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Fatty Acid Synthase Gene Regulation in Primary Hypothalamic Neurons

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Abstract

Understanding the mechanisms that regulate feeding is critical to the development of therapeutic interventions for obesity. Many studies indicate that enzymes within fatty acid metabolic pathways may serve as targets for pharmacological tools to treat this epidemic. We, and others have previously demonstrated that C75, a fatty acid synthase (FAS) inhibitor, induced significant anorexia and weight loss by both central and peripheral mechanisms. Because the hypothalamus is important in the regulation of homeostatic processes for feeding control, we have identified pathways that alter the gene expression of FAS in primary hypothalamic neuronal cultures. Insulin, glucose and AICAR (an activator of AMP-activated protein kinase) affected changes in hypothalamic FAS mRNA, which may be regulated via the SREBP1c dependent or independent pathway.

Keywords

fatty acid synthase; hypothalamus; insulin; SREBP1C; AICAR

Introduction

The hypothalamus is a region of the brain critical for the integration of peripheral signals such as insulin and leptin in order to regulate food intake [18]. Hypothalamic neurons,

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especially those in feeding pathways, must interpret peripheral and central signals to assess energy perception and modulate feeding behavior.

It has been hypothesized that fatty acid metabolic pathways contribute to the regulation of energy balance and metabolic homeostasis by distinct mechanisms in the hypothalamus [17]. C75, a synthetic fatty acid synthase (FAS) inhibitor and carnitine palmitoyltransferase-1 (CPT-1) stimulator, causes anorexia by modulating gene expression of neuropeptides in the hypothalamus of lean, diet-induced obese (DIO) and *ob/ob* mice, leading to weight loss [11, 13, 22, 23].

The fatty acid synthesis pathway is regulated via a combination of transcriptional regulation, post-translational modification, and allosteric inhibition/activation while the regulation of FAS is limited to transcriptional control [24]. In the periphery, FAS mRNA is up-regulated during energy surplus and rapidly down-regulated during energy depletion, mainly in response to different nutrient states including: diet, glucose, thyroxine, and hormonal signals, such as insulin and glucagon [19]. The sterol regulatory elements binding protein (SREBP) family of transcriptional activators plays a crucial role in both fatty acid and cholesterol homeostasis [3]. SREBP1c, a member of the SREBP family, is involved in insulin stimulation of hepatic genes such as glucokinase, acetyl-CoA carboxylase (ACC) and FAS.

During the past few years, hypothalamic AMP-activated protein kinase (AMPK) has emerged as a strong candidate energy sensor in the regulation of food intake [1, 8, 15]. AMPK is a master energy sensor acting to conserve cellular ATP levels. In addition to altering cellular metabolism, AMPK activation inhibits gene expression of FAS and ACC, while stimulating gene expression of uncoupling protein-3 (UCP-3) and mitochondrial enzymes in peripheral tissue [6].

We have previously demonstrated that enzymes of fatty acid metabolic pathways are highly expressed in hypothalamic neurons involved in regulating feeding behavior [9]. Since less is known regarding gene regulation of FAS in the hypothalamus, we investigated changes in FAS mRNA expression caused by insulin and glucose, and by pharmacological activation or inhibition of AMPK and PI3 kinase signaling pathways using an *in vitro* primary hypothalamic neuronal system.

Methods

Primary dissociated hypothalamic culture

Our hypothalamic neuronal cultures were modified from the previous work [8]. Animal experimentation was done in accordance with guidelines on care and use as established by the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee. The fetuses from a timed-pregnant (day 17 of gestation) Spague-Dawley rat (Harlan Co.) were placed in Mg^{2+}/Ca^{2+} free Hanks' Balanced Salt solution on ice. The dissected hypothalamus was transferred into Earle's Balanced Salt solution, and cells were dissociated via papain (Worthington) as per the manufacturer's protocol. The dissociated neuronal cells were re-suspended in serum free media (SFM: described in detail below) and

the cells (5×10^5 cell/ml) were plated onto 12 well plates coated with poly-D-Lysine (0.05 mg/ml), and incubated at 37°C at 5% CO₂. To limit glial cell proliferation, cytosine arabinoside furanoside (5 µM) was added to the medium 3 days after plating.

