Mechanism of the induction of brain c-Fos-positive neurons by lipid absorption

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Submitted 17 May 2006; accepted in final form 30 August 2006

Lo C-M, Ma L, Zhang DM, Lee R, Qin A, Liu M, Woods SC, Sakai RR, Raybould HE, Tso P. Mechanism of the induction of brain c-Fos-positive neurons by lipid absorption. Am J Physiol Regul Integr Comp Physiol 292: R268–R273, 2007. First published September 21, 2006; doi:10.1152/ajpregu.00334.2006.—Many gastrointestinal meal-related signals are transmitted to the central nervous system via the vagus nerve and thereby control changes in meal size. The c-Fos-positive neuron has been used as a marker of neuronal activation after lipid meals to examine the contribution of a selective macronutrient on brain neurocircuit activity. In rats fed Intralipid, the c-Fos-positive neurons were highly stimulated in the nucleus of the solitary tract (NTS) and in the hypothalamus, including the paraventricular nucleus (PVN), arcuate nucleus of the hypothalamus (ARC), and ventromedial hypothalamus at 4 h lipid feeding. However, c-Fos-like immunoreactivity was markedly attenuated in these brain regions when chylomicron formation/secretion was blocked by Pluronic L-81. After lymph was diverted from the lymph cannulated animals, the rats had a lower number of c-Fos-positive cells in the NTS and ARC. In contrast, the rats had higher c-Fos-positive neurons in PVN. The present study also revealed that c-Fos-positive neurons induced by feeding of Intralipid were abolished by CCK type 1 receptor antagonist, Lorglumide. We conclude that the formation and/or secretion of chylomicron are critical steps for initiating neuronal activation in the brain.

cholceystokinin type 1 receptor antagonist; chylomicron; hindbrain; hypothalamus

TRIACYLGLYCEROL (TG), phospholipids, and cholesterol are all dietary lipids, but TG is the major component of human dietary fat. Ingested TGs are absorbed by the small intestine, and many steps are involved in their digestion, uptake, and absorption (39). TG molecules are hydrolyzed by pancreatic lipase to form 2-monocacylglycerol (MG) and two fatty acids (FA) in the lumen of the intestine, and these are incorporated into micelles (19). The micelles facilitate the uptake of MG and FA by the intestinal epithelial cells (enterocytes) lining the intestinal villi (15). These fat digestion products are then reesterified into TG within the enterocytes and incorporated into chylomicrons, which are secreted in the lamina propria and then subsequently enter the lacteals (6, 18, 33). The chylomicrons pass from lacteals into the lymphatic duct, entering the blood circulation via the thoracic duct. As chylomicrons circulate, their stored TGs are gradually removed following lipolysis by the lipoprotein lipase located in blood vessels (31, 32). Following the ingestion of a lipid meal, gastrointestinal hormones and peptides such as CCK, apolipoprotein AIV (apo AIV), peptide YY (PYY), and glucagon-like peptide-1 (GLP-1) are also released in the lamina propria following secretion by enteroendocrine cells and the enterocytes. CCK, apo AIV, PYY, and GLP-1 have all been reported to act as satiety signals, reducing food intake (12, 13, 17, 28). Satiety signals act in the brain during meals to reduce the size of that meal. Many of these signals stimulate sensory nerves such as the vagus that act in the hindbrain (e.g., CCK) where they are integrated with other signals entering the brain directly by passing through the blood-brain barrier (e.g., leptin; see Refs. 2, 16, 37).

