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The liver-enriched transcription factor CREBH is nutritionally regulated and activated by fatty acids and PPARα

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ABSTRACT

To elucidate the physiological role of CREBH, the hepatic mRNA and protein levels of CREBH were estimated in various feeding states of wild and obesity mice. In the fast state, the expression of CREBH mRNA and nuclear protein were high and profoundly suppressed by refeeding in the wild-type mice. In ob/ob mice, the refeeding suppression was impaired. The diet studies suggested that CREBH expression was activated by fatty acids. CREBH mRNA levels in the mouse primary hepatocytes were elevated by addition of the palmitate, oleate and eicosapenonate. It was also induced by PPARα agonist and repressed by PPARα antagonist. Luciferase reporter gene assays indicated that the CREBH promoter activity was induced by fatty acids and co-expression of PPARα. Deletion studies identified the PPRE for PPARα activation. Electrophoretic mobility shift assay and chromatin immunoprecipitation (ChIP) assay confirmed that PPARα directly binds to the PPRE. Activation of CREBH at fasting through fatty acids and PPARα suggest that CREBH is involved in nutritional regulation.

Introduction

The liver-enriched bZip transcription factor, CREBH belongs to the CREB family. It activates transcription through binding to cAMP responsive element (CRE) and box B element [1]. It is a membrane-bound protein located on the endoplasmic reticulum via Leucine-zipper domain. Upon ER stress, it shifts on a Golgi, N-terminal fragment is processed by Site 1 protease and Site 2 proteases known to also work on SREBPs, traffics to the nucleus to activate transcription of acute inflammatory genes such as serum amyloid P (SAP), C-reactive protein (CRP) via CRE [2]. It also trans-activates the gluconeogenesis enzyme phosphoenolpyruvate carboxykinase (PEPCK) through direct binding to CRE. This activation can be further stimulated by cAMP and protein kinase A [3]. Although these previous studies suggest involvement of this membrane-bound transcription factor in nutrition, its precise function and nutritional regulation are not fully understood.

Non-esterified fatty acids or their CoA derivatives are the main signals involved in the transcriptional effect of long-chain fatty acids. The effects of fatty acids are mediated either directly owing to their specific binding to nuclear receptors (PPAR and HNF4α) leading to changes in the trans-activating activity of these transcription factors. During fasting, fatty acids are released from adipose tissue and travel to the liver, where they bind and activate PPARα. Activation of PPARα results in stimulation of a number of pathways, including fatty acid oxidation, ketogenesis and suppression of amino acid catabolism. In this way, endogenous ligand-activation of PPARα occurs primarily during fasting as large amounts of free fatty acids enter the blood plasma [4,5]. Dietary intake of specific fatty acids can lead to potent activation of PPARα [6]. In addition to a host of endogenous ligands, PPARα is the molecular target for fibrates, which are synthetic PPARα agonists that include bezafibrate and fenofibrate [7]. PPARα regulates gene transcription by heterodimerizing with RXR (retinoid X receptor). PPAR/RXR heterodimers bind to a specific DNA sequence, peroxisome proliferator responsive element (PPRE). The consensus sequence consists of a direct repeat element (AGGTCA) with an interspacing of one or two base pair (DR1 or DR2) with a 5′-A/T rich flanking sequence which is essential for the polarity of DNA binding of the heterodimer PPAR/RXR [8].

In the current study, nutritional regulation of hepatic CREBH expression was examined in various feeding states and diabetic mice and its promoter was investigated as a new target of PPARα.

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Materials and methods

Animal experiments. For all experiments, 8-week-old male C57BL/6 mice from CLEA Japan were used. Mice were maintained on a standard chow diet. For diet-induced obesity (DIO), C57BL/6 mice were fed high-fat/high-sucrose (HF/HS) diet for 12 weeks. For fasting/refeeding protocol, mice were fasted for 24 h and then fed various diets for 12 h. Feed ingredient contents are as follows. Standard chow diet consists of carbohydrate 60%, protein 23.6%, fat 5.3%. High-sucrose diet consists of carbohydrate 70%, protein 20%, fat-free. High-fat diet consists of carbohydrate 27.5%, protein 25.6%, fat 35%. High-fat/high-sucrose diet consists of carbohydrates 34.8%, protein 25%, fat 30% (ORIENTAL YEAST Co., Ltd.).

