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Atrazine binds to F_1F_0-ATP synthase and inhibits mitochondrial function in sperm

Yasuyoshi Hase [a], Michiko Tatsuno [a], Takeyuki Nishi [a], Kosuke Kataoka [b], Yasuaki Kabe [a], Yuki Yamaguchi [a], Nobuaki Ozawa [c], Michiya Natori [c], Hiroshi Handa [a,d,*], Hajime Watanabe [e]

[a] *Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Yokohama 226-8501, Japan*
[b] *Graduate School of Bioscience, NAIST, 8916-5 Takayama-cho, Ikoma 630-0192, Japan*
[c] *Division of Maternal Medicine, Department of Perinatology, National Center for Child Health and Development, Okura 2-10-1, Setagaya-ku, Tokyo 157-8535, Japan*
[d] *Integrated Research Institute, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Yokohama 226-8501, Japan*
[e] *Center for Integrative Bioscience, Okazaki National Research Institutes, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan*

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Abstract

Atrazine is a widely used triazine herbicide. Although controversy still exists, a number of recent studies have described its adverse effects on various animals including humans. Of particular interest is its effects on reproductive capacity. In this study, we investigated the mechanisms underlying the adverse effects of atrazine, with a focus on its effects on sperm. Here we show evidence that mitochondrial F_1F_0-ATP synthase is a molecular target of atrazine. A series of experiments with sperm and isolated mitochondria suggest that atrazine inhibits mitochondrial function through F_1F_0-ATP synthase. Moreover, affinity purification using atrazine as a ligand demonstrates that F_1F_0-ATP synthase is a major atrazine-binding protein in cells. The inhibitory activity against mitochondria and F_1F_0-ATP synthase is not limited to atrazine but is likely to be applicable to other triazine-based compounds. Thus, our findings may have wide relevance to pharmacology and toxicology.

Keywords: Atrazine; F_1F_0-ATP synthase; Herbicide; Mitochondria; Sperm; Triazine

Atrazine (ATZ), 6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine (Fig. 1A), is a triazine herbicide that inhibits photosynthesis by binding to the reaction center of photosystem II [1]. ATZ is one of the most commonly used herbicides in the world, with 30,000 tons applied annually only in the United States [2], and much of it makes its way into surface water and groundwater.

Although controversy still exists [3,4], a number of recent studies have described its adverse effects on various animals including humans. For example, ATZ reportedly increases the incidence of mammary tumor in rats [5,6] and disrupts functions of the endocrine system [2,7], the central nervous system [8], and the immune system [9]. Of particular interest is its effects on reproductive capacity. ATZ is shown to cause hermaphroditism in male frogs with a concomitant reduction in the blood testosterone level [10]. In addition, intraperitoneal injection of ATZ to male rats causes disorders of the reproductive tract with a significant decrease in sperm number and motility in epididymis [11]. Moreover, ATZ and other herbicides and insecticides are shown to reduce viability and motility of porcine sperm [12]. Consistently, epidemiological studies in the United States indicate that the level of exposure to herbicides including atrazine is inversely correlated with the concentration and motility of human sperm [13].

* Corresponding author. Address: Integrated Research Institute, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Yokohama 226-8501, Japan. Fax: +81 45 924 5145.
E-mail address: handa.h.aa@m.titech.ac.jp (H. Handa).