Both the hindbrain and the hypothalamus are involved in the trafficking and integration of the satiety signals and the regulation of food intake. The nucleus tractus solitarius (NTS) in the hindbrain receives much of the information pertinent to satiety, including vagal afferent information and gustation, and it integrates and relays this information to the hypothalamus (5, 8). Many hypothalamic nuclei are important in the regulation of food intake and energy homeostasis, including the arcuate (ARC), paraventricular (PVN) and ventromedial (VMH) nuclei (36, 43). It is believed that there is bidirectional neuronal traffic flow between these hypothalamic areas and the NTS (35, 43).

c-Fos protein is a product of the c-Fos immediate-early gene and is an intranuclear phosphoprotein, being rapidly transported in the nucleus of the cell after translation. c-Fos protein has been used as a marker for the activation of neurons in the brain since electrical or chemical stimulation results in markedly enhanced expression of c-fos mRNA as well as protein expression (23). It has been reported that c-Fos-positive protein in the NTS is strongly associated with food intake (45). Activation of c-Fos in the NTS is influenced both by the meal size (11) and the presence of different macronutrients in a meal and linoleic acid, glucose, and amino acids induce to a different degree of c-Fos-positive protein in the NTS. Intraperitoneal injection of CCK to rats induces c-fos activation in NTS in a dose-dependent manner (45).

Although dietary lipids cause the secretion of CCK (30), it is not known how lipids interact with CCK to stimulate c-Fos in the brain. The goals of this experiment therefore were 1) to determine the effect of intragastric lipid infusion on the activation of c-Fos-positive neuron in the hypothalamus and the NTS; 2) to determine if chylomicron formation is required for this activation; and 3) to determine if the CCK-1 receptor is involved in the activation of c-Fos protein by lipid absorption.

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MATERIALS AND METHODS

Sprague-Dawley rats and pelleted chow diet (LM-485) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Sodium hydroxide, paraformaldehyde, Lorglumide, hydrogen peroxide, sodium phosphate, Triton X-100, diaminobenzidine, and nickel sulfate were purchased from Sigma (St. Louis, MO). Pluronic L-81 (L-81) was kindly donated by BASF (Parsippany, NJ). Lyposyn II (20% fat) was obtained from Abbott (Abbott Park, IL). c-Fos antibody Ab-5 was purchased from Oncogene Science (West Haven, CT). Biotinylated goat anti-rabbit secondary antibody, rabbit serum, and horseradish peroxidase avidin-biotin complex were purchased from Vector Laboratories (Burlingame, CA).

Adult male rats weighing 300–350 g were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited facility in groups of two rats per cage with corn cob bedding and under conditions of controlledillumination (12:12-light-dark cycle, lights on from 0600 to 1800). They had free access to rodent chow (5.6% fat) and water. All animals were fasted for 16 h before the experiment, and each group included four rats. All procedures were conducted in an isolated quiet room to reduce variance. All animal protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

**Experiment 1: time course of infusing Intralipid on c-Fos-positive neuron in the brain.** To determine the optimal time to observe changes in c-Fos-positive neuron following the administration of lipid, experimental rats were intragastrically gavaged with 4 ml of Intralipid (2 kcal/ml; 20% liposyl II, containing 10 g safflower oil, 10 g soybean oil). Groups of lipid-treated animals were killed by an overdose of pentobarbital sodium (1 ml/rat) at 1, 2, 4, and 6 h after gavage. The brain was perfused through the left ventricle of the heart with 200 ml of 0.9% saline to wash away the red blood cells. This was followed by perfusion of 400 ml of 4% paraformaldehyde to fix the brain. The brain was removed and soaked in 4% paraformaldehyde solution overnight. Control rats received 4 ml of saline by gavage and were killed after 4 h.

**Experiment 2: effects of lipid and L-81 on c-Fos-positive neuron.** To determine if the activation of c-Fos is related to the formation and secretory activity of triglycerides, the formation of triglycerides was inhibited with L-81 (42). Although L-81 inhibits the formation of chylomicrons, it has no effect on the digestion, uptake, or reesterification of absorbed monoglycerides and FA to form TG (14). Rats received intragastric Intralipid (4 ml) by gavage with or without added L-81 (25 mg/rat). After 4 h, animals were killed as described above.

**Experiment 3: effect of lymph diversion on c-Fos-positive neuron.** To differentiate the effect of the activation of the neurons in the gut epithelium vs. possible metabolic effects of chylomicrons after enterohepatic circulation, we diverted the lymph of animals actively absorbing fat and determined c-Fos-positive neurons in the brain.

