Original Article
Lipid reduces GLP-1 production via inhibiting NF-κB p65 and IL-6 in intestinal L cells and pancreatic alpha cells

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Abstract: Diabetes mellitus, especially type 2 diabetes, is a prevalent disease adding great burdens to the patient. Glucagon-like peptide 1 (GLP-1) is reported to be promoted by interleukin 6 (IL-6), increasing insulin secretion in intestinal L cells and pancreatic alpha cells. This study aims to investigate the impact of excessive lipid intake on GLP-1 production and the potential mechanism. Mouse intestine neuroendocrine tumor cells STC-1 and pancreas alpha cell line alphaTC1 clone 9 were used to study intestinal L cells and pancreatic alpha cells, respectively. After lipid treatment, NF-κB subunit p65 and IL-6 protein levels were detected by Western blot and immunocytochemistry, and IL-6 concentration in the culture supernatants was detected by ELISA. Cell transfection was performed to over-express p65 and IL-6. Results showed that lipid reduced p65 level in nucleoprotein, IL-6 in total protein, as well as IL-6 concentration in the culture supernatants of both L and alpha cells ($P < 0.05$). p65 overexpression caused the increase of p65 in nucleoprotein and IL-6 in total protein, which abrogated the suppressive effects of lipid. Similarly, lipid inhibited GLP-1 in both L and alpha cells, but IL-6 overexpression promoted GLP-1 and abrogated the suppressive effects of lipid. These results indicated that lipid reduced GLP-1 production in L cells and alpha cells, which depended on the inhibition of NF-κB p65 and IL-6, implying the impacts of excessive lipid intake on insulin secretion. This study will provide new references for preventing and treating type 2 diabetes.

Keywords: Type 2 diabetes, lipid, glucagon-like peptide 1 (GLP-1), intestinal L cells, pancreatic alpha cells

Introduction

Diabetes mellitus (DM) is a prevalent metabolic disease caused by high blood glucose level. It is predicted by the International Diabetes Federation that the number of DM patients will increase from 382 million in 2013 to 592 million in 2035, most of which happens in low- and middle-income countries [1]. DM exhibits high risks of developing complications and even cancers [2]. DM mainly includes type 1 diabetes and type 2 diabetes. Type 1 diabetes generates from dysfunctions of pancreatic beta cells and the subsequent decreased insulin secretion [3], while type 2 diabetes occurs in the context of insulin resistance, an inability of cells to respond to insulin level changes, which is usually caused by overweight, insufficient exercises and genetic factors [4]. Type 2 diabetes accounts for about 90% of diabetes cases and burdens greatly on patients and the society. Research on key risk genes, such as transcription factor 7-like 2, has been conducted to pave the way for effective treatment strategies [5].

Glucagon-like peptide 1 (GLP-1) is a product of proglucagon gene (*GCG*), which is mainly released by intestinal L cells in response to ingestion of carbohydrates and lipids [6]. In L cells, GLP-1 is generated with the catalysis of prohormone convertase (PC) 1/3 enzymes, and the small quantity of GLP-1 secreted by pancreatic alpha cells was catalyzed by PC2 enzyme [7, 8]. GLP-1 is capable of inhibiting glucagon secretion and promoting insulin secretion in a glucose-dependent manner [9]. It also enlarges pancreatic beta cell mass and inhibits beta cell apoptosis [10]. Given its pivotal position in glucose metabolism, GLP-1 and its regulators, such as GLP-1 receptor agonists and dipeptidyl

peptidase 4, have offered promising therapeutic strategies for type 2 diabetes [11].

Excessive lipid intake may inhibit insulin secretion [12]. A previous study has shown that interleukin 6 (IL-6) promotes GLP-1 secretion in both intestinal L cells and pancreatic alpha cells, which allows the enhancement of insulin secretion [13]. Recent findings propose a potential mechanism for obesity that saturated lipids induce insulin resistance in hypothalamic cells [14]. Nuclear factor κB (NF-κB), mainly existing as a heterodimer of p65 and p50, has been reported to participate in the modulation of insulin resistance [15].