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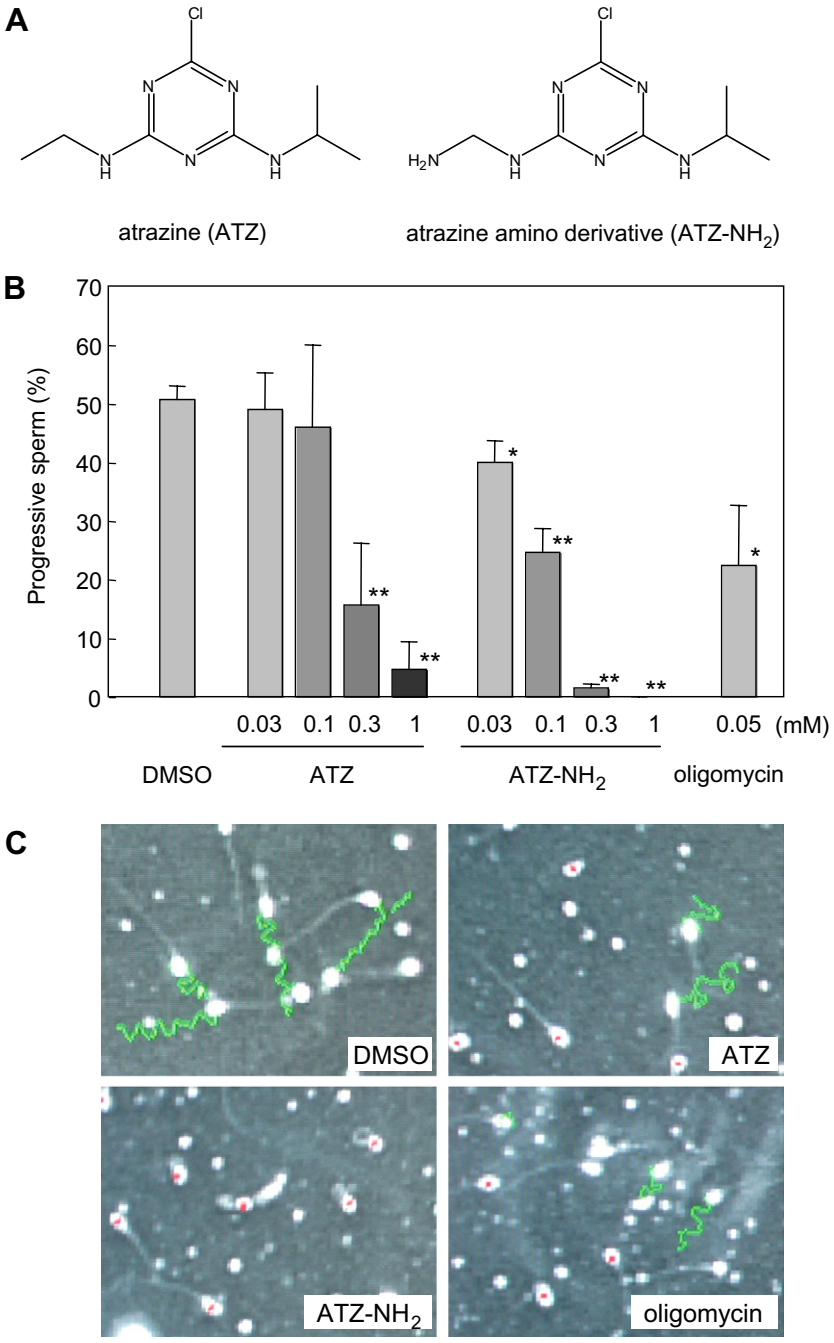

Fig. 1. ATZ reduces sperm motility. (A) ATZ and its amino derivative used in this study. (B) After incubation of sperm with the indicated compounds for 4 h, the percentage of sperm with progressive motility (velocity >25 μm/s, linearity >80%) was determined using a computer-aided sperm analyzer. Data shown are means ± SD from three-independent experiments. Student's *t*-test was used to compare values from the control (DMSO) sample and treated samples. $^{*}p < 0.05$, $^{**}p < 0.005$. (C) Representative micrographs showing motility of sperm that were incubated with 1 mM ATZ, 1 mM ATZ-NH_2, 0.05 mM oligomycin, or control DMSO for 4 h. Green lines indicate sperm tracks. Red dots denote the heads of immotile sperm.