Total RNA preparation, northern blotting and quantitative real-time PCR analysis. Total RNA from cell and tissues were prepared using Trizol reagent (Invitrogen) unless otherwise indicated. Northern blot analysis was performed as previously described [9]. cDNA probe for CREBH was prepared from reverse transcriptase for PCR using mouse liver mRNA (422–1861 bp, Genbank Accession No. NM_145365). Quantitative real-time PCR analysis was performed as described previously [10].

The primer sets were as follows; CREBH forward, 5′-CCAGAGC CCTTTACCCATACAT-3′, CREBH reverse, 5′-ATGGTTGGAGGTTAGG GTTCAG-3′, CPT1a forward, 5′-CCTGGGCATGATTGCAAAG-3′, CPT1a reverse, 5′-GGACGCCACTCACGATGTT-3′, MCAD forward, 5′-TGCTT TTGATAGAACCAGACCTACAGT-3′, MCAD reverse, 5′-CTTGGTGCTCC ACTAGCAGCTT-3′, ACO forward, 5′-CGATCCAGACTTCCAACATG AG-3′, ACO reverse, 5′-CCATGGTGGCACTCTTCTTAACA-3′.

Immunoblotting. Immunoblot analysis of nuclear extracts from livers of fasted or refed mice was as previously described [9]. For each group, livers from three mice were pooled, and aliquots (10 μg of protein) of nuclear extracts were subjected to immunoblot analysis with rabbit anti-mouse CREBH as primary antibody and anti-rabbit IgG, HRP-linked antibody (Cell signalling technology) as the secondary antibody. Anti-mouse CREBH rabbit polyclonal antibody was prepared using GST-mouse CREBH (amino acid 1–85).

Isolation and culture of hepatocytes and fatty acid administration. Primary hepatocytes were isolated from male mice and cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% fetal calf serum (FCS), 100 nM insulin, 10 nM dexamethasone, 1% penicillin/streptomycin prior to transfer to 60-mm collagen dishes at a final density 3.5×10^4 cells cm^{-2}. After incubation for 6 h for attachment, the medium was replaced with FCS free DMEM low glucose with 0.5% bovine serum albumin (BSA), 10 nM dexamethasone, 10 mM lactate for 16 h, followed by fatty acid administration for 8 h.

To examine effects of PPARα agonist and antagonist on the CREBH mRNA level in mouse primary hepatocytes, cells were supplemented with media alone or media containing PPARα antagonist (MK886) for 24 h, then after administered PPARα agonist (fenofibrate) for 8 h.

Plasmids. The expression vector for mouse PPARα was generated by inserting PCR-amplified full-length fragment into pcDNA3.1(+) (Invitrogen) containing a CMV promoter. The mouse CREBH promoter (base pairs −2000 to −1, relative to the transcriptional start site) was amplified by PCR using mouse BAC (Bacterial Artificial Chromosome). The primers were tailed with KpnI site (5′primers) or BglII site (3′primer). The PCR products were digested with KpnI and BglII and subcloned into pGL3-basic luciferase vector (Promega). Other constructs were produced by PCR using this construct as DNA template and subcloned the PCR products into the pGL3-basic luciferase vector.

Identification of the transcription start point. The transcription start point was determined by a PCR-based restricted fragment length analysis; DNA fragments amplified from mouse liver cDNA by PCR using four primer sets designed as follows; (exon 1 forward, 5′-GCTGTGGTGTGTCTCTGTCC-3′, exon1 reverse, 5′-ACAGGAAGAG CAGCTGCC-3′) (exon 1 forward, 5′-GCTGTGGTGTGTCTCTGTCC-3′, exon 2 reverse, 5′-ACTGTTTCTGTCTAGCGTCACC-3′) (exon 2 forward, 5′-AGCTGGCAACCTACCCAAG-3′, exon 3 reverse, 5′-CTTTCCA GCCGCTATGTCC-3′) (exon forward, 5′-CATCTTTGGGAGGTGGAG AC-3′, exon 4 reverse, 5′-CTCCACGTTTCTCAGGATGC-3′).

