The prolyl 4-hydroxylase inhibitor ethyl-3,4-dihydroxybenzoate generates effective iron deficiency in cultured cells

Jian Wang[a], Joan L. Buss[a], Guohua Chen[a], Prem Ponka[a,b], Kostas Pantopoulos[a,c,*]

[a] *Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, 3755 Cote-Ste-Catherine Road, Montreal, QC, Canada H3T 1E2*
[b] *Department of Physiology, Faculty of Medicine, McGill University, Montreal, QC, Canada*
[c] *Division of Experimental Medicine, Faculty of Medicine, McGill University, Montreal, QC, Canada*

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Abstract Ethyl-3,4-dihydroxybenzoate (EDHB) is commonly utilized as a substrate analog and competitive inhibitor of prolyl 4-hydroxylases. These iron-dependent enzymes have received a lot of attention for their involvement in crucial biochemical pathways such as collagen maturation and oxygen sensing. Since EDHB is also capable of chelating the enzyme-bound iron, we study here its function as a chelator. We show that the affinity of EDHB for ferric iron is significantly lower than that of desferrioxamine. Nevertheless, EDHB is sufficient to promote effective iron deficiency in cells, reflected in the activation of the iron-responsive element/iron regulatory protein regulatory network. Thus, treatment of B6 fibroblasts with EDHB results in slow activation of iron regulatory protein 1 accompanied by an increase in transferrin receptor levels and reduction of the ferritin pool.

Key words: Iron metabolism; Iron regulatory protein 1; Iron-responsive element; Ferritin; Transferrin receptor

1. Introduction

Hydroxylation of amino acid residues is emerging as an important checkpoint in various biochemical pathways. For example, it has been known for many years that proline hydroxylation is necessary for the maturation of collagen, the major protein of connective tissue, because 4-hydroxyproline is indispensable in the stabilization of collagen's triple helix [1]. More recently, it was firmly established that proline hydroxylation is involved in a mechanism for oxygen sensing. Cells of higher eukaryotes respond to reduced oxygen availability by activation of the hypoxia inducible factor (HIF) that controls the transcription of a wide array of genes involved in erythropoiesis, angiogenesis, cell proliferation/survival, glycolysis and iron metabolism. In mammals, these include, among many others, erythropoietin, vascular endothelial growth factor, transferrin and transferrin receptor [2]. The expression of HIF is regulated at the level of protein stability. Under normoxic conditions, the oxygen-sensitive subunit HIF-1α is hydroxylated at two proline residues within two functionally independent degradation domains. This modification provides a recognition site for the von Hippel–Lindau tumor suppressor protein, a component of an E3 ubiquitin ligase complex. This interaction results in ubiquitination and degradation of HIF-1α by the proteasome [3,4].

The enzymes catalyzing the hydroxylation of prolines and other amino acid residues belong to the family of 2-oxoglutarate-dependent oxygenases [5] and require ascorbate, iron and oxygen as cofactors. The prolyl 4-hydroxylases that modify HIF-1α and collagen are homologous but distinct. The former appear to function as oxygen sensors [6,7]. It is obvious that control of the activity of prolyl 4-hydroxylases is of profound biomedical relevance. A screen of several hydroxybenzene and hydroxybenzoic acid derivatives that are structurally related to 2-oxoglutarate and ascorbate has provided a first set of prolyl 4-hydroxylase inhibitors [8]. Among them, 3,4-dihydroxybenzoate displayed a strong ($K_i = 5$ μM) competitive inhibition towards 2-oxoglutarate and ascorbate. In the last 15 years, the cell-permeable ethyl ester of 3,4-dihydroxybenzoate (EDHB) has commonly been employed in cell culture experiments as a specific inhibitor of collagen synthesis [9–14].

As an *ortho*-dihydroxy derivative, 3,4-dihydroxybenzoate also has the capacity to inhibit enzyme activity in a non-competitive fashion by chelating enzyme-bound iron [8]. This notion prompted us to investigate the iron chelating properties of EDHB and its effect in cellular iron metabolism.

2. Materials and methods

2.1. Cell culture and reagents

B6 murine fibroblasts were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 ng/ml streptomycin and 10% fetal calf serum. EDHB was purchased from Sigma and desferrioxamine (DFO) from Novartis.

