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Triiodothyronine (T3) stimulates food intake via enhanced hypothalamic AMP-activated kinase activity

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ABSTRACT

Thyroid hormone regulates food intake. We previously reported that rats with triiodothyronine (T3)-induced thyrotoxicosis display hyperphagia associated with suppressed circulating leptin levels, increased hypothalamic neuropeptide Y (NPY) mRNA and decreased hypothalamic pro-opiomelanocortin (POMC) mRNA. AMP-activated kinase (AMPK) is a serine/threonine protein kinase that is activated when cellular energy is depleted. We hypothesized that T3 causes an increase in hypothalamic AMPK activity, which in turn contributes to the development of T3-induced hyperphagia. Rats that were given s.c. injections of T3 (4.5 nmol/kg) had increased food intake 2 h later without alterations in NPY and POMC mRNA levels, but with increased hypothalamic phosphorylated AMPK (169%) and phosphorylated acetyl-CoA carboxylase (194%). To determine the more chronic effects of T3, rats were given 6 daily s.c. injection of T3 or the vehicle. Food intake was significantly increased. Multiple T3 injections increased hypothalamic phosphorylated AMPK (278%) and phosphorylated acetyl-CoA carboxylase (335%) compared to the controls. Intracerebroventricular administration of compound C, an AMPK inhibitor, blocked the food intake induced by a single or multiple injections of T3. Taken together, these results suggest that enhanced hypothalamic AMPK phosphorylation contributes to T3-induced hyperphagia. Hypothalamic AMPK plays an important role in the regulation of food intake and body weight.

1. Introduction

Thyroid hormone stimulates many metabolic processes, including energy expenditure and the metabolism of lipids, carbohydrates, proteins, and minerals. These stimulatory actions, especially the increase in energy expenditure, contribute to the clinical manifestations of thyrotoxicosis. Therefore, thyrotoxicosis causes weight loss and promotes increases in food intake in both humans and rodents [1,2]. We previously reported that rats with triiodothyronine (T3)-induced thyrotoxicosis display hyperphagia associated with suppressed circulating leptin levels, increased hypothalamic neuropeptide Y (NPY) mRNA levels and decreased hypothalamic pro-opiomelanocortin (POMC) mRNA levels. The decrease in plasma leptin observed in T3-induced thyrotoxicosis suggests that leptin is the most important peripheral signal regulating food intake [3]. Recently, Kong et al. [4] demonstrated that both acute and chronic administration of the low-dose of T3 that we used could stimulate food intake without altering plasma leptin levels. These results suggested that T3 directly affects food intake. However, the mechanism for the direct regulation of food intake by T3 is not clear.

Some lines of evidence indicate that AMP-activated protein kinase (AMPK) regulates food intake [5–7]. AMPK is a major regulator of energy utilization. It has been reported that AMPK is activated by metabolic stresses that deplete cellular ATP [8,9]. When ATP levels are depleted, there is a corresponding increase in intracellular AMP levels and AMPK is activated by phosphorylation of the catalytic subunit (α) by at least one upstream AMPK kinase (AMPKK). AMPK is expressed in the hypothalamic neurons involved in the regulation of food intake [5], where it may act to restore the depleted energy. Therefore, hypothalamic AMPK has an important role in the central regulation of food intake and energy homeostasis.

We hypothesized that the hypothalamic AMPK-fatty acid synthetic pathway is activated by peripheral administration of T3. T3 may cause an increase in hypothalamic AMPK activity, which in turn contributes to the development of T3-induced hyperphagia. The aim of this study is to elucidate the role of the AMPK-fatty acid synthetic pathway in food intake in T3-treated rats. We investigated the role of hypothalamic AMPK and acetyl-CoA carboxylase (ACC) phosphorylation in promoting the increase in food intake in T3-treated rats. Furthermore, we tested the central administration of compound C, a selective inhibitor of AMPK, in T3-treated rats to determine whether the AMPK-

Abbreviations: ACC, acetyl-CoA carboxylase; AGRP, agouti-related protein; AMPK, AMP-activated protein kinase; BAT, brown adipose tissue; CPT-1, carnitine palmitoyltransferase-1; D1, *deiodinase 1*; i.c.v., intracerebroventricular; i.p., intraperitoneal; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; s.c., subcutaneous; STZ, streptozotocin; T3, triiodothyronine; T4, thyroxin; TRH, thyroid stimulating hormone-releasing hormone; TSH, thyroid stimulating hormone.