Although accumulating data suggest adverse effects of ATZ on animals, its mode of action is not well understood. While ATZ is thought to have endocrine-disrupting estrogenic activity, ATZ does not seem to affect estrogen receptors directly [14]. As a possible mechanism for its antiandrogenic effects, ATZ is shown to induce the expression of aromatase, the enzyme that converts testosterone to estradiol [15], and different studies have pointed to phosphodiesterase and the orphan nuclear receptor Ad4BP/SF-1 as ATZ targets mediating aromatase induction [14,16]. However, it is still not possible to fully appreciate the diverse effects of the herbicide. In this study, we focused on the mechanism by which ATZ affects sperm motility. Here we show evidence that ATZ binds to F_1F_0-ATP synthase and inhibits ATP synthesis in mitochondria.

Materials and methods

Computer-aided analyses of sperm. Semen samples were obtained from five volunteers (22–25 years) with their consent, who were asked to refrain from ejaculation for a week prior to sampling. Semen samples were allowed to liquefy at room temperature for 20 min and then washed with Dulbecco's modified Eagle medium to remove sperm fluid. After centrifugation, pelleted sperm were left at 37 °C for 1 h, and swim-up sperm were collected and used for subsequent analyses. Sperm placed in 20-μm-depth microcell counting chambers (Conception Technologies, La Jolla, CA, USA) were examined under light microscopy at 37 °C, and their motility parameters were measured using a computer-aided sperm analyzer (HTM-CEROS ver. 10.9i, Hamilton Throne Research, Beverly, MA, USA) under standard settings. For analysis of ATP content in sperm, sperm were incubated with the indicated compounds or left untreated for 4 h, and then lysed with ice-cold NP-40 lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1% NP-40). The ATP levels were quantified by luciferase assays using ATP determination kit (Invitrogen, Carlsbad, CA, USA).

Analyses of mitochondrial activities. Mitochondria were prepared by differential centrifugation from male Wistar/ST rat liver at 5 weeks of age. For analysis of mitochondrial membrane potential, 1 mg/ml mitochondria were preincubated with 1% dimethyl sulfoxide (DMSO), 500 μM ATZ (Wako Pure Chemical, Osaka, Japan), 500 μM ATZ-NH_2 (Shimazu, Kyoto, Japan), 50 μM oligomycin (Wako Pure Chemical, Osaka, Japan), or 4 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, Sigma, St. Louis, MO, USA) in buffer E (0.15 M sucrose, 10 mM phosphate, pH 7.4, 20 mM KCl, 5 mM $MgCl_2$, and 5 mM succinate) for 5 min at 30 °C. Then, Rhodamine 123 (Sigma, St. Louis, MO, USA) was added to a final concentration of 10 μM, and mitochondria were further incubated for 15 min at 30 °C. Mitochondria were then washed with ice-cold buffer E twice and analyzed using FACSAria (Becton–Dickinson, Tokyo, Japan). For analysis of oxygen uptake, mitochondria were preincubated with 0.5 mM ATZ, 0.1 mM atractyloside (Sigma, St. Louis, MO, USA), 5 mM sodium azide, or control DMSO in respiration buffer (0.15 M sucrose, 10 mM phosphate, pH 7.4, 20 mM KCl, 5 mM $MgCl_2$, and 4.2 mM succinate) for 5 min at room temperature. Then, ADP was added to a final concentration of 370 μM, and oxygen concentration in solution was measured real-time using an oxygen electrode with continuous stirring. For analysis of ADP transportation, mitochondria were preincubated with the indicated compounds or left untreated in buffer I (0.25 M sucrose, 10 mM Tris–HCl, pH 7.4, 10 μM EGTA, and 5 mM succinate) for 5 min. ^{3}H-labeled ADP (5 μM) was then added, and after 30 s, reactions were terminated by the addition of 500 μM atractyloside. Mitochondria were pelleted and lysed with 50 μl of 1% SDS, and incorporated tritium was measured with a liquid scintillation counter.

