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The ubiquitous environmental pollutant perfluorooctanoic acid inhibits feeding behavior via peroxisome proliferator-activated receptor-α

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Received October 4, 2007; Accepted December 5, 2007

Abstract. Perfluorinated compounds (PFCs) have been employed as surface treatment agents in a variety of products. Perfluorooctanoic acid (PFOA), a PFC that is found globally in the environment and in human tissues, has been increasing significantly in serum levels over the past 50 years. Here, we demonstrated that PFOA inhibits feeding behavior as potently as the endogenous peroxisome proliferator-activated receptor (PPAR)-α ligand, oleoylethanolamide (OEA), via the activation of PPAR-α, the vagal nerve and hypothalamic neuropeptides. Peripherally administered PFOA decreased food intake as potently as OEA. PFOA decreased gastric emptying and increased the expression level of the gene encoding urocortin 1 in the hypothalamus and the immunoreaction for urocortin 1 in the paraventricular nucleus. Vagotomy attenuated the inhibitory effects of PFOA on feeding. The inhibition of food intake and body-weight gain by PFOA was completely mitigated in PPAR-$\alpha^{-/-}$ mice. Our studies demonstrated that the ubiquitous environmental pollutant PFOA works as an imitator of OEA mimicking its action in the feeding regulatory system, providing a new mode of action as represented by environmental 'anorexigens'.

Introduction

Perfluorinated compounds (PFCs) have been industrially manufactured for over 50 years. Perfluorooctanoic acid (PFOA) is one of the most commonly occurring PFCs in the environment and human tissues (1,2). PFOA is resistant to degradation in soil, oil and water; hence, it has been employed as a surface-treatment agent in a variety of products such as metal plating and cleaning, coating formulations, fire-fighting foams, polyurethane production, inks, varnishes, vinyl polymerization, lubricants, and gasoline, and water repellents for leather, paper, and textiles (1,2). PFOA is a compound in which all carbon-hydrogen (C-H) bonds are replaced by carbon-fluorine (C-F) bonds (1,2). The C-F bond is one of the strongest bonds in nature and confers physical, thermal and chemical stabilities to most PFCs. Due to its broad application and environmental stability, PFOA is globally distributed and is bioaccumulative (3,4).

Several studies have reported the recent trend of accumulation of PFOA in individuals and pooled human serum samples from various countries (5,6). Olsen *et al* reported that 3M employees involved in PFOA production had up to 6.16 mg/l PFOA in their blood; in the US, the level of PFOA varies in the general population from <1.9-52.3 μg/l, and in children, from <1.9-56.1 μg/l (7,8). PFCs are readily absorbed but poorly eliminated. The half-life of PFOA in humans is estimated to be 3.8 years (9). It has been shown that PFC levels in human serum have increased significantly in recent years (6,10).

Animal toxicological studies have revealed that these chemicals have a potent anorexigenic effect in rodents and monkeys (11,12), but the mechanism underlying this effect is unknown. As predicted by the structural similarity of PFOA to free fatty acids and their derivative oleoylethanolamide (OEA), its high affinity to peroxisome proliferator-activated receptor-α (PPAR-α) has been confirmed (13,14). A reasonable conjecture based on its high affinity to PPAR-α predicts that the anorexic effect of PFOA is mediated by PPAR-α as that of OEA is (15). In the present study, we tested this hypothesis.

Materials and methods

Animals and chemicals. The study protocol was approved by the Animal Research Ethics Committee of Kyoto University's Institutional Review Board. Animals were kept and handled according to the guidelines of the Animal Research Committee, Graduate School of Medicine, Kyoto University.

