Postprandial increase of oleoylethanolamide mobilization in small intestine of the Burmese python (Python molurus)

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Submitted 12 September 2005; accepted in final form 19 December 2005

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The gastrointestinal tract plays a pivotal role in the regulation of vertebrate feeding and digestion, controlling multiple aspects of this process through both neuronal and humoral mechanisms. Among the humoral signals released by the gut, peptides, such as cholecystokinin, glucagon-like peptide-1, and ghrelin have attracted the most attention (4), but recent evidence suggests that lipid messengers are also involved (7, 18). One such messenger, oleoylethanolamide (OEA)—the amide of oleic acid and ethanolamine—is synthesized in a variety of rodent tissues, including the small intestine, where its levels decrease during food deprivation and increase upon refeeding (24). These periprandial fluctuations, which likely result from local changes in OEA biosynthesis and hydrolysis, are thought to represent a previously undescribed satiety-modulating signal. In support of this idea, pharmacological studies have shown that systemic administration of OEA produces profound anorexiant effects in rats and mice (11, 17, 19, 21, 24). These actions appear to be behaviorally selective, as they cannot be attributed to induction of malaise, anxiety, stress, or motor inhibition (21, 24). Furthermore, meal-pattern analyses in free-feeding rats suggest that, when administered as a drug, OEA modulates food intake by selectively prolonging the interval between successive meals, rather than by accelerating the termination of individual meals (11, 19). Thus the anorexiant effects of this lipid mediator are strikingly different from those of the gut peptide CCK, which reduces feeding by shortening meal size and duration (23).

The nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α) is a key regulator of lipid metabolism and energy balance in mammals (8, 15). Three lines of evidence indicate that this receptor, which is abundantly expressed in the intestine (2, 3), mediates the effects of OEA. First, OEA binds with high affinity to the purified ligand-binding domain of satiety-inducing mouse and human PPAR-α (K_D 37 and 40 nM, respectively) and activates with high potency PPAR-α-driven transactivation in heterologous expression systems (half-maximal effective concentration, EC_50 120 nM) (10). Second, synthetic PPAR-α agonists, such as the compounds GW7647 and Wy-14643, exert anorexiant effects that are behaviorally identical to those produced by OEA (10). Third, mutant mice in which the PPAR-α gene has been deleted by homologous recombination (PPAR-α−/− mice) do not respond to OEA or synthetic PPAR-α agonists, although they retain normal anorexiant responses to CCK octapeptide and fenfluramine (10). Together, these findings suggest that OEA is a lipid mediator produced in the small intestine, which regulates feeding behavior by activating local PPAR-α. The precise sequence of events that leads from intestinal PPAR-α activation to induction of satiety remains unclear. However, the finding that either subdiaphragmatic vagotomy or treatment with the neurotoxin capsaicin abrogates OEA-induced hypophagia suggests that this process is mediated by afferent sensory signals (9).

At present, all information on the OEA response is derived from experiments in rodents. It is unknown how primitive or phylogenetically widespread this regulatory system is. Further,
it is unknown how responsive this system is to meal size or frequency. To begin to address these issues, we examined the OEA response to feeding and digestion in an ectothermic vertebrate, the Burmese python (Python molurus). This snake species provides an excellent model organism in which to study digestive physiology on account of the great rapidity and magnitude of its physiological responses to feeding (1, 26). Most mammals, including rats and humans, consume small meals at frequent intervals and display relatively modest regulatory responses to food intake. By contrast, Burmese pythons and other ambush-hunting snakes can consume huge meals (up to 50–60% of their own body mass) after months of fasting, and, therefore, they must mount adequate regulatory responses to cope with these events (25). For example, postprandial increases in plasma CCK levels are much larger in pythons (25-fold) than they are in rats (threefold) (27). Exceedingly large postfeeding responses have also been observed with other gastrointestinal peptides, including glucagon, glucose-dependant insulinitropic peptide, and neurotensin (27).

