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Levels of *N*-acylethanolamines in O,O,S-trimethylphosphorothioate (OOS-TMP)-treated C57BL/6J mice and potential anti-obesity, anti-diabetic effects of OOS-TMP in hyperphagia and hyperglycemia mouse models

Linfang Huang [a,b], Megumi Toyoshima [b], Akihiro Asakawa [b], Kayoko Inoue [b], Kouji Harada [b], Tomomi Kinoshita [c], Shilin Chen [a], Akio Koizumi [b,*]

[a] *Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 1000193, China*
[b] *Department of Health and Environmental Sciences, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan*
[c] *Graduate School of Engineering, Kyoto University, Kyoto 606-8510, Japan*

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ABSTRACT

O,O,S-Trimethylphosphorothioate (OOS-TMP) has been shown to induce hypophagia and hypopraxia. Recent studies suggest that OOS-TMP-induced anorexia is partly mediated by its effect on the central nervous system. In this study, we examined the profiles of *N*-acylethanolamines (NEAs), including five amide-linked compounds, in the gastrointestinal system in C57BL/6J (B6) mice. The present results shown an orexigenic profile of the levels of NEAs with downregulation of the anorectic lipid, *N*-stearoylethanolamine (SEA), upregulation of the orexigenic lipid, 2-arachidonoyl glycerol (2-AG), at 2 h and upregulation of 2-AG at 24 h albeit with significant anorexia. However, the data indicated that the high level of 2-AG may be responsible for the hypopraxia. We next explored whether OOS-TMP may affect two models of hyperphagia and hyperglycemia, *ins2*$^{+/Akita}$ B6 (Akita) and B6-*lepr*^{db}/*lepr*^{db} mice (db/db). We identified potential anorexigenic effects in B6, Akita and db/db mice. Moreover, OOS-TMP was found to reduce blood glucose in Akita mice but not in db/db mice. Collectively, these findings suggest that *N*-acylethanolamines are not involved in the hypophagia but rather hypopraxia, and may play multiple physiological roles in this process. OOS-TMP might be a promising candidate for anti-obesity and anti-diabetic drug development.

1. Introduction

O,O,S-trimethylphosphorothioate (OOS-TMP) is a contaminant in a number of widely used organophosphorous insecticides. It has been recognized as a unique environmental and lung toxicant that causes emaciation, lung injury, hypercapnia, hypothermia, hyperethanolaminuria and death owing to wasting without inhibition of AchE activity in mammalian species. (Koizumi et al., 1988; Aldridge et al., 1979; Verschoyle and Cabral, 1982; Imamura et al., 1983a,b; Aldridge et al., 1985; Gandy and Imamura, 1985; Umetsu et al., 1977).

Anorexia is one of the typical symptoms of the wasting syndrome caused by OOS-TMP administered by per os, intracerebroventricular (i.c.v.) or intraperitoneal (i.p.) routes, and is accompanied by hypothermia and hypopraxia (Huang et al., 2007; Ohtaka et al., 1995; Hasegawa and Koizumi, 1990).

Regulation of appetite and body weight is mediated by a complex physiological network involving both the central and peripheral nervous systems (Schwartz et al., 2000). In our previous study, both i.c.v. and i.p. injections transiently induced hypophagia at a dose of 5 mg/kg, with mild bronchial damages without alveolar injury. Hypophagia was accompanied by upregulation of corticotropin releasing factor (CRF) in the hypothalamus. At doses higher than 5 mg/kg, i.c.v. injection induced continuous hypophagia from 20 min to 72 h after dosing, accompanied by hypothermia and lung injury. OOS-TMP is thought to induce hypophagia by enhancing the expression of CRF in the hypothalamus (Huang et al., 2007). However, the role of the peripheral gastrointestinal system in hypophagia remains unclear.

