

Roles of neuropeptides in *O,O,S*-trimethylphosphorothioate (OOS-TMP)-induced anorexia in mice [☆]

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Abstract

O,O,S-Trimethylphosphorothioate (OOS-TMP), an impurity present in various organophosphorus insecticides, has previously been shown to induce hypophagia. The major goal of this study was to investigate its mechanism of action. Both intracerebroventricular (i.c.v.) and intraperitoneal (i.p.) injection transiently induced hypophagia at a dose of 5 mg/kg within 6 h, without causing lung injury. Hypophagia was accompanied by up-regulation of corticotropin releasing factor (CRF) (2.92 ± 0.45 vs. 1.7 ± 0.5 , at 2 h after i.c.v., 3.40 ± 1.38 vs. 1.76 ± 0.41 at 6 h after i.p., $P < 0.05$) in the hypothalamus. After i.c.v. injection, hypophagia recovered by 6 h after dosing. 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 was considered to induce hypophagia through enhancing expression of CRF.

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O,O,S-Trimethylphosphorothioate (OOS-TMP) is an impurity found in a number of widely used organophosphorous insecticides [1–3]. It is suspected to have been a causative agent in the fatal accidents in malaria workers in Pakistan [4]. Toxicological investigations have unveiled the unique features of OOS-TMP: it causes lung injury and profound emaciation, a so-called wasting syndrome, without inhibition of acetylcholinesterase (AChE) activity [5–10].

Anorexia is one of the typical symptoms of the wasting syndrome caused by OOS-TMP, and is accompanied by hypothermia and lung injury. However, the underlying mechanism of this hypophagia remains an enigma. A previous study has shown that intracerebroventricular

(i.c.v.) administration in rats induced only hypophagia and slight weight loss without other symptoms [11], which suggests that the eating disorder caused by OOS-TMP is at least partly mediated by direct action on the central nervous system.

It is well established that the central nervous system plays a pivotal role in regulation of appetite and body weight. In the brain, the hypothalamus is a central feeding organ that mediates the regulation of short-term and long-term food intake and energy balance, via secretion and synthesis of various orexigenic and anorectic neuropeptides [12,13]. Among these orexigenic signals are orexin, neuropeptide Y (NPY), melanin-concentrating hormone (MCH), and agouti gene-related protein (AgRP), which potently stimulate appetite and decrease energy expenditure, whereas proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), corticotropin releasing factor (CRF), and urocortin (UCN) 1–3 are anorexigenic peptide molecules that reduce

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food intake and increase energy expenditure. To date, no information has been available on the role of the hypothalamus in the inhibition of food consumption by OOS-TMP. The principle aim of the present study was to explore OOS-TMP-induced hypophagia. We thus performed both i.c.v. and intraperitoneal (i.p.) injection; monitored food consumption, body temperature, and body weight; measured the mRNA expression levels of eating-related orexigenic and anorexigenic neuropeptides in the hypothalamus; and examined lung histopathology.

Materials and methods

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 6- to 7-week-old ddY mice from Japan SLC (Shizuoka, Japan). They were housed individually and given free access to a palletized chow and water during the acclimatization period, except when otherwise indicated. All animals were maintained at an ambient temperature of $24 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ humidity, with a controlled light/dark cycle (12:12 h).

Chemicals and OOS-TMP. All chemicals were of the purest analytical grade. OOS-TMP was synthesized and purified as previously described by Hasegawa et al. [10]. The purity of the compound was found to be $>99.8\%$ as determined by NMR (JEOL JNM-EX400KS, JEOL, Akishima, Tokyo, Japan).

LD₅₀. Mice were allowed to acclimatize to laboratory conditions for at least 3 days prior to experiment. Oral LD₅₀ of OOS-TMP at 120 h was determined by the method of Weil [14], using five doses of OOS-TMP and groups of four animals each. The selected doses were 0, 15, 30, 60, and 120 mg/kg per 2 ml corn oil. The animals which were fasted for 16 h prior to treatment received OOS-TMP by gavage between 9 and 10 a.m., and were observed for 120 h.

i.c.v. Cannulation and temperature-sensing chip. Surgical operation for i.c.v. cannulation was carried out as reported by Asakawa et al. [15]. 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 inserted 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 obturator. Temperature-sensing microchips (Digital Angel Corporation, Paul, MN, USA) were implanted in the interscapular region. The animals were allowed to recover for 1 week before any experimental manipulation.