2.2. Determination of EDHB affinity for Fe(III)

The conditional dissociation constant for the Fe–EDHB complex in phosphate-buffered saline was determined using a Cary 1 spectrophotometer, by competition with EDTA at 25°C. Data were analyzed at 490 nm, at which the only significantly absorbing species is Fe-$(EDHB)_3$. Ferric iron, prepared as a 5 mM stock solution of $FeCl_3$ in 100 mM sodium citrate, was diluted to 25 μM in the presence of 750 μM EDHB. The loss of absorbance on the addition of 25 μM EDTA was used to calculate the equilibrium constant:

$$K_{eq} = [Fe{-}(EDHB)_3][EDTA]/[Fe{-}EDTA][EDHB]^3 = K_D(EDHB)/K_D(EDTA)$$

*Corresponding author. Fax: (1)-514-340 7502.
E-mail address: kostas.pantopoulos@mcgill.ca (K. Pantopoulos).

Abbreviations: EDHB, ethyl-3,4-dihydroxybenzoate; DFO, desferrioxamine; IRP1, iron regulatory protein 1; IRE, iron-responsive element; EMSA, electrophoretic mobility shift assay; TfR, transferrin receptor

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2.3. Electrophoretic mobility shift assay (EMSA)

The cells were lysed in 'cytoplasmic lysis buffer' (1% Triton X-100, 40 mM KCl, 25 mM Tris–HCl, pH 7.4). Analysis of iron-responsive element (IRE) binding by EMSA was performed as in [15].

2.4. Western blotting

Half of the cells in each sample were lysed in 'cytoplasmic lysis buffer'. Lysates containing 30 μg of total protein were resolved by SDS–PAGE on a 7.5% gel and the proteins were transferred onto nitrocellulose filters. The blots were saturated with 10% non-fat milk in phosphate-buffered saline and probed with 1:1000 diluted antibodies against transferrin receptor (TfR; Zymed) and β-actin (Sigma). The other half of the cells were directly lysed in Laemmli sample buffer and immediately boiled for 5 min [16]. Equal aliquots of lysates were resolved by SDS–PAGE on 11% gels and transferred onto nitrocellulose filters. The blots were saturated as above and probed with 1:1500 diluted antibody against ferritin (Roche). Following a wash with phosphate-buffered saline containing 0.1% Tween 20, the blots with the TfR monoclonal antibodies were incubated with peroxidase-coupled rabbit anti-mouse IgG (1:4000 dilution). The blots with β-actin and ferritin polyclonal antibodies were incubated with peroxidase-coupled goat anti-rabbit IgG (1:5000 dilution). Detection of peroxidase-coupled antibodies was performed with the enhanced chemiluminescence method (Amersham).

3. Results

To evaluate the function of EDHB as an iron chelator, we first examined its iron binding capacity in vitro. To this end, we determined the conditional dissociation constant (K_D) of the Fe(III)–EDHB complex. It was assumed that Fe(III)–EDHB has the 1:3 stoichiometry characteristic of small bidentate ligands. The value of K_D for Fe–(EDHB)$_3$, 1.86×10^{-27} M^3, and those of Fe–EDTA and Fe–DFO are given in Table 1. The hexadentate ligands DFO and EDTA form complexes with a 1:1 Fe:ligand stoichiometry. Because the dissociation constants of bidentate and hexadentate ligands to Fe(III) have different units, direct comparison of affinities using K_D values is not simple. To make comparison possible, the parameter 'pM' is used, which is the −log of free [Fe(III)] in the presence of 1 μM total Fe(III) and 1 mM total ligand at pH 7.4. It can be inferred from the pM values shown in Table 1 that EDHB has approximately 40 times lower affinity to Fe(III) than EDTA, which in turn has approximately 2000 times lower affinity to Fe(III) than DFO. Thus, under the above conditions, EDHB is an iron chelator ~80 000 times weaker than DFO.