We hypothesized that feeding in Burmese pythons may cause an increase in intestinal OEA mobilization similar to that previously reported for gut peptides. To test this idea, we measured levels of OEA and its phospholipid precursors, N-acetyl phosphatidylethanolamine (NAPE) (6), in gastrointestinal segments of fasted and fed pythons.

MATERIALS AND METHODS

Animals and tissue collection. We obtained seven Burmese pythons (P. molurus) of undetermined sex with a body mass ranging from 0.503 to 1.286 kg (average weight, 0.729 ± 0.043 kg) from a commercial supplier (Bob Clark Captive Bred Reptiles, Oklahoma City, OK). The snakes were kept at the University of California, Irvine, animal housing facility. They were housed in separate containers at 30°C with free access to water and on a 12:12-h light-dark cycle. The animals were fed rats immediately before shipment. Upon arrival, they were fasted for 4 wk, and then were divided into two groups. The first group (n = 3) was killed at 4 wk without feeding; the second group (n = 4) was allowed to eat 1–3 rats equivalent to 25.0 ± 0.1% of their body mass and killed 48 h after feeding. Gastrointestinal segments (stomach, proximal small intestine, and colon), liver, rib, and jaw muscles were removed, rinsed in saline, cut into sections, and flash frozen in liquid nitrogen. All animal experiments were carried out under University of California, Irvine, Animal Research Protocol Number 1999–2123 to J. W. Hicks.

Chemicals. We purchased fatty-acid chlorides from Nu-Chek Prep (Elysian, MN); [3H4]-ethanolamine from Cambridge Isotope Laboratories (Andover, MA); 1-oleoyl, 2-palmitoyl-sn-glycero-phosphoethanolamine-N-arachidonoyl from Avanti Polar Lipids (Alabaster, AL). Solvents were obtained from Burdick and Jackson (Muskegon, MI), and all other chemicals were obtained from Sigma (St. Louis, MO).

Chemical syntheses. We prepared OEA and other fatty-acid ethanolamines (FAEs) by the reaction of fatty-acid chlorides with a tenfold molar excess of ethanolamine or [3H4]-ethanolamine. Reactions were conducted in dichloromethane at 0–4°C for 15 min, with stirring. The products were washed with water and dehydrated over sodium sulfate, filtered, and dried under N2. They were fully characterized by liquid chromatography/mass spectrometry (LC/MS) and 1H and 13C nuclear magnetic resonance spectroscopy. The purity was >98%.

Lipid extractions. We homogenized tissue samples and measured protein concentration using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). We spiked tissue homogenates with standard [3H4]-OEA, [3H4]-palmitoylethanolamide (PEA) and [3H4]-arachidonoylethanolamide (anandamide), and subjected them to methanol/chloroform (1:2, vol/vol) extraction. FAEs and their precursor NAPE were fractionated by open-bed silica gel column chromatography, as previously described by Cadas et al. (5). Briefly, the lipid extracts were reconstituted in chloroform and loaded onto small columns packed with silica gel G (60–230–400 Mesh ASTM; Whatman, Clifton, NJ). FAEs and NAPE were eluted with 9:1 (vol/vol) and 5:5 (vol/vol) chloroform/methanol, respectively. Eluates were dried under N2 and reconstituted in 0.1 ml of chloroform/methanol (1:4, vol/vol) for further analyses.

LC/ESI (MS) analyses. We used an 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Palo Alto, CA) equipped with ESI interface. We separated FAEs using a XDB Eclipse C18 column (50 × 4.6 mm ID, 1.8 µm, Zorbax), eluted with a gradient of methanol in water (from 85% to 90% methanol in 2.5 min) at a flow rate of 1.5 ml/min with column temperature at 40°C. MS detection was in the positive ionization mode, capillary voltage was set at 3.5 kV, and skimmer voltage was set at 40 V. N2 was used as drying gas at a flow rate of 12 l/min, temperature of 350°C and nebulizer pressure 80 psi. Capillary exit was set at 115 V. For the identification of OEA, we monitored m/z 326 [M+H]+ as precursor ion in MS/MS mode. We applied a fragmentation amplitude of 0.7 V to generate a series of characteristic fragment ions for OEA (m/z 309.3, 265.2, 247.3), using helium as a collision gas.

