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Linoleic acid and stearic acid elicit opposite effects on AgRP expression and secretion via TLR4-dependent signaling pathways in immortalized hypothalamic N38 cells

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ABSTRACT

The regulation of food intake is a promising way to combat obesity. It has been implicated that various fatty acids exert different effects on food intake and body weight. However, the underlying mechanism remains poorly understood. The aim of the present study was to investigate the effects of linoleic acid (LA) and stearic acid (SA) on agouti-related protein (AgRP) expression and secretion in immortalized mouse hypothalamic N38 cells and to explore the likely underlying mechanisms. Our results demonstrated that LA inhibited, while SA stimulated AgRP expression and secretion of N38 cells in a dose-dependent manner. In addition, LA suppressed the protein expression of toll-like receptor 4 (TLR4), phosphorylation levels of JNK and IKKα/β, suggesting the inhibition of TLR4-dependent inflammation pathway. However, the above mentioned inhibitory effects of LA were eliminated by TLR4 agonist lipopolysaccharide (LPS). In contrast, SA promoted TLR4 protein expression and activated TLR4-dependent inflammation pathway, with elevated ratio of p-JNK/JNK. While TLR4 siRNA reversed the stimulatory effects of SA on AgRP expression and TLR4-dependent inflammation. Moreover, we found that TLR4 was also involved in LA-enhanced and SA-impaired leptin/insulin signal pathways in N38 cells. In conclusion, our findings indicated that LA elicited inhibitory while SA exerted stimulatory effects on AgRP expression and secretion via TLR4-dependent inflammation and leptin/insulin pathways in N38 cells. These data provided a better understanding of the mechanism underlying fatty acids-regulated food intake and suggested the potential role of long-chain unsaturated fatty acids such as LA in reducing food intake and treating obesity.

1. Introduction

Obesity is a pandemic and serious public health challenges worldwide, with about 2 billion people being overweight and one third of them being obese [1]. The factors of obesity determinants include genetic factors, dietary intake, physical activity, environmental, eating disorders and societal influences [2]. It is indicated that the over-nutrition or hyperphagia is a major reason for inducing obesity [3]. Thus, reduced appetite or food intake will be beneficial to combat obesity [4]. The appetite or food intake in mammals is regulated by the orexigenic and anorexigenic neuropeptides in the arcuate nucleus of the hypothalamus [5]. Among the

Abbreviations: AgRP, agouti-related protein; Akt, protein kinase B; DHA, docosahexaenoic acid; FoxO1, fork-head O subfamily 1; ICV, intracerebroventricular; IKKα/β, IkB kinase α/β; IRS, insulin receptor substrate; JAK2, protein tyrosine kinase 2; JNK, Jun N-terminal kinases; LA, linoleic acid; LPS, lipopolysaccharide; OA, oleic acid; PUFA, polyunsaturated fatty acids; SA, stearic acid; STAT3, signal transducer and activator of transcription 3; TLR4, toll-like receptor 4.

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hypothalamic neuropeptides, the agouti-related protein (AgRP) promotes food intake, playing an important interface role in the communication between peripheral organs and the central nervous system [6–9].

Our previous studies demonstrated that different amino acids could affect the expression of AgRP and subsequently regulate food intake [5,10]. Meanwhile, some evidences also indicated that humans as well as rodents showed a strong preference for fat diets [11,12]. The central injection of unsaturated fatty acids such as oleic acid (OA) and docosahexaenoic acid (DHA) significantly reduced appetite and weight of rat [9]. In contrast, central administration of saturated fatty acid palmitic acid could promote food intake and weight gain in mice [13]. These results indicated that various fatty acids might exert distinct effects on food intake. However, the reason and the underlying mechanism for the opposite effects of saturated and unsaturated fatty acid remain poorly understood.

It has been implicated that TLR4 is in the center of the events that connect the consumption of dietary fats with metabolic inflammation [14,15]. The saturated fatty acids can act as non-microbial TLR4 agonists to promote the TLR4 activation, triggering its downstream inflammatory response such as IkB kinase (IKK) and Jun N-terminal kinases (JNK) signaling [16]. In contrast, the polyunsaturated fatty acids (PUFA) such as n-3 or n-6 PUFA always elicit anti-inflammatory effects [15,17]. In addition, it has been implicated that the hypothalamic inflammation might contribute to obesity pathogenesis [18–20], implying the possible involvement of hypothalamic inflammation in the regulation of food intake [21]. Furthermore, hypothalamic inflammation has been shown to induce insulin resistance [22] and leptin resistance [23], which may subsequently lead to increased food intake and obesity. For example, central administration of palmitic acid led to pro-inflammatory response and leptin resistance [24], while unsaturated fatty acids reverted diet-induced hypothalamic inflammation and resistance to insulin/leptin [25]. However, whether linoleic acid and stearic acid affect the hypothalamic inflammation and insulin/leptin signaling and subsequently influence AgRP expression and secretion in immortalized mouse hypothalamic N38 cells remain unclear.