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Key words: perfluorinated compounds, oleoylethanolamide, vagal nerve, hypothalamic neuropeptides

We used male ddy mice (34-37 g, 8-9 weeks of age; Japan SLC Inc., Shizuoka, Japan), and female PPAR-$\alpha^{-/-}$ mice (129S4/SvJae-Ppara^{tm1Gonz}, 16-22 g, 12-13 weeks of age; kindly provided by Professor F.J. Gonzalez) and wild-type 129S1/SvImJ mice (17-21 g, 8-9 weeks of age; Japan SLC Inc.). The mice were housed individually in a regulated environment (24±2˚C, 50±10% humidity, 14 h/10 h light/dark cycle with lights on at 7:00 a.m.). Food and water were available *ad libitum* except as indicated. Animals were used only once each in the experiment. Pentadecafluorooctanoic acid ammonium salt (PFOA; FW, 431.10; purity ≥98%) and oleoylethanolamide (OEA; FW, 325.5; purity ≥98%) were purchased from Fluka Chemical Corp. (WI, USA), and Cayman Chemicals (MI, USA), respectively. The Yanaihara Institute Inc. (Shizuoka, Japan) provided antibodies against urocortin 1. For acute experiments, we administered drugs or vehicles [physiologic saline containing 1% dimethylsulfoxide (DMSO) or containing 37% ethanol for PFOA and physiologic saline containing 37% ethanol for OEA; 100 μl volume administered intraperitoneally (i.p.)] to mice habituated to the experimental setting. For gavage administration, PFOA was diluted in distilled water and administered in a 10 ml/kg volume.

Feeding tests. Before feeding tests, mice were deprived of food for 16 h with free access to water, or were given free access to food and water. A standard diet (F-2, 3.73 kcal/g, Funahashi Farm Corp., Chiba, Japan) was used except in the experiment testing the effect of vagotomy on feeding suppression induced by PFOA, which used a liquid diet (Oriental Yeast Co. Ltd., Tokyo, Japan). In experiments with food-deprived mice, drugs were administered at 10:00 a.m. In dark-phase feeding studies with non-food-deprived mice, drug administrations were performed immediately before lights-off (9:00 p.m.). The drug was dissolved in physiologic saline containing 1% DMSO to a final volume of 100 μl for i.p. administration. Food intake was measured by subtracting uneaten food from initially pre-measured food at 20 min, and at 1, 2, 4, 12 and 24 h after administration, and by checking the food spillage.

Gastric emptying. Before the experiments for gastric emptying, mice were deprived of food for 16 h with free access to water. The fasted mice had free access to pre-weighed pellets for 1 h; they were then i.p. administered PFOA (30-100 μmol/kg) or vehicle. The mice were deprived of food again for 2 h after drug administration. Food intake was measured by weighing the uneaten pellets. Mice were sacrificed by cervical dislocation 3 h after the start of experiments. Immediately after sacrifice, the stomach was exposed by laparotomy, quickly ligated at both the pylorus and cardia, then removed; at this point the dry content was weighed. Contents were dried using a vacuum freeze-drying system (Model 77400; Labconco Corp., MO, USA). Gastric emptying was calculated according to the following formula: gastric emptying (%) = [1-(dry weight of food recovered from the stomach/weight of food intake)] x 100.

Real-time reverse transcription polymerase chain reaction. Mice were deprived of food for 16 h with free access to water. Mice were treated with PFOA (30 μmol/kg) or vehicle every 2 h for 4 h, with the third and final administration being 30 min before the mice were sacrificed by cervical dislocation. Immediately afterwards, the hypothalamic block was removed, frozen on dry ice, and stored at -80˚C until use in real-time reverse transcription polymerase chain reaction (real-time RT-PCR). Using an RNeasy Mini Kit (Qiagen Inc., Tokyo, Japan) RNA was isolated from the hypothalamic block. Quantification of mRNA levels was performed by SYBR-Green chemistry (Qiagen Inc.) using a one-step RT-PCR reaction on a Sequence Detection System (ABI PRISM 7700; Applied Biosystems Japan, Tokyo, Japan). Reactions were performed under the standard conditions recommended by the manufacturer. We used the mouse GAPDH gene as an internal control. All expression data were normalized to the level of GAPDH expression from the same individual sample. The following primers were used for real-time RT-PCR: GAPDH forward, ATGGTGAAGGTCGGTGTGAA and reverse, GAGTGGAGTCATACTGGAAC; neuropeptide Y (NPY) forward, TTTCCAAGTTTCCACCCTCATC and reverse, AGTGGTGGCATGCATTGGT; agouti-related protein (AGRP) forward, GAGTTCCCAGGTCTAAGT CTGAATG and reverse, ATCTAGCACCTCCGCCAAAG; orexin A forward, CGTAACTACCACCGCTTTAGCA and reverse, TGCCATTTACCAAGAGACTGACAG; melanin-concentrating hormone (MCH) forward, GGAAGATACTG CAGAAAGATCCG and reverse, ATGAAACCGCTCTC GTCGTT; cocaine- and amphetamine-regulated transcript (CART) forward, GCAGATCGAAGCGTTGCAA and reverse, TTGGCCGTACTTCTTCTCGTAGA; proopiomelanocortin (POMC) forward, GGCTTGCAAACT CGACCTCT and reverse, TGACCCATGACGTACTTCCG; corticotropin-releasing factor (CRF) forward, CGCAGCCC TTGAATTTCTTG and reverse, TCTGTTGAGATTCCC CAGGC; urocortin 1 (UCN1) forward, ACTGTCCATCGA CCTCACCTTC and reverse, AAGGCTTTCGTGACCCC ATA; urocortin 2 (UCN2) forward, CCTCAGAGAGCTC CTCAGGTACC and reverse, GGTAAGGGCTGGCTTTAG AGTTG; and urocortin 3 (UCN3) forward, CGCACCTC CAGATCAAAAGAA and reverse, GGGTGCTCCCAGCT CCAT.

