Interaction of Acetochlor with Estrogen Receptor in the Rat Uterus. Acetochlor – Possible Endocrine Modulator?

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Abstract. The aim of the present study was to investigate the ability of the chloroacetanilide herbicide acetochlor to interact with the endocrine system. The modulation of the binding of [^{3}H]estradiol-17β to protamine sulphate-precipitated uterine nuclear and cytoplasmic estrogen receptors was analysed for this purpose. Two different stages of reproductive life cycle of female rats, virgin and uniparous, were used. Our results demonstrate that acetochlor interacts in a specific manner with high-affinity limited-capacity uterine estrogen receptors. A significant difference ($p < 0.001$) in estrogen receptor density was observed between two control groups: uniparous rats ($B_{max} = 43.634 \pm 9.516$) and virgin rats ($B_{max} = 154.375 \pm 21.462$), suggesting an intrinsic difference in the nuclear estrogen receptor levels between female rats in different reproductive life cycle stages. Consequently, a different response to acetochlor treatment was noted. Exposure to acetochlor significantly ($p < 0.001$) increased estrogen receptor binding activity in the nuclear fraction of uniparous female rats ($B_{max} = 123.324 \pm 5.666$) in comparison to control ($B_{max} = 43.634 \pm 9.516$). In exposed virgin female rats, no significant difference was detected when compared to the corresponding control group. These results should prompt us to more thoroughly evaluate potential hazards of acetochlor to human and wildlife endocrine systems.

Key words: Acetochlor — Herbicides — Xenoestrogens — Estrogen receptor — Rat uterus

Introduction

One of the target sites of pesticide toxicity is the endocrine system. Alterations of endocrine functions induced by perturbation of steroid hormone biotransformation,

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by direct acting hormone agonist/antagonist on the receptor level or by synergistic operation of several mechanisms can manifest themselves as reproductive, developmental, immunological and neuro-behavioral consequences of exposure to environmental chemicals. Such chemicals have been included on a growing list of agents called endocrine disruptors. Recently, some scientists suggested that these prevailingly persistent chemicals might be responsible for the effects operating through endocrine disruption mechanism (Gray Jr. et al. 1997; Eybl et al. 1997). In humans, the end points may include breast cancer and endometriosis in women, testicular and prostate cancer in men, an abnormal sexual development and reduced fertility. In addition to human health effects biologists accumulated reports on deleterious effects on aquatic life and wildlife such as demasculinization and feminization of fish, birds, reptiles and mammals, decreased offspring survival and decreased fertility (Colborn et al. 1993; Vonier et al. 1996; Crisp et al. 1998). The experimental and epidemiological studies on some chemicals, particularly chlorinated pesticides, have, indeed, shown that these substances have estrogenic, antiestrogenic or antiandrogenic properties and that they are able to induce or to promote hormone-related disturbances (Soto et al. 1994; Wolff et al. 1996; Danzo 1997). The estrogenic activity of many of these chemicals is mediated through their interaction with estrogen receptors (Tran et al. 1996).

Acetochlor (ACT) is a recently U. S. Environmental Protection Agency (EPA) approved selective, chloroacetacetanilide herbicide used for the control of annual grasses, certain annual broadleaves and yellow nutsedge (Monsanto Company 1997). Together with alachlor, metolachlor, propachlor, propisochlor and MG-84, it belongs to the group of broadly used weed killers. ACT is an active ingredient of four pesticide products for control of corn crops, registered in the Slovak Republic (Ministry of Agriculture of the Slovak Republic 1995).

The advanced time of vaginal opening, the opening being one of the sexual maturation signs in mammals, observed in the neonatal Wistar female rats exposed to ACT (Selecká and Zeljenková 1995) prompted us to pay attention to this agent. A complete vaginal opening is the result of rising titers of estrogen produced by the ovary under hypothalamo-hypophysial axis regulation (Talamantes and Browning 1972). The significantly precocious opening in the exposed group suggested that ACT may interfere with the endocrine system. Therefore, the present study was designed to investigate the ability of ACT to influence estrogen responses. The possible interference of ACT with ligand-estrogen receptor (ER) binding was analysed for this purpose.

