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Letter

Organophosphorus insecticide dichlorvos inhibits fatty acid amide hydrolase in the male reproductive organs of rats

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ABSTRACT — Organophosphorus (OP) insecticides are used worldwide to protect agricultural crops and dwellings. These chemicals phosphorylate diverse serine hydrolases, including acetylcholinesterase. Among them, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), components of the endocannabinoid signaling system (ECS) in male reproductive organs, are candidate targets of OP insecticide-induced spermatotoxicity. The effects of OP insecticides on the ECS in male reproductive accessory organs have not yet been investigated. In the present study, we examined the potential inhibitory effect of dichlorvos (DDVP) against FAAH and MAGL in male reproductive organs. *In vitro* screening assays were conducted by activity-based protein profiling with a fluorophosphonate chemical probe using samples of the testis, epididymis, prostate, and seminal vesicle of Wistar rats. *Ex vivo* assays were then performed using organs from rats orally administered 0, 5, or 10 mg/kg DDVP, 6 days per week for 9 weeks. *In vitro* assays showed that DDVP inhibited FAAH in the proteomes of rat testis, epididymis, and prostate, but scarcely inhibited MAGL. DDVP failed to inhibit FAAH and MAGL in the seminal vesicles. *Ex vivo* assays confirmed inhibition of FAAH in the proteomes of the prostate, testis, and epididymis of DDVP-treated rats, which exhibited morphologically abnormal sperm and decreased sperm motility. In conclusion, DDVP reduced the activity of FAAH but not of MAGL in male reproductive organs excluding the seminal vesicle; prostate involvement was demonstrated for the first time. Endocannabinoid signaling inhibition in these organs might contribute to sperm abnormality via deterioration in seminal plasma quality.

Key words: Organophosphorus insecticides, Male reproductive toxicity, Fatty acid amide hydrolase, Testis, Epididymis, Prostate

INTRODUCTION

Organophosphorus (OP) insecticides are widely used to protect agricultural crops and dwelling environments from insects. The amount of OP insecticides annually used in the United States was estimated to be 33 million pounds, which accounts for 36% of the total insecticide use in 2007 (U.S. Environmental Protection Agency, 2011). OP compounds exert acute toxicity by inhibiting acetylcholinesterase (AChE) through phosphorylation of the serine hydroxyl side chain at the catalytic triad as a primary target, thereby overstimulating cholinergic neurons. Intriguingly, the OP compounds may also react with many serine hydrolases other than AChE, revealing their potential for causing secondary or unexpected biological effects (Casida and Durkin, 2013; Suzuki *et al.*, 2014).

Recently, particular interest has been directed toward the human male reproductive system as a potential target of OP insecticides (Mehrpour *et al.*, 2014). Our previous investigation showed that low semen quality was associated with indoor pesticide use (Kamijima *et al.*, 2004). In summer, a season during which pesticides are used extensively, the percentages of slow progressive and nonprogressive motile sperm were 2-fold higher in pesticide users than in control individuals. The most commonly used OP insecticides were dichlorvos (2, 2-dichlorovinyl dimethyl phosphate, DDVP). A subsequent animal experiment revealed that male Wistar rats administered DDVP subcutaneously or orally showed increased morphological abnormality and reduced motility in sperms obtained from

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the cauda epididymis (Okamura *et al.*, 2005; Okamura *et al.*, 2009). Our recent investigations suggest that OP-induced spermatotoxicity is at least partially attributable to the inhibition of fatty acid amide hydrolase (FAAH), a serine hydrolase other than AChE (Ito *et al.*, 2014; Noro *et al.*, 2013; Suzuki *et al.*, 2013). *In vivo* administration of the OP insecticide fenitrothion for 9 weeks, i.e., the duration of spermatogenesis, revealed significant linear relationships between sperm motility or morphological parameters and FAAH activity in the testes, which might indicate overstimulation of the endocannabinoid (EC) signaling system (ECS) resulting from inhibition of FAAH activity (Ito *et al.*, 2014). However, the inhibitory effects of OP on FAAH in the accessory organs of the male reproductive system have not yet been investigated.