Based on these former studies, we hypothesize that the IL-6-modulated GLP-1 production is related to the impacts of excessive lipid intake. To verify this hypothesis and reveal the impacts of lipid on GLP-1, we performed *in vitro* experiments on mouse intestine neuroendocrine tumor cells STC-1 and pancreas alpha cell line alphaTC1 clone 9, which are considered as models for intestine L cells and pancreatic alpha cells, respectively. The cells were treated with lipid, after which p65 in nucleoprotein, IL-6 in total protein and the IL-6 concentration in the culture supernatants were examined by western blot, immunocytochemistry or enzyme-linked immunosorbent assay (ELISA). p65 and IL-6 are overexpressed by cell transfection to analyze their regulation on IL-6 and GLP-1, respectively. These results will help to understand the mechanism of excessive lipid in-take in modulating GLP-1 level, facilitating research on insulin resistance and type 2 diabetes.

Materials and methods

Cell culture and treatment

Mouse intestine neuroendocrine tumor cells STC-1 and mouse pancreas alpha cells alphaTC1 clone 9 (ATCC, Manassas, VA) were designated to be L cells and alpha cells, respectively, in this study. The L cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA). The alpha cells were cultured in DMEM medium (Gibco) supplemented with 10% FBS, 15 mM HEPES buffer (Yocon, Beijing, China), 0.1 mM non-essential amino acids (Solarbio, Beijing, China), 0.02% bovine serum albumin (BSA), 2.0 g/L glucose and 1.5 g/L sodium bicarbonate (Sigma-Aldrich, Shanghai, China).

Cells were incubated in humidified atmosphere with 5% CO_2 at 37°C. Cells were divided into the lipid-treated group and the group without any treatment as a control. The cells in lipid group were treated with 10 μL of Lipid Mixture 1 (Sigma-Aldrich) per milliliter medium for 48 h before the following experiments.

Immunocytochemistry

L cells or alpha cells (2×10^5) in the control and lipid groups were seeded in 6-well plates and incubated overnight for adherence. The cells were washed in cold phosphate buffered saline (PBS) for 3 times and then fixed in cold methanol for 15 min at -20°C. After washing the cells for three times, 5% BSA was added for blockage, followed by another three-time wash. The cells were incubated in anti-p65 primary antibodies (ab32536, Abcam, Cambridge, UK) overnight at 4°C. After washed in PBS, the cells were incubated in Dylight 488 Goat Anti-Rabbit IgG (Abbkine, Redlands, CA) secondary antibodies in dark for 1 h, after which the cells were stained by DAPI (Sigma-Aldrich) for 1 min. Then the cells were immediately observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

ELISA

Before the detection, the culture medium for L cells or alpha cells in the control and lipid-treated groups was changed by serum-free medium, and the cells were cultured for another 24 h. Then the culture supernatants were collected by a pipettor and added to a 96-well plate pre-coated with the mouse IL-6-specific antibody. IL-6 concentration in the cell culture supernatants was detected using IL-6 Mouse ELISA Kit (ab100712, Abcam) according to the manufacturer's instruction. The intensity of the signal was measured at 450 nm by a microplate reader (Bio-Rad, Hercules, CA).