Under halathane anesthesia, rats were installed an indwelling silicone gastric cannula and a lymphatic cannula with clear polyvinyl chloride tubing (1 mm OD), as described previously (41). The animals were allowed to recover for 24 h in restraining cages kept in a warm chamber (30°C). The next day, the animals received either 4 ml of Intralipid or 4 ml of saline via the gastric cannula. Lymph was drained away in both groups of rats by the lymph cannulas. Over the course of 4 h following the initial Intralipid or saline administration, the animals continued to receive saline at 4 ml/h in the stomach. At the end of the experiment, the animals were killed as described above.

**Experiment 4: effect of a CCK-1 receptor antagonist on c-Fos-positive neuron.** To determine the role of CCK on c-Fos activation by lipid absorption, animals were gavaged with Intralipid with or without receiving a CCK-1 receptor antagonist. All rats received an intraperitoneal injection of saline or 1 mg/kg Lorglumide 15 min before receiving Intralipid as above. After 4 h, all animals were killed as described above.

**Immunohistochemistry for c-Fos protein-like immunoreactivity.** The brain was removed and postfixed in 4% paraformaldehyde overnight and in 30% sucrose-phosphate buffer for 2 days. Regions of the hypothalamus and hindbrain were identified according to the information provided by the rat brain stereotaxic atlas of Paxinos and Watson (27). Frozen sections (30 μm) were sliced with a microtome (cut 4055; Olympus) and were washed with hydrogen peroxide in a free-floating state. These sections were then incubated overnight with dilute primary antibody in 0.01 M sodium phosphate solution (1:30,000) and c-Fos Ab-5 in normal goat serum plus 0.3% Triton X-100 buffer. After being washed three times with sodium phosphate buffer, the tissues were incubated with biotinylated goat anti-rabbit secondary antibody (dilution 1:200) for 2 h followed by horseradish peroxidase avidin-biotin complex (dilution 1:200) for 2 h. The sections were developed by diaminobenzidine with nickel sulfate.

As negative controls, sections were incubated with nonimmune rabbit serum instead of the primary antibody to get rid of nonspecific staining.

**Statistical Analyses**

Quantitative assessment of c-Fos-positive neuron was achieved by counting the number of c-Fos-positive cells per square millimeter in selected brain regions. All slides were numbered without the treatment information to avoid any bias in counting, and each hypothalamic nuclei and hindbrain were bilaterally counted in at least 10 different sections. Cells with distinct brown nuclear c-Fos-like immunoreactivity staining in various brain regions were manually counted under light microscopy with low magnification (×40) with the aid of a 1-mm² ruler. Fos-positive neurons in PVN, VMH, and ARC were identified according to the photomicrographs depicted in plates 25–33 (bregma −1.80 to approximately −3.8 mm), and those neurons in NTS were examined from plate 70 to 76 (bregma −13.24 to approximately −14.6 mm). c-Fos-positive cells on the borderline were not repeatedly counted. Data are presented as means ± SE of the average number of cells per square millimeter. Differences between groups were determined by Student’s t-test or ANOVA. A probability of P < 0.05 was considered significant.

![Fig. 1. Time course of feeding of Intralipids on c-Fos-positive neuron in the brain. Fasted Sprague-Dawley (SD) rats were gavaged with 4 ml of Intralipid for 1, 2, 4, or 6 h or rats received 4 ml of saline for 4 h. Data are means ± SE of average no. of cells/mm² in each brain region. Different letters indicate significant difference from the other group within the same brain region (P < 0.05).](http://www.ajpregu.org)
RESULTS

Experiment 1: time course of infusing Intralipid on c-Fos-positive neurons in the brain. Following Intralipid, there was an increase in the number of cells stained with c-Fos in the NTS and some nuclei of the hypothalamus, including the PVN, ARC, and VMH (Fig. 1). As depicted in Fig. 1, c-Fos-positive neurons in the NTS were stimulated by Intralipid after 1 and 2 h, but the increase was not significantly different from what was observed in the saline control group. By 4 h, there were significantly more c-Fos-labeled neurons in the NTS and PVN following Intralipid than following saline. By 6 h, the c-Fos-positive neurons in all brain areas were attenuated relative to the 4-h samples. Based on these data, we chose 4 h after gavage as the time point for all of our subsequent studies.