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fatty acid synthetic pathway contributes to the increased food intake in T3-treated rats.

2. Materials and methods

2.1. Animal care and maintenance

Male Sprague Dawley rats (240–260 g; Saitama Experimental Animal Supply Co. Ltd., Saitama, Japan) were housed in air-conditioned animal quarters, with lights on between 0800 and 2000 h, and were given food and water *ad libitum*. The experiments were conducted according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Nippon Medical School Animal Care Research Committee.

2.2. Preparation of T3 for peripheral administration

T3 was prepared as 3,3',5-triiodo-L-thyronine (T3) (4.5 nmol/kg; Aldrich Chemical Co., Milwaukee, WI) dissolved in 5 mM NaOH. Control rats received a parallel injection with an equal volume of 5 mM NaOH. In the study of the acute effects of T3 on food intake, animals received a s.c. injection of either T3 (4.5 nmol/kg) or the vehicle (5 mM NaOH) ($n=7$ per group). Food, body weight and adiposity were weighed at 2-h and 4-h post-injection. In separate study, animals were killed by decapitation after 2-h post-injection for collection of trunk blood as described ($n=7$) [5]. In the chronic study, animals ($n=7$ per group) were given a s.c. injection of T3 or the vehicle daily for 6 days. Food intake and body weight were measured daily. On day 6, animals were decapitated after 2-h post-injection of T3. Trunk blood was collected on day 6 and the plasma was separated by centrifugation and stored at −80 °C until assayed. The hypothalami were dissected as previously described [10] and both were stored at −80 °C until assayed. Epididymal, retroperitoneal and inguinal fat pads, and interscapular brown adipose tissue (BAT) were dissected and weighed. Brains were removed and the hypothalami were snap frozen for subsequent measurement of neuropeptide mRNA expression by Northern blot and Western blot analyses (see below).

2.3. Intracerebroventricular cannulation

The animals were anesthetized with an i.p. injection of a mixture of Ketalar (ketamine HCl, 60 mg/kg; Pfizer, Tokyo, Japan) and Rompun (xylazine, 12 mg/kg; Bayer, Tokyo, Japan), and a 23-gauge stainless-steel cannula was implanted into the right lateral ventricle using a stereotaxic apparatus as previously described [10]. The upper incisor bar was set 3.3 mm below the interauricular line, and bregma was taken as A-P zero. The cannula tip was placed at A – 0.9, L – 1.2 and V – 3.6 mm and secured in place with dental acrylic. Only those rats whose cerebrospinal fluid overflowed through the cannula were used for the experiment. They were kept in individual cages and habituated by handling every day. Injections of the peptide were performed 10 days after placement of the cannula. Compound C (100 nmol in 10 µl of RPMI1640 [RPMI; Invitrogen, Carlsbad, CA]) was a gift from Merck & Co., Inc. (Whitehouse Station, NJ) [11]. In the acute study, compound C or vehicle (RPMI) was injected intracerebroventricularly (i.c.v.) just before s.c. treatment with T3. In the chronic study, compound C or RPMI was injected i.c.v. every 24 h for 6 days to control and T3-treated rats, and food intake was determined. Rats were treated 1 h before the onset of the dark cycle with either the vehicle or compound C in the chronic study.

