HepG2 cells together with plasmid pGal4/HIF-1α, which expresses HIF-1α linked to the DNA binding domain of the yeast Gal4 protein (amino acids 1–147). Because only the Gal4 fusion protein is able to bind to the Gal4 binding site, the reporter gene is transcribed only when HIF-1α has trans-activation activity. As expected, Clioquinol increased the trans-activation activity of HIF-1α in normoxic cells (Fig. 6C). We examined the levels of VEGF and EPO mRNA in Clioquinol-treated SH-SY5Y cells by Northern analysis. The results in Fig. 6D confirm that Clioquinol strongly induces the expression of both EPO and VEGF in SH-SY5Y cells.

**DISCUSSION**

We have shown that both TPEN and Clioquinol stabilize HIF-1α protein in normoxic cells by blocking its ubiquitination. The fact that the addition of Cu(II) and Zn(II) reversed the effects of Clioquinol suggests that it stabilizes HIF-1α in a Cu(II)- and Zn(II)-specific manner. Stabilization of HIF-1α is necessary but not sufficient to induce expression of its target genes (10, 16). For transactivation activity, the asparaginase of the stabilized HIF-1α needs to be not hydroxylated by FIH-1 so as to be able to interact with CBP. We found that the two chelators differed in their effect on the transactivation activity of HIF-1α. Clioquinol, but not TPEN, inhibited the activity of FIH-1. We suggest that Clioquinol does not inhibit the activity of FIH-1 by means of protein chelation for the following reasons: (i) the other Zn(II) and Cu(II) chelator TPEN fails to inhibit the activity of FIH-1, (ii) addition of Cu(II) or Zn (II) fails to reverse the inhibitory effect of Clioquinol on the asparagine hydroxylation of HIF-1α, and (iii) Zn(II) rather inhibits the activity of recombinant FIH-1 in vitro (IC50 10 μM) (24). Accordingly, TPEN-induced HIF-1α fails to interact with CBP, not because TPEN interferes directly with its interaction with CBP but because it fails to inhibit its asparagine hydroxylation by FIH-1. In contrast, Clioquinol-induced HIF-1α does interact with CBP, because it inhibits FIH-1, thus blocking asparagine hydroxylation of HIF-1α. Therefore, Clioquinol has a dual inhibitory effect blocking both ubiquitination and asparagine hydroxylation of HIF-1α. Both chelators have relatively high affinities for Zn(II) and Cu(II) and other cations. The pKa values of TPEN are: Cu(II), 20.2; Zn(II), 15.4; Fe (III), 14.4; Ca(II), 3. Those of Clioquinol are: Cu(II), 15.8; Zn(II), 12.5; Ca(II), 8.1; Mg(II), 8.6 (19, 20). Both TPEN and Clioquinol reverse Zn(II)- or Cu(II)-induced metalloprotein precipitation (12, 25).

Because Clioquinol is hydrophobic, has a low general toxicity profile, and crosses the blood brain barrier, it is being reevaluated as a prototype metal-protein-attenuating compound that decreases deposits of metalloproteins in Alzheimer, Parkinson, and Huntington diseases and the oxidative stress due to them. Clioquinol was extensively used as an antibiotic in the mid-1900s but was then withdrawn because it caused subacute myelo-optic neuropathy (13–15). In a study of APP2576 transgenic mice, which have an Alzheimer disease-type neuropathy, Clioquinol reduced both amyloid β plaques and serum levels of amyloid β without systemic adverse effects (26). A recent phase II clinical trial in 36 patients with Alzheimer disease showed that Clioquinol slowed cognitive decline and decreased plasma amyloid β concentrations, and no cases of subacute myelo-optic neuropathy were reported (27). The development and optimization of Clioquinol-like metal-protein-attenuating compounds requires careful study of other possible adverse effects. Clioquinol also causes apoptotic cell death in several human cancer cell lines (28).