N-acylethanolamines (NAEs) are a group of lipid mediator molecules with a wide range of biological effects. It is generally believed that they are formed from *N*-acylated phosphatidylethanolamines (NAPEs) (Schmid et al., 1990; Hansen et al., 2002; Schmid et al., 2002). Increasing evidence has demonstrated the ability of the *N*-acylethanolamines to control appetite, lipid homeostasis and energy balance. *N*-arachidonoylethanolamine (AEA), 2-arachidonoylglycerol (2-AG), *N*-palmitoylethanolamine (PEA), *N*-oleoylethanolamine (OEA) and *N*-stearoylethanolamine (SEA) belong

Abbreviations: OOS-TMP, O,O,S-trimethylphosphorothioate; i.c.v., intracerebroventricular; NEAs, *N*-acylethanolamines; PEA, *N*-palmitoylethanolamine; OEA, *N*-oleoylethanolamine; AEA, *N*-arachidonoylethanolamine (anandamide); 2-AG, 2-arachidonoyl glycerol; SEA, *N*-stearoylethanolamine; GC/MS, gas chromatography/mass spectrometry.

* Corresponding author. Department of Health and Environmental Sciences, Graduate School of Medicine, Kyoto University, Konoe-cho Yoshida Sakyo-ku Kyoto, 606-8501, Japan. Tel.: +81 75 753 4456; fax: +81 75 753 4458.
E-mail address: koizumi@pbh.med.kyoto-u.ac.jp (A. Koizumi).

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to the family of *N*-acylethanolamines that are generated by the enzyme *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D. The anandamide (AEA) and 2-AG, the first lipids identified to have orexigenic activities, are the main endogenous agonists of cannabinoid receptors found in the brain and other tissues associated with orexigenic effects (Pagotto and Pasquali, 2005; Engeli et al., 2005; Howlett et al., 2002). In contrast, some other anorectic lipid classes of structurally-related compounds, such as *N*-palmitoylethanolamine (PEA), *N*-oleoylethanolamine (OEA) and *N*-stearoylethanolamine (SEA), have been reported to be involved in the anorectic response (Terrazzino et al., 2004; Schmid et al., 2002).

In light of recent progress in understanding the regulation of eating behavior in the central nervous system, it is interesting to investigate the effects of OOS-TMP on the peripheral *N*-acylethanolamines levels in the gastrointestinal tract. Thus, one of the aims of the present study was to determine the levels of peripheral *N*-acylethanolamines including PEA, OEA, SEA, AEA, 2-AG that are closely associated with appetitive function, lipid homeostasis, behavior and energy balance in mice treated with OOS-TMP. In the present study, we used C57BL/6J (B6) mice, which are genetically more homogenous than ddY mice, which were not derived from an inbred mouse strain.

ins2$^{+/Akita}$ mice, a model of type 2 diabetes (Yoshioka et al., 1997), carries a C96Y mutation in the Ins2 gene on a B6 background (Wang et al., 1999). While B6-*lepr*^{db}/*lepr*^{db} (db/db) mice, a well-established genetic rodent model of hyperphagia, obesity, insulin resistance, is another model of type 2 diabetes (Hummel et al., 1966; Shao et al., 2000). As both of these diabetic models are characterized by hyperglycemia and hyperphagia, we intended to explore whether or not OOS-TMP has an influence in these mice.

2. Methods

2.1. Animals

The study protocol was approved by the Animal Research Committee of Kyoto University. All mice were handled in accordance with the Animal Welfare Guidelines of Kyoto University. We used male mice throughout this study. We purchased B6 and B6 background db/db and diabetic nonobese Akita mice(*ins2*$^{+/Akita}$) from Japan SLC, Inc. (Shizuoka, Japan). A total of 45 mice were housed individually and given free access to a pelletized chow and water during the acclimatization period, except when otherwise indicated. A standard commercial lab chow diet (F-2, 3.73 kcal/g, Funahashi Farm Corp., Chiba, Japan) was used. All animals were maintained at an ambient temperature of 24±2 °C and 50±10% humidity with a 12 h dark–light cycle (lights on at 7:00 AM).

2.2. Chemicals and OOS-TMP

All chemicals were of the purest analytical grade. OOS-TMP was synthesized and purified as previously described by Hasegawa and Koizumi (1990). The purity of the compound was found to be more than 99.8% as determined by NMR (JEOL JNM-EX400KS, JEOL Ltd. Akishima, Tokyo, Japan). PEA, OEA, AEA, 2-AG and SEA were purchased from Cayman Chemical Co. (Ann Arbor, MI).

