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Fenitrothion action at the endocannabinoid system leading to spermatotoxicity in Wistar rats

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ABSTRACT

Organophosphate (OP) compounds as anticholinesterase agents may secondarily act on diverse serine hydrolase targets, revealing unfavorable physiological effects including male reproductive toxicity. The present investigation proposes that fenitrothion (FNT, a major OP compound) acts on the endocannabinoid signaling system in male reproductive organs, thereby leading to spermatotoxicity (sperm deformity, underdevelopment, and reduced motility) in rats. FNT oxon (bioactive metabolite of FNT) preferentially inhibited the fatty acid amide hydrolase (FAAH), an endocannabinoid anandamide (AEA) hydrolase, in the rat cellular membrane preparation from the testis in vitro. Subsequently, male Wistar rats were treated orally with 5 or 10 mg/kg FNT for 9 weeks and the subchronic exposure unambiguously deteriorated sperm motility and morphology. The activity-based protein profiling analysis with a phosphonofluoridate fluorescent probe revealed that FAAH was selectively inhibited among the FNT-treated cellular membrane proteome in testis. Intriguingly, testicular AEA (endogenous substrate of FAAH) levels were elevated along with the FAAH inhibition caused by the subchronic exposure. More importantly, linear regression analyses for the FNT-elicited spermatotoxicity reveal a good correlation between the testicular FAAH activity and morphological indices or sperm motility. Accordingly, the present study proposes that the FNT-elicited spermatotoxicity appears to be related to inhibition of FAAH leading to overstimulation of the endocannabinoid signaling system, which plays crucial roles in spermatogenesis and sperm motility acquirement.

Introduction

Organophosphate (OP) insecticides are utilized throughout the world as an essential material for protecting crops, people, livestock, and companion animals from pest insect attack and disease transmission. OP compounds act on the cholinergic nervous system by inhibiting the acetylcholinesterase (AChE) as the primary target. The OP agent phosphorylates the serine hydroxy side chain at the AChE catalytic triad (Casida and Durkin, 2013). On the other hand, these compounds may secondarily react with many other serine hydrolase targets to show unexpected toxic effects (Casida and Quistad, 2004; Long and Cravatt, 2011).

Interestingly, the human male reproductive system is considered to be a secondary target for OP insecticides. Many epidemiological studies have suggested that environmental or occupational exposure to OP pesticides may cause: chromosome aneuploidy in human sperm (Padungtod et al., 1999); sperm chromatin alterations (Sanchez-Pena et al., 2004); increased DNA damage in human sperm (Meeker et al., 2004); and possible lower sperm concentration (Perry et al., 2007). Our previous report indicated that the lower semen quality (high percentages of slow progressive and non-progressive motile sperm) in the insecticide sprayers is likely relevant to pesticide applying work (Kamijima et al., 2004). In addition, animal reproductive toxicity studies demonstrated that male Wistar rats subchronically exposed (9 weeks) to an OP insecticide (dichlorvos at 5 or 10 mg/kg/day; diazinon at 3 mg/kg/day) exhibit spermatotoxicity such as broken spermatozoa (deformity), cytoplasmic droplets (underdevelopment), and reduced sperm motility, relating them to impaired function of the caput and cauda epididymides [these subchronic treatments led to 18% or 29% (dichlorvos at 5 or 10 mg/kg/day, respectively) and 32% (diazinon at 3 mg/kg/day) declines of the plasma cholinesterase activity] (Okamura et al., 2005, 2009). Further, a major OP insecticide fenitrothion (FNT) induces deleterious

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effects on the rat sperm and testes (Taib et al., 2013). However, the molecular target or mechanism for the spermatotoxicity has yet to be defined.

Our recent investigations from a chemical biology standpoint hypothesize that fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) is a potential OP target prompting spermatotoxicity in 10-week-old ICR mouse testis and epididymis cauda (Noro et al., 2013; Suzuki et al., 2013). FAAH and MAGL are hydrolyzing enzymes for endocannabinoid agonists, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), respectively, which activate the cannabinoid type-1 receptor (CB1R). The endocannabinoid signaling system, principally involving FAAH and AEA, plays crucial roles in spermatogenesis and sperm motility acquirement by regulating apoptosis or mitochondrial activity (Aquila et al., 2010; Lewis and Maccarrone, 2009; Lewis et al., 2012; Maccarrone, 2009a, 2009b). Consequently, the present study, using male rats subchronically exposed to a representative OP insecticide FNT, describes that the FNT spermatotoxicity (deteriorated sperm motility and morphology) is associated with the inhibition of FAAH leading to the overstimulation of the endocannabinoid signaling system. This may well be a possible mechanism for OP-elicited spermatotoxicity.

Material and methods

Chemicals. Sources of chemicals employed for the present investigation are listed as follows: FNT and FNT oxon (only used for potency evaluation as an inhibitor of testicular FAAH) were from Wako Pure Chemical (Osaka, Japan), fluorophosphonate (FP)-TAMRA fluorescent probe was from Pierce Biotechnology (Rockford, IL), [^{14}C]AEA and [^{14}C]mono-oleoylglycerol were from American Radiolabeled Chemicals (St. Louis, MO), anti-FAAH antibody, AEA, 2-AG, and deuterated 2-AG (2-AG-d_5) were from Cayman Chemical Company (Ann Arbor, MI), and deuterated AEA (AEA-d_4) was from Abcam (Cambridge, MA).

Animal experiment. This study was conducted according to Japanese law concerning the protection and control of animals and the guidelines for animal experiments of the Nagoya University animal center. A total of 28, 9-week-old male Wistar rats were obtained from Japan SLC (Hamamatsu, Japan). After a one-week acclimation period, they were randomly divided into 3 groups. The animal room temperature was kept at 23–25 °C, and the relative humidity was kept at 57–60%. Food and water were provided ad libitum.