We then investigated the effects of EDHB in cellular iron metabolism and the IRE/IRP regulatory system. The expression of several proteins of iron metabolism, such as TfR and ferritin, which mediate iron uptake and storage, respectively, is regulated post-transcriptionally by iron regulatory proteins (IRPs) at the levels of mRNA stability or translation. The mechanism involves binding of IRPs to IREs within the untranslated regions of the respective mRNAs [17,18]. To assess the effects of EDHB in the IRE/IRP system, mouse B6 fibroblasts were exposed to different doses of the drug for 12 h and IRE binding activity was analyzed by EMSA (Fig. 1). The concentrations of EDHB tested here (ranging from 200 to 400 μM) were previously reported to be effective for the inhibition of prolyl 4-hydroxylases in other experimental settings [9,10]. Under all these conditions, EDHB leads to a profound activation of IRP1 (lanes 1–4). The effect is comparable to a treatment with the established iron chelator DFO (lane 5). An analysis of the same extracts for IRE binding following treatment with 2-mercaptoethanol, known to activate dormant IRP1 [17,18], confirms equal loading (bottom panel).

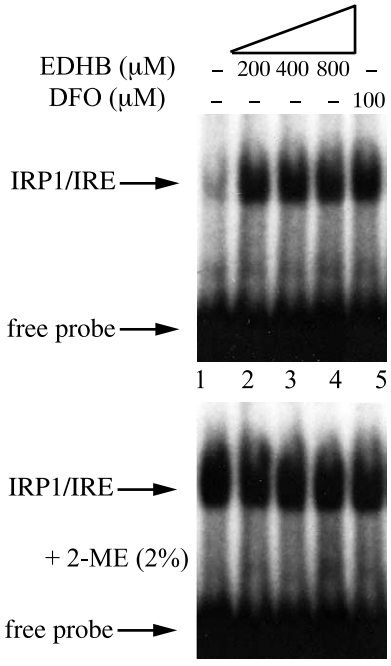

Fig. 1. Activation of IRP1 by EDHB. B6 cells (6×10^6) were left untreated (lane 1), or treated for 12 h with the indicated concentrations of EDHB (lanes 2–4) or DFO (lane 5). Cytoplasmic extracts (15 μg) were analyzed by EMSA with 25 000 cpm ^{32}P-labeled IRE probe in the absence (top panel) or presence of 2% 2-mercaptoethanol (2-ME) (bottom panel). The positions of the IRE/IRP1 complexes and of excess free IRE probe are indicated by arrows. These results are representative of those obtained from three experiments.

The effects of EDHB on IRE binding activity were further analyzed in a time course experiment (Fig. 2). As a control, B6 cells were treated with 100 μM DFO for different time intervals. A sustained treatment with DFO for >4 h results in the activation of IRP1 (lanes 1–7). These kinetics are in agreement with earlier findings [19]. Importantly, a treatment with 200 μM EDHB also leads to a complete activation of IRP1 after 4 h, while a partial activation of IRP1 within 2–4 h is also observed (lanes 8–14). The control analysis with 2-mercaptoethanol is shown in the bottom panel.

We finally examined the effects of EDHB in the expression of downstream IRP1 targets (Fig. 3). Treatment of B6 cells with 200 μM EDHB for 12 h stimulates the expression of TfR (lane 2, upper panel), and leads to a marked reduction in ferritin steady-state levels (lane 2, middle panel). As expected, similar results are obtained when the cells are treated with 100 μM DFO for 12 h (lane 3). The chelator treatments

Table 1
Affinity constants of chelators for Fe^{3+}

Chelator	K_D	pM[a]
EDHB[b]	1.86×10^{-27} M^3	23.7
EDTA[c]	5.00×10^{-23} M	25.3
DFO[c]	2.51×10^{-26} M	28.6

[a]−log [Fe^{3+}] in the presence of 1 μM Fe^{3+} and 1 mM ligand at pH 7.4.
[b]Measured in phosphate-buffered saline (pH 7.4); the experiment was performed twice and no significant variation in the values was observed.
[c]Values from Critical Stability Constants (1977), Plenum Press, New York.