The aim of the present study was to examine the potential inhibitory effects of OP insecticides on FAAH and monoacylglycerol lipase (MAGL), which hydrolyze the EC agonists, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), respectively, in the male accessory reproductive organs (i.e., the epididymis, prostate, and seminal vesicle), as well as in the testes. Inhibition was assessed via activity-based protein profiling (ABPP) with a fluorophosphonate chemical probe (fluorophosphonate-carboxytetramethylrhodamine, FP-TAMRA) to directly characterize enzyme function. We also examined whether this mode of action occurred *in vivo* by exposing the reproductive organs of male rats to DDVP, which exhibited morphologically abnormal sperms and decreased sperm motility as reported previously (Okamura *et al.*, 2009).

MATERIALS AND METHODS

In vitro screening assay

Reproductive organs obtained from male Wistar rats treated with corn oil orally for 6 days per week for 9 weeks for an *ex vivo* study (n = 10), which is described in the next section, were used for *in vitro* screening assays. Each organ was homogenized in three volumes (vol/wt) of 10 mM phosphate buffer containing 0.25 M sucrose (pH 7.4). Homogenized samples of the epididymis, prostate, and seminal vesicle (excluding testes) were centrifuged at 1,100 × *g* for 10 min at 4°C. Protein concentrations of the homogenized sample (testis) or supernatant (epididymis, prostate, and seminal vesicle) were measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan). Homogenized testis or other organ supernatants were pooled for use.

Samples containing 40 μg of membrane proteins (or 60 μg for seminal vesicles) were reconstituted in 50 mM of Tris-HCl (pH 8.0) and reacted with unlabeled DDVP (0.002-2,000 μM) in competition with the FP-TAMRA serine hydrolase probe (1 μM) (Thermo Fisher Scientific) for 30 min at 25°C. Subsequently, these samples were subjected to 10% SDS-PAGE separation to analyze fluorescence activity with a flatbed scanner Ettan DIGE Imager (GE Healthcare Life Sciences, Buckinghamshire, UK). The exposure time was 0.5 sec by using Cy3 Fluorophore (excitation filter and emission filter values were set to 540/525 nm and 595/525 nm, respectively). ECL Plex Fluorescent Rainbow Markers (GE Healthcare Life Sciences, Buckinghamshire, UK) were used as molecular weight standards.

Ex vivo assays

Ex vivo assays were conducted using reproductive tissues of DDVP-treated rats. Wistar rats were orally administered corn oil alone (vehicle) or 5 or 10 mg/kg of DDVP (22.6 and 45.3 mmol/kg, respectively) dissolved in corn oil for 6 days per week for 9 weeks (n = 10 each). On the day following the final administration, rats were killed by exsanguination from the abdominal aorta under pentobarbital anesthesia. Animal experiments were conducted in accordance with Japanese law concerning the protection and control of animals and the Guide for Animal Experimentation of Nagoya University School of Medicine. Rats treated with 5 and 10 mg/kg DDVP exhibited spermatotoxicity as reported previously (Okamura *et al.*, 2009). The membrane proteomes of reproductive tissues obtained from DDVP-treated rats were reacted with the FP-TAMRA probe for ABPP gel-based analysis in the same manner as for the *in vitro* screening assays.

RESULTS

The FP-TAMRA chemical probe is used to label diverse serine hydrolases. *In vitro* screening assays (Fig. 1A) using the ABPP approach with FP-TAMRA revealed that DDVP inhibited FAAH in the testes, prostate, and epididymis; the 63 kDa band began to disappear in the respective organs at DDVP concentration of ≥ 20 μM, ≥ 20 μM, and ≥ 2 μM, respectively. Labeled proteins clearly disappeared in a concentration-dependent manner.

