

Oleyethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR- α

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Oleyethanolamide (OEA) is a naturally occurring lipid that regulates satiety and body weight^{1,2}. Although structurally related to the endogenous cannabinoid anandamide, OEA does not bind to cannabinoid receptors and its molecular targets have not been defined. Here we show that OEA binds with high affinity to the peroxisome-proliferator-activated receptor- α (PPAR- α), a nuclear receptor that regulates several aspects of lipid metabolism. Administration of OEA produces satiety and reduces body weight gain in wild-type mice, but not in mice deficient in PPAR- α . Two distinct PPAR- α agonists have similar effects that are also contingent on PPAR- α expression, whereas potent and selective agonists for PPAR- γ and PPAR- β/δ are ineffective. In the small intestine of wild-type but not PPAR- α -null mice, OEA regulates the expression of several PPAR- α target genes: it initiates the transcription of proteins involved in lipid metabolism and represses inducible nitric oxide synthase, an enzyme that may contribute to feeding stimulation. Our results, which show that OEA induces satiety by activating PPAR- α , identify an

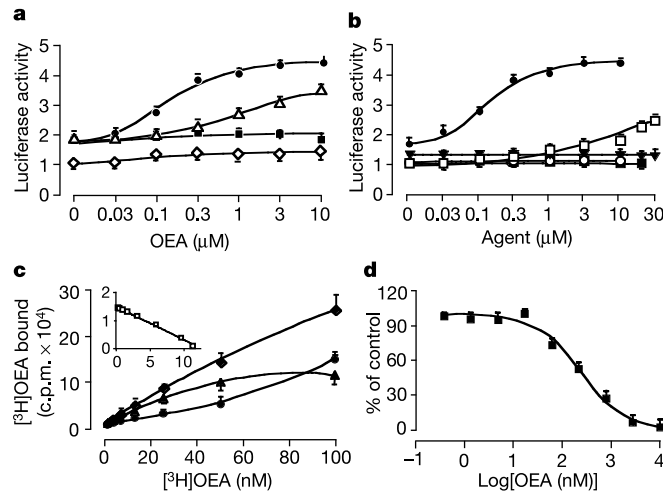


Figure 1 OEA is a high-affinity PPAR- α agonist. **a**, **b**, Activation of human PPAR- α by OEA. **a**, OEA activates human PPAR- α (filled circles) and PPAR- β/δ (open triangles), but not PPAR- γ (filled squares) or RXR (open diamonds). **b**, Effects of OEA (filled circles), oleic acid (open squares), stearyl ethanolamide (filled triangles), myristylethanolamide (filled squares) and anandamide (open circles) on PPAR- α activation. **c**, Saturation binding of [³H]OEA to purified mouse PPAR- α LBD. Shown are total binding (filled diamonds), nonspecific binding (filled circles) and specific binding (filled triangles). The inset shows a Scatchard transformation (bound – [bound/free]) of the specific binding data. **d**, Competition binding of OEA to purified mouse PPAR- α LBD. Results are the mean \pm s.e.m. of 16 (**a**, **b**) or 10 (**c**, **d**) experiments.

unexpected role for this nuclear receptor in regulating behaviour, and raise possibilities for the treatment of eating disorders.

Because OEA resembles natural compounds that activate PPARs, such as oleic acid^{3–5}, we considered that it may interact with a member of this nuclear receptor family^{6–8}. To test this idea, we engineered HeLa cells to express a luciferase reporter gene together with the ligand-binding domain (LBD) of human PPAR- α , PPAR- β/δ , PPAR- γ or retinoid-X receptor (RXR) fused to the yeast GAL4 DNA-binding domain⁹. In transactivation assays, OEA activated PPAR- α with a half-maximal concentration (EC₅₀) of 120 \pm 1 nM and activated PPAR- β/δ with an EC₅₀ of 1.1 \pm 0.1 μ M (n = 16), whereas it had no effect on PPAR- γ or RXR (Fig. 1a).