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Statistical analyses. Results are expressed as means ± SE. The significance of differences among groups was evaluated using Student’s t-test or, when appropriate, two-way ANOVA followed by Bonferroni’s post hoc test, with feeding conditions and gastrointestinal segments as the two factors. Analyses were conducted using GraphPad Prism (GraphPad Software, San Diego, CA), and differences were considered significant if P < 0.05.

RESULTS

We collected the proximal small intestine from either fasted or fed pythons and identified OEA in lipid extracts by LC coupled to tandem MS. A component eluting from the LC column with a retention time of 2.5 min yielded a precursor ion of m/z 326.3, corresponding to the molecular ion ([M+H]+)
for OEA, along with fragment ions of m/z 309.3 ([M+H]⁺-OH), 265.2 ([M+H]⁺-\text{N}_2\text{H}_2\text{C}_2\text{H}_2\text{OH}), 247.3 ([M+H]⁺-\text{N}_2\text{H}_2\text{C}_2\text{H}_2\text{OH}-\text{H}_2\text{O}) (Fig. 1A). Retention time and fragmentation pattern of the precursor ion were identical to those obtained with authentic OEA standard (Fig. 1B).

We next quantified OEA levels in different tissues by isotope-dilution LC/MS, monitoring an additional diagnostic ion of m/z 348 ([M+Na]⁺) (Fig. 2A). OEA levels were about 300-fold higher in the small intestine of fed pythons (322 ± 121 pmol/mg protein, n = 4) compared with fasted pythons (1 ± 1 pmol/mg protein, n = 3) (Fig. 2B). Two-way ANOVA gave the following results: F(conditions)₁,₁₅ = 4.990, P < 0.05; F(conditions)₁,₁₅ = 7.220, P < 0.05; F(gastrointestinal sections)₂,₁₅ = 4.778, P < 0.05. A Bonferroni’s post hoc test indicated significant differences in OEA levels in the small intestine of fed animals compared with fasted animals (P < 0.01). No such difference was noted in the stomach or colon (Fig. 2B). Moreover, no significant change in OEA levels was observed in the liver (fasted, 4.8 ± 1.9 pmol/mg protein, n = 3; fed, 15.6 ± 5.4 pmol/mg, n = 4; Student’s t-test, P = 0.13), rib muscle (fasted, 0.34 ± 0.23 pmol/mg, n = 3; fed, 0.42 ± 0.24 pmol/mg, n = 4; P = 0.33) and jaw muscle (fasted, 0.40 ± 0.22 pmol/mg, n = 3; fed, 0.70 ± 0.22 pmol/mg, n = 4; P = 0.82).

Next, to determine whether de novo OEA biosynthesis contributed to the observed increase in intestinal OEA levels, we quantified two NAPE species, which are thought to serve as OEA precursors (see Scheme 1): 1-stearoyl-2-arachidonoyl-sn-glycero-phosphoethanolamine-N-oleoyl (NAPE₁, m/z 1029) and 1-oleoyl-2-arachidonoyl-sn-phosphoethanolamine-N-oleoyl (NAPE₂, m/z 1031) (Fig. 3A). Significant postprandial increases in the levels of both NAPE₁ and NAPE₂ were observed in the small intestine of fed compared with fasted pythons (two-way ANOVA F₁,₁₅ = 26.60, P < 0.001 for NAPE₁ and F₁,₁₅ = 10.55, P < 0.01 for NAPE₂; Bonferroni’s test P < 0.01 for NAPE₁ and P < 0.05 for NAPE₂) (Fig. 3B). By contrast, no significant differences in NAPE levels were found in the stomach or colon (Fig. 3B).

