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SHORT COMMUNICATION

Duodenal cytochrome b (Cybrd 1) and HIF-2α expression during acute hypoxic exposure in mice

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Abstract
Background Recent evidence suggests that the duodenum can regulate iron absorption independently of hepcidin via the transcription factor Hif-2α acting directly on the transcription of the proteins involved in the iron transport. The current study investigates the temporal relationship between Dcytb and Hif-2α during early hypoxic stimulus in the enterocyte in vivo.
Methods Duodenal Dcytb and Hif-2α protein expression was analysed by Western blot technique while gene regulation was determined by quantitative PCR.
Results Both Dcytb and Hif-2α protein expression were increased during the first hours of hypoxic duration. A change in hepcidin expression however, was significant only at 72 h hypoxia. Increased iron absorption reported in early hypoxia could be accounted for in part by the enhancement of Dcytb expression by Hif-2α in the duodenum.
Conclusion Modulation of Hif-2α predominates over hepcidin in the regulation of intestinal iron absorption during short hypoxic duration. The intestine exerts regulatory mechanisms in the dietary absorption of iron into systemic circulation.

Keywords Iron · Hypoxia · Dcytb · Hif-2α

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Introduction

Duodenal cytochrome b [Dcytb, cytochrome b reductase (Cybrd 1)] is a mammalian transplasma ferric reductase that catalyses the reduction of ferric to ferrous ion in the process of iron absorption. Its expression is significantly responsive to modulators of iron absorption. Increased iron absorption is an adaptive response to hypoxic stimulus in mice [18] and increased iron demand in humans [8]. Although reduced oxygen levels influence the rate of red blood synthesis, the rapidly enhanced intestinal absorption of iron was shown to be independent of the confounding effect of erythropoiesis [21]. This finding was attributed to the direct modulation of gastrointestinal iron transport machinery by hypoxia [9]. However, the molecular mechanism of this regulation is not completely understood. Recent advances have, however, revealed compelling evidence that duodenal expressed HIF-2α mediates the transcription of the genes involved in iron transport in the enterocyte [11]. Using an intestine-specific double VHL/HIF-1α KO mouse model, Shah et al. [20] showed a Hif-2α specific up-regulation of both Dcytb and DMT1 (divalent metal transporter 1) as well as FPN1 (ferroportin1). This observation was confirmed in a different approach that utilised specific intestinal deletion of Hif-2α. Both intestinal Arnt KO and HIF-2α KO mice had a similar phenotype with significant reductions in mRNA levels for Dcytb and DMT1. HIF-2α KO mice were additionally shown to have decreased FPN1. Dcytb does not encode a definable IRE element and its regulation unlike that of DMTI and Fpn is exclusively transcriptional.

Hitherto, much research emphasis has been placed on hepatic hepcidin expression and its inverse relationship with iron absorption arising from its functional ubiquitination of ferroportin [12]. Interestingly, Hif-1α was

implicated in the transcriptional regulation of hepatic hepcidin expression. HIF-1α was shown to bind and suppress the hepcidin promoter, thus implying a direct repressor effect [15]. However, a recent report [25] has shown a lack of a direct transcriptional suppression of hepcidin by Hif. Moreover, in contrast to other erythropoietic mediators of hepcidin expression, the magnitude of hypoxic effector on the gene has often been marginal and fluctuates with the duration of hypoxic exposure [13]. The complexities of the physiological response to hypoxia necessitate investigation of the variables involved in iron metabolism. The current study therefore seeks to investigate the expression of Dcytb and its regulators during short durations of hypoxic exposure in mice.

Materials and methods

Reagents

All chemicals and biochemicals were purchased from either Sigma–Aldrich Co. Ltd (Poole, Dorset, UK) or BDH-Merck Ltd (Poole).

Animals

Male CD1 mice, 6–8 weeks old, were used throughout the study. Hypoxia was induced by placing mice in a steel hypobaric chamber set at 0.5 atmospheres (53.3 kPa) for various time periods. Food and water were given ad libitum throughout. The mice were killed after anaesthesia and neck dislocation after which tissues were collected. All procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Erythropietin (epo) ELISA

Mice serum Epo levels were determined with the Quantikine Mouse EPO ELISA kit (R&D Systems).