2.3. i.c.v. cannulation

The surgical operation for i.c.v. cannulation was carried out as reported by Asakawa et al. (2001). Briefly, mice were anesthetized with sodium pentobarbital by i.p. injection (80–85 mg/kg) and fixed in a stereotaxic frame (SR-6, Narishige, Tokyo, Japan). A small hole was made in the skull using a needle inserted 0.9 mm lateral to the central suture and 0.9 mm posterior to the bregma. A 24-gauge cannula beveled at one end over a distance of 3 mm (Safelet-Cas, Nipro, Osaka, Japan) was put into the third cerebral ventricle for i.c.v. injection. The stainless-steel cannula was fixed to the skull with dental cement and capped with silicon without an obtruder. The animals were allowed to recover for 1 week before any experimental manipulation.

To ascertain the injection site that the drugs were injected exactly into the cerebral ventricle, at the end of all i.c.v. experiments, diluted India ink was injected through the cannula and animals were killed immediately by anesthetic overdose. Only those animals showing uniform distribution of ink into the ventricles were used for statistical analysis.

2.4. i.c.v. injection and experimental process

Conscious 7-week old animals were gently restrained by hand. A dose of 5 mg/kg of OOS-TMP was chosen because this dose induced hypophagia by upregulating of CRF without any toxicological signs (Huang et al., 2007). Four microliters of various concentrations of OOS-TMP dissolved in 5% dimethylsulfoxide (DMSO) in 0.9% saline were injected i.c.v. using a microsyringe via PE-20 tubing fitted with a 27-gauge needle that was inserted through the guide cannula to a depth of 3 mm below the external surface of the skull. Control animals received an equivalent volume of vehicle (5% DMSO in 0.9% saline). Before feeding tests, mice were fasted for 16 h with unlimited access to water. OOS-TMP or vehicle was singly administered by i.c.v. to food-deprived mice at 10:00 a.m. Food intake was recorded at 20 min and at 1, 2, 4, 6 and 24 h, and body weights were monitored at 0, 6, 12 and 24 h after i.c.v. injection. To determine the blood glucose levels, blood samples were collected from the tail vein immediately before fasting at between 18:00 and 19:00 pm, and a further sample was obtained from the orbital sinus at 10:00 am, i.e., 24 h after i.c.v. injection. The blood glucose levels were determined using a glucometer (Glutest Ace; Arkray Factory, Shiga, Japan), which used the glucose oxidase method. At the end of observation, mice were sacrificed by rapid decapitation. The intestines were removed quickly, rinsed with 2×1 ml ice-cold saline, and then immediately stored in liquid nitrogen at −70 °C until analyzed. In addition, we obtained and stored the lung to investigate lung injury.

2.5. Gas chromatography/mass spectrometer (GC/MS) analysis of N-acylethanolamines in the intestine

The intestinal content of *N*-acylethanolamines was determined by isotope-dilution GC/MS (Giuffrida and Piomelli, 1998; Felder et al., 1996). Lipid extraction and fractionation were performed as previously described (Schmid et al., 1995; Yang and Karoum, 1999) with a slight modification. Thawed intestine (0.6 g) was homogenized in 20 ml chloroform and spiked with an isotope mixture (5 μl) containing ^{13}C labeled 2-AG, AEA, PEA, SEA and OEA (500 pmol each) as internal standards. Then, 10 ml of 0.5% saline and 10 ml of methanol were added, shaken for 1 h and centrifuged at 15,000 ×*g* for 15 min. The lipid chloroform fraction was recovered; a further 10 ml of 0.5% saline and 10 ml methanol was added, and the mixture incubated for another 20 min and centrifuged as described above. This step was repeated twice. The lipid chloroform fraction was dried on a rotary evaporator at a temperature below 50 °C. The lipid extract was redissolved in 5 ml of chloroform and applied to a column (Presep-C Florisil, Wako Pure Chemical Industries. Ltd., Osaka, Japan). The lipid extracts were washed with hexane, 0.1% acetic acid in hexane and then 20% ethyl acetate in hexane, and eluted using 2% methanol in chloroform. The fractions eluted in 2% methanol in chloroform were collected and dried with a stream of nitrogen. The dried residues were derivatized by adding100 μL O-Bis(trimethylsilyl)trifluoroacetamide. The vials were tightly capped and heated at 50 °C for 60 min. After cooling to room temperature, the derivatives were dried under nitrogen, reconstituted in 50 μL of chloroform and vortex mixed. The extracts were injected into an Agilent Technologies 6890 N Network GC equipped with an HP-5MS column (30 m×0.25 mm i.d., Hewlett-

