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Acylated ghrelin induces but deacylated ghrelin prevents hepatic steatosis and insulin resistance in lean rats: Effects on DAG/ PKC/JNK pathway

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ABSTRACT

This study investigated the molecular effects of acylated (AG) and unacylated ghrelin (UAG) or their combination on hepatic lipogenesis pathways and DAG/PKC/JNK signaling in the livers of lean rats fed standard diet. Male rats (n = 10) were classified as control + vehicle (saline, 200 µl), AG, UAG, and AG + UAG-treated groups. All treatments were given at final doses of 200 ng/kg of for 14 days (twice/day, S.C). Administration of AG significantly enhanced circulatory levels of AG and UAG turning the normal ratio of AG/UAG from 1:2.5 to 1:1.2. However, while UAG didn't affect circulatory levels of AG, administration of UAG alone or in combination with AG resulted in AG/UAG ratios of 1:7 and 1:3, respectively. Independent of food intake nor the development of peripheral IR, AG increased hepatic DAG, TGs and CHOL contents and induced hepatic IR. Mechanism of action include 1) upregulation of mRNA and protein levels of DGAT-2 and mtGPAT-1, SREBP-1 and SCD-1, and 2) inhibition of fatty acids (FAs) oxidation mediated by inhibition of AMPK/ PPAR-α/CPT-1 axis. Consequently, AG induced membranous translocation of PKCδ and PKCε leading to activation of JNK and significant inhibition of insulin signaling under basal and insulin stimulation as evident by decreases in the phosphorylation levels of IRS (Tyr^{612}) and Akt (Thr^{318}) and increased phosphorylation of IRS (Ser^{307}). However, while UAG only activated FAs oxidation in control rats, it reversed all alterations in all measured biochemical endpoints seen in the AG-treated group, when administered in combination with AG, leading to significant decreases in hepatic fat accumulation and prevention of hepatic IR. In conclusion, while exogenous administration of AG is at high risk of developing steatohepatitis and hepatic IR, co-administration of a balanced dose of UAG reduces this risk and inhibits hepatic lipid accumulation and enhance hepatic insulin signaling.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common public health problem in both children and adults of both developed and undeveloped countries around [1–4] and is characterized by a wide spectrum of liver damage ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can further progress to hepatic fibrosis, cirrhosis, and ultimately to hepatocellular carcinoma and liver failure [5,6]. A nearly consequence of NAFLD is the development of hepatic insulin resistance (IR) due to increased *de novo* synthesis and accumulation of TGs in hepatocytes [7–9], even in the absence of obesity or peripheral IR [10–12]. This conclusion is supported by the fact that reducing hepatic TGs accumulation by the use of 2,4-dinitrophenol (DNP), or by over-expression of malonyl-CoA decarboxylase or uncoupling protein 1 (UCP-1) protected against the development of NAFLD and reduced hepatic IR in high-fat diet (HFD) fed rats [7,13,14].

The glycerol-3-phosphate (G3P) pathway is the major pathway responsible for TGs synthesis the liver of mammals [15]. Lipid metabolites that mediate this pathway include acyl-CoA, lysophosphatidic acid, diacylglycerol (DAG) [15]. This pathway is regulated by the activity of two key enzymes, namely, acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol/acyl-CoA acyltransferase (DGAT). GPAT catalyzes the acylation of G3P with acyl-coenzyme A (CoA) and generates CoA and lysophosphatidic acid, while DGAT adds fatty acids (FAs) to DAG leading to the synthesis of TGs [15,16]. Independent of peripheral IR, either over-activity of any of these enzymes or accumulation of any of the above-mentioned metabolites results in steatohepatitis and hepatic IR [7,15,16]. Among all isoforms, over-activation of mitochondrial GPAT-1 (mt-GPAT-1) and DGAT-2 are implicated in the development of NAFLD, even in absence of obesity [15,17].

Indeed and independent of peripheral IR, rats fed HFD for 3 days, a model to induce hepatic IR without peripheral IR, hepatic steatosis and IR developed due to accumulation of DAG-induced membranous translocation of PKC- ε and PKC-δ and activation of their downstream target, c-Jun N-terminal kinase 1 (JNK1) [7,17]. Interestingly,

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knockdown or deletion of hepatic of PKC-ε protected the mice from TGs induced hepatic IR when fed an HFD, despite the development of hepatic steatosis, confirming the emerging roles of PKC in hepatic IR [8,18].

On the other hand, an emerging role of the gut hormones in the development and protection against hepatic steatosis and hepatic IR in NAFLD has been recently suggested [19]. In this regards, much interest is given to the role of ghrelin-ghrelin O-acyl-transferase (GOAT) system in the development and progression of NAFLD but contradictory reports do exist and the mechanisms by which GOAT acts remain unclear to date [19–25]. Ghrelin is a peptide hormone that is secreted mainly by the stomach and pancreas to controls appetite and balance of energy [25]. Ghrelin circulates in the blood with a half-life time of 30 min and is present in two forms: an acylated (AG, n-octanoylated at Ser3) and an unacylated (UAG) [25]. The main form is the UAG and both AG and UAG cross into the brain and bind in the hypothalamus [25]. Acylation is achieved by the activation of GOAT [25] and the produced AG is the active and quiet potent form of ghrelin that binds and activates the secretagogue receptor type 1a (GHS-R1a). However, UAG has been recently suggested to have multiple metabolic functions by acting on different receptors [25].

