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Cold Exposure Stimulates Synthesis of the Bioactive Lipid Oleoylethanolamide in Rat Adipose Tissue*

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Oleoylethanolamide (OEA) is an endogenous lipid mediator that inhibits feeding and stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activating receptor-type-a. Little is known about the physiological regulation of this compound outside of the gastrointestinal tract, where its production is regulated by feeding. Here we show that cold exposure increases OEA levels in rat white adipose tissue but not in liver or intestine. This change is accompanied by parallel elevations in the activity of N-acyltransferase, a key enzyme responsible for OEA synthesis, without concomitant changes in fatty acid amide hydrolase, an enzyme responsible for OEA degradation. Moreover, cold stimulates the production of two species of N-oleoylephosphatidylethanolamine OEA precursors. The changes in OEA biosynthesis are reversed by pretreatment with the β-receptor antagonist propranolol, suggesting a role for β-adrenoreceptors in this response. In agreement with these findings, the β-agonists noradrenaline and isoproterenol stimulate OEA production in isolated adipocytes, an effect that is mimicked by the adrenyl cyclase activator forskolin. Collectively, these results identify cold exposure as a natural stimulus for OEA formation in white fat and suggest a role for the sympathetic nervous system in regulating OEA biosynthesis.

The fatty acid ethanolamide (FAE) family of lipid mediators includes polysaturated species such as anandamide (1) and saturated or monounsaturated species such as oleoylethanolamide (OEA) (2, 3). Anandamide is an endogenous ligand for G-protein-coupled cannabinoid receptors (1), while OEA diffuses through the intestinal villi of rodents (4, 6) and reptiles (7), OEA levels change in response to nutrient status: they are lower in food-deprived than free-feeding animals and return to baseline values upon re-feeding (2, 4, 6, 8). Moreover, in the brain (9) and white adipose tissue (WAT) (2), OEA levels fluctuate diurnally, although the physiological significance of these oscillations remains unknown.

The biosynthesis of OEA, like that of other FAEs, is thought to occur through two sequential biochemical steps. The first consists in the transfer of a fatty acid residue from the sn-1 position of phosphatidylethanolamine to the free amine group of phosphatidylethanolamine (PE). This reaction is catalyzed by a Ca²⁺ and cyclic AMP-regulated N-acyltransferase (NAT) activity, which yields a family of N-acylphosphatidylethanolamine (NAPE) species (1). The second reaction is mediated by a NAPE-specific phospholipase D (10, 11), which cleaves NAPEs to produce OEA and other FAEs. In mammalian tissues, OEA is primarily eliminated through enzymatic hydrolysis to oleic acid and ethanolamine by one of two known enzymes, fatty acid amide hydrolase (FAAH) (12, 13) and N-acylthanolamine-hydrolyzing acid amidase (11, 14).

To explore the possible roles of OEA in WAT, we used pooled tissue samples of WAT for experiments. To determine whether OEA levels might be regulated during physiological states in which fat stores are mobilized, one such state is cold exposure, in which heightened sympathetic activity leads to increased lipolysis and fatty-acid release from WAT (15–17).

EXPERIMENTAL PROCEDURES

Animals—We used male Wistar rats (150–200 g). All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals. For cold experiments, animals were kept in a cold room maintained at +4 °C for 3–6 h in group cages (five animals per cage).

Chemicals—We purchased all chemicals from Sigma or NuCheck Prep (Elysian, MN). Drugs were dissolved in 0.9% saline and administered to rats at a volume of 1 ml-kg⁻¹.

Cell, Tissue, and Blood Preparation—We isolated epididymal adipocytes from free-feeding male Wistar rats (150–200 g) by collagenase digestion, as described (18). Isolated adipocytes were incubated with drugs for 1 h at 37 °C. Blood was collected...
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via cardiac puncture and placed into Accuspin tubes (Sigma) containing EDTA (7.2 mg), and the plasma was separated by centrifugation at 800 × g for 10 min at 22 °C. Tissues were snap-frozen in liquid N2 for LC/MS analyses.

