Targeted enhancement of oleoylethanolamide production in proximal small intestine induces across-meal satiety in rats

Jin Fu, Janet Kim, Fariba Oveisi, Giuseppe Astarita, and Daniele Piomelli


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(PLD) (14, 23, 24), which catalyzes the hydrolysis of NAPE to produce OEA. Injection of this viral vector into the duodenum of rats resulted in local increases in NAPE-PLD expression and OEA production, which were accompanied by a significant enhancement of across-meal satiety.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (250–300 g) were purchased from Charles River (Wilmington, MA). A 12-h light-dark cycle was set with lights on at 5:30 AM. Water and standard chow pellets (Prolab RMH 2500; PMI Nutrition International, Brentwood, MO) were available ad libitum. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Lipid analyses. Rats were killed with halothane, and tissues were rapidly collected and snap-frozen in liquid N2. Frozen tissues were weighed and homogenized in methanol containing internal standards (8). Cells were harvested in 50% methanol in PBS containing internal standards. OEA and other fatty-acid ethanolamides (palmitoylethanolamide, anandamide), as well as their NAPE precursors, were quantified after lipid extraction by isotope-dilution liquid chromatography/mass spectrometry, as described (8).

Adenovirus preparation. An EcoRI/NheI fragment containing full-length rat brain NAPE-PLD was cloned into the pCD515 vector (Microbix, Toronto, Ontario, Canada). Plasmids were cotransfected with adenoviral genomic plasmid into low-passage human embryonic kidney 293 (HEK293) cells using the Superfect transfection reagent (Qiagen, Valencia, CA). The shuttle plasmid pCD515 was used as a control. Cotransfected cells were harvested and subjected to three cycles of freezing and thawing. The recombinant adenovirus was recovered by centrifugation at 15000 g for 10 min. Viral particles were purified by 50,000 g ultracentrifugation for 2.5 h using cesium chloride gradient. The viral band was collected by puncture, and the purified virus was dialyzed against 3% sucrose/phosphate buffer (0.1 M). Stock virus was titrated by plaques in HEK293 cell.

Intraduodenal injections. Animals were fasted for 24 h before surgery and anesthetized by intraperitoneal injection of ketamine-xylazine (9%:1%; 1 ml/kg). A 2- to 3-cm incision was made into the ventral midline skin and abdominal muscle wall beneath the xiphoid process. The mesentery was gently retracted with sterile gauze to reveal the duodenum. The duodenum was lifted, and an adenovirus suspension (1012 pfu/1 ml PBS) was injected into the intestinal lumen ~2 cm below the pylorus using a 27-gauge needle. The ends of the scalp incision were sutured; recovering animals were kept on a heated pad at 30°C until they awoke.

Feeding behavior. Food intake was recorded in free-feeding rats by using an automated monitoring system (Scipro, New York, NY), as described previously (10). The system consists of 24 cages equipped with food baskets connected to weight sensors. The following feeding parameters were analyzed: total food intake (g/kg)—the amount of food consumed during 24 h; feeding latency (min)—the time interval from dark onset to the first eating episode; meal size (g/kg)—the amount of food consumed during the meal; postmeal interval (min)—the time interval between end of a meal and beginning of next meal; and satiety ratio (min·g−1·kg−1)—the ratio between postmeal interval and meal size.

Quantitative PCR. Total RNA was extracted from tissue and cells with TRizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized by using SuperscriptII RNase H-reverse transcriptase (Invitrogen) following the manufacturer’s instructions. Real-time quantitative PCR was performed in an Mx 3000P system (Stratagene, La Jolla, CA) by a significant enhancement of across-meal satiety.
TARGETED ENHANCEMENT OF OLEOYLETHANOLAMIDE PRODUCTION

Table 1. Effects of duodenal injections of Ad-NPLD or Ad-mock on NAPE-PLD mRNA levels in various rat tissues