Because OEA shares a common biosynthetic pathway with other biologically active FAEs, such as the endogenous cannabinoid anandamide (20) and the PPAR-α agonist PEA (16), we examined whether these lipid mediators are also present in the gastrointestinal tract of the python. The chemical structures of various FAEs identified and quantified in these analyses are reported in Table 1. Identifications were based on the detection of sodium adduct ions [M+Na]⁺ of appropriate mass (Table 1).
at the retention time of authentic standards (9). As seen with OEA, levels of saturated, mono- and diunsaturated FAEs, including PEA (16:0), stearoylethanolamide (18:0), palmitoleoylethanolamide (16:1, Δ9), and linoleoylethanolamide (18:2, Δ9–12), were higher in fed than fasted pythons (two-way ANOVA: F1/13 = 6.551, P < 0.05 for palmitoleoylethanolamide; F1/14 = 14.01, P < 0.01 for stearoylethanolamide; F1/15 = 8.607, P < 0.05 for linoleoylethanolamide; F1/15 = 4.706, P < 0.05 for palmitoleoylethanolamide) (Table 1). By contrast, polyunsaturated FAEs, such as anandamide (20:4, Δ5–8–11–14), γ-linolenoylethanolamide (18:3, Δ6–9–12), homo-γ-linolenoylethanolamide (20:3, Δ8–11–14) and docosaheXenoylethanolamide (22:6, Δ4–7–10–13–16–19) did not significantly change upon feeding (Table 1).

DISCUSSION

The major result of the present study is that feeding in the Burmese python is accompanied by a profound upregulation of OEA mobilization in the proximal small intestine. Levels of this anorexiant lipid mediator increase over 300-fold following a meal (from 13 ± 3 to 3220 ± 1415 pmol/g wet tissue). Comparison with rodents, in which feeding causes on average a threefold elevation in OEA levels (from 120 ± 15 to 350 ± 150 pmol/g wet tissue) (10, 24), highlights the exceptional magnitude of this response in pythons and suggests that intestinal OEA mobilization in python is tightly clamped during fasting and massively stimulated upon refeeding.

In mammals, the biosynthesis of OEA and other FAEs requires the activities of two separate enzymes: N-acyltransferase (NAT) and NAPE-specific phospholipase D (NAPE-PLD). NAT catalyzes the transfer of a fatty-acid group from the sn-1 position of phosphatidylcholine to the free amine of phosphatidylethanolamine (Scheme 1). This reaction leads to the formation of a family of NAPE species, which are cleaved by NAPE-PLD to produce OEA and other FAEs (Scheme 1). The finding that levels of two putative OEA precursors are significantly increased in the small intestine of fed pythons suggests that feeding stimulates OEA biosynthesis through the NAT/NAPE-PLD pathway. Because we did not measure the enzymatic activities of NAT and NAPE-PLD, we cannot completely rule out the possibility that OEA was derived from a different biochemical route or from exogenous sources such as food. However, two observations make this possibility unlikely. First, significant changes in OEA levels were observed only in the small intestine, although food remains were predominantly found in the stomach. Second, although saturated, mono- and diunsaturated FAEs increased dramatically after feeding, polyunsaturated FAEs underwent only small, insignificant changes. This finding is consistent with results previously obtained in rodents, in which a role for the NAPE-PLD...
pathway in OEA synthesis is well documented (5, 6, 30), and inconsistent with the possibility that OEA originates from the diet.

The differential regulation in the biosynthesis of FAEs with varying degrees of fatty-acid chain unsaturation, though unexplained at the molecular level, may be functionally significant. The monosaturated and saturated FAEs OEA and PEA are endogenous ligands of the nuclear receptor PPAR-\(\alpha\), which plays a central role in the control of energy balance and lipid metabolism in mammals (8, 15). Thus, the observed increase in the levels of these compounds in the python small intestine may be related to PPAR-\(\alpha\) activation. Importantly, PPAR-\(\alpha\) expression has been demonstrated in reptiles and other ectothermic vertebrates (14), although the functional roles of this receptor have not yet been investigated. In light of the present results, it would be interesting to explore such roles, as well as to determine whether reptilian PPAR-\(\alpha\) recognizes the same set of FAEs, which are recognized by mammalian PPAR-\(\alpha\). On the other hand, polyunsaturated FAEs, such as anandamide, selectively bind to G protein-coupled cannabinoid receptors (13, 22). The expression of these receptors in fish, amphibians, and birds has been demonstrated (28, 29, 31), and evidence indicates that they are implicated in multiple aspects of behavior (20, 29). The occurrence and physiological roles of cannabinoid receptors in reptiles remains unexplored.