The three groups were orally administered 5 or 10 mg/kg/day FNT dissolved in corn oil or vehicle as a control group 4 days per week for 9 weeks (an exposure condition sufficiently influencing spermatogenesis and/or sperm maturation while also minimizing OP-elicited neurotoxicity). On the day following the last administration, rats were weighed and euthanized by collecting the blood into heparinized tubes from the abdominal aorta under pentobarbital anesthesia. Testes and epididymides were removed and the cauda epidydimides were used for the evaluation of sperm count, motility, morphology, or ATP levels.

Sperm parameters. A cauda epididymis was pricked a few times with pointed forceps, and sperm were released into 2 ml of Hanks balanced salt solution (phenol red free) at 37 °C. These specimens were used for analysis of sperm motility, count and morphological parameters. The protocol was described elsewhere (Okamura et al., 2009). Percentages of motile sperm and progressive motile sperm, sperm count and normal or abnormal sperm morphology regarding shortened tail, tailless, headless, and cytoplasmic droplets were measured with a computer-assisted sperm analysis system (Hamilton Thorne Sperm analyzer HTM-IVOS, Hamilton Thorne Research, MA). Sperm ATP levels were analyzed using LuciPac and the attached lumitester (Kikkoman Biochemifa, Tokyo, Japan). ATP levels per sperm count are calculated.

FAAH and MAGL assays. FAAH or MAGL activity in rat testis was assayed by hydrolysis of the corresponding substrate [^{14}C]AEA or [^{14}C] mono-oleoylglycerol, respectively (55 mCi/mmol for both substrates) (Quistad et al., 2001, 2006). Briefly, the aliquot of cellular membrane preparation from the testis was incubated with 1 μM [^{14}C]AEA or [^{14}C] mono-oleoylglycerol for 30 min at 37 °C, and the enzymatic reaction was terminated by the addition of an organic solvent (chloroform: methanol:hexane, 1.25:1.4:1.0) and 200 mM K_2CO_3. Subsequently, the radioactivity in the aqueous upper phase, was determined in a liquid scintillation counter as the amount of the [^{14}C]arachidonic acid or [^{14}C]oleic acid produced from the enzymatic reaction. Potency of FNT oxon as an inhibitor of testicular FAAH or MAGL was also examined by the titration of FNT oxon (0.03–100 or 0.3–1000 μM for FAAH or MAGL) in the above assay systems. The half maximal inhibitory concentration (IC_{50}) value in the above assay systems was calculated by iterative nonlinear least-square regression using the Sigmaplot software Version 8.0 (SPSS, Chicago, IL).

In-gel analysis of enzyme activity. The effect of the subchronic FNT exposure on the rat testicular proteome [membrane fraction (1000 ×*g* supernatant and 20,000 ×*g* pellet) resuspended with 50 mM Tris–HCl buffer, pH 8.0) was examined by activity-based protein profiling, the in-gel analysis of enzyme activity (Long et al., 2009), with a fluorophosphonate (FP)-TAMRA fluorescent probe (Patricelli et al., 2001; Long and Cravatt, 2011). The FP-TAMRA consists of a fluorescent molecule TAMRA handle and a fluorophosphonate (FP) reactive group that specifically and covalently labels (phosphorylates) the active-site serine of enzymatically active serine hydrolases. In this manner, the FP fluorescent probe can identify the OP inhibitor-sensitive serine hydrolase(s) in diverse organs including testicular FAAH (Long and Cravatt, 2011; Long et al., 2009; Nomura and Casida., 2011; Nomura et al., 2008; Noro et al., 2013; Suzuki et al., 2013, 2014). In brief, 50 μg of the membrane protein was competitive with the FP-TAMRA serine hydrolase probe (1 μM) for 30 min at 25 °C, and the sample was then subjected to SDS-PAGE separation for analyzing the fluorescence activity by a flatbed scanner (FUJIFILM FLA-3000, Tokyo, Japan) (Noro et al., 2013; Suzuki et al., 2013, 2014). Furthermore, the aforementioned FAAH bands (0, 5, and 10 mg/kg/day FNT exposed samples) were clearly recognized by the anti-FAAH antibody (protein signals were developed using electrochemiluminescence detection reagents).

LC-MS analysis. Analysis of testicular AEA and 2-AG levels was performed on a LC-MS/MS-8030 system (SHIMADZU, Japan) consisting of a solvent delivery device (LC30AD), an autosampler, (SIL-30 AC), a system controller (CBM-20A), and a column thermostat (CTO-20A) according to the method reported by Zoerner et al. (2012) with a slight modification. Five hundred picograms of AEA-d_4 and 50,000 pg of 2-AG-d_5 were added as the internal standard to the 50 μl of the resultant testicular preparations before solvent extraction with toluene. After toluene evaporation under a stream of nitrogen, the samples were reconstituted in water–methanol (1:3), and aliquots of these solutions were analyzed by LC-MS. Separation of analytes was carried out on a Kinetex 1.7 μm XB-C18 100A column (100 × 2.1 mm) (Phenomenex, Torrance, CA).

Statistics. Statistical analysis was carried out using the EZR (Saitama Medical Center, Jichi Medical University, Japan) which is a graphical user interface for R. Comparisons were performed using the analysis of variance and Dunnett's *post-hoc* test or Jonckheere–Terpstra trend test. When the values were not distributed normally, they underwent a square-root or logarithmic conversion to attain a normal distribution. Linear regression analyses were conducted and coefficients of determination (R^2) adjusted for the degrees of freedom were shown when they were statistically significant.