<table>
<thead>
<tr>
<th>Day of Injection</th>
<th>Organs</th>
<th>Stomach</th>
<th>Colon</th>
<th>Liver</th>
<th>Fat</th>
<th>Pancreas</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>7th day</td>
<td>Ad-Mock</td>
<td>0.97±0.17</td>
<td>0.55±0.07</td>
<td>0.13±0.006</td>
<td>3.92±0.19</td>
<td>36.11±3.18</td>
<td>0.9±0.21</td>
</tr>
<tr>
<td>10th day</td>
<td>Ad-NPLD</td>
<td>1.07±0.18</td>
<td>0.74±0.21</td>
<td>0.109±0.01</td>
<td>2.82±0.4</td>
<td>23.24±5.3</td>
<td>0.99±0.07</td>
</tr>
<tr>
<td>12th day</td>
<td>Ad-Mock</td>
<td>0.877±0.18</td>
<td>0.667±0.14</td>
<td>0.125±0.01</td>
<td>2.89±0.1</td>
<td>27.6±3.18</td>
<td>1.28±0.15</td>
</tr>
<tr>
<td></td>
<td>Ad-NPLD</td>
<td>1.05±0.15</td>
<td>0.77±0.1</td>
<td>0.126±0.01</td>
<td>3.63±0.15</td>
<td>40.27±7.5</td>
<td>1.08±0.05</td>
</tr>
</tbody>
</table>

Results, in arbitrary units, are expressed as means ± SE; n = 6–8. Ad-Mock, control adenoviral vector.

Fluorescent images were captured using the SpotFlex digital imaging system (Diagnostic Instruments, Sterling Heights, MI).

Statistical analyses. Results are expressed as means ± SE. Statistical significance was evaluated using the Student’s t-test or, when appropriate, one-way ANOVA followed by the Dunnett’s post hoc test. Analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA), and differences were considered significant if P < 0.05.

RESULTS

We first verified that the adenoviral vector Ad-NPLD directs NAPE-PLD expression in intact cells. Infection of HeLa cell cultures with Ad-NPLD (10⁸ pfu, 36 h) resulted in a significant increase in NAPE-PLD mRNA levels, whereas infection with a control vector had no such effect (Fig. 1A). Heterologous NAPE-PLD expression was confirmed by both immunoblot analysis, which revealed the presence in Hela cell extracts of an appropriately sized protein band (~46 kD) (Fig. 1B), and immunocytochemistry (Fig. 1C). The characterization of the NAPE-PLD antibody used in these experiments is reported elsewhere (8). As shown in Fig. 1D, NAPE-PLD expression was accompanied by an elevation in the cellular levels of OEA and its analog palmitoylethanolamide (PEA). By contrast, we observed only a small nonsignificant change in cellular anandamide content, confirming the limited role of NAPE-PLD in the production of this endogenous cannabinoïd ligand (14, 21).

Intraduodenal injections of Ad-NPLD (10¹² pfu), but not control vector, resulted in a time-dependent elevation in jejunal NAPE-PLD mRNA content, which reached maximal levels 8 to 10 days after virus administration (Fig. 2). Confirming that this effect was restricted to the small intestine, no increase in NAPE-PLD mRNA was noted in a broad series of visceral organs and tissues, including stomach, colon, liver, epididymal fat, pancreas, and kidney (Table 1). Jejunal changes in NAPE-PLD expression were temporally associated with an increase in OEA and PEA levels (Fig. 3, A and B), but only with small...
nonsignificant alteration in anandamide levels (Fig. 3). As expected, NAPE-PLD overexpression was also accompanied by a decrease in the jejunal levels of the OEA precursor, 1-stearoyl-2-arachidonoyl-sn-glycero-phosphoethanolamine-N-oleoyl, and the PEA precursor, 1-stearoyl-2-arachidonoyl-sn-glycero-phosphoethanolamine-N-palmitoyl (Table 2).