In this study, we primarily investigated the effects of unsaturated fatty acid linoleic acid (LA) and saturated fatty acid stearic acid (SA) on AgRP expression and secretion in immortalized mouse hypothalamic N38 cells. In addition, we sought to explore the role of TLR4-dependent inflammation pathway and insulin/leptin signaling in this process. Our findings demonstrated that LA and SA elicited opposite effects on AgRP expression and secretion via TLR4-dependent signaling pathway in N38 cells.

2. Materials and methods

2.1. Chemicals and antibodies

N38 cells were obtained from Peking Union Medical Centre Laboratory (Beijing, China). Linoleic acid, stearic acid and LPS (TLR4 activator) were purchased from Sigma–Aldrich. Low Glucose-Dulbecco's minimum essential medium (LG-DMEM), fetal bovine serum (FBS), and penicillin (10,000 units/mL)/streptomycin (10,000 mg/mL) (P/S) were purchased from Gibco Biotechnology Company (GIBCO, Grand Island, NY, USA). The siRNA sequences targeting TLR4 (TLR4 siRNA) and a non-specific control small interfering RNA (siRNA) were purchased from Shanghai GenePharma Co., Ltd (Shanghai, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Polyclonal antibodies against phosphor-JAK2 (Tyr1007/1008), JAK2, phospho-STAT3 (Tyr705), STAT3, phosphor-FoxO1 (Ser256), FoxO1, IRS, phosphor-JNK (Thr183), JNK, phosphor-Akt (Thr308), Akt, phosphor-IKKα/β (Ser176/177), TLR4 and β-Actin were purchased form Cell Signaling Technology Inc. Polyclonal antibodies against phosphor-IRS (Tyr465), IKKα/β and AgRP were purchased from Santa Cruz Biotechnology Inc.

2.2. Cell culture and treatment

N38 cells were cultured in LG-DMEM containing 10% FBS, 1% penicillin-streptomycin solution in the presence of various concentrations (1, 10, 50, 100 μM) of linoleic acid or stearic acid for 4 h to investigate the dose effects of these two fatty acids. In addition, the cells were cultured with 100 μM linoleic acid and/or 1 μg/mL TLR4 activator LPS for 4 h to explore the role of TLR4 in linoleic acid-inhibited AgRP expression and secretion. Meanwhile, the N38 cells transfected with TLR4 siRNA were incubated with or without 100 μM stearic acid for 4 h to study the role of TLR4 in stearic acid-stimulated AgRP expression and secretion.

2.3. Transfection of N38 cells with siRNA

N38 cells with a confluence of 70–90% were transfected with 4 pmol siRNA specific for TLR4 or scrambled siRNA using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h, transcript levels of TLR4 were detected by quantitative RT-PCR. The result of quantitative RT-PCR indicated a transfection efficiency of about 50% 24 h post-transfection (data no shown). The transfected N38 cells were used in the experiment of stearic acid-stimulated AgRP expression and secretion.

2.4. Western blot analysis

After incubation, the N38 cells were harvested and the medium were collected. The protein expressions of AgRP, TLR4, phosphor-JNK, JNK, phosphor-IKKα/β, IKKα/β, phosphor-JAK2, JAK2, phosphor-STAT3, STAT3, phosphor-IRS, IRS, phosphor-Akt, Akt, phosphor-FoxO1, FoxO1 and β-actin were detected by western blot analysis as we previously described [26].

2.5. Statistical analysis

All data are presented as means ± standard error of the mean (S.E.M.). Statistical analysis was performed using SPASS statistics 17.0. Differences between various groups in the dose effect experiment were determined by one-way ANOVA. Differences between the control and the treated group were analyzed by Student's *t*-test. A confidence level of $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Linoleic acid and stearic acid elicited opposite effects on AgRP expression and secretion of N38 cells in a dose-dependent manner

In order to investigate the effects of linoleic acid (LA) and stearic acid (SA) on the expression and secretion of AgRP in the N38 cells, the cells were exposed to various concentrations (1, 10, 50 and 100 μM) of LA and SA for 4 h. As shown in Fig. 1A-C, linoleic acid (C18:2), a long-chain polyunsaturated fatty acid, significantly inhibited the expression of AgRP at the concentration of 100 μM. Similarly, the secretion of AgRP (the content in the culture medium) was also markedly repressed by linoleic acid in a dose-dependent manner. In line with the decreased AgRP expression and secretion in our *in vitro* results, it has been reported that central injection of linoleic acid in rat leads to a reduction of appetite and body weight [25]. In addition, the central administration of

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