Purification and identification of ATZ-binding proteins. ATZ-NH_2 was fixed to SGNEGDE beads as previously described [17]. Briefly, SGNEGDE beads (10 mg) were incubated with 500 μl of 100 mM ATZ-NH_2 in 60% 1,4-dioxane (pH 8.0) for 24 h at room temperature and then washed sequentially with 60% 1,4-dioxane (pH 8.0) and distilled water to remove unreacted ATZ-NH_2. As control, SGNEGDE beads were processed identically in the absence of ATZ-NH_2. ATZ-immobilized or control beads (0.1 mg) were equilibrated with HGKEMNDP buffer (20 mM Hepes, pH 7.9, 10% glycerol, 0.2 mM EDTA, 1.2 mM $MgCl_2$, 0.1% NP-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulphonyl fluoride) containing 50 mM KCl and were incubated with 500 μg of HeLa cell cytoplasmic or nuclear extract or rat liver submitochondrial particles for 2 h at 4 °C with gentle shaking. Submitochondrial particles were prepared by sonication of mitochondria in 20 mM Tris–sulphate, pH 8.0, 50 mM NaCl, and 2 mM $MgCl_2$ for 3 min on ice and subsequent removal of debris by centrifugation. After the beads were washed three times with HGKEMNDP buffer containing 100 mM KCl, bound proteins were eluted with HGKEMNDP buffer containing 1 M KCl. Purified proteins were resolved by SDS–PAGE, excised from the gel, and identified by LC–MS/MS essentially as described [17].

Results

ATZ reduces sperm motility and ATP content

In this study, we sought to understand more about the mechanisms underlying adverse effects of ATZ on animals, with a focus on its effects on sperm. One of our goals was to identify additional cellular targets of ATZ through affinity chromatography. Since ATZ lacks reactive groups that can be utilized for immobilization, an amino derivative of ATZ (ATZ-NH_2, Fig. 1A) was synthesized and studied in parallel with the parent compound.

First, isolated human sperm were incubated with increasing concentrations of ATZ or ATZ-NH_2, and sperm motility was examined under a microscope using a computer-aided sperm analyzer. Oligomycin, a potent inhibitor of mitochondrial F_1F_0-ATP synthase, was used as positive control since this compound is known to inhibit sperm motility [18]. In agreement with a previous study on porcine sperm [12], ATZ significantly reduced the percentage of sperm with progressive motility (sperm moving fast in a straight line) in a concentration-dependent manner (Figs. 1B and C). Remarkably, ATZ-NH_2 was more potent than ATZ in inhibiting sperm motility.

A possible reason for the low sperm motility is mitochondrial dysfunction, as mitochondria supply energy for flagellar motility. To explore this possibility, we quantified ATP content in sperm. As expected, ATZ, ATZ-NH_2, and oligomycin all reduced the intracellular ATP level, and in agreement with the above finding, ATZ-NH_2 was more potent than ATZ (Fig. 2A). These findings suggested that ATZ and ATZ-NH_2 reduce sperm motility by inhibiting ATP synthesis at the concentrations used here.

ATZ inhibits ATP synthesis in isolated mitochondria

Respiration and ATP synthesis are tightly coupled (Fig. 2B). Electrons from NADH or $FADH_2$ are transferred to molecular oxygen through the electron transport chain, during which redox proton pumps generate a proton gradient across the inner membrane. The proton gradient is then utilized for ATP synthesis by F_1F_0-ATP synthase. Given that the ATP level was reduced by ATZ and its derivative, the following possibilities were considered from the mechanisms of action of known mitochondrial inhibitors: (i) ATZ may inhibit the mitochondrial TCA cycle and the production of NADH. (ii) ATZ may inhibit one of the redox proton pumps. (iii) ATZ may collapse the proton gradient by increasing proton permeability. (iv) ATZ may inhibit the transportation of ADP, a substrate for F_1F_0-ATP synthase, into the mitochondrial matrix. (v) ATZ may inhibit F_1F_0-ATP synthase.