To determine whether virally induced overproduction of endogenous OEA is sufficient to activate PPAR-α in rat jejunum, we measured the expression of two representative PPAR-α target genes, those encoding for PPAR-α itself and for the fatty-acid transporter CD36 (9). Quantitative PCR analyses showed that, concomitantly with OEA overproduction (Fig. 3A), Ad-NPLD injection resulted in a significant elevation in jejunal PPAR-α (Fig. 4A) and CD36 mRNA (Fig. 4B). Administration of a control adenoviral vector had no effect on PPAR-α or CD36 mRNA levels (Fig. 4).

The intraduodenal injection of control vector did not alter free-feeding behavior (Figs. 5 and 6). By contrast, injection of the Ad-NPLD virus resulted in a reversible reduction in total food intake (Fig. 5), which overlapped with the time of maximal NAPE-PLD expression (Fig. 2) and OEA elevation (Fig. 3A). Meal pattern analyses on days 7–12 after virus injections revealed that this temporary hypophagia was due to a prolongation of the latency to feed (Fig. 6A) and postmeal interval (Fig. 6B), with no detectable change occurring in meal size (Fig. 6C). The expected increase in satiety ratio (postmeal interval/meal size) associated with this response pattern is illustrated in Fig. 6D. Notably, these alterations in feeding behavior were only observed during the first hours of nocturnal feeding (Fig. 7). Later nocturnal food intake or diurnal food intake were not affected by NAPE-PLD overexpression (data not shown).

**DISCUSSION**

In the present study, we examined whether artificial rises in OEA levels in the small intestine, quantitatively similar to those naturally caused by food ingestion (8), can alter feeding behavior in rats. To increase local OEA production, we constructed an adenoviral vector that directs expression of the OEA-synthesizing enzyme NAPE-PLD and injected this vector into the rat duodenum. This resulted in parallel increases in NAPE-PLD expression, PPAR-α activation, and OEA production, which were restricted to the small intestine and were

### Table 2. Effects of duodenal injections of Ad-NPLD or Ad-mock on the OEA precursor, NOPE, (m/z 1030.8 > 744.8), and the PEA precursor, NPPE, (m/z 1004.8 > 718.8) in rat jejunum

<table>
<thead>
<tr>
<th></th>
<th>Ad-Mock</th>
<th>Ad-NPLD</th>
<th></th>
<th>Ad-Mock</th>
<th>Ad-NPLD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Food intake (g/kg)</td>
<td>NOPE</td>
<td></td>
<td></td>
<td>Food intake (g/kg)</td>
</tr>
<tr>
<td>7th day</td>
<td>208.57±34.8</td>
<td>172.33±30.5</td>
<td>256.46±54.6</td>
<td>245.18±47.5</td>
<td></td>
</tr>
<tr>
<td>8th day</td>
<td>233.52±53</td>
<td>135.91±34.9</td>
<td>355.3±66.9</td>
<td>174.61±51.6</td>
<td></td>
</tr>
<tr>
<td>9th day</td>
<td>250.21±45.09</td>
<td>105.46±19.9</td>
<td>303.82±72.1</td>
<td>117.22±26.8</td>
<td></td>
</tr>
<tr>
<td>10th day</td>
<td>230.12±45.09</td>
<td>126.83±10.5*</td>
<td>303.03±67.6</td>
<td>175.13±20.7*</td>
<td></td>
</tr>
<tr>
<td>12th day</td>
<td>219.7±37.3</td>
<td>186.76±52.4</td>
<td>235.67±53.1</td>
<td>247.35±24.2</td>
<td></td>
</tr>
</tbody>
</table>

Results, in pmol/g of wet tissue, are expressed as mean ± SE; n = 3. Ad-NPLD, N-acylephosphatidylethanolamine (NAPE)-phospholipase D; NOPE, 1-stearoyl-2-arachidonoyl-sn-glycero-phosphoethanolamine-N-oleoyl; NPPE, 1-stearoyl-2-arachidonoyl-sn-glycero-phosphoethanolamine-N-palmitoyl. *P < 0.05; n = 3.

