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Up-regulation of CAR expression through Elk-1 in HepG2 and SW480 cells by serum starvation stress

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ABSTRACT

Constitutive androstane receptor (CAR) is a transcription factor regulating the expression of several genes related to drug metabolism. CAR expression was elevated in human HepG2 and SW480 cells by serum starvation. From reporter gene assays, mutagenesis, RNA interference, and chromatin immunoprecipitation assays, we identified the serum response element at −142/−139 in the *CAR* gene transactivated by Elk-1. Whereas treatment with U0126 (ERK inhibitor) enhanced CAR expression, SP600125 (stress-activated protein kinase inhibitor, SAPK) suppressed the phosphorylation of Elk-1 caused by serum-starvation stress and the elevation of CAR mRNA, suggesting that CAR expression may be mediated by phosphorylated Elk-1 via the SAPK signaling pathway.

1. Introduction

Constitutive androstane receptor (CAR, NR1I3) was originally characterized as a nuclear hormone receptor that interacts with a subset of retinoic acid response elements [1]. To date, CAR has been recognized as a xenobiotic-sensing nuclear receptor that transcriptionally regulates the expression of metabolic enzymes and transporters involved in the metabolism and elimination of endogenous and exogenous substances such as bilirubin, steroid hormones, and xenobiotics [2,3]. CAR is predominantly expressed in the liver and is localized to the cytoplasm of normal mouse hepatocytes without stimuli such as drug-treatment [2,4]. CAR is activated by phenobarbital (PB) and PB-like inducers such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene that do not bind CAR directly but activate a signal transduction pathway that results in the translocation of CAR from the cytoplasm to the nucleus [2,4].

In the previous study, we demonstrated that CAR expression changes during the cell cycle in HepG2 and SW480 cells and CAR protein accumulates during G1 in both cells [5]. Depletion of CAR by RNA interference and hepatocyte growth factor treatment in HepG2 cells resulted in decreased MDM2 expression that led to p21 upregulation and repression of HepG2 cell growth, suggesting that CAR may influence the expression of genes involved in not only the metabolism of endogenous and exogenous substances but also in cell proliferation. In this study, we found that the expression of CAR increased in serum-starved HepG2 and SW480 cells. Up to date, two pathways of the transcriptional activation of *CAR* gene mediated by hepatocyte nuclear factor 4α (HNF4α) and glucocorticoid receptor have been reported [6,7]. Treatment of primary mouse hepatocytes with the β-adrenergic agonist epinephrine has been reported to elevate intracellular cAMP levels and to induce both CAR and CAR target genes. Thus, CAR promoter is regulated by HNF4α, and the fasting- and cAMP-inducible coactivator, peroxisome proliferator-activated receptor γ coactivator 1α, coactivates HNF4α on the CAR promoter [6]. Furthermore, the distal glucocorticoid response element has been identified within the CAR promoter, which is capable of conferring transcriptional

Abbreviations: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HNF4α, hepatocyte nuclear factor 4α; PXR, pregnane X receptor; RXR, retinoid X receptor; SAPK, stress-activated protein kinase; siRNA, short interfering RNA; SRE, serum response element

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activation via the glucocorticoid pathway [7]. However, very little is known about the molecular mechanisms that induce CAR by serum starvation stress; therefore, in this study, we investigated transcriptional regulation of the CAR gene promoter and the signaling pathway of CAR expression by stress.

2. Materials and methods

2.1. Cell culture conditions

HepG2 human hepatoma cells (1×10^5 cells/ml) and Hepa1-6 mouse hepatoma cells (5×10^4 cells/ml) from RIKEN BioResource Center and SW480 human colon cancer cells (5×10^4 cells/ml) from the American type culture collection (2 ml/9.6 cm^2/well) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), and antibiotics or DMEM-Ham's F12-serum-free medium supplemented with 2 mM glutamine, 15 mM HEPES, 5 μg/ml insulin, 10 μg/ml bovine transferine, 10 ng/ml Na_2SeO_3, 2 μg/ml aminolevulinic acid, 25 mM glucose, 0.5 mg/ml linoleic acid – albumin, 1 mM pyruvate with or without human 100 ng/ml growth hormone, and antibiotics unless otherwise stated.

2.2. Cloning and plasmid

DNA fragments of the 12-kbp 5′-flanking region of human *CAR* gene [−11931/33 (12K), −11931/-5648 (6K5′), −5665/33 (6K), −5665/−2027 (4K5′), −2074/33 (2K), −2074/−1233 (2K5′), −1238/33 (2K3′), −1238/−192 (1K5′), and −212/33 (1K3′)] (the transcriptional start site of human *CAR* gene is expressed as the nucleotide number 1) were obtained from genomic DNA by PCR amplification using specific primers and cloned between the NheI and XheI sites of pGL3-tk firefly luciferase reporter plasmid [3,8]. Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene).

