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Zinc alpha2 glycoprotein alleviates palmitic acid-induced intracellular lipid accumulation in hepatocytes

Xinhua Xiao [a, 1], Han Li [a, 1], Xiaoyan Qi [a], Yadi Wang [a], Canxin Xu [b], Gexin Liu [a], Gebo Wen [a], Jianghua Liu [a, *]

[a] *Department of Metabolism and Endocrinology, The First Affiliated Hospital of University of South China, 421001, People's Republic of China*
[b] *Department of Pathology & Immunology, Developmental, Regenerative and Stem Cell Biology, Washington University in St. Louis, MO, 63110, United States*

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ABSTRACT

Zinc alpha2 glycoprotein (ZAG) plays an important role in stimulating fat mobilization and lipolysis in adipose tissue, but its role in hepatic lipid metabolism remains unclear. Palmitic acid (PA) was used to stimulate HepG2 cells with ZAG overexpression or ZAG knock down (shRNA). Overexpression of ZAG significantly inhibited lipogenesis, promoted lipolysis and fatty acid β-oxidation, and attenuated PA-induced intracellular fat accumulation. Moreover, ZAG overexpression dramatically stimulated adiponectin expression in HepG2 cells. In contrast, knockdown of ZAG notably inhibited fatty acid β-oxidation, increased lipogenesis and lipid accumulation. Collectively, these data suggest that ZAG has the potential to alleviate hepatosteatosis, making it a promising therapeutic target for fatty liver.

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1. Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) appears to be one of the most frequent causes of liver dysfunction. The incidence of NAFLD has increased markedly over these years, and is associated with the development of type 2 diabetes, atherosclerosis, hypertension, and even coronary heart disease (Sanyal, 2005; Roden, 2006). Hepatosteatosis is an important manifestation of hepatic metabolic injury, which is characterized by aberrant hepatic triglyceride (TGs) accumulation, due to increased de novo lipogenesis, increased fatty acid uptake, reduced fatty acid oxidation and export of very low density lipid (VLDL) (Anstee et al., 2013; Fabbrini et al., 2010). Thus, identifying key molecular players in the regulatory network that governs hepatic lipid metabolism is an important step toward development of treatments for hepatosteatosis.

Zinc alpha2 glycoprotein (ZAG) is widely expressed in many different tissues including adipose tissue and liver, this soluble protein can be secreted into serum and other body fluids (Burgi and Schmid, 1961; Bing et al., 2004; Mracek et al., 2010; Sánchez et al., 1999; Hassan et al., 2008). Although first identified as a potential cancer marker for some malignant tumors (Tada et al., 1991; Díez-Itza et al., 1993; Abdul-Rahman et al., 2007), ZAG has also been reported as a lipid-mobilizing factor responsible for a loss of fat mass in patients with cancer cachexia (Todorov et al., 1998). More recent evidence has suggested that ZAG impacts lipolysis and increases fat utilization, which ultimately leads to decreased adipose tissue mass (Mracek et al., 2010; Russell and Tisdale, 2011a,b; Bing et al., 2010; Mracek et al., 2010). Interestingly, previous studies have shown that levels of hepatic ZAG expression level were remarkably down-regulated in obesity obese patients (Selva et al., 2009). Although the effects of ZAG in lipid metabolism within adipose tissue has been well characterized, the role of ZAG in the regulation of TG metabolism in the liver remains unknown.

The aim of the present study was to characterize the role of ZAG in the regulation of lipid metabolism in hepatocytes. We found that

Abbreviations: ZAG, zinc alpha2 glycoprotein; NAFLD, non-alcoholic fatty liver disease; TG, triglycerides; PA, palmitic acid; SREBP-1c, sterol-regulatory element binding protein 1c; LXR, liver X receptor; FXR, farnesoid X receptor; PPARα, peroxisome proliferator-activated receptor alpha; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; SCD-1, stearoyl-CoA desaturase 1; FATP, fatty acid transport protein; CPT-1A, carnitine palmitoyltransferase 1A.

* Corresponding author. Department of Metabolism & Endocrinology, The First Affiliated Hospital University of South China, 69 Chuanshan Road, Hengyang, 421001, People's Republic of China.

E-mail address: jianghua990@163.com (J. Liu).

[1] Xinhua Xiao and Han Li contributed equally to this work.

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manipulation of ZAG expression in hepatocytes altered hepatic TG accumulation and the expression of key fatty acid oxidation genes, lipogenesis genes and adiponectin. Overall, these data indicate that ZAG is a key player in hepatic lipid metabolism in cultured HepG2 cells.

2. Materials and methods

2.1. Cell cultures

HepG2 cells (American Type Culture Collection) were cultured in minimum Eagle's medium (low glucose, Invitrogen) supplemented with 10% fetal bovine serum (BI, Germany), 100 IU/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37 °C in a 5% CO_2 incubator. Overexpression was accomplished by transfection with the pIRES2-ZsGreen1-hZAG plasmid vector for 24 h. Knockdown was accomplished by infection with LV-AZGP1-RNAi lentivirus for 48 h. Cells were then treated with 0.4 mM palmitic acid (PA) under serum-free conditions for 24 h.

2.2. Plasmids, lentivirus-RNAi transfection

Human ZAG full length DNA fragment cloned into the pIRES2-ZsGreen1 basic plasmid was obtained from Takara Bio (Dalian). Lentivirus expressing ZAG shRNA (ZAG-RNAi) was purchased from Shanghai Genechem Co. Ltd. Transfection was performed using Lipofectamine® 2000 (Invitrogen by Life Technologies) according to the manufacturer's instructions.