Cell transfection

The coding sequence of mouse *p65* (GenBank No. NM_009045) or *IL-6* (NM_031168) was cloned into pcDNA3.1 vector (Thermo Scientific, Carlsbad, CA) for overexpression of p65 and IL-6, respectively. Correct clones were screened by PCR and sequencing. The vectors were transfected into L cells and alpha cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Blank vectors were transfected as controls.
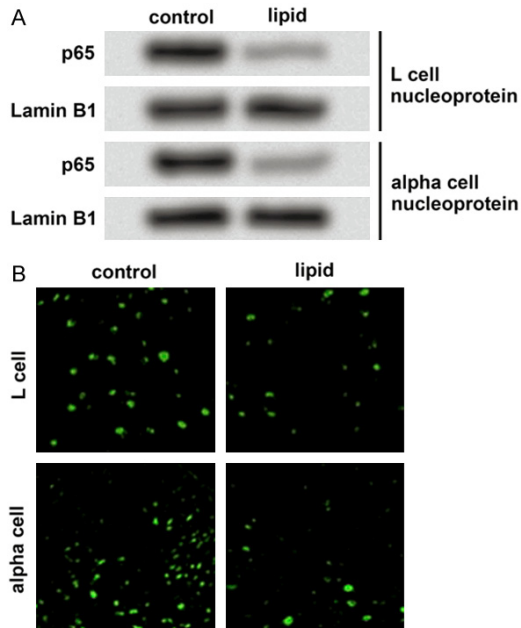
Figure 1. P65 protein expression is inhibited by lipid treatment in L cells and alpha cells. control, L cells or alpha cells without lipid treatment. Lipid, L cells or alpha cells with lipid treatment. A. Western blot shows the expression of p65 in L cell nucleoprotein and alpha cell nucleoprotein. Lamin B1 is used as an internal reference. P65 is suppressed in lipid-treated cells. B. Immunocytochemistry shows the p65-positive L cells or alpha cells with green fluorescent signals.

Briefly, the cells (1×10^5) were seeded in 6-well plates at one day before transfection. During the transfection, serum-free medium and 1.5 µg vectors were used for each well, followed by 6-h incubation at 37°C in atmosphere with 5% CO_2. Then the medium was replaced by completed culture medium and western blot were performed at 48 h post transfection.

Western blot

Cell nucleoproteins were extracted by EpiQuik Nuclear Extraction Kit (Epigentek, Farmingdale, NY), and cell total proteins were extracted by Protein Lysis Buffer (Beyotime, Shanghai, China), according to the manufacturer's instructions. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After blocked in 5% skim milk for 2 h at room temperature, the membrane was incubated in Abcam primary antibodies for p65 (ab32536), Lamin B1 (ab16048), IL-6 (ab7737), GAPDH (ab181602)

or GLP-1 (ab23468) at 4°C overnight, with Lamin B1 and GAPDH being internal references in nucleoproteins and total proteins, respectively. After washed in PBS, the membrane was incubated in horse radish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Signals were developed by ECL Plus Western Blotting Substrate (Piece, Carlsbad, CA) and analyzed by ImageJ 1.48.

Statistical analysis

Detection for each sample was repeated for 5 times. ELISA results were indicated as the mean ± standard deviation, and then analyzed by SPSS 20 with *F* test for homogeneity of variance and then *t* test for significant difference. Differences between groups were considered significant if *P* < 0.05.

Results

Lipid inhibits p65 in L and alpha cell nucleoprotein

First of all, we detected lipid-induced changes in p65 expression in both L cells and alpha cells compared to the control group. After the cells were treated by lipid, nucleoprotein samples were extracted and western blot results showed that p65 expression in the nucleoprotein of both L cells and alpha cells were downregulated (**Figure 1A**), which indicated that lipid might induce p65 suppression, suggesting the affected NF-κB activation. This suppression on p65 expression was also confirmed by immunocytochemistry using Dylight 488-conjugated secondary antibodies. Results showed that the number of p65-positive L cells was decreased, and the signal intensity was weakened in the lipid-treated group compared to the control group (**Figure 1B**). Similar results were also observed in alpha cells, indicating the suppressed p65 expression by lipid. Taken together, lipid was able to inhibit the expression of p65 in L cells and alpha cell nucleoprotein, which suggested the suppressed NF-κB activation.