Immunohistochemistry. Mice were deprived of food for 16 h with free access to water. They were then subjected to i.p. administration of PFOA (100 μmol/kg) or vehicle. The mice were anesthetized with sodium pentobarbital (80-85 mg/kg i.p.) and perfused with 4% paraformaldehyde, 0.5% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer 90 min after administration. The brains were removed and post-fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. The brains were cut into 20-μm coronal sections using a cryostat. Sections cut through the nucleus of the solitary tract (NTS) of PFOA- or vehicle-administered mice were prepared for immunohistochemistry using a c-fos antibody (rabbit polyclonal, Oncogene Research Products, CA, USA) with ABC and DAB methods. Sections cut through the paraventricular nucleus (PVN) were prepared for immunofluorescence staining for urocortin 1. Brain sections were incubated with a urocortin 1 antibody (rabbit polyclonal, Yanaihara Institute Inc.) and then incubated

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-labeled anti-rabbit IgG (Chemicon International nunoreactivity was observed by light microscopy (Olympus DX51; Olympus Optical Co. Ltd., Tokyo, Japan) or using a laser scanning microscope (LSM 510; Carl Zeiss Inc. Japan, Tokyo, Japan).

Truncal vagotomy. Four days before experiments, truncal vagotomy was performed as follows. The mice were anesthetized with sodium pentobarbital (80-85 mg/kg i.p.). After making a midline incision of the abdominal wall, the stomach was covered with sterile gauze moistened with warm saline. The lower part of the esophagus was exposed and the anterior and posterior branches of the vagal nerve were incised. At the end of the operation, the abdominal wall was sutured in two layers. In sham-operated mice, vagal trunks were similarly exposed, but not cut. Vagotomized and sham-operated mice were maintained on a nutritionally complete liquid diet. Completeness of vagotomy was verified during postmortem inspection. Mice were fixed for enzyme histochemistry and loss of acetylcholineesterase-positive fibers in the gastrointestinal tracts was determined by light microscopic observation.

Repeated administrations. Repeated oral administrations of PFOA (50 μmol/kg/day) were continued for 6 days in PPAR-$\alpha^{-/-}$ and wild-type 129S1/SvImJ mice. The mice were administered PFOA daily at 8:00 p.m. Food intake and body weight were measured daily. Serum was separated from blood obtained from the orbital sinus under ether anesthesia at the end of the experiment.

Statistical analysis. To avoid multiple comparisons, we employed ANOVA when the number of groups was larger than two. An ad hoc test using Scheffé's method or the Student's t-test was used to compare the means of two specific groups only when ANOVA was significant ($P<0.05$). When the number of groups was two, the Student's t-test was employed. Results are expressed as mean ± SE. $P<0.05$ was considered to be statistically significant.