To study the structural selectivity of PPAR- α activation, we tested several OEA analogues. As previously reported^{3–5}, oleic acid activated the receptor with micromolar potency (EC₅₀ = 10.3 \pm 0.21 μ M; n = 16), whereas stearyl ethanolamide, myristylethanolamide and anandamide¹⁰ had no effect (Fig. 1b). The synthetic agonists Wy-14643 (ref. 11) and GW7647 (ref. 12)

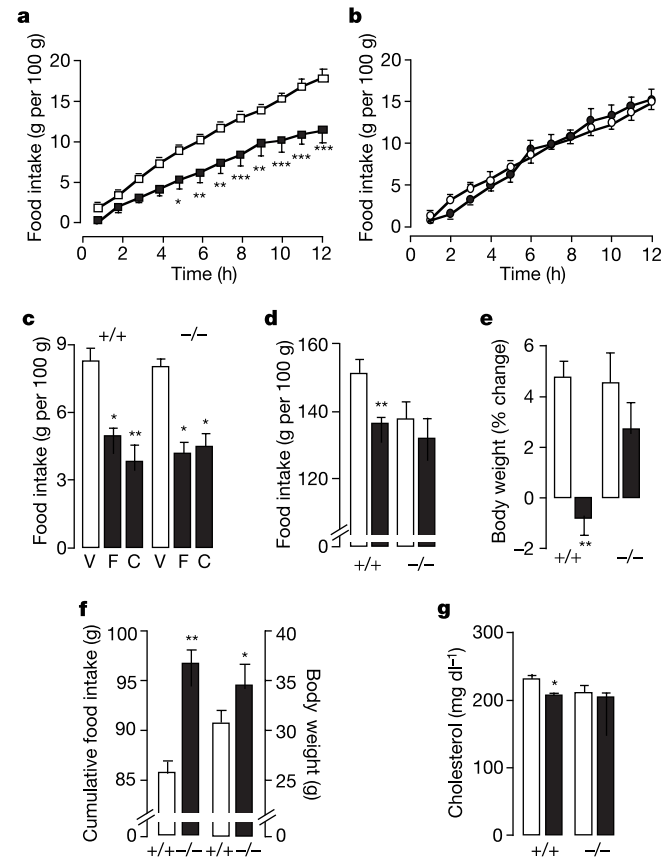


Figure 2 PPAR- α mediates the appetite-suppressing and weight-reducing effects of OEA. **a–c**, OEA reduces feeding in wild-type but not in PPAR- α ^{–/–} mice. **a**, **b**, Time course of the effects of OEA (filled squares; 10 mg per kg i.p.) and vehicle (open squares) on total food intake in wild-type (n = 6–11; **a**) and PPAR- α ^{–/–} (n = 5–8; **b**) mice. **c**, Effects of vehicle (V), *D*-fenfluramine (F; 4 mg per kg i.p.) and cholecystokinin-8 (C; 25 μ g per kg i.p.) on food intake in wild-type and PPAR- α ^{–/–} mice (n = 4–9). **d**, **e**, OEA reduces food intake and body weight gain in obese wild-type but not PPAR- α ^{–/–} mice. Shown are the effects of OEA (filled bars; 5 mg per kg i.p.) and vehicle (open bars) on food intake (**d**) and body weight gain (**e**). **f**, PPAR- α ^{–/–} mice fed with a high-fat diet develop hyperphagia. Food intake and body weight in wild-type and PPAR- α ^{–/–} mice before OEA treatment (n = 4–9). **g**, Effects of OEA (filled bars; 5 mg per kg i.p.) and vehicle (open bars) on serum cholesterol levels in obese wild-type and PPAR- α ^{–/–} mice. * P < 0.05 and ** P < 0.001 (n = 6–8) by one-way ANOVA followed by Dunnett's test or Student's *t*-test (**c–g**).