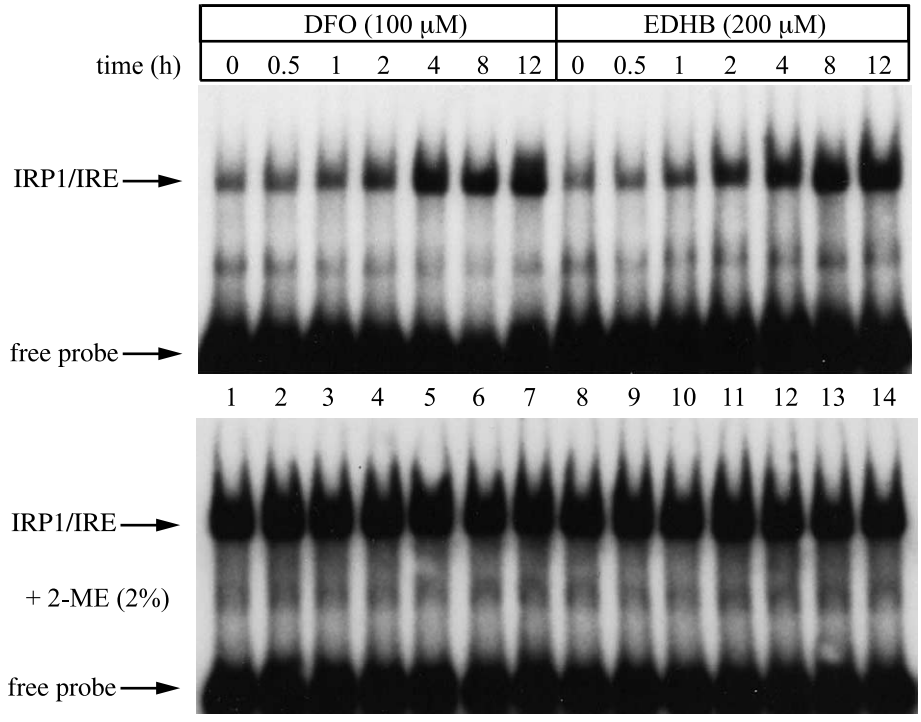

Fig. 2. Time-dependent activation of IRP1 by EDHB and DFO. B6 cells (6×10^6) were left untreated (lanes 1 and 8), or exposed for the indicated time periods to 100 μM DFO (lanes 2–7) or 200 μM EDHB (lanes 9–14). Cytoplasmic extracts (15 μg) were analyzed by EMSA with 25 000 cpm ^{32}P-labeled IRE probe in the absence (top panel) or presence of 2% 2-mercaptoethanol (2-ME) (bottom panel). The positions of the IRE/IRP1 complexes and of excess free IRE probe are indicated by arrows. These results are representative of those obtained from three experiments.

have no effect on the expression of β-actin, which serves as an internal control (bottom panel).

4. Discussion

We show here that the prolyl 4-hydroxylase inhibitor EDHB is a functional iron chelator. Its affinity for Fe(III) is substantially lower than that of DFO (Table 1). Nevertheless, when applied to cultured cells, EDHB is capable of promoting effective iron deficiency. Moreover, the cellular responses to the EDHB treatment examined here closely resemble the responses elicited by DFO. First, exposure of cells to micromolar concentrations of both EDHB and DFO trigger the activation of IRP1 (Fig. 1). Second, as with DFO, the activation of IRP1 by EDHB is relatively slow and requires treatment with the drug for at least 4 h (Fig. 2). Finally, both EDHB and DFO modulate the coordinate expression of downstream IRP1 targets, such as TfR and ferritin (Fig. 3). It should be noted that another weak iron chelator, the tridentate *N*-(2-hydroxybenzyl)-L-serine, with ~10^8 times lower K_D compared to DFO, was recently found to activate IRP with no appreciable effect on HIF-1 [20].

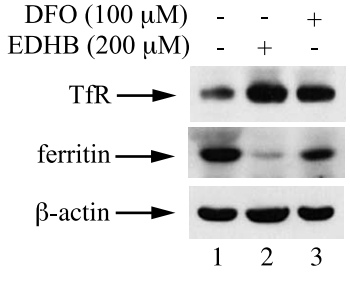

Fig. 3. Reciprocal effects of EDHB in the expression of TfR and ferritin. B6 cells (6×10^6) were left untreated (lane 1), or treated with 200 μM EDHB or 100 μM DFO for 12 h. Cell extracts were analyzed by Western blotting with antibodies against TfR (top), ferritin (middle) or β_2-actin (bottom). These results are representative of those obtained from three experiments.