Real-time PCR

Total RNA was isolated using the Trizol reagent (Invitrogen, Life Technologies, Paisley, UK) according to manufacturer's instructions. Quantitative RT PCR was carried out using two-step ABI Prism 7000HT Sequence Detection System. First, strand synthesis was performed using the ABI cDNA Synthesis Kit using 2 μg total RNA template according to manufacturer's protocol. In the second step, transcripts of the various genes were amplified with the specific primer sequences (Table 1) using the ABI SYBR Green supermix protocol [23]. The efficacy of the amplification was confirmed by a melting curve analysis and gel electrophoresis to confirm the presence of a single product.

Table 1 Primer sequences used for quantitative real-time polymerase chain reaction analysis

Primers	Sequence
mHepcidin 1-forward	AGAGCTGCAGCCTTTGCAC
mHepcidin 1-reverse	GAGGTCAGGATGTGGCTCTA
mDcytb-forward	ATGTACAGCCTGCACAGC
mDcytb-reverse	TGTCACTCCCATGAGAACC
mDMT1-IRE-forward	CTGCTGAGCGAAGATACC
mDMT1-IRE-reverse	GTAAACCATAGAAACACACTGG
m18S-forward	GAATTCCCAGTAAGTGGCGGG
m18S-reverse	GGGCAGGGACTTAATCAACG

Quantitative measurement of each gene was derived from a standard curve constructed from known concentrations of PCR product. The results were calculated by the ΔCt method that expresses the difference in threshold for the target gene relative to that of 18S. In some cases, PCR was performed for 20–30 cycles of 94 °C for 1 min, 94 °C for 10 s, 55–60 °C annealing and 72 °C for 2 min as extension time in a PTC-200 DNA Engine thermal cycler (MJ Research, USA). PCR products were stained with ethidium bromide on 1% agarose gel and visualised under UV light. Sequences of primers used, forward and reverse respectively, are shown in Table 1.

Western blot analysis

The duodenal mucosa samples were scraped with a glass slide and homogenised (in a buffer containing 50 mM mannitol, 2 mM HEPES, 0.5 mM PMSF and pH 7.2) with an Ultra Turrax (IKA, Staufen, Germany) homogeniser in (3 × 30 s pulses on full speed). The homogenate was centrifuged at 1,500*g* for 5 min and the supernatant was centrifuged for 1 h at 15,000*g* to obtain the crude membrane fraction. Nuclear extract NE-PER Kit (Pierce, Rockford, IL) was prepared using NaCl high salt buffer [1]. Protein concentration was determined using Bio-Rad reagents (Bio-Rad Laboratories, USA). Fifty micrograms of membrane extracts were loaded on a 12% gel in a SDS–PAGE. The proteins separated were then transferred to Hybond ECL-nitrocellulose membrane (Amersham Biosciences, Bucks, UK) using a Bio-Rad semidry transfer apparatus (Bio-Rad Trans-Blot^{R} SD Semi-Dry Transfer Cell). Membrane was blocked with 5% milk for 1 h and probed with Dcytb polyclonal, HIF-2α, (Novus Biologicals, USA), β-tubulin (Abcam, UK) or β-actin (Sigma, UK) antibodies diluted in 0.01% milk in TBS. Cross-reactivity

was observed with peroxidase-linked anti-IgG by using SuperSignal West Pico (Thermo Scientific, USA).

Statistical analysis

All values are expressed as mean ± SEM. Statistical differences between means were calculated with Microsoft Excel 2007 (Microsoft, Seattle, USA) by using the Student's *t*-test correcting for differences in sample variance. When multiple comparisons were necessary, one-way analysis of variance (ANOVA) was performed, using SPSS (SPSS Inc., Chicago, USA) with post-hoc test with the least significance difference.