Experiment 2: effects of lipid and L-81 on c-Fos-positive neurons. This experiment asked whether the formation and secretion of chylomicrons are necessary to mediate the increased labeling of c-Fos elicited by fat absorption. Rats receiving Intralipid but no L-81 had significantly more c-Fos-positive cells in the NTS (P < 0.05) than rats receiving Intralipid plus L-81, which had the same levels as saline controls (see Fig. 3). Similarly, c-Fos-positive neurons in the PVN and ARC were also reduced by L-81 (P < 0.01 for PVN; P < 0.05 for ARC; Figs. 2 and 3). L-81 in fact reduced c-Fos-positive cells in the hypothalamus below levels observed in saline controls, although the reduction only achieved significance in the PVN. As a control, we administered casein with or without L-81 in the stomach in a comparable group of animals and measured c-Fos after 4 h. Casein increased c-Fos...
in all of the same brain areas relative to saline, and L-81 had no effect on these levels.

**Experiment 3: effect of lymph diversion on c-Fos-positive neurons.** When lymph was diverted from the circulation by cannulation of the lymphatic duct and lipids were infused in the stomach, there was a significant reduction in c-Fos-positive cells in the NTS. Within the hypothalamus, lymph diversion had no effect on c-Fos-positive neuron in the ARC or VMH following intragastric lipid. In contrast, c-Fos-positive cells in the PVN were significantly increased by lymph diversion (Fig. 4). Rats receiving saline and lymph diversion had c-Fos levels that were comparable to saline-treated rats in the first two experiments (Fig. 4).

**Experiment 4: effect of a CCK-1 receptor antagonist on c-Fos-positive neurons.** c-Fos-protein expression following In-
tralipid was significantly attenuated by Lorglumide, a CCK-1 receptor antagonist, in all brain areas assessed, attaining sig-
nificance in the NTS, PVN, and ARC (Fig. 5).

**DISCUSSION**

The goal of these experiments was to determine the effect of the intestinal absorption of lipid emulsion, Intralipid, to induce c-Fos-positive neurons in the hypothalamus and NTS when infused in the stomach of fasted rats. We first identified an optimal time to address the issue and found that c-Fos-positive neurons increased for 4 h following intragastric feeding of Intralipid and decreased somewhat after that. Saline-infused rats had significantly less c-Fos after 4 h in similar brain areas. Using 4 h as the paradigm, we then observed that the addition of L-81, which blocks the formation of chylomicrons in intes-
tinal cells, reduced c-Fos-positive neurons, implying that chy-
lomicron formation is somehow important in the stimulation of c-Fos-positive neurons in the brain by active fat absorption. When we prevented chylomicron-rich lymph from reaching the circulation, we observed a decrease of c-Fos in the NTS but an increase of c-Fos in the PVN. Finally, when we administered a CCK-1 receptor antagonist, we attenuated the c-Fos response to Intralipid in all brain areas. We conclude that c-Fos neurons in the hindbrain and hypothalamus activated by Intralipid are dependent on chylomicron formation, as well as CCK-1 recep-
tor activation.

Administration of Intralipid in the stomach elicited differential intensity of c-Fos-positive neurons in the brain as a function of time. Administration of saline itself was associated with some c-Fos protein, but by 4 h after the procedure the levels in the NTS and the hypothalamus were significantly elevated following Intralipid relative to saline. These data are consistent with an earlier report of increased c-Fos protein in PVN and NTS in Intralipid-treated rats 4 h after an intraduodenal infusion (21). The number of c-Fos-positive cells in the brain following lipid infusion was reduced after 6 h. Based on these data, 4 h were selected as the time point for the other experiments.

