Gene 550 (2014) 165–170

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Retinoic acid regulates several genes in bile acid and lipid metabolism via upregulation of small heterodimer partner in hepatocytes

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ARTICLE INFO

Article history:
Received 5 March 2014
Received in revised form 22 June 2014
Accepted 8 July 2014
Available online 9 July 2014

Keywords:
Retinoic acid
Small heterodimer partner
AML12 cell line

ABSTRACT

Retinoic acid (RA) affects multiple aspects of development, embryogenesis and cell differentiation processes. The liver is a major organ that stores RA suggesting that retinoids play an important role in the function of hepatocytes. In our previous studies, we have demonstrated the involvement of small heterodimer partner (SHP) in RA-induced signaling in a non-transformed hepatic cell line AML 12.

In the present study, we have identified several critical genes in lipid homeostasis (Apoa1, Apoa2 and ApoF) that are repressed by RA-treatment in a SHP dependent manner, in vitro and also in vivo with the use of the SHP null mice. In a similar manner, RA also represses several critical genes involved in bile acid metabolism (Cyp7a1, Cyp8b1, Mdr2, Bsep, Baat and Ntcp) via upregulation of SHP. Collectively our data suggest that SHP plays a major role in RA-induced potential changes in pathophysiology of metabolic disorders in the liver.

1. Introduction

Retinoic acid (RA) is an active metabolite of vitamin A. RA exerts regulatory effects on gene expression by binding to retinoic acid receptors, members of nuclear receptor family. Both in vivo and in vitro studies have established that RA functions as a signaling molecule in varied biological processes including development, growth, cell proliferation, cell differentiation, and different aspects of metabolism (Duester, 2008; Mark et al., 2006). Vitamin A is primarily stored in the liver tissue as retinyl esters (O'Byrne and Blaner, 2013). Although the liver is an important target of RA, the molecular mechanisms by which RA exerts its action in the liver are not clearly understood. Recently, we have described several novel RA responsive genes in murine hepatocytes (Mamoon et al., 2008). Among these, nuclear receptor small heterodimer partner (SHP) appears to be considerably up-regulated by RA. The up-regulation of SHP by RA indicates an interaction between retinoid and SHP pathways. SHP is an atypical nuclear receptor that lacks the conserved DNA binding domain (Seol et al., 1996). However, it contains the dimerization and ligand-binding domain common to all members of nuclear receptor superfamily, and interacts with a wide variety of other nuclear receptors. These interactions lead to repression of an array of target genes in several metabolic pathways (Seol et al., 1996).

Importantly, SHP has been demonstrated to be involved in the control of bile acid, cholesterol, triglyceride, glucose, and drug metabolism pathways (Båvner et al., 2005; Zhang et al., 2011). Dysregulation of bile acids has been linked to various components of metabolic syndrome like visceral obesity, insulin resistance and dyslipidemia (Hylemon et al., 2009; Lefebvre et al., 2009). Genetic mutations and variations of human SHP have also been linked to similar components of metabolic syndromes like increased birth weight, obesity, and diabetes (Hung et al., 2003). The liver plays a key role in insulin resistance and in the pathogenesis of diabetes, and it has been increasingly recognized that there are links between obesity, insulin resistance, and liver diseases. Along these lines, the administration of retinoid derivatives to humans has been demonstrated to increase serum triglyceride levels, decrease serum HDL-cholesterol levels, and lead to liver damage (Bershad et al., 1985; Geubel et al., 1991; Lestringant et al., 1997). Hypertriglyceridemia and low HDL are markers of insulin resistance and have been linked to cardiovascular disease.

Developing a detailed understanding of the mechanism of action of nuclear receptors is essential in light of the fact that dysregulation of nuclear receptor-mediated transcription pathways is implicated in numerous human pathologies. The interactions of SHP and RA pathways open up possibilities of targeting SHP for therapeutic interventions. Our previous studies point towards the interaction of SHP and RA pathways in hepatocytes. For a better understanding of this interaction, we studied the role of SHP in the RA-mediated regulation of bile acid and lipid metabolism. Experiments were carried out in the murine hepatocyte, AML12 cell line, in which the expression of SHP was down-regulated by siRNA. In vivo experiments were also performed in the SHP null mice.

Abbreviations: RA, retinoic acid; SHP, small heterodimer partner; AML12, alpha mouse liver 12; VAD, vitamin A deficient; TTNPB, 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid.

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http://dx.doi.org/10.1016/j.gene.2014.07.017
0378-1119/

2. Materials and methods

2.1. Cell culture

The non-transformed murine hepatocyte AML 12 cells were grown in modified Eagles's/Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 nM dexamethasone and ITS (insulin, transferrin and selenium, Invitrogen, Carlsbad, CA) at 37 °C in an atmosphere with 5% CO_2.

2.2. Quantitative, real-time PCR (qPCR)

First-strand cDNA for real-time quantitative PCR analysis was synthesized from 5 μg of total RNA (prepared as previously described by us, Mamoon et al., 2008) using random primers and SuperScript™ III Reverse Transcriptase kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions.

Quantitative PCR reactions were performed in a DNA Engine Opticon 2 System (Bio-Rad, formerly MJ Research Inc.). Samples were prepared using the DyNAmo™ SYBR® Green qPCR kit from Finnzymes (New England Biolabs, Beverly, MA). Reaction mixes consisted of 10 μl of SYBR green-containing master mix, 1 μl of 1:5 dilution of cDNA prepared as described above, and 1 μl of 10 μM amplification primers for selected genes (20 μl total reaction volume). The sequences of gene-specific primers designed for qPCR are presented in Table 1. Thermocycling conditions have been previously described (Mamoon et al., 2008). Calculations of relative gene expression in treatment samples versus controls (normalized to Gapdh as reference control gene) were performed using Genex Macro™ version 1.1 software (Bio-Rad Laboratories). Each gene was tested in multiple PCR reactions, and the mean of at least three reactions was used to calculate expression levels.

