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Saturated fatty acids-induced miR-424–5p aggravates insulin resistance via targeting insulin receptor in hepatocytes

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ABSTRACT

The excessive intake of saturated fatty acids (SFA) causes obesity and liver steatosis, which are major risk factors for insulin resistance and type 2 diabetes. Although the expression of certain microRNAs (miRNAs) targeting the insulin signaling molecules are regulated aberrantly in SFA-induced obesity, their implications on hepatic insulin resistance are largely unknown. This study examined the associations of miR-424–5p, which is induced by SFA, with the development of insulin resistance. SFA palmitate (PA)-treated HepG2 cells and high fat diet (HFD)-induced obese mouse livers showed an impairment of insulin signaling due to a significant decrease in INSR and IRS-1 expression. Based on expression profiling and *q*RT-PCR analysis, miR-424–5p, which presumably targets the 3′UTR of *INSR*, was upregulated in both PA-treated HepG2 cells and the liver of HFD-fed mice. miR-424–5p was found to target the 3′UTR of *INSR* directly and downregulated INSR expression at the post-transcriptional step. Furthermore, the overexpression of miR-424–5p suppressed INSR expression significantly, leading to impaired insulin signaling and glycogen synthesis in hepatocytes. A novel mechanism for how SFA-induced miR-424–5p impairs insulin signaling through the targeting of INSR is reported. In addition, the crucial role and underlying mechanism of miR-424–5p in the obesity-induced hepatic insulin resistance is explained.

1. Introduction

The excess dietary intake of saturated fatty acids (SFA), such as palmitate (PA), has long been considered a leading cause of obesity, which frequently promotes ectopic lipid accumulation [1]. As the liver is essential for whole-body metabolic homeostasis, lipid accumulation in hepatocytes is linked causally to the dysregulation of insulin sensitivity and nutrient utilization, consequently provoking hepatic insulin resistance, metabolic syndrome, and type 2 diabetes (T2DM) [2]. Insulin signaling in the liver is mediated by a relay of signal transduction processes initiated from the cell-surface insulin receptor (INSR). Insulin binding to INSR signals the insulin receptor substrate (IRS), which activates multiple distinct downstream pathways to modulate the liver functions [3]. Hepatic insulin resistance generally accounts for insufficient insulin sensitivity or insulin signaling in the liver to regulate proper glycogenolysis and glucose production, aggravating both the decrease in whole-body glucose utilization and disposal [4].

Although an in-depth knowledge of obesity-induced hepatic insulin resistance is intricate and still not completely understood, several mechanisms have been proposed to explain how SFA can result in an impairment of insulin signaling [2,4]. The emerging evidence from various experimental and clinical studies suggest that a high intake of fat, particularly SFA, promotes the accumulation of significant amounts of diacylglycerol and ceramide, which interfere with insulin signaling via serine phosphorylation and the downregulation of IRS [4]. Although perturbations in the expression and modification of IRS are considered major detrimental factors in insulin sensitivity, there is evidence suggesting that the suppression of INSR is also linked to the aggravation of insulin resistance and T2DM [5–7]. Several clinical studies have found that patients with T2DM have lower than normal levels of cellular INSR [7]. In addition, reduced INSR and IRS-1 expression concomitant with the development of insulin resistance have been observed in the livers of a high fat diet (HFD)-fed mice and exogenous PA-treated hepatocytes [4]. Furthermore, whole-body INSR knockout mice developed rapid and severe metabolic disorders, such as

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insulin resistance, hyperglycemia, hyperinsulinemia, and ketoacidosis [6]. Moreover, liver-specific INSR-deficient mice exhibited impaired hepatic glucose homeostasis, resulting in marked hyperglycemia and hyperinsulinemia [5]. Although INSR plays a key role in regulating the cellular and whole-body insulin sensitivity as well as glucose and lipid homeostasis, the mechanism by which SFA downregulates INSR in hepatocytes remains elusive.

MicroRNAs (miRNAs) are a class of highly conserved endogenous small non-coding RNAs, an average of 22 nucleotides long, which negatively regulate gene expression by targeting the 3′UTR of the target mRNA for transcripts degradation or translational repression at the post-transcriptional step [8]. Although the molecular targets and roles of the individual miRNAs are not understood completely, growing evidence suggests that miRNAs play an important role in a wide range of normal and pathophysiological cellular processes, such as the cellular metabolism, growth, proliferation, and survival/death [8,9]. In this regard, the aberrant expression of miRNAs has been connected intimately with a number of diseases, including diabetes, neurodegeneration, and cancer [8,9]. Over the last few decades, there has been growing interest in the implications of certain miRNAs in the pathogenesis of obesity-induced insulin resistance and T2DM [10]. These miRNAs modulated by SFA-induced obesity play important roles in insulin secretion, insulin signaling, and the regulation of important metabolic pathways, such as glycogen synthesis, gluconeogenesis, and fatty acid utilization [10]. Despite the advances in knowledge, the emerging mechanism for how miRNAs are interconnected to the development of hepatic insulin resistance by SFA or obesity is poorly understood.

