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Research Paper

Hypoxia enhances H_2O_2-mediated upregulation of hepcidin: Evidence for NOX4-mediated iron regulation

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ABSTRACT

The exact regulation of the liver-secreted peptide hepcidin, the key regulator of systemic iron homeostasis, is still poorly understood. It is potently induced by iron, inflammation, cytokines or H_2O_2 but conflicting results have been reported on hypoxia. In our current study, we first show that pronounced (1%) and mild (5%) hypoxia strongly induces hepcidin in human Huh7 hepatoma and primary liver cells predominantly at the transcriptional level via STAT3 using two hypoxia systems (hypoxia chamber and enzymatic hypoxia by the GOX/CAT system). SiRNA silencing of JAK1, STAT3 and NOX4 diminished the hypoxia-mediated effect while a role of HIF1α could be clearly ruled out by the response to hypoxia-mimetics and competition experiments with a plasmid harboring the oxygen-dependent degradation domain of HIF1α. Specifically, hypoxia drastically enhances the H_2O_2-mediated induction of hepcidin strongly pointing towards an oxidase as powerful upstream control of hepcidin. We finally provide evidences for an efficient regulation of hepcidin expression by NADPH-dependent oxidase 4 (NOX4) in liver cells. In summary, our data demonstrate that hypoxia strongly potentiates the peroxide-mediated induction of hepcidin via STAT3 signaling pathway. Moreover, oxidases such as NOX4 or artificially over-expressed urate oxidase (UOX) can induce hepcidin. It remains to be studied whether the peroxide-STAT3-hepcidin axis simply acts to continuously compensate for oxygen fluctuations or is directly involved in iron sensing per se.

1. Introduction

Hepcidin has emerged as central regulator of systemic iron homeostasis since its first identification as an antimicrobial peptide [1,2]. The 25 amino acid peptide is primarily expressed in hepatocytes [3]. Deletion of hepcidin causes massive iron overload and many iron overload diseases are associated with suppressed hepcidin [4,5]. In contrast, overexpression of hepcidin causes rapid decrease of serum iron ultimately leading to anemia [6] by binding to the iron exporter ferroportin (FPN1) finally leading to its internalization and subsequent proteasomal degradation [7,8]. The impact on iron levels is conducted by the concerted blockage of FPN1 which is expressed on duodenal enterocytes (iron absorption), macrophages (iron recycling) and hepatocytes (iron storage).

Hepcidin is strongly induced by IL-6 and microbial molecules, such as lipopolysaccharide rapidly leading to the so called anemia of chronic disease [9]. The inflammation-mediated depletion of iron is thought to function as anti-bacterial defense mechanism. More recently, we could identify H_2O_2, a cellular key reactive oxygen species and important inflammatory cofactor, as potent inducer of hepcidin [10]. Interestingly, hepcidin shows a bivalent response to H_2O_2 depending on the peroxide level. Thus, H_2O_2 induces hepcidin in hepatocytes independent of IL-6 when exposed to very low and continuous non-toxic levels [10]. In contrast, artificial bolus treatment with high and toxic levels, drastically blocked hepcidin expression [11,12] most likely due to unspecific inhibition of the mRNA transcription machinery [10].

So far, it remains poorly understood how exactly hepcidin is controlled by iron levels despite the discovery of various upstream regulators of hepcidin such as C/EBPα, BMP6, TFR1 and 2, Matriptase-2, IL-6, CREBH, CHOP, and recently PDGF-BB [13]. Controversial and

Abbreviations: β2mg, β2-microglobulin; BMP, Bone morphogenetic protein; CAT, Catalase; FPN1, ferroportin 1; GOX, Glucose Oxidase; HIF, Hypoxia inducible factor; IL-6, Interleukin 6; JAK, Janus kinase; NOX, NADPH oxidase; ODD, Oxygen-dependent degradation domain; PHD2, Prolyl hydroxylase domain protein 2; Prx2, Peroxiredoxin 2; SIH, Salicylaldehyde isonicotinoyl hydrazone; SOCS3, Suppressor of cytokine signaling 3; STAT3, Signal transducer and activator of transcription 3; UOX, Urate oxidase

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partly conflicting findings have been especially reported on the role of hypoxia in regulating hepcidin. Thus, in vivo studies in rodent models have demonstrated that hypoxia leads to increased duodenal iron absorption and reduced hepcidin expression [12,14–17], suggesting a direct action of HIFs on hepcidin promoter via downregulation of CCAAT/enhancer binding protein alpha [18]. However, recent studies have refuted these conclusions showing that HIF-mediated suppression of hepcidin occurs indirectly through EPO-induced erythropoiesis [19,20]. Interestingly, not all studies have demonstrated a decreased hepcidin expression in vitro. Thus, no diminished hepcidin levels or even an induction was seen in cultured HepG2 and Huh7 under hypoxic conditions [21,22]. Although a direct role of HIF1α or HIF2α on hepcidin regulation has been suggested by several authors [18,23–25], recent studies performed in hepatoma cell lines or in primary hepatocytes could not confirm former conclusions [12,19,21,22].

A major challenge of in vivo studies on hypoxia is the very complex and difficult to interpret overlay by many adaptive responses of living organisms at various levels. For example, systemic hypoxia stimulates renal erythropoietin release which induces red blood cell formation and hence depletion of serum iron [19]. Moreover, hypoxia has important physiological adaptive effects such as an elevated cardiac output and a hyperventilation. In addition, hypoxic studies are sometimes performed using to high, aerobic levels of oxygen that are not mimicking physiological conditions found in specialized tissues. In fact, hepatocytes are usually surrounded by 4% and 8% in the pericentral and periportal area, respectively [26–31]. In contrast, cultured cells are often exposed to artificially high oxygen levels of 21% instead of physiological low oxygen. Another pitfall with regard to ROS studies and hypoxia is the usage of artificially high peroxide bolus concentrations in the range up to 100 μM [32,33].