2.4. RNA extraction and Northern blot analysis

Total tissue RNA was extracted with the TRizol reagent (Life Technologies, Inc., Gaithersburg, MD). The protocol for Northern blot analysis has been described previously [12,13]. Briefly, samples (10–20 µg of RNA per lane) were electrophoresed through a 1.5% agarose gel containing 2.2 M formaldehyde, and then transferred by capillary blotting onto a nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ). Membrane blots were prehybridized in 1 M NaPO4, 20% SDS and 0.1% BSA for 2 h at 65 °C. 32P-Labeled specific riboprobes for NPY, agouti-related protein (AGRP), POMC or β-actin were added and the membranes were hybridized overnight at 65 °C. The membranes were washed at 65 °C in 2X SSC for 30 min; 2X SSC and 0.1% SDS for 30 min; and 0.5X SSC and 0.1% SDS for 30 min, and then exposed to Kodak XAR film at −70 °C for either 72 h for NPY and POMC, or 24 h for β-actin. The hybridization signal was determined from the autoradiograms using an NIH image analysis system.

2.5. Western blot analysis

In separate experiments, another pair of hypothalami were homogenized in lysis buffer (50 mM Tris–HCl [pH 7.5], 250 mM sucrose, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 50 µg/ml leupeptin, and 50 µg/ml soybean trypsin inhibitor). Protein concentration was determined with the DC protein assay (Bio-Rad Laboratories, Hercules, CA). The samples (40 µg) were mixed with 2X Laemmli sample buffer and then SDS was added to a final concentration of 0.2%. The lysates were boiled for 5 min, size separated on 4–15% Tris–HCl SDS Ready Gels (Bio-Rad Laboratories), and electrophoretically transferred to Hybond enhanced chemiluminescence nitrocellulose membranes (ECL, Amersham Biosciences). The blots were blocked in 5% nonfat dry milk (wt/vol) dissolved in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) and then washed four times for 5 min each in PBS-T. The blots were incubated overnight with primary antibodies against phospho-AMPKα (α1 and α2, Thr^{172}) (1:1000; Cell Signaling, Danvers, MA), phospho-ACC (Ser^{79}) (1:1000, Cell Signaling), anti-ACC (ACC1, 1:1000; Upstate Biotechnology, Lake Placid, NY) and anti-AMPKα (1:1000; Cell Signaling) in PBS-T and 5% BSA. The blots were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for phospho-AMPK α, AMPKα, phospho-ACC, and ACC (Amersham Biosciences; 1:20,000 dilution in PBS-T) for 1 h, washed again and then exposed for 1 min to the ECL Western Blotting Detection Reagent (Amersham Biosciences). Blots were exposed to Hyperfilm ECL (Amersham Biosciences) and developed. The films were developed, scanned and the image was analyzed using an NIH image analysis system.

2.5.1. Plasma hormone measurements

Plasma leptin [13], T3, thyroxin (T4), and thyroid stimulating hormone (TSH) [5] were measured with radioimmunoassays as previously described. Plasma desacyl and acyl ghrelin were measured with an ELISA (SCETI, Tokyo, Japan).

2.6. Data analysis

All data are presented as the mean ± SEM. Statistical analyses were carried out using an ANOVA, with the *post hoc* Fisher's Least Significant Difference method or an unpaired *t*-test (SigmaStat Ver. 3.5; Systat Software, Inc., Richmond, CA). $P<0.05$ was considered significant.

3. Results

3.1. Acute effects of T3 injection on food intake and plasma hormones

To examine the acute effect of T3 on food intake, rats received a single injection of T3. Two hours after the s.c. injection of T3, food intake increased (2.5 ± 0.5 [T3] vs. 0.4 ± 0.8 g [control], $P<0.05$) (Fig. 1A). The orexigenic effect of T3 was time-dependent and was no longer evident when assessed at 4 h after the administration of T3 (2.7 ± 1.1 [T3] vs. 0.5 ± 0.9 g [control]). There was no difference in body weight