By contrast, the inhibitory potential of DDVP on MAGL was weak; the 35 kDa band began to disappear at DDVP concentration ≥ 2,000 μM in the testes, epididymis, and prostate. In seminal vesicles, DDVP failed to inhibit FAAH and MAGL at concentrations up to 2,000 μM. Expression levels of FAAH and MAGL in the seminal vesicle were quite low and varied among rats. We

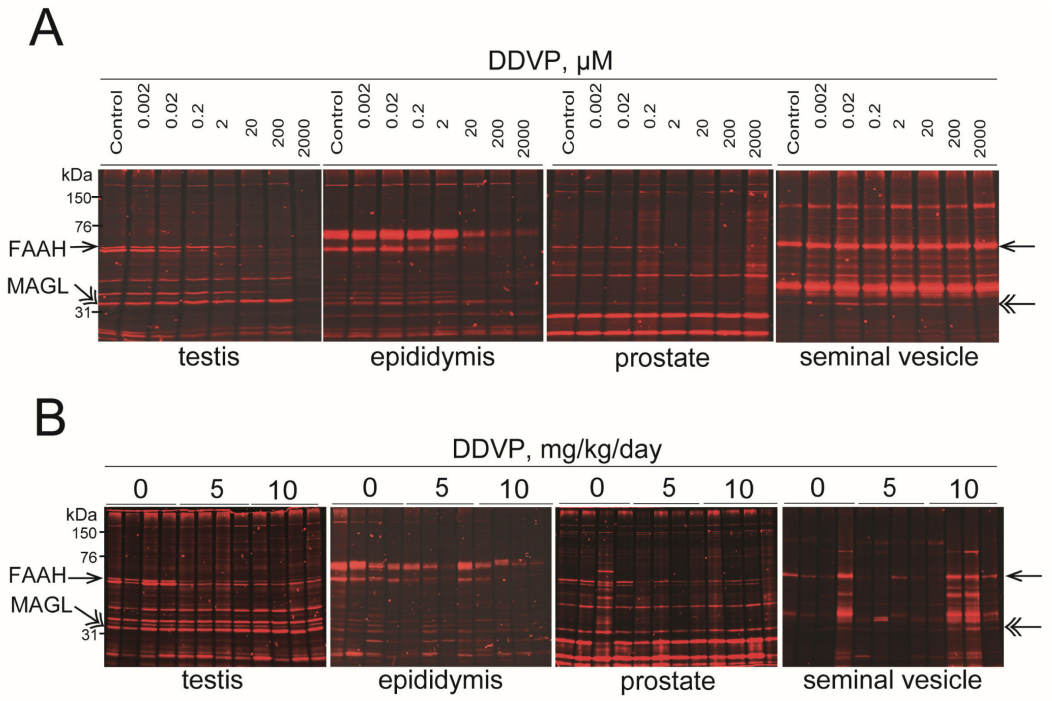

Fig. 1. **A.** *In vitro* screening of target molecule(s) of the anticholinesterase compound DDVP in the membrane proteomes of rat male reproductive tissues. Arrows and two-headed arrows show the bands representing FAAH and MAGL, respectively. The activity-based protein profiling (ABPP) approach shows in-gel inhibition of enzyme activity with a fluorophosphonate probe (FP-TAMRA), which competitively phosphorylates diverse serine hydrolases, revealing concentration-dependent disappearances of the bands for fatty acid amide hydrolase (FAAH) (63 kDa) and monoacylglycerol lipase (MAGL) (35 kDa) in testes, epididymis, and prostate, but not in seminal vesicles. **B.** *Ex vivo* assays of the effect of DDVP treatment (6 days per week for 9 weeks via oral gavage) on the serine hydrolase proteomes of rat male reproductive tissues. Membrane preparations of rat male reproductive tissues were reacted with the FP-TAMRA chemical probe for ABPP gel-based analysis. ABPP was conducted in all mice (n = 10/group) and representative ABPP gel images are shown.

could not detect the bands unless we increased membrane protein loading on the gel by 50%.

Consistently, in *ex vivo* assays, the ABPP analyses of membrane proteomes obtained from DDVP-treated rats revealed inhibition of FAAH but not of MAGL (Fig. 1B); DDVP inhibited FAAH in testes, epididymis, and prostate (the band disappeared in 5 and 10 mg/kg DDVP groups), but failed to inhibit FAAH in the seminal vesicles.

In both *in vitro* and *ex vivo* studies, DDVP also inhibited serine hydrolases other than FAAH and MAGL. These were not identified in the present study.

DISCUSSION

To the best of our knowledge, this is the first study to show that the OP insecticide DDVP potentially inhibited FAAH in rat prostate as well as in the testes and epididymis, whereas inhibition of MAGL in the organs tested was weak. The inhibition of either EC-metabolizing enzyme was not detected in seminal vesicles.