2.3. Oil Red O staining and intracellular triglyceride assay

Lipid accumulation in HepG2 cells was evaluated by Oil Red O staining and the measurement of triglyceride (TG) content. Briefly, samples were fixed with 4% paraformaldehyde then stained with Oil Red O for 15 min. After washing in PBS repeatedly, staining was examined by light microscopy. Intracellular TG was extracted with chloroform/methanol (2:1) as described previously (Folch et al., 1957) and the TG concentration was determined using a triglyceride commercial reagent kit (GPO-POD; Applygen Technologies Inc., Beijing, China) (Zhang et al., 2010).

2.4. RNA isolation, quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from HepG2 cell lysates was extracted with Trizol reagent (Sangon, Biotech Co., Ltd. Shanghai, China). cDNA was synthesized from 1 μl RNA in a total volume of 20 μl using random primer (Fermentas Life Sciences, EU) and reverse transcriptase (Invitrogen). Primers were designed using Primer5 input software and specificity of each primer was determined by a BLAST search. Quantitative real-time PCR was performed in a 25 μl reaction volume using Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific) following the manufacturer's instructions. Results were analyzed by using $2^{-\Delta\Delta Ct}$ method described previously (Livak and Schmittgen, 2001). The mRNA levels were normalized to β-actin. Primer sequences are listed in Table 1.

Table 1
Primers used for quantitative real-time PCR.

Gene	Forward primer:5′–3′	Reverse primer:5′–3′
ZAG	AGGGAAGGTTTGGTTGTG	GGCTGGGATTTCTTTGTT
Adiponectin	GACCAGGAAACCACGACTCA	CGATGTCTCCCTTAGGACCA
FXR	CCTGGATTTCTTCTGGACATT	GGACCTGCCACTTGTTCTGT
PPARα	GCTCACTGTTTTCGTGGTTGTA	GACTCTGTTTTCTGGGCTCTTC
SREBP-1c	TGCATTTTCTGACACGCTTC	CCAAGCTGTACAGGCTCTCC
LXR	TTGCTAAACAGCTACCCGGCT	ATCACCTCGATCGCAGAGGT
FAS	TGCGTGGCCTTTGAAATGTGCT	ACACGCTCCTCTAGGCCCTTCA
ACC	TCTCCTCCAACCTCAACCAC	AAGCAGCCCATCACTTCATC
SCD-1	CTCACTGGGAAGAAGCAAGG	TGTGTTCAGCAGGGTTTGTG
FATP	GGATTCTCCCTGTTGTTCCTC	ACCTTTGCCTTCACCTTCAG
CPT-1A	CGCTACTCCCTGAAAGTG	CTTGACCATACCCATCCAG
β-Actin	CATCCTGCGTCTGGACCTGG	TAATGTCACGCACGATTTCC

2.5. Protein preparation and western blot

Total protein extracts from cultured hepatocytes were prepared as described previously, and protein concentrations were determined with the Mini Protean 3 Cell (Bio-Rad). Proteins were separated on 10–20% Tris-glycine gels (Invitrogen) and transferred to PVDF membrane (Millipore) for 1.5–2 h. Membranes were blocked in Tris-buffered saline with Tween (TBST; 20 mM Tris, 150 mM NaCl, pH 7.4, plus 0.15% Tween 20) containing 2.5% horse serum for 2 h at room temperature, and then incubated with primary antibodies at 4 °C overnight. Membranes were washed with 1% TBST (TBS containing 1% Tween-20) three times before incubation with horseradish peroxidase-conjugated secondary antibodies (Invitrogen Company, Shanghai, China) for 1 h at room temperature. Finally images were acquired using ECL Plus (Beyotime institute of Biotechnology, China). The optical density of the bands was analyzed with Quantity One software (Ahphinmager2200, Leica, Germany). Protein levels were normalized to β-actin.

2.6. Statistical analysis

Data are expressed as mean ± SEM. All experiments in vitro were carried out in triplicate and on three separate occasions. Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with Student's *t*-test was used to determine differences between groups. A value of $p < 0.05$ was taken as statistically significant.

3. Results

3.1. ZAG negatively regulates hepatic intracellular lipid accumulation

Evidence has suggested that ZAG may reduce lipid content in adipose tissue by increasing lipid utilization (Bing et al., 2010; Russell and Tisdale, 2011a,b). We examined the effect of ZAG on lipid content in liver cells by stimulating HepG2 cells that overexpress (ZAG expression plasmid, Fig. S1) or are deficient in (lentivirus shRNA, Fig. S2) ZAG after exposure to PA. Cells transfected with either pIRES2-GFP or GFP RNAi served as controls for the overexpression and knock down, respectively. Oil Red O staining showed that exposure to PA led to intracellular lipid accumulation in pIRES2-GFP and GFP-RNAi transfected HepG2 cells, cellular TG content was obviously increased. While the cellular fat droplets and TG content were dramatically decreased especially in PA-induced HepG2 cells (Fig. 1A and B). On the other hand, knockdown ZAG by infection of lentivirus shRNA (LV-siRNA-ZAG) markedly increased triglyceride in hepatocytes compared to the control scrambled shRNA (LV-siRNA-scr) in both unstimulated and PA-stimulated HepG2 cells (Fig. 1C and D). These observations suggested that ZAG can negatively regulate unstimulated and PA-induced hepatic intracellular lipid accumulation.

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