engaged PPAR- α with EC₅₀ values of $1.4 \pm 0.1 \mu\text{M}$ and $150 \pm 20 \text{ nM}$, respectively ($n = 5$). Saturation binding studies showed that [³H]OEA associates with the purified LBD of mouse and human PPAR- α (Fig. 1c), with dissociation constants (K_d) of $37.4 \pm 0.1 \text{ nM}$ (mouse, $n = 10$) and $43.3 \pm 1.6 \text{ nM}$ (human, $n = 8$). Binding competition experiments yielded a half-maximal inhibitory concentration (IC₅₀) for OEA of $120.0 \pm 10.7 \text{ nM}$ ($n = 10$; Fig. 1d). The results indicate that OEA is a high-affinity agonist of PPAR- α .

To test whether PPAR- α activation contributes to the appetite-suppressing properties of OEA, we used mice deficient in PPAR- α (PPAR- $\alpha^{-/-}$ mice)^{13,14}. OEA reduced feeding in wild-type mice, but not in PPAR- $\alpha^{-/-}$ mice (Fig. 2a, b), although the two strains showed comparable tissue concentrations of OEA (Supplementary Table 1) and similar responses to other appetite suppressants, such as D-fenfluramine and cholecystokinin-8 (Fig. 2c). In addition to its short-term effects on feeding, OEA produces in rats a sustained inhibition of body weight gain¹. To determine the role of PPAR- α in this response, we fed wild-type and PPAR- $\alpha^{-/-}$ mice with a high-fat chow and, once they had become obese, treated them daily with OEA for 4 weeks. OEA reduced total food intake and suppressed body weight gain in wild-type mice, whereas it had no effect in PPAR- $\alpha^{-/-}$ mice (Fig. 2d, e). Notably, whereas mutant mice receiving a standard chow maintain normal body weight^{13,14}, those fed with a high-fat diet ate more and gained more weight than did wild-type mice (Fig. 2f). Thus, PPAR- α expression may not be necessary only for the satiety-inducing and weight-reducing actions of OEA, but also for the maintenance of feeding homeostasis.

Does OEA modulate feeding through direct activation of PPAR- α ? To address this question, we used three synthetic PPAR- α agonists of different potency^{11,12}: Wy-14643 and GW7674, which engage PPAR- α *in vitro* with potencies comparable to that of OEA, inhibited food intake in C57BL/6J mice (Fig. 3a), whereas the weaker agonist clofibrate did not (25–100 mg per kg body weight; data not shown). Meal pattern analyses showed that the effects of Wy-14643 and GW7647 were due to prolonged eating

latency, rather than to changes in meal size or post-meal interval (Fig. 3b). This response is identical to that elicited by OEA (Fig. 3b) and is indicative of a satiety-inducing action².

OEA is thought to produce satiety by activating peripheral sensory fibres¹. Accordingly, in rats in which these fibres had been removed either by cutting the vagus nerve below the diaphragm or by treatment with the neurotoxin capsaicin¹, OEA had no effect on food intake (Fig. 3c and data not shown). These procedures also prevented the effect of Wy-14643 (Fig. 3d, e, and Supplementary Table 2), whereas they did not affect that of D-fenfluramine, which reduces appetite through a central mechanism (Fig. 3c).

The close correspondence between the effects of OEA and those of synthetic PPAR- α agonists is reinforced by two findings. First, potent agonists of PPAR- β/δ (GW501516)¹⁵ and PPAR- γ (ciglitazone)¹⁶ did not affect feeding in C57BL/6J mice (Fig. 3f); and second, PPAR- $\alpha^{-/-}$ mice did not respond to Wy-14643 (Fig. 3g, h). We cannot exclude the possibility that PPAR- β/δ , which weakly recognizes OEA *in vitro* (Fig. 1a), may contribute to some OEA responses; however, the fact that the PPAR- β/δ agonist GW501516 does not affect food intake implies that the role of PPAR- β/δ in OEA signalling, if any, is distinct from that of PPAR- α .