Results

To investigate whether or not PFOA influences feeding behavior, we first examined the effects of the intraperitoneal injection of PFOA on feeding in food-deprived mice. PFOA produced inhibitory effects on feeding behavior in a dose-dependent manner (Fig. 1A). Next, we compared the inhibitory effects of PFOA and OEA at the same molar concentration (100 μmol/kg) on feeding in the dark-phase. By 4 h after administration, PFOA decreased the level of feeding as potently as OEA (Fig. 1B). We also examined whether or not PFOA influenced the gastric emptying rate. In this study, PFOA administered i.p. significantly delayed gastric emptying 2 h after administration (Fig. 1C).

In order to evaluate the possible action of PFOA through the hypothalamic pathway, we examined the effects of PFOA administered i.p. on the expression of genes encoding hypothalamic neuropeptides. Real-time RT-PCR analysis revealed an increase in the level of urocortin 1 expression by 51.2% compared with controls (Fig. 2A) after i.p. PFOA

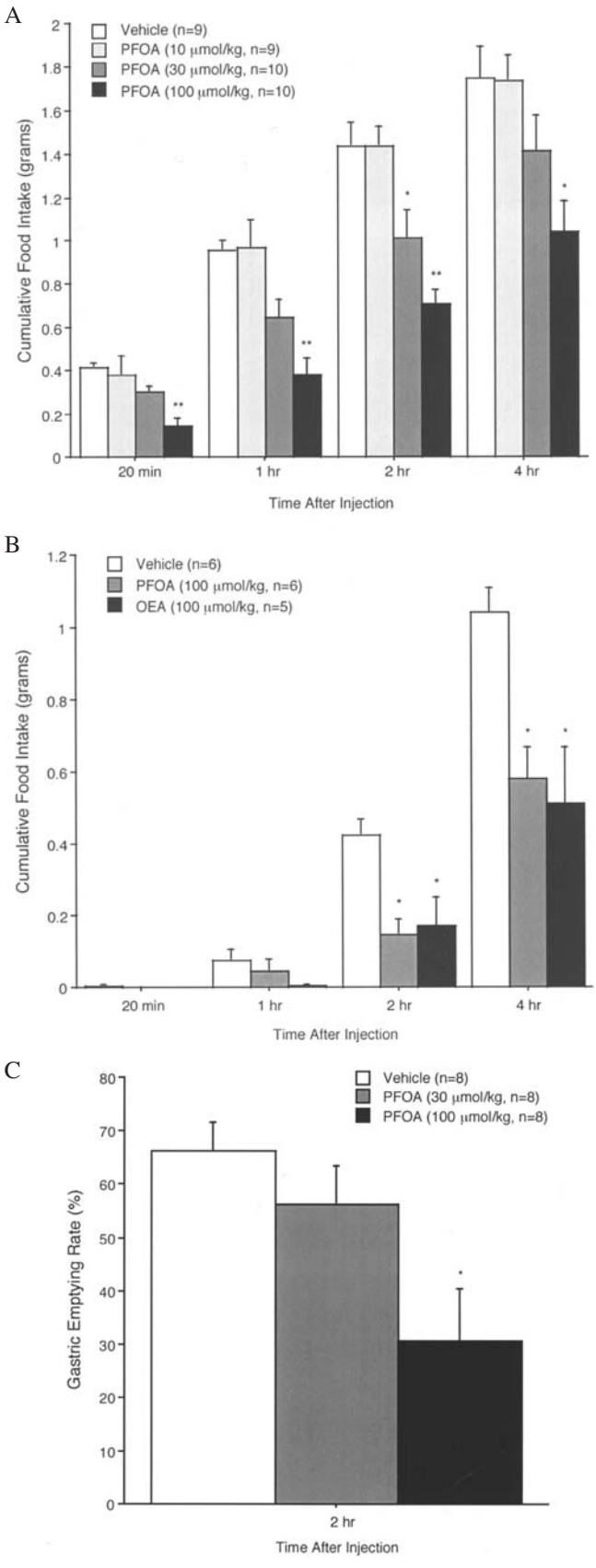
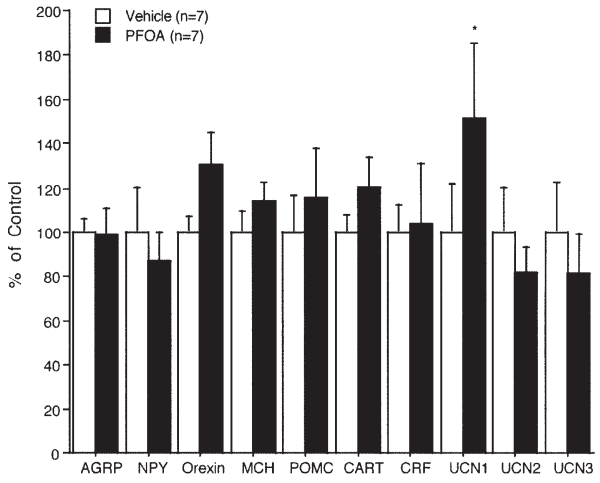


