Biochimica et Biophysica Acta 1811 (2011) 1045–1053

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Suppression of retinol-binding protein 4 with RNA oligonucleotide prevents high-fat diet-induced metabolic syndrome and non-alcoholic fatty liver disease in mice

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ARTICLE INFO

Article history:
Received 21 December 2010
Received in revised form 20 September 2011
Accepted 23 September 2011
Available online 1 October 2011

Keywords:
Retinol-binding protein 4
Anti-RBP4 RNA oligonucleotide
RBP4 inhibition
High fat diet fed mice
Metabolic syndrome
Non-alcoholic fatty liver disease

ABSTRACT

Conflicting data have been reported regarding the role of retinol-binding protein (RBP4) in insulin resistance, obesity, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). In this study, we used pharmacological methods to investigate the role of RBP4. RNA oligonucleotide against RBP4 (anti-RBP4 oligo) was transfected into 3T3-L1 adipocytes. RT-PCR analysis showed that RBP4 mRNA expression decreased by 55% ($p<0.01$) compared with control cells. Validated RNA oligo was used in an *in vivo* study with high fat diet (HFD) fed — mice. 14 weeks of HFD feeding increased RBP4 expression (associated with elevated serum levels measured with immunoblotting and ELISA) by 56% in adipose tissue ($p<0.05$) and 68% in the liver ($p<0.01$). Adipose RBP4 levels were significantly reduced after 4 weeks treatment with anti-RBP4 oligo (25 mg/kg, $p<0.01$) and rosiglitazone (RSG, 10 mg/kg, $p<0.05$) compared with scrambled RNA oligo (25 mg/kg) treated mice. Only anti-RBP4 oligo significantly inhibited RBP4 protein ($p<0.01$) and mRNA expression ($p<0.01$) in the liver and reduced serum RBP4 levels. Anti-RBP4 oligo and RSG showed comparable effects on impaired glucose tolerance, hyperinsulinaemia and hyperglycaemia. Anti-RBP4 oligo significantly enhanced adipose-GLUT4 expression ($p<0.01$) but did not increase muscle-GLUT4. Both RSG and anti-RBP4 oligo significantly reduced hepatic phosphoenolpyruvate carboxykinase expression (both $p<0.05$). Histological analysis revealed that anti-RBP4 oligo ameliorated hepatic steatosis and reduced lipid droplets associated with normalized liver function. Histological and pharmacological results of this study indicate that RBP4 is not only an adipocytokine, but also a hepatic cytokine leading to metabolic syndrome, NAFLD and type 2 diabetes.

1. Introduction

Experimental and clinical studies have shown pathways that lead to type 2 diabetes from obesity through a variety of fat-derived proteins termed “adipokines” [1,2]. These adipokines and their signaling pathways represent potential therapeutic targets for preventing or treating obesity and/or type 2 diabetes. Retinol-binding protein 4 (RBP4), which is a 22–25 kDa protein synthesized in the liver and adipose tissue, has recently been identified as one of the most important adipokines in the insulin resistance associated with obesity and type 2 diabetes [3,4]. It has been shown that RBP4 mRNA in adipose tissue is up-regulated in mice models of insulin resistance with adipocyte-specific ablation of GLUT4 resulting in increased concentrations of serum RBP4 [5]. An up-regulation of RBP4 in adipose tissue has also been observed in other insulin-resistant mouse models such as *ob*/*ob* mice and high-fat diet (HFD) fed mice [5]. RBP4 gained much clinical attention after the early notion that it played an important role in mediating adipose tissue communication with other insulin target tissues of rodent models of insulin resistance and diabetes. The clinical investigations have revealed a positive correlation between circulating RPB4 levels and the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance and type 2 diabetes [6–8]. Furthermore, the severity of glucose intolerance in women with previous gestational diabetes is also associated with high RBP4 concentrations [9]. Several recent studies also demonstrated that RBP4 is associated with elevated liver fat and liver damage [10–12], indicating RBP4 may be a reliable circulating marker of non-alcoholic fatty liver disease (NAFLD) [13]. These findings suggest that the increased secretion of RBP4 by adipose tissue is likely to have an important role in the pathogenesis of metabolic syndrome, type 2 diabetes and fatty liver disease; however, not all studies showed an association between elevated circulating RBP4 concentrations and insulin resistance