To test further the ability of OEA to activate PPAR- α , we examined its effects on the expression of PPAR- α target genes. We focused first on the small intestine, which is a likely site of action of OEA¹ and contains large amounts of PPAR- α ^{17,18}. In the jejunum of C57BL/6J mice, OEA increased the expression of three genes regulated by PPAR- α (Fig. 4a–c): those encoding PPAR- α , fatty acid translocase (FAT/CD36) and fatty acid transport protein 1 (FATP1)^{17,19}. A similar effect was observed in the liver (Fig. 4e–g) and the duodenum, but not in the ileum (Supplementary Figs 1 and 2). By contrast, OEA did not affect the expression of three additional genes that are not controlled by PPAR- α (Fig. 4d).

Underscoring the role of PPAR- α in these responses, we found that Wy-14643 mimicked the effects of OEA (Fig. 4a–g) and that neither OEA nor Wy-14643 changed gene expression in PPAR- $\alpha^{-/-}$ mice (Fig. 4a–c, e–g). In addition to stimulating transcription, PPAR- α also represses genes such as that encoding inducible nitric

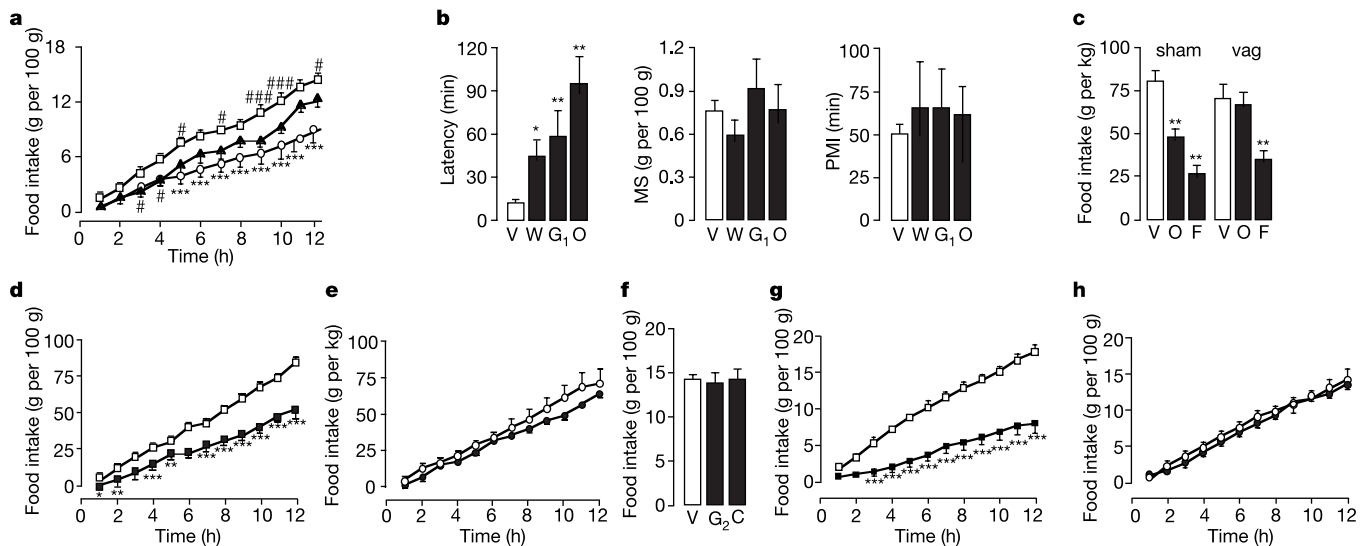


Figure 3 PPAR- α agonists mimic the appetite-suppressing effects of OEA. **a**, Effects of vehicle (open squares), Wy-14643 (filled triangles; 40 mg per kg i.p.) and GW7647 (open circles; 20 mg per kg i.p.) on food intake in C57BL/6J mice (vehicle, $n = 40$; drugs, $n = 4-7$). **b**, Effects of vehicle (V), Wy-14643 (W; 40 mg per kg i.p.), GW7647 (G₁; 20 mg per kg i.p.) and OEA (O; 10 mg per kg i.p.) on feeding latency, first meal size (MS) and first post-meal interval (PMI) in C57BL/6J mice (vehicle, $n = 40$; drugs, $n = 4-7$). **c**, Effects of vehicle (V), OEA (O; 10 mg per kg i.p.) and D-fenfluramine (F; 3 mg per kg subcutaneous) on food intake in control (sham) and vagotomized (vag) rats. **d, e**, Time