**Fig. 5.** NAPE-PLD overexpression reduces food intake in free-feeding rats. Time-course of the effects of Ad-NPLD or control vector on daily food consumption. D5-D12, day after virus injection. Open bars, control adenoviral vector; closed bars, Ad-NPLD vector. Results, in g/kg of body weight, are expressed as mean ± SEM; *P < 0.05, n = 11–12.
temporally associated with a significant reduction in food intake. Importantly, the reversible hypophagia associated with NAPE-PLD overexpression was behaviorally identical to that elicited by exogenous OEA (10, 15), as it was due to a prolongation of feeding latency and postmeal interval rather than to a decrease in meal size. Also, of interest is the finding that the anorexic effect induced by NAPE-PLD overexpression was restricted to the first hours of nocturnal feeding, when rats eat the first and largest of their daily meals. These results support the hypothesis that feeding-induced fluctuations in small-intestinal NAPE-PLD activity and OEA production are sufficient to influence across-meal satiety (3, 8).

Previous studies have shown that food ingestion stimulates OEA mobilization in the mucosal layer of the duodenum and jejunum but exerts no such effect in the serosal layer from the same intestinal segments, in other sections of the gastrointestinal tract (stomach, ileum, colon) or in a broad series of internal organs and tissues (e.g., liver and brain) (8, 18). Furthermore, food intake does not alter OEA concentrations in portal or cardiac blood (8, 18). These observations suggest that OEA may act as a local—that is, autocrine or paracrine—satiety signal rather than as a blood-borne hormone. The present experiments show that a localized increase in OEA mobilization in the small intestine can both activate PPAR-α—as evidenced by the induction of two key PPAR-α target genes (PPAR-α itself and CD36)—and reduce food intake, thus providing a critical piece of evidence in support of a local messenger role of OEA. Further support for this hypothesis comes from studies showing that surgical resection of the vagus nerve below the diaphragm or pharmacological ablation of peripheral sensory fibers each abrogates the anorexic effects elicited by intraperitoneal administration of OEA (18).

Our experiments raise several questions, which remain to be answered. The first pertains to the identity of the fatty-acid ethanolamides involved in the regulation of feeding behavior. Both food intake (3, 8) and NAPE-PLD overexpression (present results) increase the levels of multiple fatty-acid ethanolamides along with OEA. At present, the functional roles played by each member of this family of compounds, if any, remain unclear. Because both OEA and PEA activate PPAR-α and reduce feeding (11, 18), the possibility that PEA contributes to the hypophagic effect of NAPE-PLD overexpression should be taken into consideration. The second question relates to the cell types in the small intestine (5) responsible for OEA biosynthesis. Our viral approach allowed us to restrict NAPE-PLD expression to the upper gastrointestinal tract, but not to direct it to a specific cellular locale. Therefore, additional
experiments using cell-type-specific deletion or overexpression of the NAPE-PLD gene (or those encoding other OEA-metabolizing enzymes) are necessary to fully elucidate the functional anatomy of OEA signaling in the upper gut. Another question concerns the possible roles of OEA in aspects of intestinal physiology other than feeding regulation. Both exogenous OEA administration (18) and endogenous OEA overproduction (present results) stimulate expression of the PPAR-α-regulated protein CD36. This multifunctional cell-membrane glycoprotein is highly expressed in the small intestine and has been implicated in the transport of fatty acids across the enterocyte membrane (7, 12), confirming the suggestion that OEA signaling at PPAR-α regulates lipid absorption along with food intake (25). A final consideration is the time course of the anorexic effects of OEA overproduction. The finding that such effects are limited to the first hours of the night, when rodents consume a significant proportion of their daily food, suggests that OEA signaling might be controlled by circadian regulators, which remain to be discovered.

Despite these open questions, the present results provide important new evidence for a role of OEA as a local satiety messenger in the small intestine and further validate the use of viral technology as a tool for the local delivery of pharmacologically active agents to organs and tissues.

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GRANTS

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