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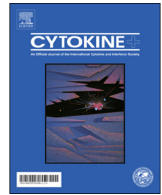

JNK facilitates IL-1β-induced hepcidin transcription via JunB activation

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ABSTRACT

Hepcidin, a liver-derived hormone, negatively regulates circulating iron levels through an increase in its expression in response to iron overload. Inflammation also increases production of hepcidin, potentially leading to inflammatory anemia. We previously revealed that proinflammatory cytokine interleukin (IL)-1β increased hepcidin expression through its transcriptional stimulation in hepatocytes. Induction of CCAAT-enhancer-binding protein (C/EBP) δ and IL-6 in response to IL-1β treatment stimulated hepcidin transcription via the C/EBP-binding site (C/EBP-BS) and signal transducer and activator of transcription (STAT)-BS on the hepcidin promoter, respectively. Here, we show an additional pathway responsible for IL-1β-induced hepcidin transcription. IL-1β stimulated phosphorylation of c-Jun N-terminal kinase (JNK) and its substrates c-Jun and JunB. SP600125, a JNK inhibitor, blocked IL-1β-induced phosphorylation of c-Jun and JunB as well as IL-1β-induced expression and transcription of hepcidin. Reporter assays for hepcidin transcription revealed that reporters with mutations of cAMP response element (CRE) site B, a putative Jun binding element, decreased responsiveness to IL-1β, and that activated JunB, but not c-Jun, conferred IL-1β-induced hepcidin transcription. Furthermore, binding of JunB to hepcidin promoter was increased by IL-1β. The present study indicated that IL-1β activates JNK and subsequently stimulates JunB activation, leading to hepcidin transcription via CRE site B on the hepcidin promoter. The present experiment provides novel insights into the molecular mechanisms underlying induction of hepcidin by inflammation and alteration of iron homeostasis.

1. Introduction

Disturbance of iron metabolism is frequently observed in various diseases. Anemia of inflammation is the most common anemia in hospitalized patients [1]. Tissue accumulation of iron has been reported in patients with cardiovascular diseases and neurodegenerative disorders [2,3]. The dysregulated iron metabolism contributes to the pathogenesis of various diseases [4], but the detailed mechanisms largely remain unknown.

Hepcidin, a liver-derived peptide hormone, negatively regulates plasma iron levels through internalization and degradation of the sole iron exporter ferroportin localized on enterocytes, macrophages, and hepatocytes [5,6]. Hepcidin expression is primarily regulated by systemic iron status; expression levels of hepcidin paralleled the intake levels of iron [7]. Iron overload increased expression of bone morphogenetic protein (BMP) 6 [7], which stimulated hepcidin transcription via BMP-response elements (BMP-REs) [8].

However, hepcidin expression is not only regulated by iron status. Inflammatory cytokines, such as interleukin (IL)-6 and oncostatin M, activated hepcidin transcription through binding of signal transducer and activator of transcription (STAT) 3 on the STAT-binding site (STAT-BS) in the hepcidin promoter [9]. Hepcidin transcription was also stimulated by serum via 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-response element (TRE) through induction of activator protein (AP)-1 such as c-Fos and JunB [10]. Therefore, hepcidin induction by factor(s) other than iron status is likely to lead to disturbance of the systemic iron metabolism.

Recently, we revealed that in addition to up-regulation of IL-6, IL-1β increased expression of CCAAT-enhancer-binding protein (C/EBP) δ, which stimulated hepcidin transcription through binding to C/EBP-BS on the hepcidin promoter [11]. In this study, we identified an additional element on the hepcidin promoter that is needed for IL-1β-induced maximal transcription; hepcidin transcription was stimulated via the cAMP response element (CRE) site B through the c-jun N-terminal kinase (JNK) pathway in response to IL-1β. Transcriptional stimulation of hepcidin mediated through multiple modes is likely one of the causes of iron-metabolism disturbance resulting from overproduction of hepcidin by non-iron stimuli.

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2. Materials and methods

2.1. Materials

The following reagents were purchased and used: recombinant human IL-1β was from RayBiotech, Inc. (Norcross, GA, USA); SP600125 was from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA); SB203580 was from Millipore (Bedford, MA, USA); rabbit polyclonal antibodies against phospho-extracellular signal-regulated kinase (ERK) (Thr202/Tyr204) (#9101), ERK (#9102), phospho-JNK (Thr183/Tyr185) (#9251), JNK (#9252), phospho-p38 (Thr180/Tyr182) (#9211), p38 (#9212), phospho-c-Jun (Ser63) (#9810), phospho-JunB (Thr102/Thr104) (#8053), and JunB (#3753S) were from Cell Signaling Technology (Danvers, MA, USA); rabbit monoclonal antibody against phospho- cAMP response element-binding protein (CREB) (Ser133) (87G3) was from Cell Signaling Technology; a mouse monoclonal antibody against β-actin (AC-15, ab-6276) was from Abcam (Cambridge, MA, USA); a rabbit polyclonal antibody against C/EBPδ (M-17, sc-636) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and siRNA transfection

HepG2 human hepatoma cells were maintained in Dulbecco's modified Eagle medium with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. For siRNA experiments, HepG2 cells (3×10^4 per well) were seeded onto 24-well plates. After attachment, cells were transfected with 2 µL of lipofectamine RNAi Max (Invitrogen) and 40 pmol of siRNA. At 44 h after transfection, cells were serum-starved with 0.2% FBS medium and treated with IL-1β (2 ng/mL). The following nucleotide sequences were used for dsRNA: 5′- CAGCUCAAAC AGAAGGUCA$_d$T$_d$T -3′ for JunB and 5′- GUUCAGCGUGUCCGGC GAG$_d$T$_d$T -3′ for GFP.