Figure 1. (A) Inhibitory effects of ip-administered PFOA (10-100 μmol/kg) on cumulative food intake in food-deprived mice. (B) The inhibitory effects of PFOA and OEA (i.p., 100 μmol/kg) on cumulative food intake in non-food-deprived mice, in dark-phase feeding studies. (C) Inhibitory effects of PFOA (i.p., 30 and 100 μmol/kg) on the gastric emptying rate during the first 2 h after administration. Each bar represents the mean ± SE. n, number of mice used. $^{*}P<0.05$; $^{**}P<0.01$ compared with the control group by Scheffé's method after ANOVA.

A



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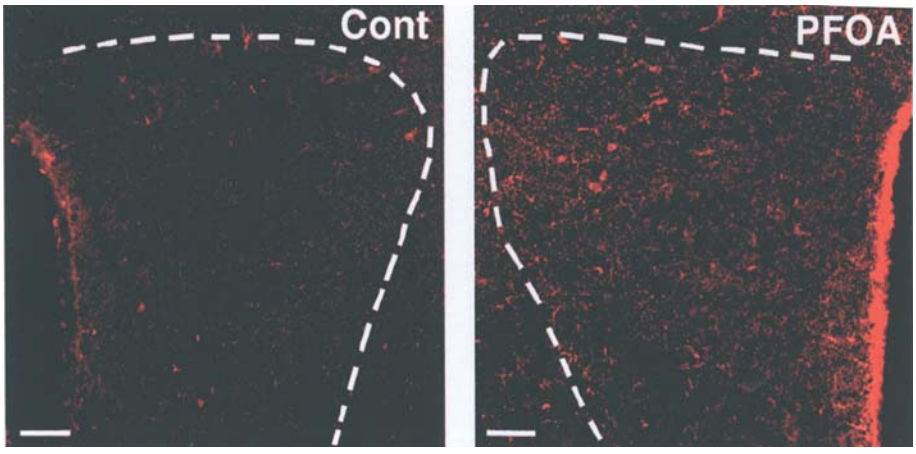

Figure 2. (A) Effects of PFOA (i.p., 100 μmol/kg) on hypothalamic peptide mRNA levels as assessed by real-time RT-PCR in food-deprived mice, and expressed as a percentage of the level in vehicle-treated controls. Each bar represents the mean ± SE. n, number of mice used. *P<0.05 compared with the control group by the Student's t-test. (B) Photomicrographs of an immunohistochemical demonstration of urocortin 1 in the PVN (n=3-4). Scale bars, 50 μm.

administration. We then tested whether or not PFOA increased the level of urocortin 1 in the PVN. As expected, immunohistochemical studies for urocortin 1 showed that, compared with controls, PFOA increased the immunoreactivity for urocortin 1 in the PVN (Fig. 2B).

Using mice that had undergone truncal vagotomy, we investigated whether the inhibitory effect of PFOA on feeding is associated with a vagally mediated pathway. As shown in Fig. 3A, vagotomy attenuated the inhibitory effects of i.p. PFOA administration on feeding. Moreover, exogenous PFOA increased the level of c-fos expression [40.9±5.33 vs. 20.5±1.97 number/section (control), n=4, P<0.02] in the NTS (Fig. 3B).