Culture medium

We adapted a primary hypothalamic culture system for our studies as published previously using SFM as a culture media [14]. SFM contains DMEM/F12, 1 mg/ml fatty acid free-BSA, 0.04 mg/ml transferrin, 0.1 µM corticosterone, 0.001 µM 17β-estradiol, 10 µM putrescine, 10 nM sodium selenite, 10 nM triiodo-L-thyronine, 5 nM 17α-hydroxyprogesterone, 50 U/ml gentamycin, 100 µg/ml kanamycin, and 4 mM glutamine without insulin.

Insulin treatment and PI3 kinase inhibitor experiment

Primary hypothalamic neurons were plated onto 12 well plates in SFM containing 100 nM insulin (SFM+ins) for 7 days. On DIV (day *in vitro*) 7, cultures were incubated for an additional 18 h in the absence of insulin and then insulin was added in varying concentrations (0 to 1000 nM) to the SFM. At the specified times, the cells were collected and analyzed. A PI3 kinase inhibitor, LY294002, was added 45 min prior to insulin treatment at a final concentration of 50 µM. The cells pre-treated with or without LY294002 were incubated with 500 nM insulin for 24 h.

Glucose and AICAR treatments

The cells cultured in SFM+insulin for 7 days were divided into aliquots, which were incubated with either 5.5 mM glucose in SFM or 25 mM glucose in SFM without insulin for 18 h. Then the cells were treated with 500 nM insulin or 1 mM AICAR (5-aminoimidazole-4-carboxamide riboside) for 24 hr.

Immunocytochemistry

Primary hypothalamic cells (DIV7) were fixed in a solution of methanol/acetone and exposed to primary antibody at the appropriate concentrations (TUJ1/anti-NST monoclonal antibody 1:1000, BabCo; anti-FAS rabbit antibody 1:50, Santa Cruz) overnight at 4°C. Cells were washed with PBS and incubated with fluorescence conjugated-secondary antibody (FITC conjugated anti-mouse 1:100, and rhodamine conjugated anti-rabbit antibodies 1:100, Jackson ImmunoResearch).

Real-time quantitative PCR

Real-time quantitative RT-PCR was performed following our method as described previously [23]. Gene-specific primer pairs for FAS, SREBP1c and cyclophilin were designed using Primer3 software. The forward and reverse primers used are as follows: TCGACCTGCTGACGTCTATG and TCTTCCCAGGACAAACCAAC (FAS), GGACCATGGATTGCACATT and AGGAAGGCTTCCAGAGAGGA (SREBP1c), TATCTGCACTGCCAAGACTGA and CCACAATGCTCATGCCTTCTTTCA (Cyclophilin).

Western immunoblot

Immunoblot of FAS was performed with cell extracts separated on a 4–15% gradient SDS-PAGE gel. Cells cultured in SFM+ins medium in 6 well plates were minced in TES buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 250 mM sucrose, protease inhibitors cocktail, 0.1 mM PMSF) with 1% Triton X-100. Cells were incubated in either a polyclonal antibody to FAS (1:1000, Santa Cruz) or a monoclonal antibody to β -tubulin (1:5000, Sigma). Protein signal was detected by ECL (Pierce) and quantified using Image J software (NIH).

Statistical analysis

All values are presented as mean $\pm$ S.E. Statistical significance was determined by one-way ANOVA analysis for multiple comparisons or t-test analysis for unpaired samples (LY treatment) as appropriate.

Results

FAS is expressed in cultured hypothalamic neurons

We determined FAS expression in hypothalamic neurons using NST (neuron-specific Class III β tubulin) as a neuronal marker. FAS protein was detected by immunocytochemistry in cells that expressed NST, indicating that FAS and NST are co-localized in hypothalamic neurons cultured in SFM+ins (serum-free media containing 100 nM insulin) for 7 days (Fig. 1).

Changes in FAS gene expression during development in serum-free media

Since serum contains sufficient amounts of insulin in high glucose media, the addition of insulin does not further induce FAS expression levels [7]. However, insulin is an essential factor for neuronal growth and development. As such, FAS mRNA and protein levels were examined from cells cultured in SFM+ins and collected on DIVs 1, 3, 7 and 10 (Fig. 2A). In parallel with mRNA measurements, the protein level of FAS was also analyzed. Based on quantitative analysis, FAS protein levels were consistently related to mRNA expression levels, indicating that there is no significant change in FAS expression of mRNA or protein by DIV10 (Fig. 2B).