In this study, miR-424−5p was induced in SFA-treated HepG2 cells and HFD-fed mouse liver. Mechanistically, miR-424−5p targets the 3′UTRs of *INSR* mRNA directly, and inhibits the expression of INSR, resulting in an impairment of insulin signaling in hepatocytes. Overall, miR-424−5p induced by SFA and obesity is linked causally to the development of hepatic insulin resistance and may be an important novel mediator in obesity-induced hepatic insulin resistance, which in turn increases the risk of T2DM and metabolic diseases.

2. Materials and methods

2.1. Cell culture and PA treatment

HepG2 cells, a cell line derived from human hepatocellular carcinoma (ATCC #77400), and L6-GLUT4myc cells, which is a L6 skeletal muscle cell line stably expressing GLUT4myc (Kerafast, Inc., Boston, MA, USA), were harvested in growth medium (MEMα supplemented with 10% FBS and 1% penicillin-streptomycin). For the PA treatment, a BSA-conjugated PA solution was prepared according to a previous report [11]. The HepG2 cells were then cultured with a BSA-conjugated PA solution (0−0.5 mM) for 0−18 h and treated for the last 30 min in the presence or absence of insulin (100 nM, Sigma).

2.2. Animal and HFD-induced insulin resistance

All animal studies were conducted with the approval of the Animal Use and Care Committee at Dongguk University (approval IACUC). C57BL/6N male mice were obtained from OrientBio (Seongnam, Gyeonggi, Korea). Six-week old mice were given access to a normal fat diet (NFD, 11% calories from fat) or a high fat diet (HFD, 60% calories from fat; Dyets Inc., Bethlehem, PA, USA) *ad libitum* for 14 weeks. At the end of the experiment, the mice were fasted overnight, treated with insulin (1 U/kg body weight) and subjected to biochemical analysis.

2.3. RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

The total RNA was purified using a miRNeasy Mini Kit (Qiagen). The cDNAs were synthesized using a miScript II RT Kit (Qiagen). RT-PCR or *q*RT-PCR was carried out using the specific primers (Supplementary Table 1) [12].

2.4. Transfection of miRNA mimic and vectors

HepG2 cells were reverse-transfected with miRNA and/or miRNA inhibitor mimic using G-fectin (Genolution, Seoul, Republic of Korea) according to the manufacturer's protocol. For the Dual-luciferase target validation assay, L6-GLUT4myc cells were cotransfected with 200 nM miR-424−5p mimic or scrambled control miRNA (Genolution) and the vectors containing the luciferase reporter genes using Lipofectamine 2000 (Invitrogen).

2.5. Plasmid constructs for dual-luciferase reporter gene assay

Using the primer sets described in Table S1, the human *INSR* 3′UTR (252 nt) was amplified by RT-PCR from HepG2 cells. The miR-424−5p binding sites were mutated by site-directed mutagenesis and subcloned to pmirGLO (Promega). Dual-luciferase target validation assays were performed as described [12].

2.6. Cell lysis, immunoblotting and antibodies

The cell lysates were prepared using a lysis buffer and Laemmli solution, as described elsewhere [13]. SDS-gel electrophoresis and immunoblotting analysis were conducted using the specific antibodies (Supplementary Table 2) [14]. The proteins were visualized using an ECL Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) and quantified by densitometry using an analytical scanning system (Alpha Imager HP; Alpha Innotech, San Leandro, CA, USA).

2.7. Glycogen assay

The HepG2 cells were serum-starved for 4 h in MEMα and incubated with DMEM high glucose in the presence or absence of insulin (100 nM) for 3 h. The glycogen contents of the cells were analyzed using a glycogen colorimetric assay Kit II (Biovision) according to the manufacturer's instructions.

2.8. Database and statistical analysis

The target genes and sites of the miRNAs were analyzed computationally using publicly available algorithms (TargetScan: www.targetscan.org, Pictar: pictar.mdc-berlin.de). The values of all experiments are expressed as the mean ± SEM from at least three independent experiments. Where applicable, the significance of the difference was analyzed using a Student's *t*-test for unpaired data.

3. Results

3.1. PA inhibits insulin signaling and upregulates miR-424−5p in HepG2 cells

To identify miRNAs involved directly in SAF-induced hepatic insulin resistance, HepG2 hepatocytes were treated with PA and the correlations between the impairment of insulin signaling and dysregulation of miRNAs were evaluated. As shown in Fig. 1, the treatment of HepG2 hepatocytes with PA (0.5 mM) decreased the expression of INSR and IRS-1 significantly compared to the vehicle-treated control. On the other hand, the PA treatment did not alter

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