To determine whether and how ATZ and its derivative affect mitochondrial function, we used mitochondria isolated from rat liver in a subsequent study. First, we examined whether ATZ and its derivative have any effect on

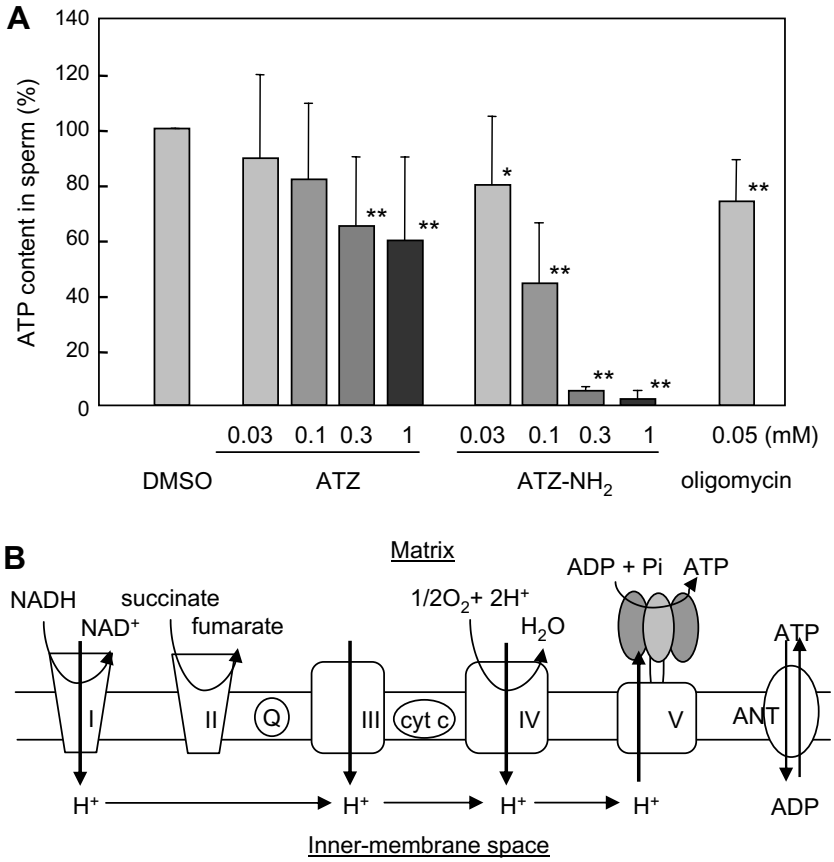

Fig. 2. ATZ reduces ATP content in sperm. (A) After incubation of sperm with the indicated compounds for 4 h, ATP content in sperm was measured as described in Materials and methods. Each value was expressed as % of the control sample. Data shown are means ± SD from seven-independent experiments. $^{*}p < 0.05$, $^{**}p < 0.005$. (B) Schematic drawing of ATP synthesis in mitochondria. ANT, adenine nucleotide translocase.

mitochondrial membrane potential. Rhodamine 123, a mitochondria-specific, membrane potential-sensitive fluorescent dye, was incubated with mitochondria, and its incorporation was examined by FACS analysis. FCCP, a protonophore uncoupler that abolishes the membrane potential, strongly decreased the incorporation of Rhodamine 123 (Fig. 3A). By contrast, ATZ and ATZ-NH_2 did not decrease, but in fact slightly increased its incorporation. These results eliminated the possibility that ATZ may collapse the proton gradient.

We next measured oxygen uptake by mitochondria with an oxygen electrode. Since respiration and ATP synthesis are tightly coupled, both are strongly enhanced when ADP is added to mitochondria. However, if the coupling is abolished by the loss of proton gradient, for example by FCCP, rapid respiration occurs without any concomitant ATP synthesis. Indeed, the addition of ADP significantly increased oxygen uptake (Fig. 3B, top left panel). Here, atractyloside, an inhibitor of adenine nucleotide translocase that is responsible for ADP transportation into the mitochondrial matrix, was used as control. As reported previously [19], ADP-induced oxygen uptake was abolished by prior incubation with atractyloside (bottom left panel). When mitochondria were preincubated with ATZ instead, ADP-induced oxygen uptake was also reduced significantly (top right panel). Since the proton gradient is maintained in the presence of ATZ (Fig. 3A), the above observation can be taken as evidence that ATZ inhibits ATP synthesis in isolated mitochondria. In addition, since succinate was used as electron donor in the assays, the production of NADH is unlikely to be the target of ATZ (see Fig. 2B).