We also examined whether or not i.p.-administered PFOA inhibits food intake in PPAR-$\alpha^{-/-}$ mice. While food intake (Fig. 4A) and body weight (Fig. 4B) decreased significantly in wild-type mice after oral PFOA administration, neither food intake nor body weight decreased (Fig. 4C and D) in PPAR-$\alpha^{-/-}$ mice. There were no significant differences in serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) between PFOA-administered animals (mean ± SE, IU/l): 162±62.9 for wild-type mice and 127±23.3 for PPAR-$\alpha^{-/-}$ mice (ANOVA, P>0.05) for ALT; 330±120 for wild-type mice and 149±14.1 for PPAR-$\alpha^{-/-}$ mice (ANOVA, P>0.05) for AST.

Discussion

We demonstrated that peripherally administered PFOA inhibited feeding behavior. It has been reported that OEA, an anorexigenic lipid mediator (15,16), is produced in a variety of tissues, including the small intestine, where the endogenous levels decrease during fasting and increase upon re-feeding (15-17). OEA, administered i.p. or by an oral route, decreases the level of food intake in mice and rats (15-17). Prior to the onset of the dark phase, OEA also decreases the level of food intake in non-food-deprived mice (15). In our study, PFOA administered i.p. decreased food intake in the dark phase as potently as OEA. It has been shown that compounds that cause anorexia and cachexia delay gastric emptying as OEA inhibits the intestinal motility in mice (18-20). As expected, exogenous PFOA significantly decreased gastric emptying rate.

The discovery of leptin and ghrelin has helped elucidate the mechanisms underlying the regulation of food intake at

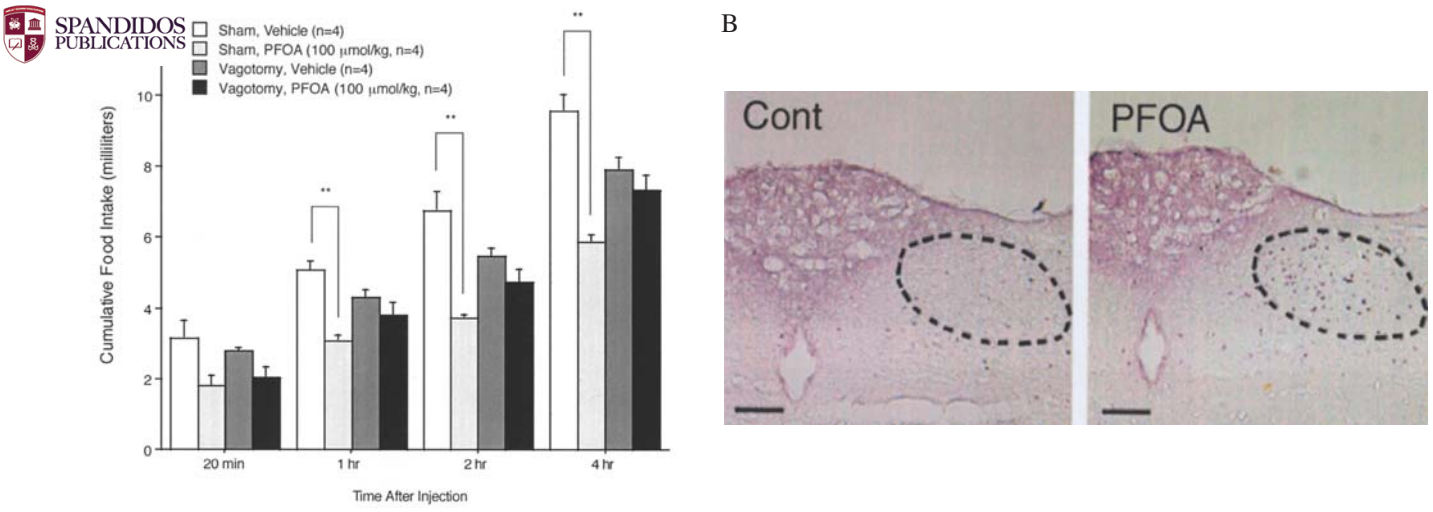

Figure 3. (A) Attenuation of the inhibitory effect of PFOA (ip, 100 μmol/kg) on food intake by vagotomy in food-deprived mice. Each bar represents the mean ± SE. n, number of mice used. **P<0.01 compared with the control group by Scheffé's method after ANOVA. (B) Stimulatory effects of i.p.-administered PFOA (100 μmol/kg) on c-fos expression in the NTS 90 min after administration (n=4). Scale bars, 50 μm.