HIF-1 is a master regulator that attenuates ischemic injury by inducing several genes required for angiogenesis, erythropoiesis, glycolysis, and vasodilation. Activation of HIF-1α also promotes the survival and progression of various cancers (29, 30). In contrast, it has beneficial effects on ischemic injury to the heart and brain, suggesting that a low molecular weight activa-

**FIGURE 5. Effect of Clioquinol on Asn hydroxylation.** A–D, mass spectrophotometric analysis for measuring FIH-1 activity. 4 μM F-HIF-1α (788–822) peptide was incubated without (A and C) or with (B and D) 2.8 μg of His-FIH-1 in a buffer containing 400 μM α-ketoglutarate and 100 μM ascorbic acid in a total volume of 50 μl. To investigate the effect of Clioquinol, the FIH-1 hydroxylation reaction was performed in the absence (A and B) or presence (C and D) of Clioquinol. MALDI-TOF spectra were obtained after purifying the reactants with ZipTipC18 (Millipore). Note that the indicated molecular weights correspond to the peptides with detached fluorescein isothiocyanate in a sodium-added form during the MALDI-TOF measurements.
Clioquinol-induced HIF-1α and Its Targets

A

B

C

D

FIGURE 6. Effect of Clioquinol on the transactivation activity of HIF-1α. A, effects of Clioquinol on CBP recruitment by HIF-1α in vivo. HepG2 cells were exposed to TPEN, MG132, or Clioquinol or to hypoxia for 6 h. 200-μg samples of whole cell lysates were immunoprecipitated with anti-CBP antibody overnight at 4°C. The resulting immunocomplexes or total lysates (30 μg) were analyzed using human HIF-1α antibody or anti-CBP antibody. One representative of at least two independent experiments with similar results is shown. B, effects of Clioquinol on the interaction between HIF-1α and CBP in vitro. 35S-labeled HIF-1α (20 μl) was incubated with resin-bound GST-CBP-N (amino acids 1–450 of CBP) in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM dithiothreitol) for 1 h at 4°C in the presence of the indicated amount of Clioquinol or TPEN with or without ZnCl2 (20 μM). The resin was washed as described under “Experimental Procedures.” C, effect of Clioquinol on transactivation of HIF-1α. S × 104 HepG2 cells were transfected with 100 ng of pGALO-HIF-1α plasmid and 100 ng of Ga4-driven reporter plasmid (gal4-tk-Luc) together with plasmid pCHO110 (50 ng), which encodes β-galactosidase. 16 h prior to harvest, the cells were treated with TPEN and Clioquinol or hypoxia. 48 h after transfection, luciferase activities were analyzed using the reporter gene assay system (Promega) and normalized by β-galactosidase activities. Values represent means and S.D. of at least three independent experiments. D, SH-SY5Y cells and HepG2 cells were treated with Clioquinol (indicated dose) or hypoxia for 16 h. 10 μg of total RNA were used for Northern analysis. 25 ng of DNA fragments encoding VEGF (AF022375, 700–948 bp) and EPO (NM_000799, 595–895 bp) were labeled by random priming (Promega). 105 cpm/μg of cDNA was used for Northern hybridizations (16). VEGF and EPO mRNAs were visualized by autoradiography.

induced by this preconditioning program was lost in HIF-1α−/− mice (37). EPO administration increased functional recovery and decreased apoptosis in isolated hearts subjected to ischemia 24 h later. The cardiac protection induced by hypoxic preconditioning is critically dependent on HIF-1α and EPO (34). Interestingly, EPO and its receptor are expressed in the brain and contribute to neuroprotection from ischemic damage (38, 39). In a first clinical trial of EPO in patients with acute stroke, EPO-treated patients had significantly reduced size of infarct and improved clinical outcome (40). In addition to EPO, administration of the angiogenic cytokine VEGF improves tissue perfusion via neovascularization in animal models of myocardial and limb ischemia. Besides its angiogenic activity VEGF had a novel protective activity on damaged neurons and glia (41), and transplantation of VEGF-transfected neural stem cells into rat brain provided neuroprotection after transient focal cerebral ischemia (42).

However, effective delivery of VEGF or EPO remains a challenge. Our finding that the lipophilic and less toxic small compound Clioquinol activates HIF-1α and its target genes, in particular VEGF and EPO, in normoxic cells suggests that Clioquinol might provide preconditioning protection against myocardial, neuronal, and limb ischemic injuries and other neurodegenerative diseases.

