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Induction of hypoxia inducible factor (HIF-1α) in rat kidneys by iron chelation with the hydroxypyridinone, CP94

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ABSTRACT

Hypoxia inducible factor (HIF-1α) is a master regulator of tissue adaptive responses to hypoxia whose stability is controlled by an iron containing prolyl hydroxylase domain (PHD) protein. A catalytic redox cycle in the PHD's iron center that results in the formation of a ferryl (Fe^{+4}) intermediate has been reported to be responsible for the hydroxylation and subsequent degradation of HIF-1α under normoxia. We show that induction of HIF-1α in rat kidneys can be achieved by iron reduction by the hydroxypyridin-4 one (CP94), an iron chelator administered intraperitoneally in rats. The extent of HIF protein stabilization as well as the expression of HIF target genes, including erythropoietin (EPO), in kidney tissues was comparable to those induced by known inhibitors of the PHD enzyme, such as desferrioxamine (DFO) and cobalt chloride ($CoCl_2$). In human kidney cells and *in vitro* PHD activity assay, we were able to show that the HIF-1α protein can be stabilized by addition of CP94. This appears to inactivate PHD; and thus prevents the hydroxylation of HIF-1α. In conclusion, we have identified the inhibition of iron-binding pocket of PHD as an underlying mechanism of HIF induction *in vivo* and *in vitro* by a bidentate hydroxypyridinone.

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1. Introduction

At the molecular level, the transcriptional factor hypoxia-inducible factor (HIF) orchestrates the cellular response to low O_2 tension [1,2]. The importance of the HIF pathway can be inferred from the fact that it is present in virtually all cell types and all higher eukaryotes [3]. HIF is a heterodimer that consists of one of the O_2-regulated α-subunits and a constitutively expressed β-subunit (HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator or (ARNT)). Three structurally related α subunits (HIF-1α, HIF-2α, and HIF-3α) have been identified to date [4]. Upon stabilization by hypoxia, iron chelation or oxidative stress, HIF-α translocates to the nucleus, dimerizes with HIF-β [5,6], binds to hypoxia response elements (HRE), and induces the expression of its transcriptional targets via recruitment of p300/CBP [6,7]. HIF-1α expression can thereby be controlled by O_2-dependent and O_2-independent mechanisms. In the presence of O_2, the overall levels of α subunits are low due to the rapid degradation of HIF-α, which is initiated by hydroxylation of either of the two prolines in the oxygen degradation domain (ODD) (P402 and P564 in HIF-1α) by the prolyl hydroxylase domain proteins (PHDs) [8–10]. PHD1, PHD2, and PHD3 have closely related catalytic domains and belong to the superfamily of 2-oxoglutarate (2OG)-dependent oxygenases. In order to be active, PHDs require O_2, the citric acid cycle intermediate 2OG as a co-substrate, plus Fe^{+2} and ascorbate as cofactors [11,12]. Hydroxylated prolines enable specific recognition of HIF-α by the von Hippel–Lindau (VHL) protein [13,14]. Binding of hydroxylated HIF-α by VHL is followed by rapid polyubiquitylation and proteasomal degradation [15].

HIF-α levels can be also regulated in an O_2-independent manner by various factors through the phosphatidylinositol-3-kinase (PI3K)-FKBP rapamycin-associated protein signaling pathway [16]. HIF transcriptional machinery is responsible for coordinating a host of cellular responses to low O_2 levels in animals by controlling a large array of target genes. In addition, the involvement of the HIF system in the pathogenesis of a range of cardiovascular pathologies makes it an ideal target for therapeutic manipulation.