ATZ probably inhibits mitochondrial F_1F_0-ATP synthase activity

To discriminate the remaining possibilities, we examined oxygen uptake in the presence of FCCP, wherein respiration occurs independently of ATP synthesis. ATZ should block oxygen uptake even under the condition if its target is a step leading to the generation of proton gradient, such as the electron transport chain. Conversely, ATZ should not block oxygen uptake under the condition if its target is a step after the generation of proton gradient, such as ADP transportation and F_1F_0-ATP synthase. FCCP-induced oxygen uptake was not inhibited by atractyloside, consistent with its role as an inhibitor of ADP transportation (Fig. 3B, bottom left panel). Here, sodium azide was used as another control, which inhibits the electron transport chain at complex IV (cytochrome *c* oxidase). As

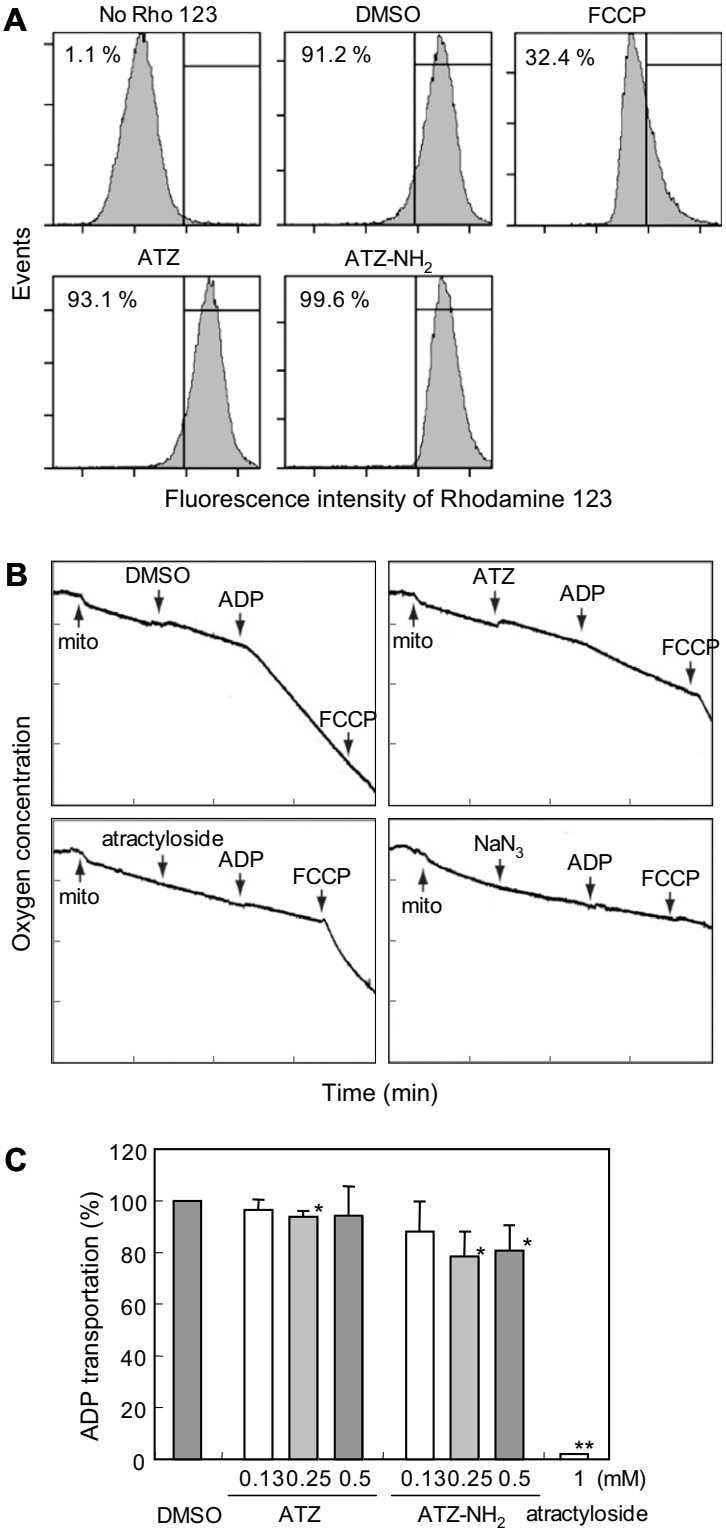

Fig. 3. ATZ inhibits ATP synthesis in isolated mitochondria. (A) The membrane potential of mitochondria was determined by incorporation of Rhodamine 123 (Rho 123). The percentage of mitochondria above a fluorescence threshold is shown at the top left corner of each panel. (B) After incubation of mitochondria (mito) with the indicated compounds for 5 min, ADP was added, and oxygen concentration in solution was measured real-time. Where indicated, the protonophore FCCP was added to further activate oxygen uptake. (C) After incubation of mitochondria with the indicated compounds for 5 min, incorporation of ^{3}H-labeled ADP into mitochondria was measured and expressed as % of the control sample. Data shown are means ± SD from three-independent experiments. $^{*}p < 0.05$, $^{**}p < 0.005$.

expected, FCCP-induced oxygen uptake was inhibited by sodium azide (bottom right panel). As for ATZ, it behaved similarly to atractyloside, and FCCP accelerated oxygen consumption even in the presence of ATZ (top right panel). These results suggested that ATZ inhibits a step after the generation of proton gradient, either ADP transportation or F_1F_0-ATP synthase.