Abbreviations: AUC, areas under the curve; AOC, areas over the curve; GLUT4, glucose transporter 4; HFD, high fat diet; ipGTT, intraperitoneal glucose tolerance test; ipITT, intraperitoneal insulin injection; NEFA, non-esterified fatty acids; NAFLD, non-alcoholic fatty liver disease; PEPCK, phosphoenolpyruvate carboxykinase; RBP4, retinol-binding protein 4; RNA oligo, RNA oligonucleotide; RSG, rosiglitazone; TAG, triacylglycerol

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1388-1981/$ – see front matter.
doi:10.1016/j.bbalip.2011.09.011

in type 2 diabetes patients [14,15]. For example, a clinical study in Spain showed that serum RBP4 is not increased in obese patients with or without type 2 diabetes [16]. Neither plasma RBP4 levels nor RBP4 expression in adipose tissue was increased in obese women, and RBP4 levels did not correlate with insulin sensitivity [17]. Recent studies in Hispanic-Americans demonstrated that increased RBP4 levels are only associated with impaired glucose tolerance, but not with whole body and hepatic insulin resistance [18]. Conflicting data has also been reported about the role of RBP4 in various features of NAFLD [19].

These contradictory observations raise some questions regarding the actual role of RBP4 in the pathogenesis of obesity linked with insulin resistance, type 2 diabetes and NAFLD. In this study, we used a mouse model to investigate whether over-expression and secretion of RBP4 is correlated with metabolic syndrome and contributes to the development of type 2 diabetes. We hypothesized that suppressing RBP4 gene expression will reduce secretion of RBP4 from adipocytes and the liver into the bloodstream. Lowering the concentration of circulating RBP4 will improve metabolic abnormalities in obesity associated with insulin resistance. This hypothesis was tested using synthesized anti-RBP4 nucleotide to block RBP4 mRNA expression in HFD mice.

2. Research design and methods

2.1. In vitro study

2.1.1. RNA oligonucleotide

RNA oligonucleotide (RNA oligo) had been designed to complement the 5′-untranslated region, start codon or termination region of the RBP-4 genes. In order to target the RBP4 gene, the sequence of the synthetic RNA oligo was determined based on RBP4 mRNA (NM_011255) from free energy analysis. The RNA oligo was modified with 2′-O-methyl and a 3′-butanol cap to increase the *in vivo* stability of the RNA. The sequences of RNA oligo against RBP4 (anti-RBP4 oligo) and the scrambled control RNA oligo (control oligo) were 5′-CAT CCT AGA CGT TGC TAC-3′ and 5′-AGA CCT CTC ATA GCA GCT GAT-3′, respectively. The anti-RBP4 oligo and the scrambled control oligo were synthesized and purified through high-pressure liquid chromatography at Oligos Etc Inc. (Wilsonville, OR).

2.1.2. Transfection of synthetic RNA oligonucleotide into 3T3-L1 adipocytes

The differentiation of 3T3-L1 fibroblasts into adipocytes was induced prior to transfection as described previously [20]. RBP4 mRNA expressions are also detectable when adipocytes attain confluence [21]. 3T3-L1 adipocytes were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin in an atmosphere of 5% CO_2. To facilitate anti-RBP4 oligo transfection into 3T3-L1 cells, Lipofectamine™ 2000 (Invitrogen Life Technologies Corporation, CA) was used as a lipid-based carrier. One day before transfection, 0.5 ml of cells (3×10^5/ml) in growth medium without antibiotics was seeded in each well of a 24-well plate. For each transfection, anti-RBP4 oligo and control oligo were diluted in 100 μl of Opti-MEM I Reduced Serum Medium without FBS. The final concentration of RNA when added to the cells was 2 μM. The transfection procedure was as described previously [22]. The efficiency of transfection was determined using an FITC-labeled anti-RBP4 nucleotide and analyzed on a FACS Lalibur (BD Pharmingen, CA, USA) for uptake of fluorescence.