Activated IRP binds to multiple IREs in the 3′-untranslated region of TfR mRNA protects it against degradation, whereas IRP binding to a single IRE in the 5′ untranslated region of ferritin mRNA inhibits its translation [17,18]. Surprisingly, the inhibitory effect of EDHB on ferritin expression appears to be stronger than that of DFO (Fig. 3, middle panel), at least under the experimental conditions tested (treatment with the drugs for 12 h). The reason for this is unclear, but it may be related to differences in cell permeability between the two drugs. Nevertheless, we have observed in the past that an overnight (~16 h) treatment of B6 cells with 100 μM DFO almost completely abolishes cellular ferritin content [21]. We conclude that the iron chelating capacity of EDHB is sufficient to modulate the IRE/IRP regulatory system and to promote cellular responses to iron starvation. Importantly, the time scale of these effects is well within the time scale of experiments in which EDHB has been employed to inhibit collagen synthesis (it ranges from a few hours up to several days [9–14]).

Our data suggest that EDHB is not as specific an inhibitor for prolyl 4-hydroxylases as previously thought. Moreover, its capacity to perturb cellular iron metabolism could potentially be associated with various pleiotropic effects. Therefore, many data obtained using this inhibitor should be interpreted with

caution. Along these lines, any strategies for the clinical application of EDHB for the treatment of fibrotic diseases should take into account the function of the drug as an iron chelator.

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References

[1] Pihlajaniemi, T., Myllyla, R. and Kivirikko, K.I. (1991) J. Hepatol. 13, S2–S7.
[2] Semenza, G.L. (2001) Cell 107, 1–3.
[3] Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin Jr., W.G. (2001) Science 292, 464–468.
[4] Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) Science 292, 468–472.
[5] Schofield, C.J. and Zhang, Z. (1999) Curr. Opin. Struct. Biol. 9, 722–731.
[6] Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J. and Ratcliffe, P.J. (2001) Cell 107, 43–54.
[7] Bruick, R.K. and McKnight, S.L. (2001) Science 294, 1337–1340.
[8] Majamaa, K., Gunzler, V., Hanauske-Abel, H.M., Myllyla, R. and Kivirikko, K.I. (1986) J. Biol. Chem. 261, 7819–7823.
[9] Sasaki, T., Majamaa, K. and Uitto, J. (1987) J. Biol. Chem. 262, 9397–9403.
[10] Nandan, D., Clarke, E.P., Ball, E.H. and Sanwal, B.D. (1990) J. Cell Biol. 110, 1673–1679.
[11] Ibrahimi, A., Bonino, F., Bardon, S., Ailhaud, G. and Dani, C. (1992) Biochem. Biophys. Res. Commun. 187, 1314–1322.
[12] Rocnik, E.F., Chan, B.M. and Pickering, J.G. (1998) J. Clin. Invest. 101, 1889–1898.
[13] Chernoff, E.A., Clarke, D.O., Wallace-Evers, J.L., Hungate-Muegge, L.P. and Smith, R.C. (2001) Tissue Cell 33, 97–110.
[14] Nakajima, I., Muroya, S., Tanabe, R.I. and Chikuni, K. (2002) Differentiation 70, 84–91.
[15] Mueller, S. and Pantopoulos, K. (2002) Methods Enzymol. 348, 324–337.
[16] Wang, J. and Pantopoulos, K. (2002) Mol. Cell. Biol. 22, 4638–4651.
[17] Hentze, M.W. and Kühn, L.C. (1996) Proc. Natl. Acad. Sci. USA 93, 8175–8182.
[18] Eisenstein, R.S. (2000) Annu. Rev. Nutr. 20, 627–662.
[19] Pantopoulos, K., Weiss, G. and Hentze, M.W. (1996) Mol. Cell. Biol. 16, 3781–3788.
[20] Creighton-Gutteridge, M. and Tyrrell, R.M. (2002) Free Radic. Biol. Med. 33, 356–363.
[21] Caltagirone, A., Weiss, G. and Pantopoulos, K. (2001) J. Biol. Chem. 276, 19738–19745.