Lipid inhibits IL-6 production and secretion from L and alpha cells

NF-κB is an important transcription factor and is supposed to regulate IL-6 expression, thus we examined IL-6 protein expression and secre-
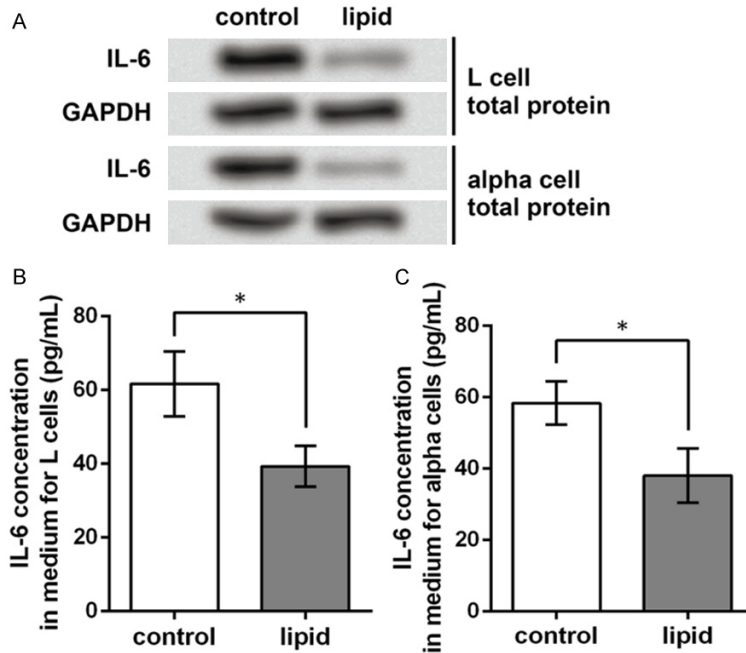
Figure 2. IL-6 protein expression and secretion are inhibited by lipid treatment in L cells and alpha cells. control, L cells or alpha cells without lipid treatment. lipid, L cells or alpha cells with lipid treatment. A. Western blot shows the expression of IL-6 protein in L cell total protein and alpha cell total protein. GAPDH is used as an internal reference. IL-6 is suppressed in lipid-treated cells. B. ELISA shows the concentration of IL-6 in culture supernatants of L cells (n = 5), suggesting lipid treatment inhibits IL-6 secretion from L cells. C. ELISA shows the concentration of IL-6 in culture supernatants of alpha cells (n = 5), suggesting lipid treatment inhibits IL-6 secretion from alpha cells. *$P < 0.05$. IL-6, interleukin 6.

tion from L cells and alpha cells. Western blot indicated that IL-6 expression in total protein of both L cells and alpha cells were inhibited by lipid treatment (**Figure 2A**), suggesting that lipid could suppress the expression of IL-6. ELISA in the culture supernatants was performed to analyze IL-6 secretion from L cells and alpha cells, and results showed that both L cells and alpha cells liberated less IL-6 into the extracellular culture medium after lipid treatment, with significant differences compared to the control group ($P < 0.05$, **Figure 2B** and **2C**). Taken together, excessive lipid might induce the suppression on IL-6 production and secretion from L cells and alpha cells.

P65 promotes lipid-suppressed IL-6 in L and alpha cells

Based on the above results, we further investigated the relationship between NF-κB p65 and IL-6 by transfecting p65 overexpression vector. Then western blot was performed to examine p65 protein levels in nucleo-protein and IL-6 protein levels in total protein. Results showed that in L cells, the nuclear p65 protein level was up-regulated by p65 overexpression as predicted (**Figure 3**), which might be resulted from the induced the translocation of NF-κB to nucleus. Also, the IL-6 protein level in total L cell protein was elevated by p65 overexpression compared to the transfection control group. It was noteworthy that when p65 was overexpressed in the lipid-treated L cells, the nuclear p65 level and total IL-6 level remained almost unchanged compared to the transfected cells without lipid treatment. Together with the above findings that lipid could suppress the level of nuclear p65 and production of IL-6 in L cells and alpha cells, these results implied that p65 overexpression could abrogate the effect of lipid treatment in L cells. The same detection procedures were also performed on alpha cells and similar results were acquired. These findings indicated that p65 promoted the expression of IL-6 and abrogated effects of lipid, emphasizing that the lipid-modulated IL-6 expression depended on NF-κB p65.

