Biochemical and Biophysical Research Communications 406 (2011) 268–272

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Retinoids synergized with insulin to induce *Srebp-1c* expression and activated its promoter via the two liver X receptor binding sites that mediate insulin action

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ARTICLE INFO

Article history:
Received 29 January 2011
Available online 18 February 2011

Keywords:
Insulin
Metabolism
Primary hepatocytes
Retinoids
Srebp-1c expression
Retinoid X receptor

ABSTRACT

We have reported that the rat liver lipophilic extract (LE) synergized with insulin to induce *Gck* and *Srebp-1c* in primary rat hepatocytes. After identification of retinol and retinal in LE, only their effects in the absence or presence of insulin on *Gck*, but not that on *Srebp-1c*, were investigated subsequently. The retinoid effects on the *Srebp-1c* expression and the activation of its promoter were examined with real-time PCR and reporter gene assays, respectively. In primary hepatocytes, retinal and retinoic acid (RA) synergized with insulin to induce *Srebp-1c* expression. This induction was followed by the elevation of its target gene, fatty acid synthase. Activation of retinoid X receptor, but not retinoic acid receptor, was responsible for the induction of *Srebp-1c* expression. RA, but not retinal, also induced *Srebp-1c* expression in a dose dependent manner in INS-1 cells. The RA responsive elements in *Srebp-1c* promoter were determined as previously identified two liver X receptor elements responsible for mediating insulin action. We conclude that retinoids regulate hepatic *Srebp-1c* expression through activation of retinoid X receptor. The RA- and insulin-induced *Srebp-1c* expression converged at the same sites in its promoter, indicating the roles of vitamin A in regulation of hepatic gene expression.

1. Introduction

Elevation of hepatic vitamin A (VA, retinol) content in patients with diabetes was observed more than 70 years ago (1937) [1]. Subsequently, depletion of hepatic glycogen content in VA deficient (VAD) rats was reported in 1957 [2]. When isotretinoin, 13-*cis* retinoic acid, was used to treat patients with acne, some of them developed isotretinoin-induced hypertriglyceridemia [3]. All these early observations suggested that VA status affected glucose and lipid homeostasis, a topic remained to be investigated.

As an essential micronutrient, VA plays crucial roles in the general health of an individual [4]. Therefore, retinol homeostasis must be delicately maintained to meet optimal physiological requirements. This is achieved by a network of enzymes and proteins involved in the transport, production, and catabolism of retinoids [5]. The regulation of this system can be attributed to the control of the expression levels of some of these enzymes by the active metabolite of retinol, retinoic acid (RA) [6]. RA exists in multiple isomeric forms, such as all-*trans* RA and 9-*cis* RA, and RA regulates gene expression through activation of two families of nuclear receptors, retinoic acid receptors (RARα, β and γ) activated by all-*trans* RA, and retinoid X receptors (RXRα, β and γ) activated only by 9-*cis* RA [7].

Insulin resistance, diabetes and other metabolic abnormalities are associated with profound changes of hepatic lipid and glucose metabolism. These can be attributed to the altered expression of genes involved in glucose and lipid metabolism [8]. Insulin responsive elements in the *Srebp-1c* promoter have been identified as two liver X receptor (LXR) binding sites and one sterol regulatory element [9,10]. This implies that insulin regulates the expression of its responsive genes after it stimulates the synthesis of endogenous agonists for nuclear receptor activation. When we analyzed the effects of the lipophilic extract (LE) from rat livers on insulin-regulated gene expression, we found that the LE synergized with insulin to induce glucokinase gene (*Gck*) and *Srebp-1c* expression in primary rat hepatocytes with different induction patterns [11]. The existence of retinol and retinal in LE was confirmed later, and their effects on *Gck*, but not *Srebp-1c*, were examined in that study [11]. It has been reported that SREBP-1c mediated the retinoid-dependent increase in fatty acid synthase (*Fas*) promoter activity in HepG2 [12]. Therefore, we hypothesized that retinoids may regulate the expression of *Srebp-1c* in primary hepatocytes.

In this study, we report that retinoids transiently synergized with insulin to induce the expression of *Srebp-1c* in primary rat hepatocytes via the activation of RXR, but not RAR. The retinoic acid responsive elements (RAREs) in its promoter are the

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doi:10.1016/j.bbrc.2011.02.031

previously identified two LXR responsive elements that mediated insulin-induced *Srebp-1c* transcription.

2. Materials and methods

2.1. Reagents

The reagents for primary hepatocyte isolation and culture have been published [13]. Reagents for cDNA synthesis and real time PCR were obtained from Applied Biosystems (Foster city, CA). Source of LG268 was reported previously [11]. All other compounds or enzymes were purchased from Sigma (St. Louis, MO) unless described otherwise.

2.2. Animals and diets

Sprague–Dawley rats (for hepatocytes) were purchased from Harlan Breeders (Indianapolis, IN). Rats were housed in colony cages, and fed a standard rodent diet before isolation of primary hepatocytes. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee at Knoxville.