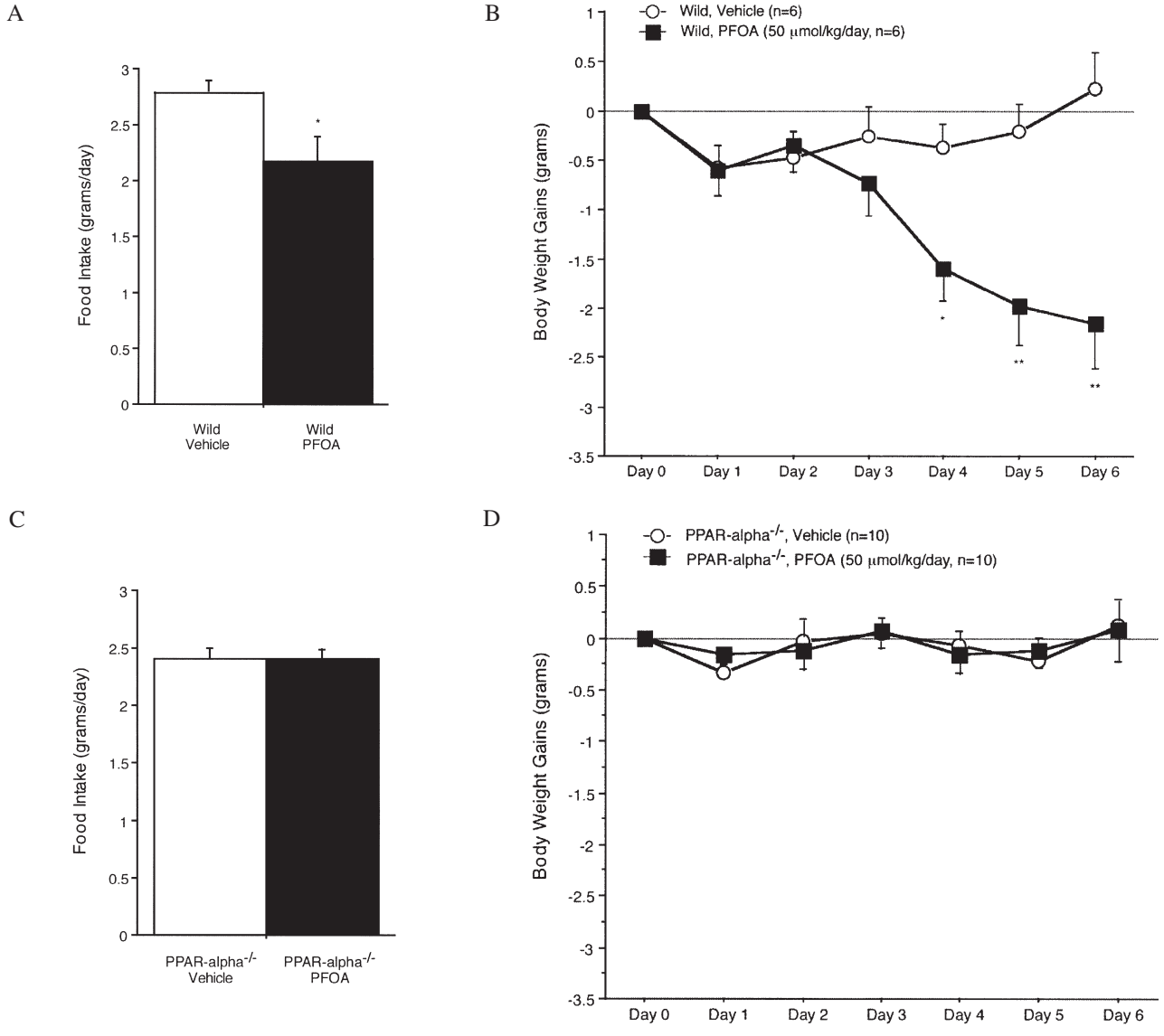

Figure 4. Inhibitory effects of PFOA (ip, 50 μmol/kg/day) on (A) average daily food intake and (B) body weight in wild-type 129S1/SvImJ mice. Effects of administered PFOA (i.p., 50 μmol/kg/day) on (C) average daily food intake and (D) body weight in PPAR-$\alpha^{-/-}$ mice. Each bar represents mean ± SE. n, number of mice used. *P<0.05; **P<0.01 compared with the control group by the Student's t-test.