In mammals, paradoxical physiological effects of AG and UAG has been reported on various metabolic pathways in liver, muscles and adipose tissue. AG has been found to be a pro-diabetic factor whereas UAG was shown to be an anti-diabetic and is able to antagonize the metabolic effects induced by AG [26]. It has been shown that exogenous administration of AG *in vitro* and *in vivo* increased hepatic glucose output and impaired hepatic and systemic insulin sensitivity, independent of food intake [27–32]. Short-term administration of AG for 4 days (200 ng/kg/day, twice/day), increased hepatic lipid accumulation by inhibiting AMP-activated protein kinase (AMPK)-dependent fat oxidation [31]. Similarly, administration of AG for longer periods (i.e. 11 nmol/kg/d, for 14 days) induced hepatic steatosis and hepatic IR via activation of the mammalian target of rapamycin (mTOR) and peroxisome proliferator-activated receptor-γ (PPARγ) signaling [23] or via activation of P53 (30 μg/day, for 10 days) [33]. Paradoxically, exogenous AG administration for 4 days to rodents with pre-established NAFLD (200 μg/day, twice/day) or in combination with HFD for 8 weeks (10 ng/kg, 3times/week) limited liver triglyceride content, oxidative stress and inflammation in a rat model of HFD-induced obesity [22,32]. This variation could be time and dose-dependent and could be explained by alterations in food uptake. On the other hand, studies on the effect of UAG on hepatic lipogenic pathways are limited and only the short-term administration of UAG (20 nmol/kg) for 6 h showed beneficial effect toward improving oxidative phosphorylation and enhancing FAs oxidation [26]. In a separate study, UAG had no effect on hepatic TGs accumulation in control rats [34].

In light of the presented and occasionally conflicting evidence on the mechanism of action of AG and UAG on hepatic lipogenesis and hepatic IR in NALFD animal model, it is clinically significant to elucidate the pathogenesis of NALFD and hepatic IR and provide potential targets for the treatment as well as the prevention from NALFD and IR. The effect of AG and UAG on the key regulatory enzymes of the G3P pathway as well as DAG-PKC-JNK pathway in control rat's hepatocytes fed standard diet was never investigated. Therefore, this study focused on investigating the short-term effect (i.e. 14 days) of administering AG and/or UAG to control rats fed standard diet on hepatic levels of DAG, TGs, mtGPAT-1 mRNA, DGAT-2 mRNA and the expression of PKC/JNK signaling pathway.

2. Materials and methods

2.1. Animals

Adult healthy male Wistar rats (170 ± 10 g) of the same genetic background (5th generation of breeding) were supplied by the animal house of the College of Medicine at King Khalid University (KKU), Abha, Saudi Arabia. During the adaptation period for one week and during the experimental procedure, all animals were kept in a controlled environment (22 ± 1 °C, 12:12-h light-dark cycle) and fed a standard commercial chow diet (2018S Harlan-Teklad) containing 18% protein and 5% fat with an energy density of 3.4 Kcal/g. Animal care, handling, and treatments were operated in accordance with the Institutional Animal Care and Use Committee of KKU University, School of Medicine which are in accordance with guidelines established by National Institutes of Health Guide for Care and Use of Laboratory Animals, 1996 (7th edition).

2.2. Experimental procedure

Four groups, 10 rats each, were divided as follows: A control, AG, UAG, an AG + UAG-treated groups. AG (Cat. No. G8903 was purchased from Sigma, Aldrich, UK. UAG was purchased (Cat No. 2951) from Tocris Bioscience, USA. All treatments were given for 14 consecutive days. Control rats received subcutaneous injections of normal saline (200 μl). Both AG and UAG treatments were administered at a final dose of 200 ng/kg in 200 μl subcutaneous injections (2 injections/day, given at 8:00 P.M and 8:0 A.M). The dose of AG selected here was based on Barazzoni et al. [31] who showed that this dose induced body weight increase as well as the elevation of hepatic TGs without changing food intake. In our labs, this was found to be true in preliminary experiments carried out on three animals that received the same treatment protocol. Based on this, we decided to use the same dose of UAG which has been previously demonstrated by Gauna et al., [29] to restore reduced insulin sensitivity induced by AG. Food intake was monitored daily at 8:00 A.M. However, in a separate set of experiments where we investigated the effect of AG and UAG on hepatic insulin signaling, insulin was administered to the above-named groups before tissue collection (discussed below).

2.3. Oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (ITT)

Both OGTT and ITT were carried out individually on days 14 and 15, respectively. For each test, all rats were fasted for at 12 h and then received either glucose solution (2 g/kg) via oral gavage (OGTT) or were injected with insulin of (0.75 U, i.p.) (ITT). During each test, blood samples (250 μl) were collected in EDTA Eppendorf tubes (10 μL of 10% of K3-EDTA per one ml blood) at 0, 15, 30, 60, 90 and 120 min, and then centrifuged at 5000 rpm/min for 10 min to collect plasma. Plasma glucose and insulin levels were determined for each time interval during OGTT and plasma glucose levels were also determined for each time interval during ITT. Data were graphed and areas under the curve (AUC) for both glucose and insulin levels obtained by each treatment in both tests were calculated and relatively presented as compared to AUC of the control rats.

2.4. Blood and tissue collection

After the last treatment on day 14, all animals were fasted for 4 h and were anesthetized using pentobarbital sodium i.p. (60 mg/kg). Compared to other types of anesthetics, pentobarbital sodium was selected as it interferes less with insulin secretion and glucose metabolism under fed and fasted conditions [29,35,36]. In addition, blood and tissue sample collection commenced after short-term fasting to avoid potential confounding effects of fasting-related hyperghrelinemia [31]. One ml samples of direct blood were collected by cardiac puncture into EDTA-laced tubes (10 μL of 10% of K3-EDTA per 1 ml of blood) to separate plasma. At the same time, other 2 ml of blood samples were collected into plain tubes, centrifuged at 5000 rpm/min for 10 min to collect serum, stored at −20 °C and used later for the determination of serum lipid levels. Then, animals were killed by cervical dislocation and

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