Insulin changes FAS mRNA expression

To determine if there were insulin-induced changes in FAS expression, DIV7 cells cultured in SFM+ins were placed into SFM, cultured for 18 h, and re-fed SFM+ins for an additional 24 h. Insulin caused a significant increase in FAS mRNA, approximately 1.6-fold at 2 h, indicating that FAS mRNA induction by insulin is time-dependent (Fig. 3A). A range of insulin concentrations (100–1000 nM) stimulated FAS mRNA expression in a dose-dependent manner with a slight decrease at the 1000 nM insulin concentration for 2 h treatment after 18 h insulin withdrawal (Fig. 3B).

Glucose changes FAS mRNA expression

We measured FAS mRNA levels following insulin treatment in different glucose concentrations of SFM (Fig. 3B). Importantly, glucose regulated FAS expression both in the

presence and in the absence of insulin. High glucose (25 mM) stimulated FAS expression in SFM, and high insulin (100 and 500 nM) had a synergistic effect on FAS mRNA levels.

PI3 kinase inhibition decreases FAS mRNA expression

It has been demonstrated that insulin stimulation of the FAS gene is mediated by the PI3 kinase-Akt/PKB pathway in 3T3-L1 adipocytes [25]. To determine the contribution of this pathway to hypothalamic FAS expression, neurons were treated with a PI3 kinase inhibitor, LY294002, and its effect on insulin induction in FAS gene expression was examined (Fig. 3C). The inhibitory effect on FAS mRNA expression was shown at the 2 h time point in 500 nM insulin treated cells.

SREBP1c is changed by insulin and a PI3 kinase inhibitor

The rise in SREBP1c mRNA after insulin treatment underlines a key role for FAS gene expression in hypothalamic neurons (Fig. 4). The kinetic result of SREBP1c mRNA is consistent with the change in FAS by 2 h, suggesting that the down-regulation of SREBP1c is involved in the decrease in FAS expression at the same time point. SREBP1c; however, was significantly down-regulated at 4 and 6 h, and recovered at 24 h. LY294002 causes a more significant reduction in SREBP1c during 24 h compared to FAS (Fig. 4B). LY294002 was demonstrated to have a strong inhibitory effect on FAS and SREBP1c expression at a concentration of 50 μ M, implicating a role for the PI3 kinase signaling pathway in the regulation of both genes. As SREBP1c has been shown to be an upstream regulator of FAS, the effect of PI3 kinase inhibition on FAS may be a secondary effect of a reduced SREBP1c level by LY294002.

AICAR changes FAS and SREBP1c expression

AICAR mimics the effect of AMP, and has been used to activate AMPK [20]. We determined whether activation of AMPK by AICAR changes FAS gene expression in hypothalamic neurons (Fig. 5A). AICAR (1 mM) significantly reduced FAS mRNA in hypothalamic neurons. The effect of AICAR on FAS mRNA levels was similar in both low (5.5 mM) and high (25 mM) glucose SFM. Interestingly, a combined treatment of insulin and AICAR resulted in an increase in FAS mRNA level compared to AICAR treatment only. Consistent with FAS expression, AICAR decreases hypothalamic SREBP1c mRNA in both low and high glucose SFM (Fig. 5B). In addition, high glucose also stimulated hypothalamic SREBP1c expression, which is similar with an increase in FAS expression. The difference with FAS gene expression was the complete lack of effect on SREBP1c expression with combined insulin and AICAR treatment.

Discussion

Previous studies have demonstrated the importance of insulin and glucose in the induction of peripheral SREBP1c, which leads to the up-regulation of downstream lipogenic enzymes, including FAS. Nonetheless, whether similar regulation occurs centrally remains unknown. FAS is induced by both insulin and glucose in cell types such as the hepatocyte, while SREBP1c is induced by insulin only, with no significant change in SREBP1c mRNA in high glucose concentrations (25 mM) [2]. However, one study found that high glucose (25 mM)

induces SREBP1c mRNA levels in the mouse hepatic cell line, H2-35 [7]. In the present study, we have found that hypothalamic FAS and SREBP1c genes are significantly induced by both insulin and glucose, suggesting that insulin and glucose regulate FAS gene expression via a SREBP-1c dependent pathway in the hypothalamus. In contrast, the expression of both FAS and SREBP1c is repressed by AICAR, which is consistent with the results shown in hepatocytes and pancreatic islets [4, 12].