Results

Enhancement of Dcytb mRNA expression in the duodenum was significant after 18 h of hypoxia (Fig. 1a). Protein level, however, was remarkably increased during the early hours of low oxygen tension (Fig. 1b, c). DMT1-IRE mRNA expression showed a time course increase that was significant ($p \leq 0.05$) at 24 h of hypoxic exposure (Fig. 1d). Similarly, Hif-2α protein expression in the duodenum exhibited a progressive increase over the time course of hypoxia (Fig. 2). Hypoxic stimulus slightly enhanced liver hepcidin mRNA expression at 6 h before a gradual downregulation at longer hypoxic exposure. Inhibition of hepatic hepcidin was significant only after 72 h of hypoxic exposure (Fig. 3). In agreement with previous studies [19], serum Epo level exhibited a sharp initial increase at 6 h of hypoxic exposure and remained elevated significantly over the duration of the exposure to hypoxia, albeit at lower magnitude (Fig. 4).

Discussion

The current study demonstrates the time-course of increased induction of Dcytb expression (Fig. 1), which

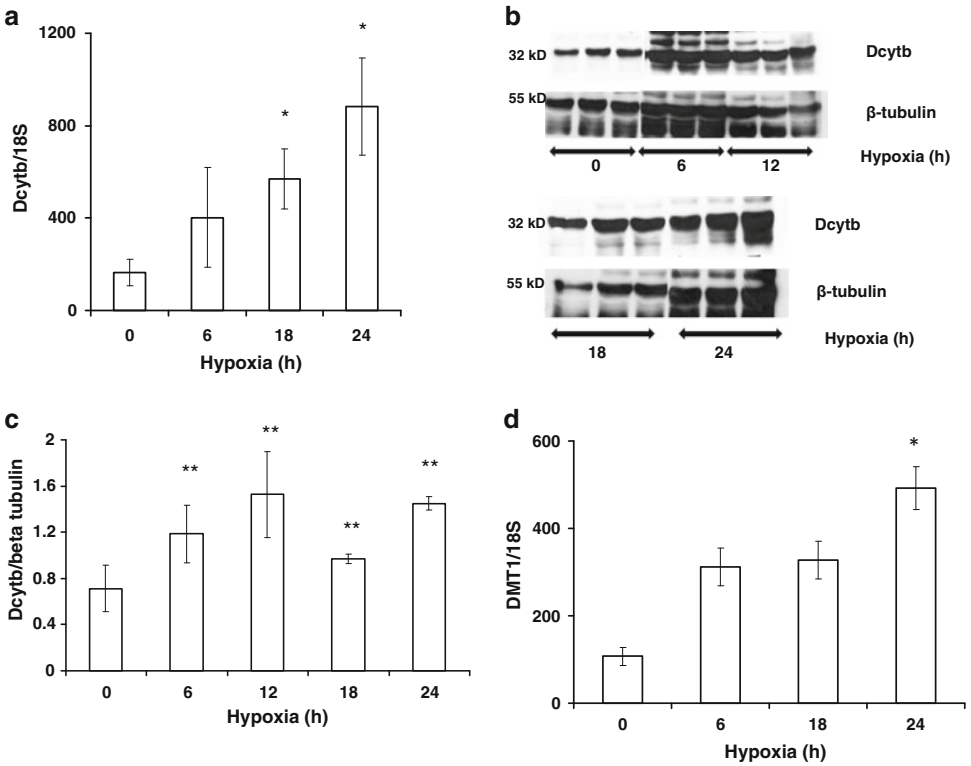

Fig. 1 *Dcytb* mRNA and protein expression in duodenal tissues from mice exposed to hypoxia. **a** Relative mRNA expression levels in duodenum of mice at different duration of hypoxia. *Dcytb* mRNA expression was determined using quantitative real-time PCR. Data are mean ± SEM of four observations in each group and are expressed as a ratio of *18S* mRNA in arbitrary units. Data are representative of two experiments. **b** Western blot analysis of Dcytb protein expression in mice duodenal tissues at different duration of hypoxic exposure. Expression was normalised to *β*-tubulin protein levels in the samples. **c** Semi-quantitative densitometry data of Western blot of Dcytb protein expression in duodenal tissues of mice after exposure to hypoxia. Data are means ± SEM, $n = 3$. **d** Relative *DMT1-IRE* mRNA expression levels in duodenum of mice at different duration of hypoxia. *DMT1-IRE* mRNA expression was determined using quantitative real-time PCR. Data are mean ± SEM of four observations in each group and are expressed as a ratio of *18S* mRNA in arbitrary units