course of the effects of vehicle (open symbols) and Wy-14643 (filled symbols; 40 mg per kg i.p.) on food intake in control (**d**) and vagotomized (**e**) rats. **f**, Lack of effect of the PPAR- β/δ agonist GW501516 (G₂; 5 mg per kg i.p.) and PPAR- γ agonist ciglitazone (C; 15 mg per kg i.p.) on food intake in C57BL/6J mice ($n = 4-6$). **g, h**, Time course of the effects of vehicle (open symbols) and Wy-14643 (filled symbols; 40 mg per kg i.p.) on food intake in wild-type (**g**) and PPAR- $\alpha^{-/-}$ (**h**) mice ($n = 7-11$). * $P < 0.05$, ** $P < 0.001$ and *** $P < 0.0001$ by one-way ANOVA followed by Dunnett's test or Student's *t*-test with Bonferroni's correction.

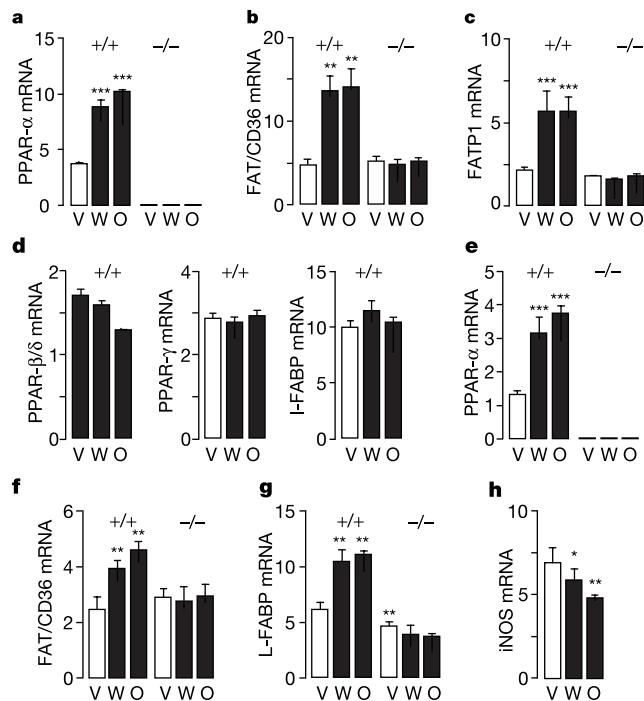


Figure 4 OEA regulates gene expression through PPAR- α activation. **a–d**, Effects of vehicle (V), Wy-14643 (W; 30 mg per kg i.p.) and OEA (O; 10 mg per kg i.p.) on messenger RNA levels of PPAR- α (**a**), FAT/CD36 (**b**), FAFP1 (**c**) and PPAR- β/δ , PPAR- γ and L-FABP (**d**) in the jejunum of wild-type and PPAR- $\alpha^{-/-}$ mice ($n = 5$). **e–g**, Effects of vehicle (V), Wy-14643 (W; 30 mg per kg i.p.) and OEA (O; 10 mg per kg i.p.) on mRNA levels of PPAR- α (**e**), FAT/CD36 (**f**) and L-FABP (**g**) in the liver of wild-type and PPAR- $\alpha^{-/-}$ mice ($n = 5$). **h**, Transrepression of iNOS expression by OEA (O; 10 mg per kg i.p.) and Wy-14643 (W; 30 mg per kg i.p.) in the jejunum of C57BL/6J mice ($n = 5$). mRNA levels are expressed in arbitrary units. * $P < 0.05$ and ** $P < 0.001$ by one-way ANOVA followed by Dunnett's test.

oxide synthase (iNOS)²⁰. Accordingly, in the jejunum of C57BL/6J mice, OEA and Wy-14643 decreased iNOS expression (Fig. 4h). These results indicate that OEA closely mimics the genomic actions of PPAR- α agonists.