The ECS responds to the endogenous ligands AEA and 2-AG via the cannabinoid receptors CB1R and CB2R. The degradation of these ligands is regulated by FAAH and MAGL. The presence of ECS components in the male reproductive system has been identified in seminal plasma, male reproductive tissues, and Leydig and Sertoli cells, as well as in the male germ cells, spermatogonia to mature spermatozoa (Cobellis *et al.*, 2006; Lewis and Maccarrone, 2009; Lewis *et al.*, 2012; Maccarrone, 2009; Rapino *et al.*, 2014).

FAAH is the major enzyme responsible for the catabolism of AEA. It also targets noncannabinoid fatty acid amides, including *N*-palmitoyl ethanolamine (PEA), *N*-oleoyl ethanolamine (OEA), oleamide (Cravatt *et al.*, 1996; Cravatt *et al.*, 2001), and the *N*-acyl taurines (Saghatelian *et al.*, 2006). AEA, PEA, and OEA have been

detected in picomolar to nanomolar levels in human seminal plasma (Amoako *et al.*, 2013; Schuel *et al.*, 2002). The source of AEA, PEA, and OEA in seminal plasma remains unknown, but the contribution by the epididymis, prostate, and seminal vesicles is possible (Amoako *et al.*, 2014). Notably, the prostate and seminal vesicles produce 15 to 30% and 50 to 80%, respectively, of seminal fluid in rodents (Turton and Hooson, 1998). Maintenance of normal EC and AEA congeners such as PEA and OEA tone is necessary to preserve normal sperm function and male fertility (Amoako *et al.*, 2014; Maccarrone *et al.*, 2015). Taken together, the inhibition of FAAH in the prostate and epididymis may result in elevated levels of AEA, PEA, and OEA in seminal plasma.

AEA elicits inhibitory effects on male reproduction (Lewis and Maccarrone, 2009; Maccarrone *et al.*, 2003; Ricci *et al.*, 2007; Rossi *et al.*, 2007). AEA in the Sertoli cells of the testis, which regulate spermatogenesis by providing nutrients and hormonal signals required for the development of germ cells, reportedly induced apoptosis of the Sertoli cells, indicating that AEA may be a regulator of cell death or survival (Maccarrone and Finazzi-Agro, 2003; Rossi *et al.*, 2007). In addition, AEA inhibited sperm motility in the epididymis in a study using CB1R-knockout mice (Rossi *et al.*, 2007), whereas the CB1R antagonist rimonabant clearly increased sperm motility in the epididymis via a CB1R-dependent mechanism (Aquila *et al.*, 2010). Thus, downregulation or inhibition of FAAH and the resulting elevation in AEA levels overstimulate EC signaling, which could lead to apoptosis of testicular cells and disruption of sperm function.

In our previous studies, inhibitory effects of OP on EC ligand degrading enzymes, especially FAAH, were postulated to be a triggering mechanism for OP-induced spermatotoxicity (Noro *et al.*, 2013; Suzuki *et al.*, 2013). Nine-week administration of the OP insecticide fenitorothion in rats elicited spermatotoxicity in association with inhibited testicular FAAH activity (Ito *et al.*, 2014). The inhibition of testicular FAAH activity accounted for 44% and 63% of aggravation of indexes for normal morphology and cytoplasmic droplets (indices of sperm deformity and underdevelopment), respectively, in fenitorothion-administered rats (Miyake *et al*., submitted). Meanwhile, the inhibitory effects of OP on FAAH activity in reproductive organs other than the testes and epididymis, have not been elucidated yet except within the current study.

In summary, this study identified inhibitory effects of OP on FAAH in the male accessory reproductive organs *in vitro*. We first detected OP-induced inhibition of FAAH in the prostate. *In vivo*, the activity of FAAH was also inhibited, although FAAH expression was lower in prostates of rats exhibiting sperm deformity and reduced sperm motility than in the testes and epididymis. This suggests that lowered FAAH in the prostate might contribute to aggravation of sperm parameters via deterioration of seminal plasma quality.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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