REFERENCES

1. Masson, N., and Ratcliffe, P. J. (2003) J. Cell Sci. 116, 3041–3049
2. Seagroves, T. N., Ryan, H. E., Lu, H., Wouters, B. G., Knapp, M., Thibault, P., Laderoute, K., and Johnson, R. S. (2001) Mol. Cell. Biol. 21, 3436–3444
3. Bruck, R. K., and McKnight, S. L. (2001) Science 294, 1337–1340
4. Epstein, A. C., Gleadle, J. M., McNeill, L. A., Waterson, R. A., O’Rourke, I., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. L., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J. L., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2001) Cell 107, 43–54
5. Ivan, M., Kondo, K., Yang, H., Kim, W., Vialiando, I., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G. J. (2001) Science 292, 464–468
6. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. L., Gielbert, J., Gaskell, S. J., Kriegesheim, A., Hebestreit, F. H., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Science 292, 468–472
7. Iwai, K., Yamakawa, K., Kamura, T., Minato, N., Conaway, R. C., Conaway, J. W., Klaussmer, D., and Pause, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2436–12441
8. Kamura, T., Koepp, D. M., Conrad, M. N., Skowrya, D., Moreland, R. J., Ilipoulous, O., Lane, W. S., Kaelin, W. G. Jr., Eldledge, S. J., Conaway, R. C., Harper, J. W., and Conaway, J. W. (1999) Science 284, 657–661
9. Mahon, P. C., Hori, K., and Semenza, G. L. (2001) Genes Dev. 15, 2675–2686
10. Choi, S. M., Choi, K.-O., Lee, N., Oh, M., and Park, H. (2006) Biochem. Biophys. Res. Commun. 343, 1002–1008
11. Choi, K.-O., Lee, T., Lee, N., Kim, J., Yang, E., Yoon, J., Kim, J., Lee, T., and Park, H. (2005) Mol. Pharmacol. 68, 1803–1809
12. Raman, B., Ban, T., Yamaguchi, K., Sakai, M., Kawai, T., Naiki, H., and Goto, Y. (2005) J. Biol. Chem. 280, 16157–16162
13. Cherry, R. A., Atwood, C. S., Xilinas, M. E., Gray, D. N., Jones, W. D., Raman, B., Ban, T., Yamaguchi, K., Sakai, M., Kawai, T., Naiki, H., and Goto, Y. (2005) J. Biol. Chem. 280, 16157–16162
14. Cherry, R. A., Atwood, C. S., Xilinas, M. E., Gray, D. N., Jones, W. D., McLean, C. A., Barnham, K. J., Volitakis, I., Fraser, W. F., Kim, Y., Huang, X., Goldstein, L. E., Moir, R. D., Lim, J. T., Beyreuther, K., Zheng, H., Tanzi, R. E., Masters, C. L., and Bush, A. I. (2001) Neuron 30, 665–676
15. Kaur, D., Yantiri, F., Rajagopalan, S., Kumar, J., Mo, J., Boonplueang, R., Viswanath, V., Jacobs, R., Yang, L., Beal, M. F., DiMonte, D., Volitakis, I., Ellerby, L., Cherry, R. A., Bush, A. I., and Andersen, J. K. (2003) Neuron 37, 899–909
16. Nguyen, T., Hamby, A., and Massa, S. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 11840–11845
16. Hur, E., Chang, K. Y., Lee, E., Lee, S., and Park, H. (2001) *Mol. Pharmacol.* **59**, 1216–1224
17. Cockman, M. E., Masson, N., Mole, D. R., Jaakkola, P., Chang, G.-W., Clifford, S. C., Maher, E. R., Pugh, C. W., Ratcliffe, P. J., and Maxwell, P. H. (2000) *J. Biol. Chem.* **275**, 25733–25741
18. Arany, Z., Huang, L. E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M., Bunn, H. F., and Livingston, D. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 12969–12973
19. Cherny, R. A., Legg, J. T., McLean, C. A., Fairlie, D. P., Huang, X., Atwood, C. S., Beyreuther, K., Tanzi, R. E., Masters, C. L., and Bush, A. I. (1999) *J. Biol. Chem.* **274**, 23223–23228
20. Agrawal, Y. K., and Patel, D. R. (1986) *J. Pharm. Sci.* **75**, 190–192