If OEA enhances expression of PPAR- α target genes, it should also reproduce the corrective effects of PPAR- α agonists on serum lipid levels^{21–23}. Consistent with this prediction, in genetically obese Zucker (*fal*) rats, a 2-week regimen of OEA administration decreased serum cholesterol and triglyceride along with food intake and body weight gain (Supplementary Fig. 3). In addition, 4 weeks of OEA treatment lowered cholesterol in obese wild-type mice²⁴, but not in PPAR- $\alpha^{-/-}$ mice (Fig. 2g). We conclude that long-term OEA administration causes metabolic changes similar to those produced by PPAR- α agonists, which are abrogated by PPAR- α deletion.

The functional similarity between OEA and PPAR- α agonists suggests that OEA is a natural ligand for PPAR- α . This possibility is supported by the concerted regulation of OEA synthesis and expression of PPAR- α and iNOS. In the small intestine of C57BL/6J mice, OEA concentrations were significantly lower at night (01:30), when the mice feed, than during the day (16:30), when they are satiated and resting (Fig. 5a, b). The expression of PPAR- α paralleled OEA concentrations, whereas that of its transrepression target iNOS showed the opposite pattern (Fig. 5c, d). Notably, daytime intestinal concentrations of OEA were in the range needed to activate PPAR- α fully *in vitro* (~300 nM), suggesting that they may be adequate to engage the receptor *in vivo*.

In conclusion, our findings indicate that OEA shows all of the defining characteristics of an endogenous PPAR- α agonist: first, it binds with high affinity to PPAR- α ; second, it mimics the actions of

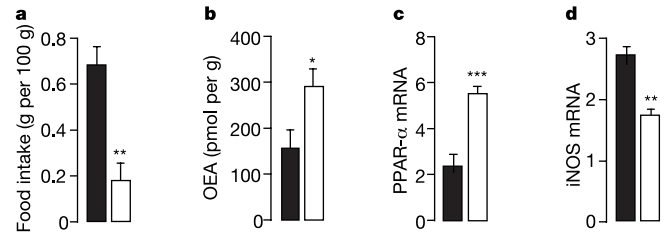


Figure 5 Circadian regulation of intestinal OEA synthesis and PPAR- α expression. Food intake (**a**), OEA content (**b**), and PPAR- α (**c**) and iNOS (**d**) mRNA levels at 01:30 (filled bars) and 16:30 (open bars) in free-feeding C57BL/6J mice maintained on a 12-h/12-h dark/light cycle.

synthetic agonists in a PPAR- α -dependent manner; and last, under appropriate conditions, it reaches tissue concentrations that are sufficient to activate PPAR- α . The results also suggest that PPAR- α activation not only mediates OEA-induced weight stabilization, which may be expected from the metabolic roles of this receptor^{6–8}, but is also responsible for OEA-induced satiety, a behavioural role that was not previously attributed to PPAR- α . The molecular mechanism underlying this response is still undefined, but one possibility is that it may involve the transcriptional regulation of intestinal NO production. Intestinal epithelial cells contain the enzyme iNOS and generate significant amounts of its product, NO, which may act as a peripheral appetite-stimulating signal^{25,26}. The ability of OEA to reduce iNOS expression through PPAR- α suggests that downregulation of NO signalling may contribute to the persistent satiety-inducing actions of this lipid mediator. □

Methods

Chemicals

We synthesized GW7647 (ref. 12) and GW501516 (ref. 27) by modifications to the published procedures (see Supplementary Information) and synthesized OEA as described²⁸. All other chemicals were purchased from Sigma or Tocris.