This finding prompted us to examine whether ATZ has any effect on ADP transportation. For this, mitochondria were briefly incubated with ^{3}H-labeled ADP, and its incorporation was measured with a liquid scintillation counter. As a result, ATZ did not affect ADP transportation appreciably, although ATZ-NH_2 slightly reduced ATP transportation (Fig. 3C). These results suggested that the target of ATZ in mitochondria is not ADP transportation but F_1F_0-ATP synthase.

ATZ directly interacts with mitochondrial F_1F_0-ATP synthase

Next, we sought to identify molecular targets of ATZ directly by affinity chromatography. ATZ-NH_2 was immobilized to SGNEGDE beads through epoxy groups on the surface and was used as a ligand for purification. SGNEGDE beads are submicrometer-sized organic particles that have been developed in our laboratory and proven superior to conventional agarose-based matrices in terms of purification yield and non-specific protein binding [17].

First, nuclear and cytoplasmic extracts from human cervical carcinoma HeLa cells were used as input material for affinity purification. Silver staining revealed about a dozen polypeptides in the cytoplasmic extract that were selectively associated with ATZ-immobilized beads (Fig. 4A). When the same procedure was performed with the nuclear extract, these and other polypeptides were found associated with ATZ beads negligibly (data not shown). Then, these proteins were excised from the gel and subjected to MS/MS analysis, and 18 unique peptide sequences were obtained. Strikingly, all these sequences were found to match various subunits of human mitochondrial F_1F_0-ATP synthase (Supplementary Table 1). Affinity purification was also carried out with submitochondrial particles from rat liver, and a set of polypeptides that are very similar in composition to those from HeLa cytoplasmic extract were obtained (Fig. 4B). MS/MS analysis identified 28 unique peptide sequences, and all these sequences were found to match various subunits of rat mitochondrial F_1F_0-ATP synthase (Supplementary Table 2). The identity of these polypeptides was further confirmed by immunoblot analysis (Fig. 4C). Thus, the biochemical analysis also pointed to the role of F_1F_0-ATP synthase as a molecular target of ATZ.