IL-6 promotes lipid-suppressed GLP-1 in L and alpha cells

Next we wanted to make connections between IL-6 and GLP-1 to reveal the effects of lipid on GLP-1 production. IL-6 was overexpressed by transfection and GLP-1 protein expression in total protein of L cells and alpha cells was detected by western blot. Results showed that in L cells, lipid treatment could suppress GLP-1 protein expression compared to the transfection control group without lipid treatment (**Figure 4**), and IL-6 overexpression led to the up-regulated GLP-1 protein level compared to the transfection control group, suggesting the inhibitory effect of lipid and the promotive effect of IL-6 on GLP-1 expression. Moreover,
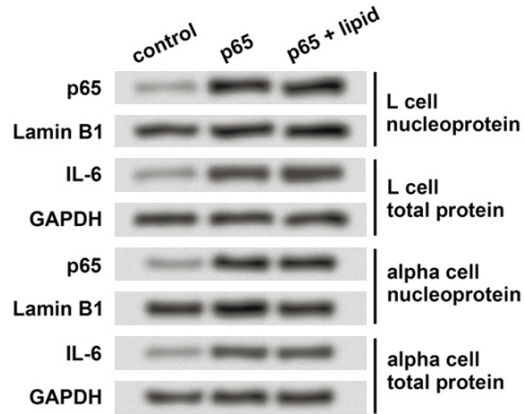
Figure 3. The effect of lipid on IL-6 depends on p65. P65 overexpression vector is transfected in L cells and alpha cells to up-regulate p65 expression. Control, cells transfected with blank vectors as the transfection control. P65, cells transfected with p65 overexpression vector. p65 + lipid, cells treated with lipid and transfected with p65 overexpression vector. Western blot shows that p65 overexpression up-regulates nuclear p65 levels in L cells and alpha cells, and that the inhibitory effect of lipid is abrogated by p65 overexpression. Lamin B1 and GAPDH are used as the internal reference in nucleoprotein and total protein, respectively. IL-6, interleukin 6.
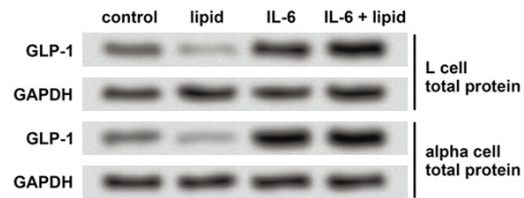
Figure 4. GLP-1 is inhibited by lipid and promoted by IL-6 overexpression. IL-6 overexpression vector is transfected in L cells and alpha cells. Control, cells transfected with blank vectors as the transfection control. lipid, cells treated with lipid and transfected with blank vectors. IL-6, cells transfected with IL-6 overexpression vector. IL-6 + lipid, cells treated with lipid and transfected with IL-6 overexpression vector. Western blot is performed to detect GLP-1 levels in the four groups, and GAPDH is used as an internal reference. IL-6, interleukin 6. GLP-1, glucagon-like peptide 1.

IL-6 overexpression in the lipid-treated L cells promoted GLP-1 expression compared to the cells only treated with lipid, suggesting that IL-6 overexpression could reverse the effect of lipid on GLP-1. Similar GLP-1 expression pattern was also observed in alpha cells. Taken together, these findings implied that lipid suppressed GLP-1 production in L cells and alpha cells, which was in an IL-6-dependent manner.

Discussion

This study treats mouse STC-1 and alphaTC1 clone 9 cells with lipid to reveal the mechanism of excessive lipid on regulating GLP-1 level in intestinal L cells and pancreatic alpha cells. We find that lipid treatment inhibits nuclear p65 protein level, cellular IL-6 protein level, as well as IL-6 secretion from L cells and alpha cells. p65 overexpression promotes IL-6 expression, which can abrogate the inhibitory effect of lipid on IL-6. IL-6 elevates GLP-1 level, which also reverses the suppressive effect of lipid on GLP-1.