2.3. Western blot analysis

Nuclear fractions of proteins were extracted from AML12 cells 3 h and 24 h after incubation with 1 μM RA (or vehicle) using a NE-PER reagent kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Pierce-Thermo Fisher Scientific, Rockford, IL). Proteins were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following separation, proteins were transferred to Immunoblot PVDF Membrane (Bio-Rad, Hercules, CA). The membranes were probed with goat polyclonal antibodies that recognize SHP (Sc-22040, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200. Secondary antibody was bovine anti-goat IgG-HRP (Sc-2350, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:5000. The bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce-Thermo Fisher Scientific, Rockford, IL).

Table 1
Primers for genes studied.

Apoa1:	GTCACCCACACCCTTCAG
	CGCATCCACATACACATTAGC
Apoa2:	TGC AGC ACA GAA TCG CAG CA
	CAG CAG TGC GAC CAT TGC GA
ApoF:	GCA AAG GCC CTG GCC AAT GT
	AGG GGC TGG GCC AAT TGC TA
Bsep:	AAA TCG GAT GGT TTG ACT GC
	GGC AAT GGC TTC ATC AAT TT
Baat:	CCA GGG GTC ATT GAC TTG TT
	CCA GAG CTA AGG TGG CAA AG
Cyp7a1:	CAA CGG GTT GAT TCC ATA CC
	ATT TCC CCA TCA GTT TGC AG
Cyp8b1:	TGG CCT CTT TCA CTT CTG CT
	CGG AAC TTC CTG AAC AGC TC
Mdr2:	CAC TCA CAG AAG ATA CTG GAC
	CAG GAA CCA AGA ATT CTT CTG
Nrip1:	TGG TGA GCA ACG AAA GAT G
	AAC TCG GGT GCA GAC TAC
Ntcp:	TTC AGC AAG ATC AAG GCT CA
	CCA GAA GGA AAG CAC TGA CG
Pgc1:	GCA GGC CTA ACT CCT CCC ACA
	GTG GCA CCA CGG TCT TGC AA

2.4. SiRNA interference

Targeted silencing of gene expression was conducted by using a Lipofectamine RNAiMAX kit from Life Technologies. One day before transfection, AML12 cells were plated in growth medium without antibiotic so that they were 40–50% confluent at the time of transfection. For each well, RNAi duplex-Lipofectamine RNAiMAX was prepared in Opti-MEM I Reduced Serum Medium (Life Technologies, Grand Island, NY). Cells were transfected in triplicates in 6 well plates with SHP siRNAs AAG AAG AGA UCU ACC AGA AGG GUG C and UUG AAG AGG AUC GUG CCC UUC AGG U at increasing concentrations (0, 20, 40, or 80 nM). Forty eight or 72 h later, RNA was isolated using the Cells to cDNA II kit (Life Technologies, Grand Island, NY) followed by real time quantitative PCR. Twenty four hours prior to end of incubation plates were treated with either 1 μM all-*trans* RA or vehicle. Gapdh and actin were used as controls for specificity. The siRNA 12935-300 (Life Technologies, Grand Island, NY) was also used as a negative control.

2.5. Intraperitoneal administration of RA

SHP −/− mice were kind contribution from Amgen, Thousand Oaks, CA and Dr. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX). During breeding and maintenance of the colonies lack of SHP gene expression was confirmed by PCR. WT control mice (C57BL/6) were purchased from Harlan Spague Dawley Inc. Mice were housed in climate controlled room on standard 12-hour light/dark cycle. Mothers were placed on a vitamin A deficient (VAD) diet (TD.86143, Harlan Teklad Co., Madison, WI) at birth of the experimental mice and then the pups were weaned on the same VAD diet until the age of 7 weeks. This regimen has been shown to deplete the hepatic stores of vitamin A (retinol and retinyl esters), which is considered the gold standard measurement for vitamin A deficiency (Tanumihardjo, 2004). We confirmed vitamin A deficiency status by measuring retinol and retinyl esters by HPLC from the liver of the mice on this VAD regimen (data not shown). At the end of the 7 week period on VAD diet, prior to the experiments animals were weighed and separated into male and female groups reflecting WT and SHP −/− mice ($n = 8–12$ in each group). Intraperitoneal injections were delivered to conscious animals using a 32 g needle. Animals were restrained by holding the skin behind the neck by the thumb and index finger while wrapping another finger around the tail. The needle was inserted into the abdomen just above the knees, slightly to the right of the midline and care was taken not to puncture any organ. Control groups received the vehicle, cottonseed oil while experimental groups received 0.5 mg/kg all-*trans* RA. All mice were sacrificed 3 h or 24 h following the injections, and liver tissue was harvested for RNA isolation and quantitative real time PCR. All animal experiments were conducted according to the *Guide for the Care and Use of Laboratory Animals, 8th edition* (prepared by National Academy of Sciences and published by NIH).

3. Results and discussion

We have demonstrated that RA induces a novel RA-responsive gene, SHP in AML12 cells at the mRNA level (Mamoon et al., 2008) and at the protein level (Fig. 1). We have also demonstrated a correlation between RA-induced genes and genes induced by RAR-specific ligand, TTNPB {4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid} (Mamoon et al., 2008).

Along with SHP, several putative secondary genes involved in lipid metabolism were also repressed at 24 h but not at 3 h after incubation with RA. We speculated that SHP was a primary RA-responsive gene