Table 1
Effects of a single i.c.v. injection of OOS-TMP (5 mg/kg) on gut lipids (B6)

			Anrexigenic lipids (pmol/g tissue)			Orexigenic lipids (pmol/g tissue)	
Time after dosing		Number of mice	PEA	OEA	SEA	AEA	2AG
2 h	Case	5	63.61±19.59	1.11±1.56	37.85±7.02 *	7.38±0.87	1719.58±599.69
	Controls	5	59.73±11.09	0.74±0.73	52.99±9.00	6.1±0.88	1290.49±119.90
24 h	Case	5	184.93±35.16	85.4622±89.83	13.362±4.97	0.497±0.59	3565.74±1164.00 *
	Control	5	232.83±68.56	200.43±103.35	12.41±5.05	0.93±0.92	1698.21±450.79

$n=5$ per group *$p<0.05$ vs. control.
*Significant ($p<0.05$).

Packard, Palo Alto, CA) in the splitless mode. Mass spectral data were acquired using the Agilent 5973 Network Mass Selective Detector. The oven temperature was increased from 150 °C to 280 °C at a rate of 10 °C per min. The M-15 ions were monitored using the selected ion monitoring mode.

2.6. Statistical analysis

For the levels of *N*-acylethanolamines, statistical analysis was performed using the Wilcoxon signed-rank test. One-way analysis of variance (ANOVA) was used for the cumulative food intake, relative body weight, and blood glucose level. When ANOVA was significant, the Duncan procedure was performed for multiple comparisons, All analyses were done using STATISTICA™ software (StatSoft®, Japan). A p value <0.05 was considered to be significant.

3. Results

3.1. Effects of a single i.c.v. injection of OOS-TMP on the levels of N-acylethanolamines in the intestine of B6 mice at 2 h and 24 h

Previous studies have shown that anorexia generated by OOS-TMP is partly mediated by its action on the central nervous system, while the profile of the peripheral gastrointestinal system is currently unclear. Therefore, we determined the levels of *N*-acylethanolamines (NEAs) including PEA, OEA, SEA. AEA, 2-AG fatty acid amide compounds in intestines (peripheral organ) to investigate whether OOS-TMP acts on the gastrointestinal system resulting in anorexia, hypopraxia.

We determined the levels of *N*-acylethanolamines (NEAs) in the intestines at 2 h and 24 h after administration of OOS-TMP. As shown in Table 1, at 2 h, quantification of NEAs by GC/MS revealed that that the level of SEA, an anorexic lipid mediator, was significantly decreased after treatment with OOS-TMP at a dose of 5 mg/kg compared with the control group, while its congeners, the other two types of orexic lipid mediators AEA and 2-AG, were slightly increased. On the other hand, at 24 h, the level of 2-AG, an orexigenic lipid mediator, was further increased (Table 1), while the levels of PEA and OEA, an anorexic molecule, declined to a lesser extent. No detectable changes were observed in SEA compared with the controls (Table 1).

The present results indicated that the orexigenic profile of the endocannabinoid levels was strengthened in the intestine with OOS-TMP at a dose of 5 mg/kg at 2 h and 24 h albeit with significant anorexia.

3.2. Effects of OOS-TMP on cumulative food intake, body weight gain and blood glucose in B6, Akita and db/db mice

Next, we investigated whether OOS-TMP induces comparable anorexigenic effects in the hyperphagic mouse models Akita and db/db mice. OOS-TMP significantly inhibited feeding in B6 at all time points, while feeding in the Akita and db/db mice had recovered somewhat by 4 h, indicating that the effects of OOS-TMP were less intensive in these hyperphagic mice. However, of interest is that at 24 h, OOS-TMP significantly inhibited food intake in these mice (OOS-TMP vs control (g); B6 mice: 0.28±0.4 vs 4.55±1.54; Akita: 1.02±0.45 vs 3.38±0.65; db/db:1.85±0.21 vs 5.35±0.86; Fig. 1A, B, C). It should be also pointed out that food consumption by Akita mice and db/db mice did not showed hyperphagia when compared with controls. However, the cumulative food consumption at 24 h in treated mice was significantly larger in Akita or db/db than in C57BL/6 ($p<0.05$).