2.1.3. Assay of RBP4 mRNA expressions in 3T3-L1 adipocytes

To determine efficacy of anti-RBP4 oligo on down-regulation of RBP4 gene, the transfected cells were collected for determination of RBP4 mRNA expression using real-time PCR. The total RNA from cells was extracted by using SV Total RNA Isolation System (Promega, WI) in accordance with the manufacturer's instructions. After determination of RNA concentrations by measuring the absorbance at 260 nm, 200 ng RNA as template was reversely transcribed to cDNA by using Quanti®Tect Reverse Transcription Kit (QIAGEN, AL). The resulting RT products (cDNA samples) were stored at −20 °C for real-time PCR. RBP primer pair was used to amplify 223 bp DNA from mouse mRNA designed by Oligo Etc Inc. (Wilsonville, OR). The sense primer was 5′ AAG ATC CTG CCA AGT TCA AGA 3′, the antisense primer was 5′ CGT TGC CTC ACC AGC CTC 3′′ and β-actin as reference genes from Promega (Madison, WI). The amplification was carried out using Mastercycler *ep realplex* (Eppendorf, CA) with fastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions. The relative RBP4 mRNA levels in anti-RBP4 oligo transfected cells were presented as percentage of RBP4 mRNA levels in control-oligo transfected cells.

2.2. In vivo study

2.2.1. Animals and treatment

Male C57BL/6 mice (8 week old) supplied by the Animal Resources Center (Perth, Australia) were acclimatized in communal cages at 22 °C, with a 12 h light, 12 h dark cycle (lights on at 0700) for 1 week and had access to a standard chow diet or a HFD (59% fat, 21% protein, 20% carbohydrate by energy composition) [23] for 10 weeks to induce metabolic syndrome. Body weight and food intake were measured weekly. Rosiglitazone (RSG) was purchased from the GlaxoSmithKline (Boronia, VIC, Australia). RSG was dissolved in medical saline at the ratio of 10 mg per 5 ml. RNA oligos were freshly prepared in medical saline at the ratio of 25 mg per 5 ml, and the solution was sterilized through a 0.2-μm filter. After 10 weeks feeding, HFD mice were randomly divided into four groups (n = 8 per group) and treated with saline (5 ml/kg of body weight) or RSG (10 mg/kg of body weight) daily by oral gavage, and scrambled control oligo (25 mg/kg of body weight) or anti-RBP4 oligo (25 mg/kg of body weight) via intraperitoneal (ip) injection every second day for 4 weeks. HFD feeding was continued during 4 weeks of treatment with oligos. Eight chow fed mice were treated with saline (5 ml/kg of body weight) by oral gavage as a control group.

All experimental procedures were approved by the Joint Royal North Shore Hospital/University of Technology Sydney Animal Care and Ethics Committee and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

2.2.2. Intraperitoneal glucose and insulin tolerance tests

At the end of 4-weeks treatment, all mice were subjected to the intraperitoneal glucose tolerance test (ipGTT) following an overnight fast (12 h). An intraperitoneal glucose bolus (2 g/kg of body weight) was administered to the conscious, unrestrained mice. The blood glucose levels were determined from tail blood samples taken at 0 (prior to glucose administration) and 15, 30, 60, 90, 120 and 150 min after the glucose injection using One Touch Profile glucometer. For insulin tolerance tests, mice were subjected to a 6-h fast, followed by intraperitoneal insulin injection (ipITT, 1 U/kg of body weight). Blood glucose levels were measured in the same manner as ipGTT. Results of ipGTT were expressed as integrated areas under the curves (AUC) over 150 min for glucose calculated using the Trapezoidal Rule with software of Graphpad prism 4 (Graphpad Prism, CA). Results of ipITT were calculated the areas over the curves (AOC) with a baseline set from initial glucose value, which represented the integrated reduced level of blood glucose during the insulin tolerance test [24].

2.2.3. Tissue collection

At the end of the experiment, mice were sacrificed using CO_2 after 12 h of fasting. Blood samples were collected from the heart and stored on ice until centrifuged (10 min at 1400 *g*) to separate the