Transfection and luciferase assays. HepG2 cells were transfected with 200 ng each of indicated luciferase reporter and expression plasmids (CMV-PPARα or basic plasmid CMV7 as negative control) and a pRL-SV40 plasmid as a reference (Promega) using FuGENE6 (Roche). After a 24 h incubation at 37 °C, we measured the amount of firefly luciferase activity and normalized it to the amount of renilla luciferase activity.

Electrophoretic mobility shift assay (EMSA). Human PPARα and RXRα proteins were generated from the full-length human PPARα and RXRα expression vectors (pCI-PPARα and CMX-RXRα), respectively, using the TNT T7 Quick-coupled transcription/translation system (Promega). The probes for PPARα binding site were as follows: rat PEPCK promoter (−908 to −873 bp), gtcactcccacGGCC AAAGGTCAtgagaagggaatt; mouse CREBH promoter (−188 to −156 bp), gagctgtaagtAGGGGAGAGGTCAcacagacccg. Double-stranded oligonucleotides used in EMSA were labelled with [α-^{32}P]dCTP

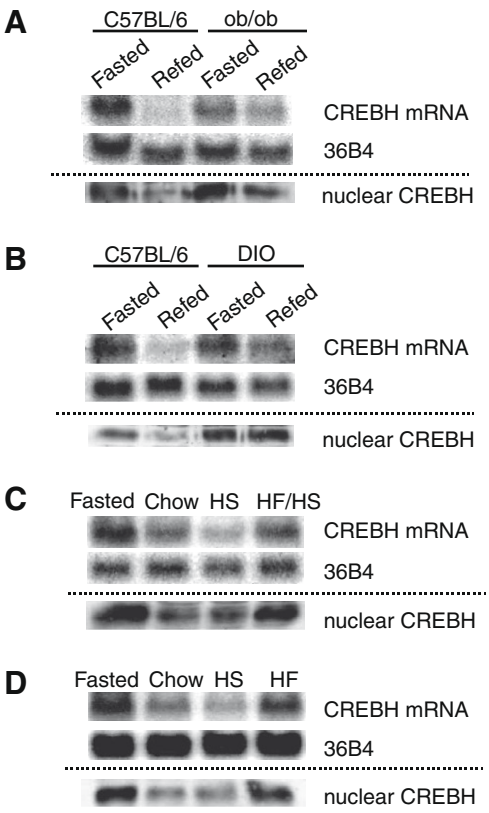

Fig. 1. Hepatic mRNA and protein levels of CREBH in various feeding states and obesity mice. (A) The expression of CREBH in the livers of wild-type and ob/ob mice. Mice were fasted for 24 h or fasted for 24 h/refed for 12 h. Both mice were fed HS diet during the refeeding period. (B) The expression of CREBH in the livers of wild-type and DIO (HF/HS diet for 12 weeks) mice. Mice were fasted for 24 h or fasted for 24 h/refed for 12 h. Wild-type mice were fed HS diet, DIO mice were fed HF/HS diet during the refeeding period. (C) The expression of CREBH in the livers of wild-type fed various diets. Mice were fasted for 24 h or fasted for 24 h/refed for 12 h. Mice were fed standard chow, HS and HF/HS diet during the refeeding period. (D) The expression of CREBH in the livers of wild-type fed various diets. Mice were fasted for 24 h or fasted for 24 h/refed for 12 h. Mice were fed standard chow, HS and HF diet during the refeeding period. Upper panel shows Northern blot, lower panel shows Western blot in nuclear extracts (nuclear CREBH). (n = 3 per group).