**LC/MS Analyses**—We solvent-extracted FAEs from tissues or media and quantified them by isotope dilution LC/MS, using methods delineated elsewhere (4, 19, 20). Briefly, we first homogenized tissue samples in methanol spiked with the standards, [3H]OEA, [3H]palmitoylethanolamide (PEA), and [3H]stearoylethanolamide and subjected them to methanol/chloroform (1:2, v/v) extraction. For NAE quantification the samples were spiked with the internal standard, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N(2,4-dinitrophenyl), and quantified against the external synthetic standard, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine-N-arachidonyl. FAEs and their precursor NAPEs were fractionated by open-bed silica gel column chromatography. The lipid extracts were loaded onto small columns packed with silica gel G (60–230–400 Mesh ASTM; Whatman, Clifton, NJ). FAEs and NAPEs were eluted from the columns with 9:1 and 5:5 (v/v) chloroform/methanol, respectively. Eluates were dried under N2 and reconstituted in 0.1 ml of chloroform/methanol (1:3, v/v). LC/MS analyses were conducted using an HP1100 series high performance liquid chromatography/MS equipped with a Hewlett Packard octadecylsilica (ODS) Hypersil column (100 × 4.6 mm inner diameter, 5 μm). LC/MS conditions were the same as those described previously (19).

**Enzyme Assays**—We measured NAT and FAAH activities in membrane fractions under conditions that were linear with respect to protein concentration and time as described previously (21, 22). Briefly, NAT assays were performed in 50 mM Tris buffer, pH 7.4, containing protein (4 mg/ml), 0.1% Triton X-100, 3 mM CaCl2 (or 10 mM EGTA), and [14C]dipalmitylphosphatidylcholine (1 μCi/ml, 80–120 mCi-mmol⁻¹). After 1 h of incubation, reactions were stopped with methanol, and the lipids were extracted. The samples were applied to silica gel G-columns, [14C]NAPE-containing fractions were eluted with chloroform/methanol (5:5, v/v), and radioactivity in the fractions was measured by liquid scintillation counting. FAAH assays were performed under linear conditions, except that [3H]anandamide (arachidonyl-[1-3H]ethanolamide; 60 Ci mmol⁻¹) was included as a substrate and radioactivity was measured in the aqueous phase after chloroform extraction.

**Statistics**—Results are expressed as mean ± S.E. of n separate experiments. The significance of differences among groups was evaluated using Student’s t test or analysis of variance followed by a Tukey’s multiple comparisons test or a Dunnett’s post hoc test, as appropriate.

**RESULTS**

We used isotope dilution LC/MS to measure tissue OEA content in rats that had been maintained at 4 °C for up to 6 h. Visceral fat depots isolated from these rats had significantly greater levels of OEA and its analog PEA than did those from control animals, which had been kept at room temperature for the same time (Fig. 1, A and B). This increase was time-dependent (Fig. 1C) and restricted to WAT: the cold challenge did not affect OEA levels in liver and intestine and caused a small, albeit significant OEA decrease in skeletal muscle and plasma (Fig. 1D).

OEA accumulation in WAT may have resulted from increased biosynthesis and/or decreased degradation. We found that cold exposure enhanced NAT activity in fat (Fig. 2A), while it had no effect on intestinal or liver NAT activity and lowered it in skeletal muscle (Fig. 2B). In contrast to NAT, FAAH activity in fat was not significantly affected by the cold challenge (Fig. 2C), indicating that the rise in adipose OEA levels reflected increased formation rather than decreased elimination. In further support of this possibility, we found that the rise in adipose NAT activity was accompanied by a concomitant increase in the levels of various NAPE species, which may serve as OEA precursors, including alk-1-palmitoyl-2-arachidonoyl-sn-glycerophosphoethanolamine-N-oleoyl (OAE-N-oleoyl-PE 1; Fig. 2D) and alk-1-palmitoyl-2-arachidonoyl-sn-glycerophosphoethanolamine-N-oleoyl (OAE-N-oleoyl-PE 2; Fig. 2E).