the molecular level (21,22). Thus far, hypothalamic neuropeptides, including NPY, AGRP, orexin, MCH, POMC, CART, CRF, and urocortin, have been shown to be involved in the regulation of food intake (23). We found that the level of urocortin 1 expression was increased after i.p. PFOA administration. Urocortin 1 was the second ligand for the CRF receptor to be identified (24). Whereas CRF is mainly involved in the regulation of stress-related behavior and colonic motility, urocortin 1 is mainly involved in the regulation of feeding behavior and gastric motility (25). Previous studies have shown that urocortin 1 potently suppresses food intake and gastric emptying (25). Additionally, Rodriguez de Fonseca *et al* have reported that i.p.-administered OEA increases the level of c-fos expression in the PVN, which is the primary site for the coordination of central anorexigenic signals (16). In our study, PFOA increased the immunoreactivity for urocortin 1 in the PVN. Taken together, these results indicate that the anorexigenic activity of PFOA may be mediated, at least in part, via the activation of urocortin 1 in the PVN.

Previous studies have shown that peripheral satiety signals, including cholecystokinin, peptide YY, and pancreatic polypeptide, produce inhibitory effects on food intake via the activation of peripheral sensory fibers (19). Peripherally administered OEA fails to decrease food intake when those fibers have been removed either by truncal vagotomy or treatment with capsaicin (16). We found that vagotomy attenuated the inhibitory effects of PFOA administration on feeding suggesting a vagus pathway that transfers anorexigenic signals induced by PFOA to the PVN. Moreover, PFOA increased the level of c-fos expression in the NTS, which processes vagal sensory input to the central nervous system. Increased c-fos expression in the NTS also supports the vagal transfer of the anorexigenic signals elicited by PFOA as seen with OEA (16).

The PPARs belong to the superfamily of nuclear hormone receptors that are ligand-activated transcription factors and play a crucial role in the metabolism of lipids and carbohydrates (26). It has been suggested that PPAR-α has evolved to function as a key modulator of fatty acid catabolism during cellular fasting (26,27). It is now recognized as a predominant target for the fibrate class of antihyperlipidemic drugs, including bezafibrate and clofibrate (26). In addition, previous studies have shown that PPAR-α exhibits a high affinity for both PFOA and OEA (13,14). Several transactivation assays, reporter systems, and studies using different cell types have shown that human, mouse and rat PPAR-α are activated by PFOA (13,14). Moreover, PFOA activation is suppressed by PPAR-α antagonists (14). These data indirectly suggest that the anorexigenic effect of PFOA is mediated by PPAR-α. Thus, we tested the involvement of PFOA using PPAR-$\alpha^{-/-}$ mice. While oral PFOA administration decreased food intake and body weight in wild-type mice, neither food intake nor body weight decreased in PPAR-$\alpha^{-/-}$ mice. These observations indicate that PFOA elicits an anorexigenic effect through the binding of PPAR-α.

Our studies indicate that the ubiquitous environmental pollutant PFOA potently inhibits feeding behavior. This effect is mediated by PPAR-α, transferred by the vagal nerve, and is accompanied by increased expression of the anorexic peptide urocortin 1, and delays in gastric emptying. The total global historical industry-wide production of total PFOA for the period 1951-2004 is estimated to be 3600-5700 tons (28). The present study clearly demonstrates that PFOA may mimic the endogenous PPAR-α ligand, OEA (26,29). The present evidence, in turn, raises concern about the pharmacological action of PFOA in humans, since its effects might include various behavioral changes, not only in food intake but also in other reproductive and motor activities similar to OEA (30). We believe that further efforts are required to monitor the level of PFOA currently found ubiquitously worldwide.

Acknowledgements

This study was supported by a Grant-in-Aid for Health Sciences Research from the Ministry of Health, Labor and Welfare of Japan (H15-Chemistry-004), the Asahi Breweries Foundation and the Showa Shell Sekiyu Foundation (2005-A038).

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