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HUMAN PLACENTAL Ca^{2+}-ATPase: IN VITRO INHIBITION BY DDT HOMOLOGS

(DDT homologs; placental microvilli; fetal viability)

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SUMMARY

In vitro inhibition of Ca^{2+}-ATPase by DDT homologs was studied using maternal brush-border membranes from human term placentas as an enzyme source. At 10 μM concentration many of the compounds tested inhibited this enzyme. The order of effectiveness of inhibition was as follows: *p,p'*-DDE > *p,p'*-DDD > *p,p'*-DDT > methoxychlor(mec). Both *p,p'*-DDOH and *p,p'*-DDA did not inhibit the placental Ca^{2+}-ATPase. Assays using varying concentrations (0.3 μM to 0.1 mM) of *p,p'*-DDT were also performed. The inhibition of human placental Ca^{2+}-ATPase ranged from 12% for 0.3 μM *p,p'*-DDT to 69% for 30 μM *p,p'*-DDT. Higher concentrations of this pesticide failed to cause further enzyme inhibition.

INTRODUCTION

Calcium is integral for fetal development and survival. It has many important functions in the fetus, including the regulation of cellular metabolism, blood homeostasis, and skeletal development. It has been shown to be transported across

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Abbreviations: *p,p'*-DDA, bis(*p*-chlorophenyl)acetic acid; *p,p'*-DDD, 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethane; *p,p'*-DDE, 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene; *p,p'*-DDOH, 2,2-bis(*p*-chlorophenyl)ethanol; *p,p'*-DDT, 1,1-bis(*p*-chlorophenyl)-2,2,2-tri-chloroethane; EGTA, ethyleneglycolbis(β-aminoethyl ether) *N,N,N,N*-tetraacetic acid; mec, methoxychlor; SDS, sodium dodecyl sulfate.

the placenta against a concentration gradient where it is present in higher concentration in fetal blood than in that of maternal circulation [1]. The definitive mechanism for this calcium transport has not yet been fully established and is under investigation [2–4]. It has been demonstrated, however, that an ATP-dependent calcium uptake process as well as a Ca^{2+}-ATPase exist in the brush-border membrane fraction of human placenta [3,4]. As with many other tissues, it has been suggested [3] that placental Ca^{2+}-ATPase may be involved in the regulation of intracellular calcium concentration of the placental cells, as well as in the transport of calcium across the placenta to the fetus.

Several epidemiological surveys have clearly documented a positive correlation between the amount of organochlorine pesticide residues in maternal or cord blood, placenta, and fetal tissues and an increased incidence of spontaneous abortion [5,6], missed abortion [7], fetal prematurity [6,8], induction of early labor [6,9,10], premature delivery [11,12], and stillbirths [12–14]. The underlying biochemical mechanism(s) responsible for these reproductive failures are still unknown. Based on animal data, it is hypothesized that induction of maternal hepatic enzymes, estrogenicity [15], or induction of prostaglandin biosynthesis [16] may be involved. However, due to the lack of necessary human data, the validity of these postulates remains to be established.

During the past 15 years, many reports have shown several organochlorine pesticides to inhibit many types of ATPases isolated from tissues of different animal species, including Mg^{2+}-ATPase, Na^+,K^+-ATPase, and Ca^{2+}-ATPase [17–21]. Earlier the inhibition of Ca^{2+}-ATPase was proposed as a mechanism of organochlorine pesticide neurotoxicity [20], and the same may be applicable to toxicity to other tissues including placenta. It is proposed that following the exposure of pregnant women to these pesticides, a significant inhibition of placental Ca^{2+}-ATPase may occur which could alter placental biochemistry and result in its dysfunction that may contribute to the decreased viability of the fetus. To date, the in vitro inhibition of human placental Ca^{2+}-ATPase by organochlorine pesticides has not been reported. The results of this study indicate that Ca^{2+}-ATPase present in the brush-border membranes of human term placenta is sensitive to inhibition by DDT homologs.

EXPERIMENTAL PROCEDURES

Materials

ATP (disodium salt) and mec were purchased from Sigma Chemical Co., St. Louis, MO, while *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, *p,p'*-DDA, and *p,p'*-DDOH were of the highest purity available from Aldrich Chemical Co., Milwaukee, WI.

Preparation of brush-border membranes

The procedure followed for the isolation of brush-border membranes was essentially that outlined by Booth et al. [22]. Upon delivery, full-term placentas were immediately placed on ice and processed within 1 h. 100 g of lobular villous tissue were removed and washed several times in 50 mM $CaCl_2$ to remove as much blood as possible. The tissue was then manually teased and stirred in 0.15 M NaCl with 1.0 mM-EGTA for 1 h. After filtration through gauze, the preparation was centrifuged at $800 \times g$ for 10 min and the resulting supernatant centrifuged at $10\,000 \times g$ twice for 10 min each. The resulting supernatant was then centrifuged at $100\,000 \times g$ for 1 h to obtain microvillous brush-border membranes (microvilli). Suspensions of microvilli (approx. 1 mg/ml) were stored frozen ($-20°C$) for up to 2 weeks in buffer (10 mM mannitol, 2 mM Tris–HCl, 1 mM EGTA, pH 7.1) with no significant decrease in enzyme activity. The microvilli (1 ml) were then washed in 30 ml storage buffer containing no EGTA before use to remove EGTA from the enzyme preparation. Protein determination was by the Lowry method using bovine serum albumin as the standard [23].