i.c.v. Injection and experimental process. Conscious animals were gently restrained by hand. Four microliters of various concentrations of OOS-TMP, dissolved in 5% dimethylsulfoxide (DMSO) in 0.9% saline, were injected intracerebroventricularly 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 injection of vehicle. Before feeding tests, mice were fasted for 16 h with unlimited access to water. A standard diet (F-2, 3.73 kcal/g; Funahashi Farm Corp., Chiba, Japan) was used. OOS-TMP or vehicle was administered by a single i.c.v. injection to food-deprived mice at 10:00 a.m. Food intake and body temperature were recorded at 20 min and 1, 2, 4, 6, 24, 48, and 72 h, and body weight was monitored at 0, 6, 24, 48, and 72 h after i.c.v. injection. At the end of observation, blood samples were obtained from the orbital sinus under diethyl ether anesthesia. Mice were sacrificed by rapid decapitation. The hypothalamus was removed quickly and frozen at -70°C . Whole brains were frozen in liquid nitrogen immediately and kept at -70°C until use. Blood samples were centrifuged at 3000g for 15 min at 4°C , and sera were separated and

frozen at -70°C until analyzed. Lungs were excised from the carcass and fixed in 10% (v/v) neutral-buffered formalin.

i.p. Injection and experimental process. After fasting for 16 h, conscious mice received i.p. injection of OOS-TMP 5 mg/kg. Control animals received an equivalent dose of 5% DMSO in 0.9% saline. Food intake was recorded at 20 min and 1, 2, 4, and 6 h following i.p. injection. At 6 h, mice were sacrificed by rapid decapitation. The hypothalamus was dissected out quickly and frozen at -70°C until use. Lungs were excised from the carcass and fixed in 10% (v/v) neutral-buffered formalin.

Real-time RT-PCR analysis of gene expression in the hypothalamus. Hypothalamic gene expression was examined as previously reported [16]. Total RNA was extracted from the hypothalamus using an RNeasy Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan). Aliquots (10 ng) were amplified using QuantiTect SYBR Green RT-PCR (Qiagen, Tokyo, Japan). Quantification of amplified products was performed on an ABI PRISM 7700 Sequence Detection System purchased from Applied Biosystems Japan (Tokyo, Japan). All expression data were normalized to the expression level of mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) from the same individual samples.

Lung histopathology. Fixed lungs were processed routinely, embedded in paraffin wax, and their sections were stained with hematoxylin–eosin (HE).

Clinical chemistry. Serum CRP, LDH, and AchE were determined by conventional methods. In addition, we also determined brain AchE activity using brain homogenates in five volumes of phosphate buffer (pH 7.5) standardized to protein concentrations, as reported previously [10].

Statistical analysis. Data are presented as means $\pm$ SD. Statistical analyses of cumulative food intake and mRNA expression for neuropeptides were performed by the *t* test. As for the dose–response of cumulative food intake, body temperature change, and relative body weight, we performed one-way analysis of variance (ANOVA). When ANOVA was significant, the Duncan procedure was performed for multiple comparison. Statistical analysis used STATISTICA software (Stat-Soft, Japan). $P < 0.05$ was considered to be significant.

Results

LD₅₀

The 120 h oral LD₅₀ of OOS-TMP for mice was found to be 60 mg/kg (95% CI 40–89 mg/kg). Death occurred at 3–4 days following treatment. At all doses, animals presented with severe hypophagia and body weight loss (data not shown).

Dose–response and time course of cumulative food intake after i.c.v. injection

A dose–response relationship between food intake and dose of OOS-TMP is shown in Fig. 1A and B. All the treated animals had inhibited food intake. Mice dosed with OOS-TMP at 5 mg/kg ate significantly less food during the first 2 h in comparison with control mice, but recovered by 4 h. By contrast, at doses >5 mg/kg, animals revealed hypophagia and continued up to 72 h.

Changes in body temperature and weight after i.c.v. injection

The change in body temperature is summarized in Fig. 2A and B. OOS-TMP-induced hypothermia at 20 min, except at a dose of 5 mg/kg. At doses between 5 and 15 mg/kg, body temperature recovered by 6 h, but again, declined irreversibly. At

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