21. Dann, C. E., III, Bruick, R. K., and Deisenhofer, J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 15351–15356
22. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. (2002) *Science* **295**, 858–861
23. Freedman, S. J., Sun, Z. Y., Poy, F., Kung, A. L., Livingston, D. M., Wagner, G., and Eck, M. J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 5367–5372
24. Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y. M., Bullock, A. N., Weldon, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M., Ratcliffe, P. I., Pugh, C. W., and Schofield, C. J. (2002) *J. Biol. Chem.* **277**, 26351–26355
25. Maynard, C. J., Cappai, R., Volitakis, I., Cherny, R. A., White, A. R., Beyreuther, K., Masters, C. L., Bush, A. I., and Li, Q.-X. (2002) *J. Biol. Chem.* **277**, 44670–44676
26. Doraiswamy, P. M., and Finefrock, A. E. (2004) *Lancet Neurol.* **3**, 431–434
27. Ritchie, C. W., Bush, A. I., Mackinnon, A., Macfarlane, S., Mastwyk, M., MacGregor, L., Kiess, L., Cherny, R., Li, Q. X., Tammur, A., Carrington, D., Mavros, C., Volitakis, I., Xilinas, M., Ames, D., Davis, S., Beyreuther, K., Tanzi, R. E., and Masters, C. L. (2003) *Arch. Neurol.* **60**, 1685–1691
28. Ding, W. Q., Liu, B., Vaughn, J. L., Yamauchi, H., and Lind, S. E. (2005) *Cancer Res.* **65**, 3389–3395
29. Semenza, G. L. (2003) *Nat. Rev. Cancer* **3**, 721–732
30. Li, L., Lin, X., Staver, M., Shoemaker, A., Semizarov, D., Fesik, S. W., and Shen, Y. (2005) *Cancer Res.* **65**, 7249–7458
31. Natarajan, R., Salloum, F. N., Fisher, B. J., Kukreja, R. C., and Fowler, A. A., III (2006) *Circ. Res.* **98**, 133–140
32. Siddiq, A., Ayoub, I. A., Chavez, J. C., Aminova, L., Shah, S., LaManna, J. C., Patton, S. M., Connor, J. R., Cherny, R. A., Volitakis, I., Bush, A. I., Langsetmo, I., Seeley, T., Gunzler, V., and Ratan, R. R. (2005) *J. Biol. Chem.* **280**, 41732–41743
33. Grimm, C., Wenzel, A., Groszer, M., Mayser, H., Seeliger, M., Samardzija, M., Bauer, C., Gassmann, M., and Reine, C. E. (2002) *Nat. Med.* **8**, 718–724
34. Calvillo, L., Latini, R., Kajstura, J., Leri, A., Anversa, P., Ghezzi, P., Salio, M., Cerami, A., and Brines, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 4802–4806
35. Ferriero, D. M. (2005) *Epilepsia* **46**, 45–51
36. Simons, M., and Ware, J. A. (2003) *Nat. Rev. Drug Discov.* **2**, 863–871
37. Cai, Z., Manalo, D. J., Wei, G., Rodriguez, E. R., Fox-Talbot, K., Lu, H., Zweier, J. L., and Semenza, G. L. (2003) *Circulation* **108**, 79–85
38. Digiacaylioglu, M., Bichet, S., Marti, H. H., Wenger, R. H., Rivas, L. A., Bauer, C., and Gassmann, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 3717–3720
39. Ehrenreich, H., Aust, C., Krampf, H., Jahn, H., Jacob, S., Herrmann, M., and Siren, A. L. (2004) *Metab. Brain Dis.* **19**, 195–206
40. Ehrenreich, H., Hasselblatt, M., Dombowski, C., Cepek, L., Lewczuk, P., Stiefel, M., Rustenbeck, H. H., Breiter, N., Jacob, S., Knerlich, F., Bohn, M., Poser, W., Ruther, E., Kochen, M., Gefeller, O., Gleiter, C., Wessel, T. C., de Ryck, M., Itri, L., Prange, H., Cerami, A., Brines, M., and Siren, A. L. (2002) *Mol. Med.* **8**, 495–505
41. Rosenstein, J. M., and Krum, J. M. (2004) *Exp. Neurol.* **187**, 246–253
42. Zhu, W., Mao, Y., Zhao, Y., Zhou, L. F., Wang, Y., Zhu, J. H., Zhu, Y., and Yang, G. Y. (2005) *Neurosurgery* **57**, 325–333