To identify neuroendocrine factors that contribute to the stimulation of OEA biosynthesis in response to cold, we focused on noradrenaline for two reasons. First, during the acute phase of cold adaptation noradrenaline is released from sympathetic fibers that innervate WAT (17, 23). Second, by engaging β-adrenergic receptors on adipose cells, noradrenaline initiates fatty acid mobilization and modulates the formation of adipose-derived signaling molecules such as leptin and tumor necrosis factor-α (15–17, 24). If noradrenaline mediates the cold-induced stimulation of OEA synthesis, β-adrenergic antagonists should block this response. In agreement with this prediction, systemic administration of the β-antagonist propranolol (25 mg kg⁻¹, intraperitoneal, 30 min before cold exposure) prevented cold-induced changes in adipose levels of OEA (Fig. 3A), NAT activity (Fig. 3B) and OEA precursor NAPEs (Fig. 3C). The β-antagonist did not affect, however, adipose OEA content in animals that had been kept at room temperature (data not shown).

As the effects of propranolol in vivo might be indirect, to further test the contribution of noradrenaline to OEA synthe-
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**DISCUSSION**

The sympathetic nervous system plays a key role in the metabolic adaptation to low ambient temperatures (15–17, 23). Its activation by cold heightens noradrenaline release in adipose tissue (17) and enables, through β-receptor-dependent phosphorylation of hormone-sensitive lipase, and possibly other lipases (25), the release of free fatty acids from triglyceride stores. Moreover, sympathetic activity contributes to the regulation of adipose signaling by changing expression of adipokines (such as leptin) and cytokines (such as tumor necrosis factor-α) (15–17, 24). Here, we show that acute cold exposure (3–6 h at 4 °C) up-regulates OEA synthesis in WAT. This response is prevented in vivo by the β-antagonist propranolol and reproduced in vitro by noradrenaline and the β-adrenergic agonist isoproterenol, suggesting that noradrenaline may evoke OEA synthesis through direct stimulation of β-receptors on white adipose cells.

Beyond the plausible implication of cAMP, implied by forskolin’s ability to increase OEA levels, the molecular mechanism linking β-receptor occupation to OEA synthesis remains undefined. In particular, a critical feature of this mechanism, which warrants explanation, is its remarkable tissue specificity. We found, indeed, that cold exposure does not increase OEA synthesis in intestine and liver and even reduces it in skeletal muscle, a phenomenon that might be responsible for the lowering of plasma OEA levels observed after cold challenge but whose mechanistic underpinnings and functional significance...
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are unclear. These questions notwithstanding, the finding that low ambient temperature selectively enhances adipose OEA synthesis suggests that this lipid mediator, which was previously shown to stimulate lipolysis in WAT (5), may also be involved in adipose signaling. Interestingly, cold exposure stimulates the synthesis of the OEA analog PEA, which has also been shown to activate PPAR-\(\alpha\) (7) and stimulate lipolysis in WAT (5).

The physiological function of OEA in WAT during cold exposure remains to be defined and will be the subject of future inquiry. One possibility is that OEA may help contribute to the adrenergic mobilization of fat stores during a cold challenge. When sympathetic activity is enhanced, as it happens during acute cold exposure, occupation of \(\beta\)-adrenergic receptors may generate OEA in fat, which may participate in \(\beta\)-receptor-dependent lipolysis. We have previously shown that OEA-induced lipolysis occurs in a PPAR-\(\alpha\) dependent manner (5). This result suggests that PPAR-\(\alpha\), which is present in WAT in small but significant levels (27), may also contribute to the response of adipose tissue to cold challenges. Of note, however, OEA also activates, at least under pharmacological conditions, PPAR-\(\beta\) \textit{in vitro} (but not PPAR-\(\gamma\)) (6), TRPV-1 (28), and GPR119 (29), raising the possibility that these receptors may also serve as targets for OEA during cold challenges. Irrespective of the answers to these questions, our finding that cold stimulation induces OEA biosynthesis in white adipose tissue provides a physiological framework for the actions of this lipid-derived mediator in adipocytes.

REFERENCES