After administration of OOS-TMP, mice displayed weight loss and decreased food intake. In particular, from 12 to 24 h a significant body weight reduction was observed in Akita mice, and db/db mice showed significant body weight loss at 24 h ($p<0.001$). The mean and SD of

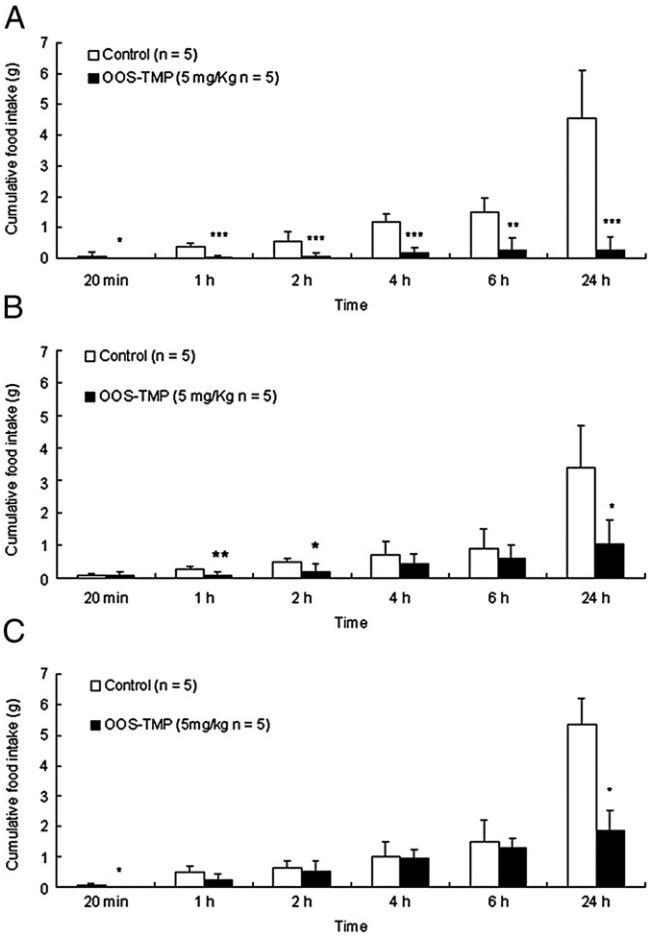

Fig. 1. Effects of a single i.c.v. injection of OOS-TMP (5 mg/kg) on cumulative food intake in B6, Akita and db/db mice from 20 min to 24 h. A. Effect of OOS-TMP on cumulative food intake in food-deprived B6 mice. $n=5$; *$p<0.05$ *vs.* control, **$p<0.01$ *vs.* control, ***$p<0.001$ *vs.* control; B. Effect of OOS-TMP on cumulative food intake in food-deprived Akita mice. $n=5$; *$p<0.05$ *vs.* control, **$p<0.01$ *vs.* control, ***$p<0.001$ *vs.* control; C. Effect of OOS-TMP on cumulative food intake in food-deprived db/db mice $n=5$; *$p<0.05$ *vs.* control, **$p<0.01$ *vs.* control, ***$p<0.001$ *vs.* control.

body weight (g) at 24 h were 23.4±2.25 for B6 mice, 19.98±0.99 for Akita mice and 44.97±1.00 for db/db mice (Fig. 2A,B,C).

Histological examination of the lungs form these mice revealed mild desquamation of the Clara cells in the bronchi as previously reported in ddY mice (Huang et al., 2007) and an absence of alveolar damage at 24 h (data not shown).

Blood glucose levels were significantly decreased at 24 h after OOS-TMP administration in Akita mice (n=5); 414.00±96.71 mg/dL before treatment and 138.00±139.40 mg/dL at 24 h after treatment (p<0.01). In contrast, blood glucose levels in Akita mice without treatment (n=5) were (509.00±90.21 mg/dL) before vehicle treatment and (538.75±70.74 mg/dL) at 24 h after vehicle treatment. In db/db mice, blood glucose levels were (n=5); 258.20±74.16 mg/dL before treatment and 347.60±108.34 mg/dL at 24 h after dosing (p>0.05) while in db/db control mice, they were (277.75±111.33 mg/dL) and (407.25±157.31 mg/dL) respectively (p>0.05).