In conclusion, the identification of OEA and other endogenous FAEs in the gastrointestinal tract of the Burmese python reveals that this class of lipid messengers may be widespread across vertebrate groups and possibly of ancient evolutionary origin. The signaling pathways engaged by these mediators might thus represent a common mechanism for the regulation of energy balance in vertebrates.

**ACKNOWLEDGMENTS**

We thank Mr. K. Eng for the help with lipid extractions, Dr. J. LoVerme for insightful discussions and Dr. F. Ahmed for consultancy on chromatographic separation. Lipid analyses were conducted at the Agilent/UCI Analytical Discovery Facility, Center for Drug Discovery.

**Table 1. Chemical structures and tissue levels of various FAEs in the gastrointestinal tract of Python molurus**

<table>
<thead>
<tr>
<th>FAE</th>
<th>m/z</th>
<th>Structure</th>
<th>Feeding Condition</th>
<th>Stomach</th>
<th>Small Intestine</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2, (\Delta^{6-12})</td>
<td>346</td>
<td><img src="structure1.png" alt="image" /></td>
<td>Fasting</td>
<td>3 ± 1</td>
<td>4 ± 2</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fed</td>
<td>2 ± 2</td>
<td>325 ± 101‡</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>18:3, (\Delta^{6-9-12})</td>
<td>344</td>
<td><img src="structure2.png" alt="image" /></td>
<td>Fasting</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fed</td>
<td>1 ± 1</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>18:0</td>
<td>350</td>
<td><img src="structure3.png" alt="image" /></td>
<td>Fasting</td>
<td>11 ± 1</td>
<td>5 ± 2</td>
<td>8 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fed</td>
<td>86 ± 21</td>
<td>222 ± 62†</td>
<td>42 ± 14</td>
</tr>
<tr>
<td>16:0</td>
<td>322</td>
<td><img src="structure4.png" alt="image" /></td>
<td>Fasting</td>
<td>26 ± 4</td>
<td>48 ± 28</td>
<td>15 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fed</td>
<td>115 ± 24</td>
<td>335 ± 137*</td>
<td>70 ± 29</td>
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<tr>
<td>16:1, (\Delta^{9})</td>
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<td><img src="structure5.png" alt="image" /></td>
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<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fed</td>
<td>36 ± 15</td>
<td>232 ± 104*</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>20:3, (\Delta^{6-11-14})</td>
<td>372</td>
<td><img src="structure6.png" alt="image" /></td>
<td>Fasting</td>
<td>9 ± 1</td>
<td>4 ± 1</td>
<td>18 ± 14</td>
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<tr>
<td></td>
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<td>18 ± 12</td>
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<td>1 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Fed</td>
<td>12 ± 9</td>
<td>17 ± 6</td>
<td>7 ± 5</td>
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<td>22:6, (\Delta^{6-7-10-13-16-19})</td>
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<td>1 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fed</td>
<td>8 ± 5</td>
<td>14 ± 6</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; \(n = 3–4\). *\(P < 0.05\), †\(P < 0.01\), ‡\(P < 0.001\); n.d. = nondetectable (two-way ANOVA followed by Bonferroni’s post hoc test, fed vs. fasting). FAE, fatty-acid ethanolamides; m/z, mass-to-charge ratio. Tissue levels are given in pmol/mg protein.
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R1412

OEA IN SMALL INTESTINE OF BURMESE PYHTONS

GRANTS

This work was supported by National Institute of Health Grant DK-070347 (to D. Piomelli) and by National Science Foundation Grant IB-N0091308 (to A. F. Bennett).