2.3. Biochemical procedures

Quantitative reverse transcription PCR, Western blot analysis, transfection assays, chromatin immunoprecipitation assay, and short interfering RNA (siRNA)-mediated protein knockdown were performed according to standard protocols. Details can be found in Supplementary data.

3. Results

3.1. Serum starvation stress induces CAR expression

In the previous paper [5], we demonstrated that CAR protein levels vary significantly throughout the cell cycle and CAR accumulates during G1 in human SW480 and HepG2 cells. In this study, we examined whether the expression of CAR increased in cells arrested at the G1 phase by serum starvation. In cells cultured in serum-free medium with insulin for 24 h, >70% of the cells were in G1. The expression of CAR mRNA and protein significantly increased, but that of pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) and retinoid X receptor (RXR) did not (Fig. 1). Consistent with the increased expression of CAR, UGT1A1 and CYP2B6 proteins increased to 5.5- and 1.6-fold of the control, respectively (data not shown). Treatment with epidermal growth factor (EGF) suppressed the expression of CAR (Fig. 1).

3.2. Transcriptional activities of various human CAR 5′-flanking fragments

Little is known about the mechanism of transcriptional regulation of human *CAR* gene, except that CAR promoter is directly regulated by GR [6] and HNF4α [7] through GR and HNF4α response elements identified in the mouse and human promoter gene. In order to locate the serum-starvation stress response element in the *CAR* gene, various DNA fragments generated from a 12-kbp 5′-flanking region of the gene were generated and placed in front of the reporter luciferase gene. These deletion constructs were examined for their transcriptional activity in HepG2 cells in DMEM containing 200 μM BSA without serum. The 0.2K fragment (−212/33) displayed prominent transcriptional activity, whereas the other 5′-fragments containing the 0.2K fragment were slightly activated in serum-free medium (Fig. 2A). Nucleotide sequence analysis of the 0.2K fragment revealed two potential binding sites of the Ets family [serum response element (SRE) 1, −142/−139; SRE2, −110/−107] (Fig. 2B). To define the roles of each SRE in the 0.2K fragment, these motifs were mutated singly or simultaneously. The mutated DNAs were constructed into luciferase reporter gene plasmids, and subjected to transient transfection assays in HepG2 cells (Fig. 2B). Mutation of SRE1 (−142/−139) resulted in a 42% decrease of the original activity, while that of SRE2 (−110/−107) retained

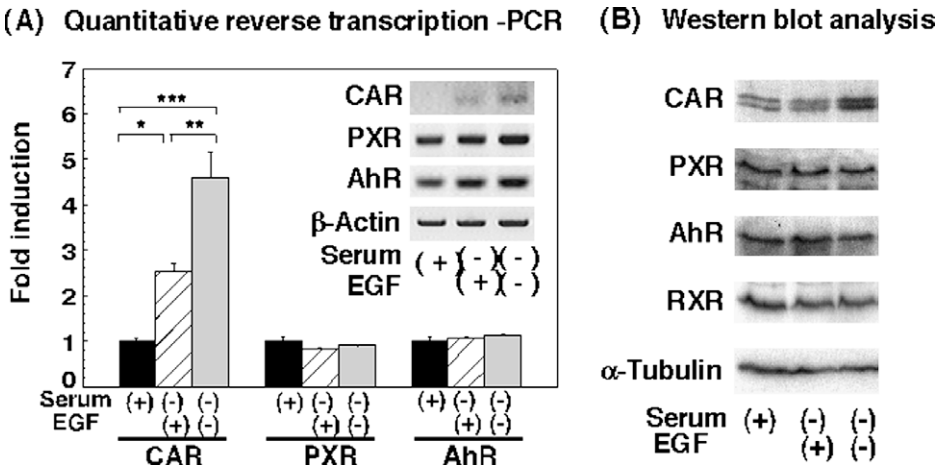

Fig. 1. Effect of serum starvation stress on the expression of transcription factors in HepG2 cells. Cells were cultured in serum-plus medium or serum-free medium supplemented with insulin in the presence or absence of 100 ng/ml human EGF for 24 h. In (A), the mRNA level was normalized to the β-actin mRNA level. The mRNA levels in cells cultured in serum-plus medium were calculated as 1. Inserted figures express PCR products of each gene. Data represent the mean ± S.E. of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.