A number of small molecule drug prototypes that specifically target the PHD enzyme responsible for HIF-1α degradation and inactivation have been reported [17,18]. These molecules either competitively inhibit or chelate iron, an essential cofactor for PHD enzymatic activity. PHD inhibition has emerged as a potential therapeutic strategy in a number of pathophysiological conditions; including myocardial ischemia, cerebrovascular disease, and anemia [19]. Recent work has shown that HIF-1α protein is regulated through posttranslational hydroxylation of specific prolyl and asparaginyl residues [15,20]. Hydroxylation is catalyzed by specific O_2-dependent enzymes that belong to the 2-oxoglutarate-

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dependent dioxygenase superfamily. In the resting state, ferrous iron (Fe^{+2}) at the active site is coordinated by three residues from the enzyme (normally two histidine residues and one aspartate or glutamate residue) and by two–three water molecules. During the enzymatic cycle, splitting of molecular O_2 is coupled both to hydroxylation of HIF-1α and to oxidative decarboxylation of 2-oxoglutarate to succinate and carbon dioxide. The reaction cycle proceeds by the formation of a highly reactive ferryl (Fe^{+4}) intermediate that oxidizes the HIF-1α amino acid residue [21]. In the absence of HIF-1α substrate, uncoupled turnover leaves the iron center of PHD in an inactive oxidized, ferric (Fe^{+3}) state. Thus, the accessibility of iron in this enzyme accounted for the ability of iron chelators to inhibit the PHD enzymatic activity by direct iron chelation [22] or possibly by an electron transfer to recycle Fe^{+4} back to Fe^{+3}.

Bidentate hydroxypyridinones are a class of low molecular weight Fe^{+3} chelators of considerable clinical interest developed as orally active compounds for the treatment of iron overload. The hydroxypyridinone CP20, commercially known as Deferiprone is currently in clinical use as iron chelator [23,24]. CP20 and related analogs were shown recently to protect against oxidative stress through the reduction of the ferryl iron (Fe^{+4}) of myoglobin and the prevention of heme-to-protein crosslinking, an index of oxidative side reactions associated with hemoglobin and myoglobin redox chemistries [25]. Given their ability to reduce the Fe^{+4} iron in myoglobins and hemoglobins, we decided to investigate, 1,2-diethyl-3-hydroxypyridin-4-one (CP94), a close analog of CP20 in influencing HIF enzymatic machinery, *in vivo* and *in vitro*. The synthesis and physicochemical characterization of the bidentate CP94 and CP20 has been described previously [26].

2. Materials and methods

2.1. Animals and surgical preparation

Male Sprague–Dawley rats weighing 250–300 g were purchased from Charles Rivers Laboratories (Wilmington, MA) and acclimated for 1 week upon arrival to the CBER/FDA animal care facility. All animals were fed normal diets throughout the acclimation period and weighed 300–350 g at the time of study. Animal protocols were approved by the FDA/CBER Institutional Animal Care and Use Committee with all experimental procedures performed in adherence to the National Institutes of Health guidelines on the use of experimental animals. Rats were dosed intra-peritoneally (i.p.) on days 1 through 6 with 1,2-diethyl-3-hydroxypyridin-4-one (CP94, 75 mg/kg/day) desferrioxamine (DFO, 25 mg/kg/day), cobalt chloride ($CoCl_2$, 15 mg/kg/day) and vehicle (phosphate buffered saline, PBS) as volume of 0.35 ml each prepared daily. On study day 7 (24 h after the last dose), rats were euthanatized via the i.p. route with a single dose of Euthasol (100 mg/kg). Animals underwent necropsy for gross morphological changes and tissues were collected for further evaluation.

2.2. Cell culture

Human embryonic kidney (HEK 293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Mediatech Inc., VA) supplemented with 10% fetal bovine serum (FBS; Mediatech Inc., VA), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Mediatech Inc., VA) at 37 °C in a humidified incubator under 5% CO_2/95% air. Bovine aortic endothelial cells (BAECs) were used between passages 3 and 15 (Cell Applications, San Diego, CA) and cultured with the culture media from HyClone (Logan, UT) and supplemented with EGM-MV BulletKit from Lonza (Walkersville, MD) and Glutamax from Invitrogen (Carlsbad, CA). The cells were maintained at 37 °C in a 5% CO_2, 95% air incubator. For *in vitro* experiments described here, 50 mM stock solutions of CP94 and CP20 were prepared in water. Synthesis of CP20 and CP 94 were carried according to previously published methods [26–28].