Therefore, we here study in detail hepcidin expression under hypoxia (physiological oxygen levels found the liver). We primarily focus on the cellular level in hepatoma and primary liver cells to avoid systemic overlaying responses. In addition, we use both the hypoxia chamber and the recently established enzymatic hypoxia system, which allows the independent control of oxygen and peroxide levels [10,34,35]. Our data show a clear induction of hepcidin by physiological hypoxia from 1% to 7%. While a direct role of HIF1α is ruled out, promotor and signaling studies point towards the STAT3 signaling pathway. Moreover, we provide evidence for a drastically enhanced upregulation of hepcidin by peroxide under hypoxic conditions linking iron regulation directly to oxidases. Finally, our studies demonstrate that peroxide-generating oxidases such as NOX4 or UOX are able to efficiently control the expression of hepcidin via the STAT3 axis. Since hepcidin control is not only restricted to NOX4 but also other oxidases, our findings suggest that oxidase-mediated expression of hepcidin may be involved in iron sensing per se.

2. Material and methods

2.1. Cell culture and primary cells isolation

Huh7 cells from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan) were cultured under standard conditions using Dulbecco's modified Eagle medium (Sigma-Aldrich, Taufkirchen, Germany), 25 mM glucose and 10% fetal calf serum under 21% oxygen and 5% CO_2 [10]. Primary human hepatocytes were isolated from healthy liver region of patients with liver metastasis undergoing partial liver resection using a collagenase-based protocol as published previously [36].

2.2. Chemicals and reagents

Actinomycin D, Rotenone and Cobalt (II) chloride were all purchased from Sigma-Aldrich (Taufkirchen, Germany). SIH was a kind gift of Dr. P. Ponka (McGill University, Montreal, Canada). Stock solutions prepared in DMSO were further diluted in culture medium with a final concentration of 0.1% DMSO.

2.3. Exposure of cells to H_2O_2 and/or hypoxia

Steady-state H_2O_2 (H_2O_2ss) treatment was performed using the glucose oxidase and catalase system (GOX/CAT system) as described previously [10,34]. Briefly, glucose oxidase (GOX) and catalase (CAT) were purchased from Sigma-Aldrich (Taufkirchen, Germany) and the activities were determined using a sensitive chemiluminescence technique [33,34,37,38]. During all experiments k_{GOX} was kept at 4×10^{-8} M/s, while k_{CAT} was adjusted to reach the final H_2O_{2ss} concentration of 2.5 μM. Since GOX metabolizes glucose and oxygen stochiometrically to H_2O_2 and δ-gluconolactone, attention was payed to decrease glucose during 24 h by no more than 3 mM. For hypoxia chamber treatments, cell culture plates were placed in a sealed chamber flushed with 5% O_2, 5% CO_2 and 90% N_2 or with 1% O_2, 5% CO_2 and 94% N_2. The hypoxia chamber was incubated at 37 °C for a maximal period of 24 h. In some experiments, the GOX/CAT system was used in combination with the hypoxia chamber to generate additional H_2O_2.

2.4. Hypoxia imaging

The cells were grown on chamber slides at 21, 5 or 1% O_2. During the last 2 h, pimonidazole was added at a final concentration of 200 μM (Hypoxyprobe Plus Kit; HPI inc., Burlington, MA, USA). The slides were then washed with PBS and fixed in 100% methanol. Pimonidazole adducts were stained with the FITC-labeled mouse antibody according to the manufacturer's instructions. Cells were visualized using a 20 × objective on a Zeiss Axiovert 200M microscope (Zeiss, Göttingen, Germany) and the images were processed using Fiji ImageJ.

2.5. RNA isolation, cDNA synthesis and real time quantitative PCR analysis

RNA was isolated with Trifast (Peqlab biotechnology GmbH, Erlangen, Germany) according to the manufacturer specifications. Reverse transcription and the real time quantitative PCR reactions were performed as previously described [10]. Primers and probes were designed using the Probefinder software (Roche, Mannheim, Germany) and the sequences are provided in Supplemental Table S1.

2.6. Transfection experiments

Huh7 cells were transfected with an ODD containing plasmid construct, NOX4, UOX or hepcidin promoter plasmid constructs wild-type, truncated or mutated promoter constructs as previously described [39–41] using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). As previously described, the ODD containing plasmid (pCMV-ODD-GFP vector) was used for the competition experiments and the same vector without the ODD insert was used as control [31]. NOX4 cDNA was cloned into the *Kpn*I and *Not*I sites of a pcDNA3 vector, resulting in antisense orientation in respect to the cytomegalovirus (CMV) promoter (Invitrogen, Carlsbad, CA). UOX cDNA was cloned into the *Eco*RI site of a pBluescriptSK vector. Hepcidin promoter constructs containing firefly luciferase and a control plasmid containing renilla luciferase were co-transfected at a ratio of 1:16 as published previously [10]. NOX4 and hepcidin promoter construct transfections were carried out for 48 h. For hypoxia studies, cells were then transferred to 5% O_2 in a hypoxia chamber for additional 24 h.

2.7. RNA silencing

Huh7 cells were transfected using Lipofectamine 2000 and 10 nM siRNA. For the assessment of hepcidin promoter activity, the cells were co-transfected with hepcidin wild type promoter containing firefly luciferase construct, *Renilla* control plasmid and siRNA directed against