The most striking finding is that there is no correlation of combined treatment with insulin and AICAR to FAS and SREBP1c expression (Fig. 5). This suggests that insulin may affect a SREBP1c-independent regulatory activator for its transcriptional regulation of FAS while SREBP1c expression is repressed by AICAR. It is also possible that insulin may be involved in the transcriptional activity of SREBP1c through other regulatory mechanisms. Recent studies indicate that glycogen synthase kinase-3 (GSK-3) could phosphorylate SREBP1c, resulting in its ubiquitination and proteasomal degradation [21]. Insulin is known to inhibit GSK-3 activity via PKB dependent phosphorylation [5]. Therefore, insulin may elevate the level of SREBP1c not only by transcriptional induction, but also by protein stabilization. This mechanism could account for the difference in SREBP1c gene expression and its transcriptional activity for FAS seen with the combined treatment of insulin and AICAR. Even though AICAR represses SREBP1c mRNA expression levels, insulin may stabilize its protein levels, which could increase FAS gene expression. In addition, based on the findings that the insulin-PI3 kinase pathway can inhibit AMPK [10, 16], insulin signaling could attenuate AMPK's inhibitory action on FAS expression, which should be further addressed in hypothalamic neurons.

We present evidence that insulin regulation of the hypothalamic FAS gene is mediated by the PI3 kinase signaling pathway. Glucose-mediated induction of SREBP1c and FAS suggests that glucose can be taken up in a dose-dependent manner, which might be through an insulin-dependent synergistic mechanism. It may be that insulin facilitates gene induction by glucose through the induction of glucokinase genes or the translocation of glucose transporters.

Mechanistic cellular and molecular studies for the regulation of fatty acid pathways in the brain are of vital importance in establishing strategies for obesity and other related disease therapies. We have found that insulin and glucose regulate hypothalamic FAS gene expression, at least in part by SREBP1c, which could be a similar mechanism in the periphery. However, the mechanisms by which AMPK activity affects FAS gene expression and the possible interactions between insulin signaling and the AMPK pathway need to be further investigated.

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Fig. 1. Immunocytochemistry of FAS and NST in primary hypothalamic neuronal cultures
Immunostaining of DIV7 cells with FAS (rhodamine) and NST (FITC).

Fig. 2. FAS expression in hypothalamic neurons cultured in SFM+ins mRNA (panel A) and protein levels (panel B) of FAS are examined on DIV 1, 3, 7 and 10 cells.

Fig. 3. Changes in FAS mRNA levels by insulin and a PI3 kinase inhibitor

FAS mRNA levels were measured from the cells cultured in SFM+ins for 24 h (panel A), in either 5.5 mM or 25 mM glucose SFM for 2 h (panel B), and cells pre-treated with LY294002 (LY) for 45 min then treated with 500 nM insulin for 24 h in 5.5 mM glucose SFM (panel C). Statistical significance was demonstrated by p value: $P < 0.05$ (* or #), $P < 0.01$ (**), $P < 0.001$ (***). Comparison of control with insulin treatment in either 5.5 mM or 25 mM glucose (*) and comparison within 25 mM glucose (#) are shown (panel A and

B). In panel C, comparisons of control with insulin treatment (*) were performed by one-way ANOVA and comparison with LY treatment (#) was performed by t-test.

Fig. 4. Changes in SREBP1c mRNA level by insulin and a PI3 kinase inhibitor
 SREBP1c mRNA levels (panel A and B) were determined from cells cultured in the same condition as shown in Fig. 3A and 3C, respectively. Statistical significance was demonstrated by p value: $P < 0.05$ (* or #), $P < 0.01$ (##), $P < 0.001$ (***) or (###). Comparison of control with insulin treatment (*) and comparison with LY treatment (#) are performed as in Fig. 3A and 3C.

Fig. 5. Changes in hypothalamic FAS and SREBP1c mRNA levels by insulin and AICAR
 FAS (panel A) and SREBP1c (panel B) mRNA levels were shown from cells treated with insulin and/or AICAR in either 5.5 mM or 25 mM glucose SFM. Statistical significance was demonstrated by p value: $P < 0.05$ (* or #), $P < 0.01$ (** or ##), $P < 0.001$ (***) or (###). Comparison of control with insulin treatment in either 5.5 mM or 25 mM glucose (*) and comparison within 25 mM glucose (#) are performed by one-way ANOVA.