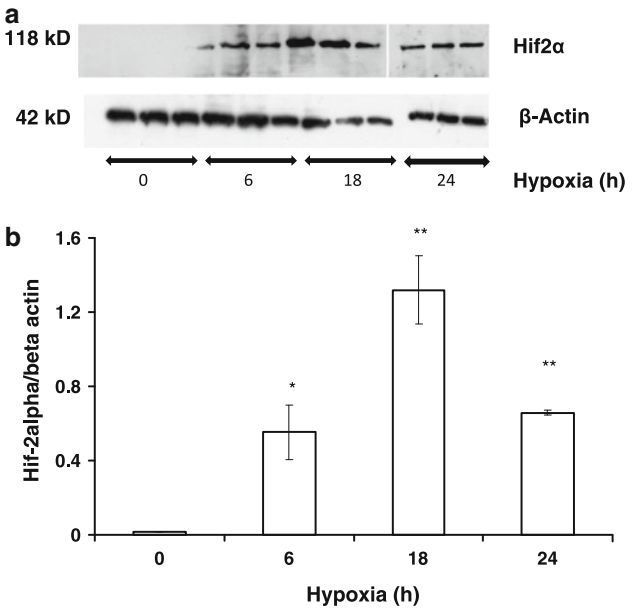

Fig. 2 Hif-2α protein expression in duodenal tissues of mice exposed to hypoxia. **a** Western blot analysis of Hif-2α protein expression in mice duodenal tissues at different durations of hypoxic exposure. Expression was normalised to β-tubulin protein levels in the samples. **b** Semi-quantitative densitometry data of Western blot of Hif-2α protein expression in duodenal tissues of mice after exposure to hypoxia. Data are means ± SEM, $n = 3$

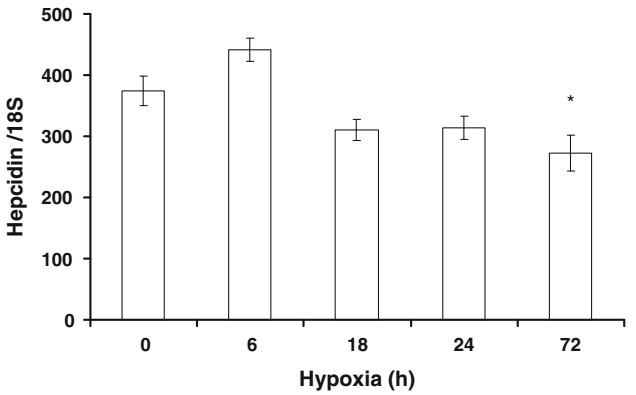

Fig. 3 *Hepcidin* mRNA in hepatic tissues of mice exposed to hypoxia. *Hepcidin* mRNA expression was determined using quantitative real-time PCR. Data are mean ± SEM of four observations in each group and are expressed as a ratio of *18S* mRNA in arbitrary units

might contribute in part to enhanced iron uptake during the early stages of hypoxic exposure [19]. The molecular mechanism underlying this response is shown to be consistent with increased expression of Hif-2α protein in the duodenum of mice exposed to hypoxia (Fig. 2). These findings confirm that the enhanced duodenal Hif-2α expression seen in rat acute hypoxia models [26] also occurs in the mouse iron absorption hypoxia model as studied by Raja et al. [19]. Furthermore, our findings are in