4. Discussion

Appetite and energy homeostasis are regulated by various stimulatory (orexigenic) and inhibitory (anorexigenic) signaling pathways involving the central nervous system and the gastrointestinal system. Our previous study demonstrated that OOS-TMP induced anorexia, hypopraxia and enhanced the expression of corticotropin releasing factor (CRF), anorexigenic signalling, in the hypothalamus without apparent toxicity (Huang et al., 2007). However, the effects of *N*-acylethanolamines on the intestine have not been investigated. In this paper, sensitive and specific GC/MS assay was used to determine changes in intestinal levels of *N*-acylethanolamines, including PEA, OEA, SEA, AEA and 2-AG.

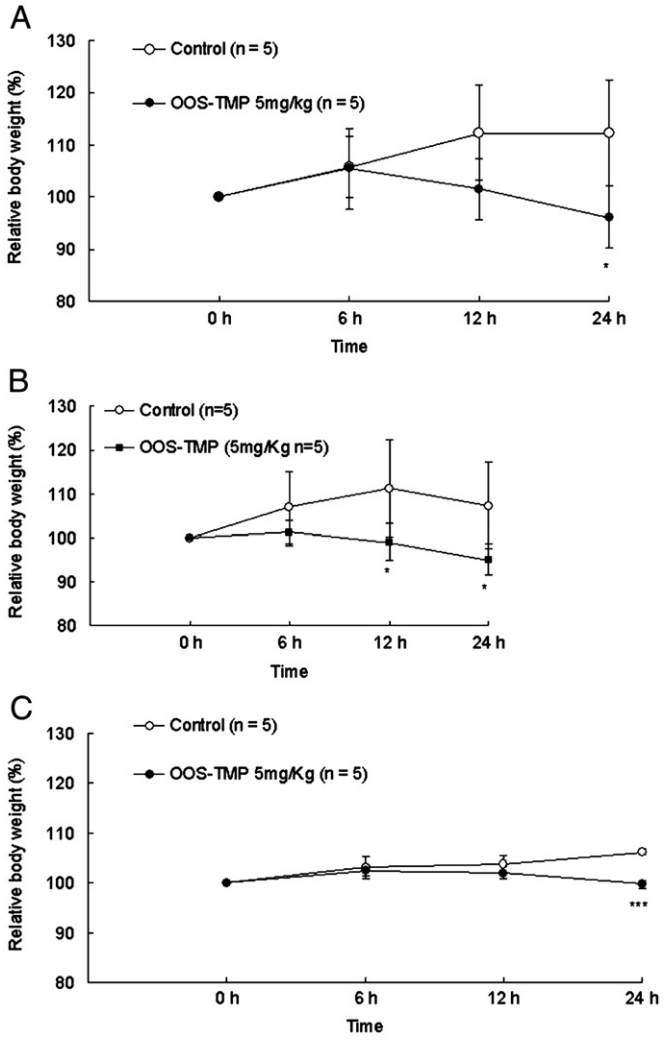

Fig. 2. Effects of a single i.c.v. injection of OOS-TMP (5 mg/kg) on relative body weight of B6 mice, Akita mice and db/db mice from 0 to 24 h. A. Effect of OOS-TMP on relative body weight in food-deprived *B6* mice. n=5; *p<0.05 *vs.* control, **p<0.01 *vs.* control, ***p<0.001 *vs.* control; B. Effect of OOS-TMP on relative body weight in food-deprived Akita mice. n=5*p<0.05 *vs.* control, **p<0.01 *vs.* control, ***p<0.001 *vs.* control; C. Effect of OOS-TMP on relative body weight in food-deprived db/db mice. n=5; *p<0.05 *vs.* control, **p<0.01 *vs.* control, ***p<0.001 *vs.* control.

It appears that *N*-acylethanolamines signalling via cannabinoid receptors modulates food intake, lipid homeostasis, behaviour and energy balance (Pacher et al., 2006; Cota et al., 2003; Kirkham et al., 2002; Onaivi et al., 2002). Endocannabinoids are implicated in appetite and body weight regulation. 2-AG was the first endocannabinoids to be identified, and is the arachidonate ester of glycerol, which activates both the CB_1 and CB_2 receptors (Mechoulam et al., 1995; Sugiura et al., 1995). Surprisingly, the present study revealed an orexigenic pattern in the intestine at both 2 h and 24 h. Importantly, 2-AG levels remained constantly higher than control at 2 and 24 h conditions, especially, at 24 h, with twofold increase in mice small intestines.