The data of experiment 2 indicate that c-Fos-positive neu-
rons induced by Intralipid is dependent on chylomicron for-
mation, since the Intralipid- plus L-81-treated animals had
relatively low levels of c-Fos-positive cells in both the hindbrain and the hypothalamus. L-81 blocks the movement of prechylomicrons from the endoplasmic reticulum and Golgi within intestinal cells, but it does not affect digestion, uptake, or resynthesis of TG by small intestinal cells (24, 39). Consequently, chylomicron secretion is inhibited, and fat droplets accumulate within the intestinal enterocytes (14). Experiment 2 also implies that formation of chylomicrons, but not that of very low density lipoproteins (VLDLs), is a critical step in inducing c-Fos-positive cells in the brain. Two different types of TG-rich lipoproteins, chylomicrons, and VLDLs, are produced in intestinal enterocytes (41). Infusion of oleate or linoleate results mainly in the formation of chylomicrons. In contrast, lymphatic VLDL is the major intestinal lipoprotein secreted during fasting or following L-81 treatment (4, 26, 41), and L-81 does not block the formation of VLDLs (39).

The L-81 data suggest that the c-Fos-positive neurons induced by Intralipid do not result from the detection of lipids by some sort of membrane receptor lining the lumen since L-81 does not block events within the lumen and does not interfere with the uptake of lipids in intestinal cells (40). After their formation in enterocytes, chylomicrons are released and transported in the lymphatic vessels via lacteals in the mucosa (39). Vagal afferents are abundant in the lacteals and other lymph vessels of the submucosa (9, 29). It is therefore possible that nerves in the lamina propria are activated by the passage of chylous lymph and its components and that they project signals to the NTS (1, 9, 29). Of course, there is a possibility the c-Fos activation is induced by the metabolic effects of the chylomicrons loaded with TG entering the general circulation.

The L-81-treated animals had fewer c-Fos-positive cells in the hypothalamic nuclei than the saline-treated rats. We have previously observed that animals administered Intralipid plus L-81 have comparable levels of VLDL in the lymph relative to the saline group (41). This suggests that the decrease of c-Fos following L-81 is probably not because of altered factors other than the reduction of chylomicrons. It could also be the case that L-81, by itself, reduces the c-Fos-positive neuron. However, this is probably not the case because, when L-81 is administered along with protein in the stomach, there is no change of c-Fos-positive neuron in the brain. The molecular mechanism for the reduced c-Fos-positive neuron in the Intralipid- plus L-81-treated rats is therefore unclear in the present study.

Experiment 3 asked whether chylomicrons elicit brain c-Fos-positive neurons by activating some mechanism downstream of the intestine (e.g., metabolic effect). To assess this, we diverted the chylous lymph out of the body, thus circumventing reaching potential downstream targets. This procedure attenuated but did not eliminate lipid-induced c-Fos in the NTS, suggesting that chylomicrons may act in more than one site to impact the hindbrain. Within the hypothalamus, lymph diversion had no effect on c-Fos-positive neuron in the ARC or VMH, and it significantly increased it in the PVN. Compared with the PVN and the VMH, the ARC is considered to have a relatively permeable blood-brain barrier and therefore is a potential entry point for circulating peptides and nutrients to influence neural activity (2, 8, 16, 44). However, there was a relatively low number of c-Fos-positive cells in the ARC of the lymph-diverted group, implying that direct stimulation of the brain may not be a mechanism for chylomicron-elicited c-Fos-positive neurons by activating some mechanism downstream of the intestine (e.g., metabolic effect). To assess this, we diverted the chylous lymph and its components and that they project signals to the NTS (1, 9, 29). Of course, there is a possibility the c-Fos activation is induced by the metabolic effects of the chylomicrons loaded with TG entering the general circulation.

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LIPID ABSORPTION AND BRAIN c-Fos EXPRESSION