Calcium-stimulated ATPase assay

The calcium-stimulated ATPase activity was measured by monitoring the release of inorganic phosphorous (P_i) by colorimetric determination of a phosphomolybdate complex as described by Ames [24]. The standard reaction mixture for the assay contained 10–60 μg of brush-border membrane protein, 1.0 mM $CaCl_2$, and sufficient buffer (30 mM Tris–HCl, pH 8.2) to attain a final volume of 1.0 ml. After preincubation for 10 min at 37°C, the reaction was initiated by the addition of 2.0 mM ATP. The assays were run at 37°C for 30 min and the reaction was terminated by the addition of 3.0 ml of 2% SDS. Control incubations were performed as above with SDS added before initiation of the reaction. Ca^{2+}-stimulated ATPase activity was determined by subtracting values obtained in the absence of calcium from those in its presence. Ca^{2+}-ATPase activity is expressed as μmol of P_i released/min/mg protein. Under these conditions, the enzyme activity measured was linear with respect to the time and protein concentrations used at the pH optimum of 8.2. For the inhibition studies, the standard assay conditions described above were used with differing concentrations of DDT analogs (0 to 1×10^{-4} M in 10 μl acetone) present. I_{50} values were determined using linear regression evaluation of the data. When applicable, the data were statistically evaluated using Student's paired *t*-test.

RESULTS

The basal Ca^{2+}-ATPase activities observed in this study are in agreement with those reported for human placental microvilli [3]. Human placental Ca^{2+}-ATPase was significantly inhibited ($P < 0.01$) by four of the six organochlorine compounds

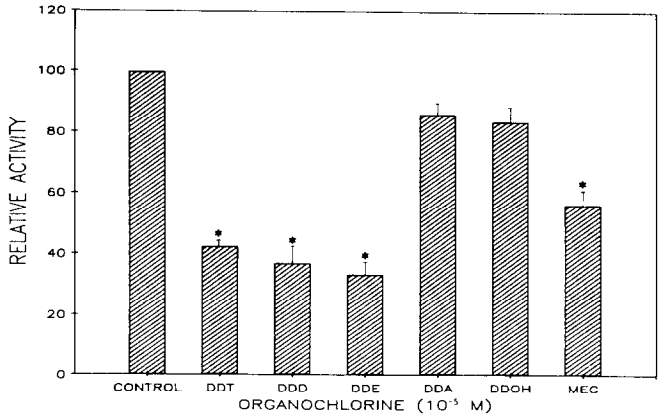

Fig. 1. In vitro inhibition of human placental Ca^{2+}-ATPase by 6 organochlorine compounds. The data are represented as % control of enzyme activity remaining upon exposure to 0 (control = 100%) or 10 μM concentrations of DDT or DDT analogs in vitro (M ± S.E.M.). The experiments were performed using standard assay conditions detailed in experimental procedures. The asterisks denote values significantly different from control at $P<0.01$. Control activity = 1.18 ± 0.25 μmol P_i released/min/mg protein (n = 4).

tested in vitro (Fig. 1). At 10 μM concentration, the degree of enzyme inhibition observed was dependent on the specific organochlorine compound used. Thus, *p,p'*-DDT, *p,p'*-DDD, and *p,p'*-DDE were the most inhibitory compounds with 33% to 42% of control activity remaining. Methoxychlor was intermediate with

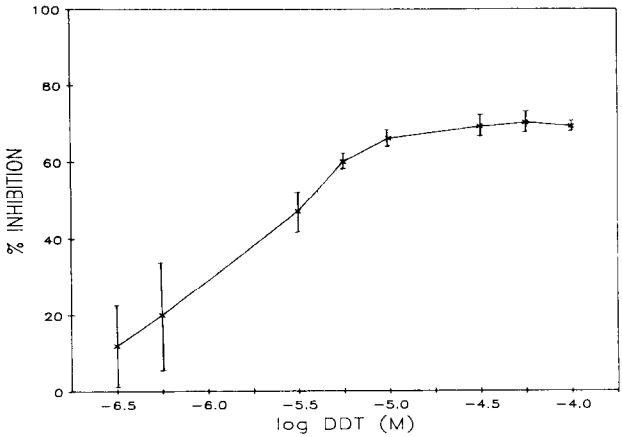

Fig. 2. In vitro inhibition of human placental Ca^{2+}-ATPase activity by varying concentrations of *p,p'*-DDT. The data are represented as % control of enzyme activity remaining in the presence of 3×10^{-7} M to 1×10^{-4} M concentrations of *p,p'*-DDT (M ± S.E.M.). The assays were performed using standard assay conditions detailed in experimental procedures. Control activity = 1.09 ± 0.21 μmol P_i/min/mg protein (n = 4).

about 50% inhibition, whereas *p,p'*-DDA and *p,p'*-DDOH, did not significantly inhibit the enzyme, in vitro.