2.3. Western blotting

Rats were euthanized and tissues were harvested 24 h after the sixth dose of CP94, DFO and PBS for protein isolation. Kidney and muscle tissue (100–200 mg) were homogenized in ice cold RIPA lysis buffer (Millipore, Temecula, CA) containing protease inhibitors (Roche, Germany). Samples were separated on 4–12% Tris-glycine (TG) gels (Invitrogen, Inc., Carlsbad, CA) and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk and incubated with primary antibodies against HIF-1α, HIF-2α (R&D Systems, Minneapolis, MN), and erythropoietin (Cell Signaling, Inc.). After multiple washes in TBS-T, the membranes were incubated with relevant HRP-conjugated secondary antibodies and bands were detected using the ECL Plus chemiluminescence kit (Amersham, Arlington Heights, IL). Densitometry analysis was performed using NIH Image J software. Equal protein loading was verified by stripping and reprobing membranes for β-actin.

2.4. Prolyl hydroxylase domain-2 (PHD) activity assay

Proline hydroxylation of HIF-1α by PHD2 is crucial for O_2-dependent degradation in which iron redox cycling properties is known as a key step for PHD activity [21,29]. Thus, we tested CP94 on the hydroxylation of HIF-1α using a modified PHD activity assay [30,31].

To prepare unmodified HIF-1α, cells were incubated in hypoxia (1% O_2) for 4 h. Under these conditions, PHD is inactive resulting in an accumulation of the unhydroxylated HIF-1α protein. The cells were then lysed with lysis buffer containing 20 μM of MG132, a proteasome inhibitor; and the cell lysates were used as a source for unhydroxylated HIF-1α substrates. The lysates containing unhydroxylated HIF-1α were subjected to a hydroxylation reaction by incubating in reaction buffer (40 mM Tris–HCl, pH 7.5, 0.1 mM 2-oxoglutarate, 0.25 mM ascorbate, 0.4 mg/ml catalase, 0.5 mM dithiothreitol, and 20 μM MG132) with or without CP94 and 50 or 150 μM $FeSO_4$ at 37 °C under normoxic condition. After 1.5 h incubation under normoxic condition the reaction mixtures were subjected to immunoprecipitation using an anti-HIF-1α antibody. The precipitants were analyzed by immunoblot assay using antibodies specifically recognizing hydroxy-Pro564 HIF-1α (Rockland, Gilbertsville, PA), HIF-1α, and PHD2 (Novus Biologicals, Littleton, CA) [29].

2.5. Real-time PCR and data analysis

Total RNA isolated from HEK 293 cells or rat kidneys was used for cDNA synthesis. Real-time PCR reactions were performed using 7600HT PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The specific TaqMan® gene probes were purchased from Applied Biosystems. Real-time PCR for each gene were performed in triplicate and normalized to 18S rRNA expression values. Results are presented as fold change and expressed as means ± S.E.M. Statistical analysis real-time PCR was performed on raw $\triangle C_t$ data. Within-group differences for all comparisons were determined by ANOVA with a post hoc analysis for determination of differences between groups. Significance was set at $p \leq 0.05$.

2.6. Luciferase assays

Bovine aortic endothelial cells (BAECs) were cultured in 24-well plates and were allowed to reach 75% confluency prior to transient transfection. Cells were transfected using Lipofectamine and Lipofectamine Plus Reagent, both from Invitrogen (Carlsbad, CA), and used according to the manufacturer's directions. The HIF-1α luciferase reporter and renilla control vector were from SA Biosciences (Frederick, MD) and were used at 75 ng DNA per well. Prior to addition of DNA for transfection, BAEC media was changed to Opti-