Recently, it has been suggested that 2-AG reduces spontaneous locomotor activity and rearing frequency in a dose-dependent manner (Darmani, 2002). Other studies in rodents have also shown that larger doses of 2-AG and AEA are motor suppressive and both compounds are equipotent in reducing spontaneous locomotor activity (Mechoulam et al., 1995). The present study confirmed that the reduction in locomotor activity requires larger doses of 2-AG as the 2-AG level was1719.58±599.69 pmol/g at 2 h with mice exhibited low locomotor activity and the level at 24 h was 3565.74±1164.00 pmol/g and the mice displayed typical hypopraxia. Given that one of important finding of the present investigation is that the high concentration of 2-AG may be responsible for the reduction in locomotor activity observed, there are two contradictory statuses, in that there was anorexigenic dominance in the central nervous system and orexigenic dominance in the gastrointestinal system. Taken together, it can be concluded that anorexigenic signals in the central nervous system may predominate eating behaviour. By contrast, the endocannabinoid system in the gastrointestinal system may be involved in the locomotor suppression reduced by OOS-TMP.

N-stearoylethanolamine (SEA) has recently been reported also to inhibit food intake by downregulating of liver gene expression of stearoyl-coenzyme A desaturase 1, while PPARα, PPARβ, and PPARγ expression was unaffected (Terrazzino et al., 2004). In the present study, however, we observed an orexigenic profile with significantly declined amount of SEA in intestines at 2 h but unchanged at 24 h after OOS-TMP treatment.

The possible physiological and pathological significance of the changes of *N*-acylethanolamines in the feeding-associated brain region including the hypothalamus, brain stem and limbic forebrain remains to be determined in the future. Particularly, the 2-AG level in the brain needs to be determined because it is found in both the gastrointestinal tract and the brain (Mechoulam et al., 1995).

The present study confirms the previously reported anorexigenic effect of OOS-TMP (Huang et al., 2007; Hasegawa and Koizumi, 1990; Koizumi et al., 1988). However, there are subtle inconsistencies: B6 control mice continued to be hypophagic at 24 h while early recovery in eating behavior was observed in ddY mice (Huang et al., 2007). This discrepancy may indicate a genetic difference in susceptibility to OOS-TMP between these two widely used mice strians. In fact, B6 mice are more genetically homogeneous than ddY mice. Another inconsistency may be related to the absence of hyperphagia in the Akita and db/db control mice that underwent i.c.v. surgery, but without administration of OOS-TMP, when compared with their counterpart control mice. This may be associated with susceptibility to surgery: Akita and db/db might be more susceptible to the damage associated with i.c.v.

surgery. However, when these three types of mice were compared at the 24 h timepoint, the Akita and db/db mice treated with OOS-TMP consumed more diet than the B6 mice ($p<0.05$). Therefore, these results are consistent with our previous observation in that the anorexigenic effects of OOS-TMP in hyperphagic mice, Akita (Toyoshima et al., 2007) and db/db mice were ameliorated compared with wild-type B6 mice.

In our experiment, there was a striking drop in blood glucose in the Akita diabetic mice and a loss of body weight at 24 h, the db/db, Akita and B6 mice after i.c.v. administration of OOS-TMP. This observation suggests that OOS-TMP may have potential anti-obesity and anti-diabetic-like effects in a variety of animal models of eating-related disorders. Comprehensive understanding of the molecular and biochemical mechanism of OOS-TMP-induced anorexia warrants further exploration.

In summary, the present data indicate that peripherally NEAs are not involved in the hypophagia but instead hypopraxia caused by OOS-TMP. We have also shown that the endocannabinoid 2-AG is the most sensitive to variation during feeding. Overall, these findings are consistent with previous reports and support the role of endocannabinoids in the physiological regulation of appetite, body weight and behavior control. Although we observed changes in endocannabinoid levels in the gastrointestinal system after OOS-TMP administration, the exact mechanisms for this remain unknown: it simply represents the fasting conditions in the gastrointestinal system and the effect of OOS-TMP on endocannabinoid metabolizing enzymes left unclear. Further studies are necessary.

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