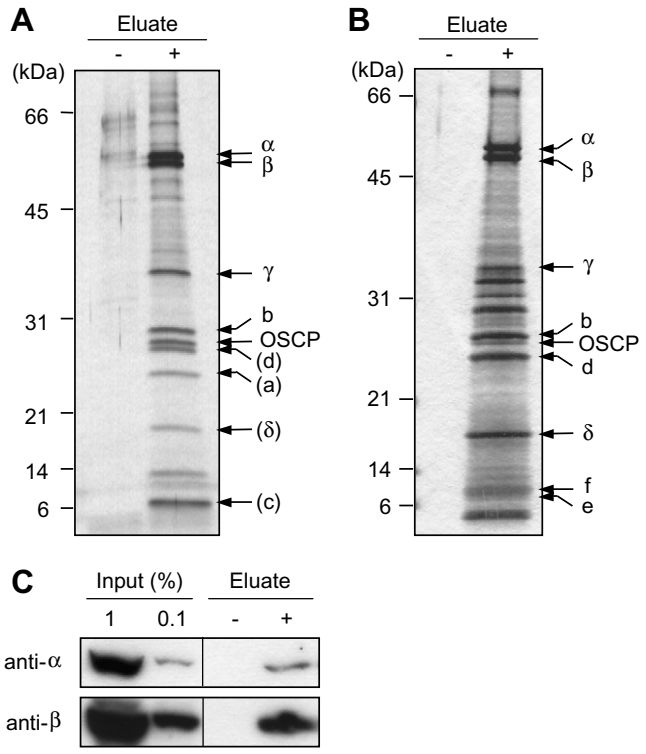

Fig. 4. Purification and identification of ATZ-binding proteins. (A,B) Purification was carried out with ATZ-immobilized (+) or control (−) SGNEGDE beads using HeLa cell cytoplasmic extract (A) or rat liver submitochondrial particles (B) as input. Aliquots of eluate fractions were analyzed by SDS–PAGE and silver staining. The identity of the polypeptides shown in parentheses was not determined by MS/MS but was estimated from their molecular weights. (C) Aliquots of input and eluate fractions from (B) were subjected to immunoblot analysis with antibodies against the α and β subunits of F_1F_0-ATP synthase.

Discussion

In this study, we provided evidence that ATZ has potential to inhibit mitochondrial function by binding to mitochondrial ATP synthase. The ATP synthase has two major structural parts known as F_0 and F_1. F_0 is the membrane-bound component that conducts protons across the inner membrane and consists of ten subunits named a, b, c, d, e, f, g, F6, OSCP, and A6L. F_1 is the soluble component that catalyzes ATP synthesis and the reverse reaction ATP hydrolysis, and has a subunit composition of $\alpha_3\beta_3\gamma\delta\varepsilon$. The mechanical energy of proton flow is converted into the chemical energy of ATP through the combined action of F_0 and F_1. Since many of the subunits belonging to F_1 and F_0 were identified, it is likely that F_1F_0-ATP synthase was purified as a holoenzyme by the above procedure. However, it remains to be determined exactly how ATZ binds to F_1F_0-ATP synthase and modulates its activity, which will be the subject of our next study.

This study was motivated by the idea that ATZ applied to fields may cause a reduction of sperm count and motility to humans and other animals. However, the effect of ATZ on sperm motility was seen at >100 μM, exposure levels that probably do not result from normal use of the herbicide. It is therefore uncertain whether the observed phenomena are ecologically relevant.

Nevertheless, our findings have another important implication. The triazine scaffold has various applications

in medicine and industry because of its ease of manipulation and structural similarity to purine and pyrimidine [20]. For example, atrazine and other triazine-based compounds, such as simazine and cyanazine, are widely used as herbicides. The triazine compound named vardenafil is a phosphodiesterase inhibitor that is widely used in the treatment of erectile dysfunction. Lamotrigine is another triazine-based drug that is used in the treatment of epilepsy and bipolar disorder. We therefore examined several other triazine compounds and found that one reduces sperm motility and inhibits F_1F_0-ATP synthase activity more potently than ATZ (our unpublished data). Williams et al. have also shown that a set of triazine compounds (different from those we tested) directly bind to F_1F_0-ATP synthase, although they have not studied in detail whether and how these compounds may affect mitochondrial function [20]. Thus, the finding that certain triazine compounds affect F_1F_0-ATP synthase probably can be generalized. This point may be worth considering in the development and risk assessment of triazine-based chemicals or drugs, especially if the compounds will be used at high doses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.107.

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