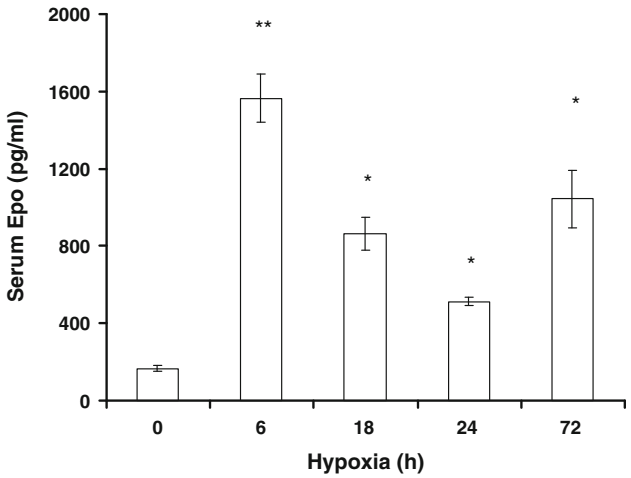

Fig. 4 Serum Epo levels in mice exposed to hypoxia. Data are means ± SEM, $n = 4$. Data are representative of two experiments

agreement with the previous reports of Shah et al. [20] and Mastrogiannaki et al. [11] in which Dcytb, expressed at the apical membrane of the enterocyte, is mediated by Hif-2α regulation.

Hif-2α activation induces the expression of both Dcytb and DMT1 and this possibly partly explains the molecular mechanism of increased iron uptake in iron deficient animals [10]. The situation in early hypoxic conditions has been shown to be similar in the current study. Duodenal iron levels tend to decrease during hypoxia [17], and consequently, the stability and activity of Hif-2α may be driven and sustained by hydroxylation changes that are effected by the iron [2] levels as well as by hypoxia.

Moreover, Hif-2α activation in early hypoxia is accounted for possibly by both the intrinsic low oxygen tension of the enterocyte and the exogenous effect of the hypobaric chamber. Hif-2α was shown to bind directly to two consensus hypoxic response elements (HREs) within the promoters of DMT1 and Dcytb [20].

Increased iron absorption during early hypoxia exposure possibly precedes changes in hepcidin regulation (Fig. 4). Hepcidin response during hypoxic stimulus seems to be significant after 24 h. Reduced hepatic hepcidin mRNA expression was reported at 24 h of hypoxia exposure albeit in Balb/c mice [3]. Mouse strain differences have been reported in the expression and regulation of iron metabolism genes [4]. Although Epo has been shown [16] to inhibit hepcidin expression, it is apparent that this is not operational during early hypoxia. Epo and other stimuli emanating from enhanced erythropoiesis might have a direct effect on iron absorption independent of hepcidin regulation. Similarly, increased erythropoiesis induced by phenylhydrazine injection reduced hepcidin expression only after 3 days [5]. Enhanced iron efflux by Fpn could then create a negative balance in the enterocyte iron levels,

which subsequently enhances Dcytb and DMT1 expression. This implies the operation of independent mechanisms of iron absorption during short or long duration of hypoxic exposure [14]. The biphasic kinetics of iron transport during early hypoxia is modulated by Hif-2α, which activates the expression of brush border proteins, Dcytb and DMT1 [11, 20].

Ferroportin (Fpn) protein expression was only slightly increased after 24 h of hypoxia (GO Latunde-Dada, unpublished result). The responsiveness of the Fpn during vectorial transport of iron has often been subtle under different modulators of iron absorption [6]. It has recently become apparent that the regulation of Fpn comprises transcriptional existence in two isoforms, namely, Fpn 1A-IRE and Fpn 1B- non-IRE, [27], as well as translational IRE-IRP binding and the post-translational interactions with hepcidin. Other regulatory mechanisms presumably act in concert with hepcidin in modulating dietary iron absorption in the enterocyte [7, 24]. Multiple levels of regulation encompass basal regulation by IRE-IRP, local sensing and activation of Hif-2α and the feedback ferroportin-hepcidin modulation. The complexity of the relative effects of the modulators and the temporal or spatial boundaries of functionality awaits further delineation. Hif-2α exerts a transcriptional regulatory control on the expression of Dcytb, DMT1 and ferroportin. The emphasis in the current study is on Dcytb as it is the only member of the iron transport machinery that lacks the IRE response element and seems solely transcriptionally regulated. While DMT1 and ferroportin exhibit multiple regulatory mechanisms, it is their non-IRE isoforms that are presumably significantly modulated by Hif-2α [22]. The control of iron acquisition and assimilation through the regulation of Dcytb and DMT1 by Hif-2α represents an important gauge for maintaining optimal iron homeostasis in mammals.

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