It is generally accepted that the predominant form of NF-κB is the heterodimer composed of two REL-related DNA binding subunits, p65 and p50 [16, 17]. Endogenous stable homodimers of p65 or p50 subunits exist in cellular environment [18, 19], but the two kinds of homodimers exhibit distinct functions in NF-κB-directed transcriptional regulation [20]. NF-κB activation and translocation to cell nucleus allow its modulation on various genes by recognizing and binding to the κB sequence in gene promoter [21]. Thus NF-κB is an important transcription factor playing vital roles in regulating gene expression. In this study, nuclear level of p65, one of the most common subunits of NF-κB, was detected to indicate activated NF-κB, which helped us to speculate the expression change of downstream IL-6.

In both L cells and alpha cells, we found lipid treatment inhibited nuclear p65 protein level, cellular IL-6 protein level and subsequently led to the reduction in IL-6 secretion to the culture supernatants. Moreover, the suppressive effects of lipid on IL-6 were abrogated by p65 overexpression, which implied that the influence of lipid in these cells was via the regulatory functions of p65. Numerous studies have reported the regulatory relationship between NF-κB p65 subunit and IL-6. For example, the increase in IL-6 expression is dependent on the activation of p65 during retinal ischemia/reperfusion injury [22] and in prostate cancer [23]. The NF-κB binding site in IL-6 promoter is relatively conserved from lower vertebrates to human, allowing the effective regulation by NF-κB, especially p65 subunit [24]. Together with these former studies, results of this study again confirm that p65 subunit of NF-κB is

capable of regulating IL-6 protein expression in L cells and alpha cells under high lipid conditions, which further decreases IL-6 secretion from these cells.

Similarly, we verified the function of lipid on GLP-1 via IL-6 overexpression and found that IL-6 could promote GLP-1 level and reverse the suppressive effects of lipid treatment on GLP-1. As reported by previous studies, GLP-1 is increased because of the up-regulated proglucagon level induced by administration or elevated concentration of IL-6 [13], and impaired GLP-1 is responsible for insulin resistance and liver fat accumulation [25]. With regard to IL-6 and insulin, IL-6-knockout mice display obesity, hepatosteatosis and insulin resistance compared with normal mice [26]. Besides, mice with IL-6 receptor α deficiency is prone to possess the disruption of glucose homeostasis when fed a high-fat diet [27]. Based on these findings and the results of this study, it is reasonable to deduce that lipid treatment in L cells and alpha cells is capable of suppressing IL-6 production and further inhibits GLP-1 level.

According to the above arguments, the mechanism of lipid treatment in L cells and alpha cells can be summarized as a series of modulated factors including the suppressed NF-κB activation and the following inhibited IL-6 production, which leads to decreased proglucagon and GLP-1 levels. GLP-1 and its receptors are promoters of insulin secretion [28, 29], and the excessive expression of its receptors causes severe hypoglycemia in a type 2 diabetes case [30]. GLP-1 treatment can improve insulin sensitivity as confirmed in human and rodents [31, 32]. So it can be deduced that the lipid-induced GLP-1 decrease found in L cells and alpha cells is possible to reduce insulin secretion from beta cells, which may further lead to insulin resistance and contribute to type 2 diabetes. Hence, excessive lipid intake from daily diet may greatly impact on GLP-1 production in intestinal L cells and pancreatic alpha cells, thus suppressing insulin secretion and increasing the risk of type 2 diabetes. Future studies will focus on the verification of proglucagon and insulin changes for more convincing evidence.

In summary, this study indicates the effects of lipid on suppressing GLP-1 expression via regulating NF-κB p65 subunit and IL-6, which is a possible cause of excessive lipid intake-induced decrease of insulin secretion. These findings provide a possible explanation for the molecular mechanism of type 2 diabetes and offer new perspectives for prevention and treatment of this disease.

Disclosure of conflict of interest

None.

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