Animals

We purchased male C57BL/6J, PPAR- $\alpha^{-/-}$ (129S4/SvJae-*Ppara*^{tm1Gonz}) and wild-type 129S1/SvImJ mice (Jackson Laboratories), male Zucker rats (Charles River) and vagoatomized Sprague-Dawley rats (Zivic Laboratories). Animals were maintained on a 12-h/12-h light/dark cycle and had free access to water and RMH 2500 chow (Prolab).

Transactivation assays

We generated plasmids containing the LBD of human PPAR- α (nucleotides 499–1,407), PPAR- β/δ (412–1,323), PPAR- γ (610–1,518) and RXR (403–1,389) fused to the DNA-binding domain of yeast GAL4 under control of the human cytomegalovirus promoter and to a neomycin resistance gene to provide stable selection with 0.2 mg ml⁻¹ G418 (Calbiochem). We cultured HeLa cells in DMEM medium supplemented with fetal bovine serum (10%), transfected them with 3 μ l of Fugene 6 (Roche) containing 1 μ g of pFR-luc plasmid (Stratagene), and replaced the media after 18 h with DMEM containing 0.1 mg ml⁻¹ hygromycin (Calbiochem). After 4 weeks, we isolated surviving clones, analysed them by luciferase assay, and selected for transfection a cell line that showed the highest luciferase activity. Cells were maintained in DMEM containing hygromycin and G418.

For transactivation assays, we seeded cells in six-well plates and incubated them for 7 h in DMEM containing hygromycin and G418, plus appropriate concentrations of test compounds. We used a dual-luciferase reporter assay system (Promega) and an MIX Microtiter plate luminometer (Dynex) to determine luciferase activity in cell lysates.

Binding assays

We constructed a pGEX- α plasmid containing the gene encoding glutathione S-transferase (GST) fused to the LBD of human PPAR- α by subcloning the LBD fragment into pGEX-4T (Amersham Biosciences) digested with *Eco*RI. *Escherichia coli* strain BL21 (Novagen) was cultured in 2XYT medium and induced for overexpression by the addition of 0.1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG). The IPTG-induced culture was grown at 30 °C for an additional 6 h and cells were collected by centrifugation. The GST-LBD fusion protein was purified from the cell pellet by using glutathione-Sepharose beads (Amersham Biosciences), as recommended by the manufacturer.

For binding assays, the GST-LBD fusion protein (50 nM) was incubated at 25 °C for 2 h in buffer containing 50 mM HEPES, pH 7.0, 50 mM KCl, 5 mM EDTA, 10 mM dithiothreitol (DTT), 0.1% bovine serum albumin and 15 Ci mmol⁻¹ [³H]OEA (ARC). Free and bound [³H]OEA were separated on a Sephadex G-25 spin column (Amersham Biosciences) and radioactivity in the bound [³H]OEA fraction was measured. Nonspecific binding was determined in the presence of non-radioactive OEA (50 μ M).

RNA isolation and complementary DNA synthesis

We stored tissues in RNALater (Ambion), extracted total RNA with TRIzol (Invitrogen) and quantified it with Ribogreen (Molecular Probes). We synthesized cDNA with SuperscriptIII RNase H reverse transcriptase (Invitrogen).

Polymerase chain reaction

Reverse transcription of 2 µg of total RNA was carried out with 0.2 µg of Oligo(dT)₁₂₋₁₈ primer for 50 min at 42 °C, and real-time quantitative polymerase chain reaction was done with an ABI PRISM 7700 sequence detection system (Applied Biosystems). We designed primer/probe sets with Primer Express software (Applied Biosystems) and gene sequences available from GenBank database. Primers and fluorogenic probes were synthesized by TIB Molbiol.