2.3. RNA isolation and RT-quantitative PCR

Total RNA isolation, cDNA synthesis, and quantitative PCR were performed as described previously [11]. The following primers were used for qPCR: 5′-CTGTTTTCCCACAACAGACG-3′ and 5′-TTCGCCTCT GGAACATGG-3′ for hepcidin, 5′-GGACATAGGAGCGCAAAGAA-3′ and 5′-GCTTCTCTCGCAGTTTAGTGG-3′ for C/EBPδ, 5′-AGGATAGTGCGAT GTTTCAGG-3′ and 5′-GACTTCTCAGTGGGCTGTCC-3′ for c-Jun, 5′-ATACACAGCTACGGGATACGG-3′ and 5′-GCTCGGTTTCAGGAGTT TGT-3′ for JunB, 5′-CAGTTCCTCTACCCCAAGGTG-3′ and 5′-TTCTGCT TGTGTAAATCCTCCA-3′ for JunD, and 5′-TGACCTTGATTTATTTTGCA TACC-3′ and 5′-CGAGCAAGACGTTCAGTCCT-3′ for HPRT1. The ΔΔCt method was used to normalize the levels of target transcripts to HPRT1 levels [12].

2.4. Western blot

Western blot analyses were performed as previously described [13]. The immunoreactive proteins were visualized using Chemi-Lumi One Ultra (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. The band intensity was quantified by use of Image J software, and the representative result is shown.

2.5. Reporter assay

Luciferase-based reporter assays were performed in HepG2 cells as previously described [11]. The reporter with a mouse hepcidin promoter fragment (nt −2018 to −35, hepcidin(-2018)-luc) was previously described [10]: the translation initiation site is numbered as +1. The mutant reporters were prepared from hepcidin(-2018)-luc as the parent vector. The reporter with mutated TRE (mTRE) was previously described [10]. The mutated CRE (mCRE) site A and mCRE site B constructs contain the following base substitutions in hepcidin (-2018)-luc: 5′-TGACACAA-3′ to 5′-acACACAA-3′ and 5′-TGACATCA-3′ to 5′-gcACATCA-3′, respectively; the mutated nucleotides are shown in small characters. The nucleotide sequences of the constructs were verified by DNA sequencing.

2.6. Chromatin immunoprecipitation (ChIP)

HepG2 cells (1.8×10^7) were cross-linked by the addition of formaldehyde to reach a final concentration of 1%. After an 8 min incubation at room temperature, glycine was added to a final concentration of 0.125 M. Cells were washed twice with phosphate-buffered saline (PBS), scraped from the plates, and centrifuged at 3000 rpm. The cell pellets were resuspended in 5 mL of hypotonic buffer (10 mM HEPES (pH 7.5), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM PMSF, 1% (v/v) aprotinin, 1 mM Na_3VO_4) containing 0.5% NP-40, and vortexed. Subsequently, the suspension was kept on ice for 10 min at 4 °C, followed by centrifugation. The pellets were washed with hypotonic buffer and resuspended in 1 mL of nuclear lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.2% SDS, 0.1% sodium deoxycholate, 1 mM PMSF, 1% (v/v) aprotinin, 1 mM Na_3VO_4). After incubation for 10 min on ice, the cell lysates were sonicated using a Handy Sonic model UR-20P (Tomy Seiko, Tokyo, Japan) with six sets of 10 s pulses (power level: 5), and centrifuged to remove cell debris. The supernatant was divided into two tubes, and diluted with 500 µL of buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 1% (v/v) aprotinin, 1 mM Na_3VO_4). After incubation with 50 µL of 25% (v/v) protein G agarose beads for 2 h at 4 °C, the supernatants were immunoprecipitated for 16 h at 4 °C with 8 µL of anti-JunB antibody or normal rabbit serum, followed by incubation with 30 µL of ChIP-grade protein G agarose beads (Cell Signaling Technology, #9007) for 2 h at 4 °C. Subsequently, the beads were washed with RIPA buffer, RIPA buffer containing 500 mM NaCl, and LiCl buffer (10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate). The beads were further washed with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA twice, followed by elution in 200 µL of elution buffer (10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, 0.5% SDS) and incubated for 5 min at 95 °C. DNA fragments were extracted with phenol-chloroform-isoamyl alcohol. DNA was precipitated and resuspended in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, followed by qPCR analysis using 5′-TCCCGATCAGCAGA ATGACATC-3′ and 5′-ACCGAGTGACAGTCGCTTTT-3′ as the primers.

2.7. Statistical analyses

Data are expressed as the mean ± standard error (SE). Data on gene expression were log-transformed to provide an approximation of a normal distribution before analysis. Differences of gene expression in the cells were examined using unpaired *t*-tests. Differences of $P < 0.05$ were considered significant.

3. Results

3.1. IL-1β activates the JNK pathway, which is required for hepcidin transcription

We previously showed that IL-1β stimulated expression of C/EBPδ and IL-6, leading to transcriptional activation of the hepcidin gene via C/EBP-BS and STAT-BS on the promoter, respectively [11]. However, a reporter with mutated C/EBP-BS and mutated STAT-BS still responded to IL-1β [11], which suggested that IL-1β-induced hepcidin transcription could not be explained only by these two elements and that an additional element(s) is required for IL-1β-induced hepcidin transcription.

IL-1β has been shown to stimulate a nuclear factor κ-light-chain-

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