2.3. Primary hepatocytes, RNA extraction and quantitative real-time PCR

Methods for preparation of primary hepatocytes and analysis of RNA were described previously [11]. The real time PCR primer sets for detecting *Fas* (from Dr. Bruce Spigelman's group in Harvard Medical School), *Gck*, *Cyp26a1* [14], *Srebp-1c* [11] have been published. The primers for *Rarb* (forward 5′-GGCCTCTGGGACAAATTCAG-3′, and reverse 5′-GCAGACGCTTGGCGAACT-3′) were designed using Primer Express software (Applied Biosystems). The gene expression level was normalized to that of 36B4 unless described otherwise. Data were presented as the fold induction calculated from the ΔΔCt values [13] using 36B4 as the invariable control gene [11].

2.4. INS-1 cell culture, and reporter gene constructs and assay

INS-1 cells (833/15) were maintained as described previously [15]. Standard protocols (Molecular Cloning) were followed in all recombinant DNA engineering procedures. The reporter gene constructs reported previously [10] were transfected into INS-1 cells using Fugene 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacture's manual. The activation of reporter gene constructs were measured using dual luciferase assay as described previously [10] and reported as fold induction.

2.5. Statistics

Data were presented as means ± SD. The number of experiments represented the independent experiments using hepatocytes isolated from different animals on different days. Levene's test was used to determined homogeneity of variance among groups using SPSS 17.0 statistical software and where necessary natural log transformation was performed before analysis. Multiple comparisons were analyzed by one-way ANOVA. The independent sample *t*-test was used to compare two conditions. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Retinal and retinoic acid synergized with insulin to induce Srebp-1c expression

Since we have observed that rat liver LE which contained retinol (ROL) and retinal (RAL) synergized with insulin to induce *Gck* and *Srebp-1c* expression with different induction patterns, we only reported the effects of ROL, RAL and RA on *Gck* expression in the previous publication [11]. We decided to check the direct effects of retinoids on the expression level of *Srebp-1c*, a key transcription factor controlling the hepatic fatty acid biosynthesis [16]. Primary rat hepatocytes were treated with increasing concentrations of ROL, RAL and RA in the absence or presence of insulin. As shown in Fig. 1, retinol up to 20 μM did not induce *Srebp-1c* expression without or with insulin. In the absence of insulin, RAL up to 20 μM did not affect *Srebp-1c* expression. RAL synergized with insulin to induce *Srebp-1c* expression when its concentration reached 20 μM. Without insulin, RA at 20 μM induced *Srebp-1c* expression. RA at 2 and 20 μM synergized with insulin to induce *Srebp-1c* expression. All these results demonstrated that RAL and RA had the ability to synergize with insulin to induce *Srebp-1c* expression in primary rat hepatocytes.

3.2. RA transiently synergized with insulin to induce the expressions of Gck and Srebp-1c differently, and resulted in elevation of SREBP-1c target gene, Fas

Since RA induced the expression of both *Gck* and *Srebp-1c*, it is important to determine whether their induction patterns are similar or not. The expression levels of *Gck*, *Srebp-1c* and *Fas*, a target gene of SREBP-1c, were examined by real time PCR at 0, 3, 9, 12 and 24 h after treatment of 5 μM RA in the absence or presence of 1 nM insulin. As shown in Fig. 2A, RA robustly synergized with insulin to induce *Gck* expression as early as three hours. The fold induction started to decline at 6 h after the stimulation, and the synergy lasted for at least 12 h. Fig. 2B showed that hepatocytes treated with 5 μM RA had significantly higher levels of *Srebp-1c* mRNA than control cells did at 3 (2.1 ± 0.5- vs 0.71 ± 0.07-fold) and 6 (0.53 ± 0.09- vs 0.26 ± 0.02-fold) hours after treatment. RA also robustly synergized with insulin to induce *Srebp-1c* expression at 3 h and the fold induction began to drop at 6 h, similar to the pattern of *Gck* expression. However, at 9 h after the stimulation, the synergistic induction of RA and insulin to *Srebp-1c* expression no longer

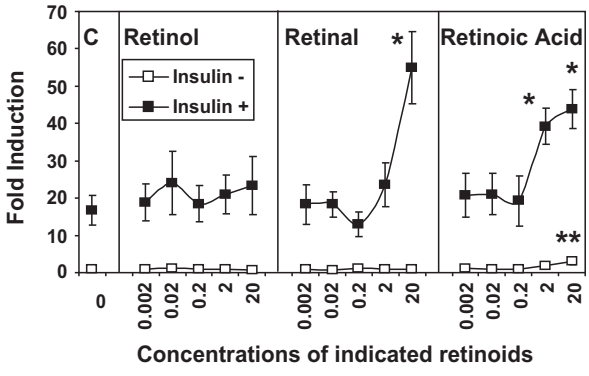

Fig. 1. Retinal and retinoic acid synergized with insulin to induce *Srebp-1c* mRNA expression in rat primary hepatocytes. Hepatocytes were treated with indicated ligands without or with 1 nM insulin for 6 h. Total RNA was isolated and subjected to real-time PCR analysis. *Srebp-1c* mRNA level in vehicle control group was assigned a value of 1 (mean ± SD, $n = 3$, $*$ for comparing indicated groups with control in the presence of insulin; $**$ for comparing retinoic acid group with control group in the absence of insulin; all $P < 0.05$).