The primer/probe sequences for the mouse genes were as follows. For PPAR-α: forward (F), 5'-CTTCCCAAAGCTCCTTCAAAAA-3'; reverse (R), 5'-CTGCGGATGCTCCGGTG-3'; probe (P), 5'-TGGTGGACCTTCGGCAGCTGG-3'. For PPAR-β/δ: F, 5'-GATGACA GTGACCTGGCGCT-3'; R, 5'-AGGCCTGGCCGGTCTC-3'; P, 5'-TTCATCGCGCCAT CATCTGTGT-3'. For PPAR-γ: F, 5'-AGTGAGACCGCCAGG-3'; R, 5'-GCAGCAGG TTGCTTGGATGT-3'; P, 5'-TTGCTGAACGTGAAGCCCATCGA-3'. For FAT/CD36: F, 5'-CGCGATGAGAAAGCAGAA-3'; R, 5'-CAACCAGGCCAGGAGC-3'; P, 5'-TGTTCAGAAACCAAGTGACCGGAAATAA-3'. For FATP1: F, 5'-GCACAGCAGG TACTACCGCA-3'; R, 5'-GGCGGACGATGC-3'; P, 5'-TGCTGCCTTGGCCACCA TTCCTA-3'. For L-FABP: F, 5'-TCACCATCACCTATGGACCA-3'; R, 5'-TCCAGTTCCG CACTCTCC-3'; P, 5'-AGTGGTCCGCAATGAGTTCACCC-3'. For GAPDH: F, 5'-TCACTGGCATGGCCTCC-3'; R, 5'-GGCGGACGTCAGATCC-3'; P, 5'-TTCCTA CCCCNAATGTGTCCTCGTCC-3'. RNA levels were normalized by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard, and measurements were made as described²⁹.

Feeding experiments

For acute experiments, we administered drugs or vehicles (saline for CCK-8 and D-fenfluramine, dimethylsulphoxide (DMSO)/saline (70/30) for all other agents; 4 ml per kg intraperitoneally (i.p.)) at 17:00–17:30 to free-feeding animals habituated to the experimental setting. Feeding was monitored for 12 h by an automated system (SciPro)².

For chronic experiments, we fed male wild-type and PPAR-α^{-/-} mice with a D12492 high-fat diet (60 kcal % fat; Research Diets) for 7 weeks. Body mass indices³⁰ were 0.36 ± 0.01 g cm⁻² for wild-type (n = 13) and 0.41 ± 0.01 g cm⁻² for PPAR-α^{-/-} (n = 15) mice. We divided the mice into four groups (n = 7–8 each) and treated them for 4 weeks with vehicle (saline/polyethylene glycol/Tween 80 (90/5/5); 1 ml per kg) or OEA (5 mg per kg i.p., once daily). In a separate experiment, we treated obese Zucker rats for 2 weeks with vehicle or OEA (5 mg per kg i.p., once daily), while maintaining them on RMH 2500 regular chow (ProLab). We measured food intake and body weight daily.

For capsaicin deafferentation, male Wistar rats were treated with capsaicin as described¹. The rats were deprived of food for 24 h and given Wy-14643 (2–40 mg per kg i.p.) or vehicle. Food pellets and spillage were measured manually 0.5–4 h after drug injection.

Biochemical analyses

We measured OEA levels by high-performance liquid chromatography coupled with mass spectrometry³, and serum lipids with a commercial kit (Wako).

Statistical analyses

Results are expressed as the mean ± s.e.m of n separate experiments. The significance of differences between groups was evaluated by one-way analysis of variance (ANOVA) followed by a Dunnett's test for multiple comparisons, or Student's *t*-test with or without Bonferroni's correction. Analyses were done with GraphPad Prism software (GraphPad).

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Proton-sensing G-protein-coupled receptors

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Blood pH is maintained in a narrow range around pH 7.4 mainly through regulation of respiration and renal acid excretion^{1,2}. The molecular mechanisms involved in pH homeostasis are not completely understood. Here we show that ovarian cancer G-protein-coupled receptor 1 (OGR1), previously described as a receptor for sphingosylphosphorylcholine³, acts as a proton-sensing receptor stimulating inositol phosphate formation. The receptor is inactive at pH 7.8, and fully activated at pH 6.8—site-