The inhibition of enzyme activity was dependent upon the concentration of organochlorine compound employed (Fig. 2). The degree of inhibition increased with increasing concentration of *p,p'*-DDT from 3×10^{-7} M to 3×10^{-5} M where maximum inhibition, reaching 69%, occurred. A further increase of *p,p'*-DDT concentration to 1×10^{-4} M did not yield higher inhibition of enzyme activity. The apparent I_{50} value of human placental Ca^{2+}-ATPase for *p,p'*-DDT was determined to be $5.68 \pm 1.27 \times 10^{-6}$ M (n = 4).

DISCUSSION

Numerous studies on the susceptibility of different ATPases to organochlorine pesticides have been reported [17–21]. The studies indicate that the degree of enzyme inhibition depends upon the type of ATPase, tissue, animal species, and compound used. To our knowledge, the susceptibility of placental ATPases to *p,p'*-DDT and related compounds has not been examined to date in any animal species including man.

Specifically, inhibition of Ca^{2+}-ATPase has been proposed as a mechanism of neurotoxicity of organochlorine pesticides [20], and the possibility exists that a similar mechanism of toxicity may be operational in other tissues. However, Ca^{2+}-ATPases from different tissue sources do not appear to be equally sensitive to inhibition by organochlorine compounds. For example, *p,p'*-DDT was found to cause a high degree of Ca^{2+}-ATPase inhibition in preparations of lobster peripheral nerves [20] and flounder sarcoplasmic reticulum [19], while studies on avian reproductive tissues gave varied results. Furthermore, Ca^{2+}-ATPase in egg shell gland preparations of Pekin ducks was found to be inhibited by *p,p'*-DDE, while that of chicken was not [21]. In view of these results, possible inhibition of placental Ca^{2+}-ATPase by DDT homologs was examined.

The results presented indicate the in vitro sensitivity of human placental Ca^{2+}-ATPase to *p,p'*-DDT and specific DDT analogs. With respect to placental Ca^{2+}-ATPase, the greatest inhibition occurs with *p,p'*-DDT, *p,p'*-DDD, and *p,p'*-DDE which are stored in human adipose tissue [15], while the urinary metabolites of *p,p'*-DDT, namely *p,p'*-DDA and *p,p'*-DDOH cause no enzyme inhibition. Although the use of an unpurified enzyme preparation prohibits the unequivocal determination of the mechanism involved in inhibition, the fact that these compounds are not specific inhibitors for one type of ATPase suggests the possibility that more than one factor may be involved. Similarly, the degree of lipophilicity of these compounds alone cannot explain the observed inhibition, due to the reported insensitivity of rat liver mitochondrial Mg^{2+}-ATPase to the highly lipophilic compound mirex [25].

The results also indicate that human placental Ca^{2+}-ATPase, as with most other

ATPases studied, is not completely inhibited by *p,p'*-DDT [20]. Only oligomycin-sensitive mitochondrial Mg^{2+}-ATPase has been shown to be completely inhibited by this compound [26] and thus appears to be the most sensitive of the ATPases with respect to inhibition by this compound. The failure to observe total enzyme inhibition by *p,p'*-DDT of lobster peripheral nerve Ca^{2+}-ATPase has been attributed to the presence of two enzyme activities (DDT-sensitive and -insensitive) [20]. Due to the lack of necessary evidence for the same, Cutkomp et al. [17] have suggested the existence of only one enzyme activity which is partially inhibited by *p,p'*-DDT. The present work does not substantiate the presence of two enzyme activities in human placental microvilli with respect to inhibition by DDT homologs.

The fact that *p,p'*-DDT and its metabolites which are stored in human fat depots were found to be most inhibitory to human placental Ca^{2+}-ATPase may be of toxicological significance. It has been documented that due to maternal mobilization of lipids during pregnancy [27], these chemicals are transported across the placenta and are found in high concentrations in fetal tissues and in the placenta. It is proposed that inhibition of placental Ca^{2+}-ATPase activity may represent one of many biochemical events responsible for the reported unfavorable pregnancy outcomes associated with exposure to this class of compounds. Since *p,p'*-DDT and related pesticides are still in use today in different parts of the world, the opportunity for prenatal exposure to these chemicals exists. This is of concern when one realizes the well documented [5–14] relationship between increased organochlorine pesticide exposure and decreased fetal viability. Further mechanistic studies are needed to clearly establish the relationship, if any, between the inhibition of human placental Ca^{2+}-ATPase and the fetotoxicity seen with specific organochlorine pesticides.

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