The leaching behaviour of ACT in different types of soil connected with the potential for contamination of groundwater (Cohen et al. 1995; Balinova 1997) and the preservation of its activity in the water for months (Kovrižnych and Urbančíková 1998) suggested the possible route of the chronic exposure of humans and wildlife to ACT. These facts supported our effort to broaden the information profile of this herbicide.

To our knowledge, this is the first report on the determination of the estrogen potential of ACT at the ERs level.

Materials and Methods

Chemicals and reagents

Acetochlor, ($C_{14}H_{20}ClNO_2$, m. w. 269.8) chloroacetanilide herbicide, technical grade 92.2%, was obtained from Nitrokemia Industries, Fuztogyartelep, Hungary, (LD50 for female rats: 768 mg/kg; LD50 for male rats: 1063 mg/kg (Khalkova et al. 1990; Antov et al. 1991)). Radiolabeled [2,4,6,7-^{3}H]estradiol, purity 98.2% and specific activity 81.0 Ci/mmol, 3 TBq/mmol, was purchased from Amersham (Buckinghamshire, UK). Scintilation counting fluid (Bray solution) was supplied as a complete reagent by Chemopetrol (Neratovice, Czech Republic).

Animals and herbicide exposure

Adult female SPF rats of the Wistar strain (280–300 g b.w.), were obtained from Charles River AnLab, Inc., (Prague, Czech Republic). There are several stages of the reproductive life cycle of female mammals (fetal, prepubertal, cycling adult, pregnant, lactating, reproductively senescent). Therefore, it is important to examine each stage thoroughly before the assumption can be made that the females are not influenced by an environmental endocrine disruptor (Crisp et al. 1998). For this reason, virgin female rats (VFR) and uniparous female rats (UFR) on day 5 after the end of 3-week lactation period were used in the experiment. The female animals were housed in a controlled environment under 12 h light-dark cycle and with free access to food and tap water. Experimental groups were treated with ACT suspension in sunflower seed oil (dose volume 5 ml/kg b. w.) with the dose equal to 20% LD_{50}, once a day for six days by oral gavage. The control rats of both stages received vehicle only. After six-day administration the rats were killed by decapitation under ether anaesthesia. The uteri were removed, cleaned of the adhering fat, weighted and quickly frozen in liquid nitrogen.

Binding assay

Binding of estrogen to the estrogen receptors was assessed using *in vitro* estrogen exchange assay on protamine sulphate-precipitated receptors in nuclear and cytosol uterine fractions. The assay on protamine precipitates of the classic ER$s\alpha$ is described in detail in the reports by Zava et al. (1976) for nuclear fraction; Chamness et al. (1975) for cytosol, and modified in the report by Alexandrová and Soloff (1980). This biochemical method is included in the Screening Battery (Tier 1 or T1S) recommended by Endocrine Disrupters Screening and Testing Advisory Committee (EDSTAC) for detection of potential estrogenicity of chemicals (Gray Jr. 1998).

Preparation of cytoplasmic and nuclear fractions

The frozen tissue was pulverized and homogenized in 12 ml/g wet wt ice-cold TED buffer containing 10 mmol/l Tris HCl, pH 7.4/4℃, 1.5 mmol/l EDTA, 0.5 mmol/l dithiothreitol (DTT). The homogenate was centrifuged at 3,000 × g. The obtained supernatant was then centrifuged at 105,000 × g for 1 h, and the supernatant

prepared in this way and referred to as cytosol was the source of cytoplasmic ERs. The crude 3,000 × *g* pellet was resuspended in TED buffer and recentrifuged at 3,000 × *g*. The nuclear receptors were extracted with KTED buffer (0.6 mol/l KCl in TED buffer, pH 8.5) from the pellet and separated from the mixture of disrupted nuclei by ultracentrifugation at 105,000 × *g* for 1 h.

Protamine assay

Cytosol and nuclear proteins were vortexed with protamine sulphate solution (1 mg/1 ml of TED buffer) and precipitates were sedimented by centrifugation at 3,000 × *g*. The precipitates in duplicates were incubated with increasing concentrations of [^{3}H]estradiol-17β: 0.08; 0.24; 0.56; 0.8; 1.2; 2.0 nmol/l; cytosol receptors at 22℃ for 22 h, nuclear receptors overnight at 4℃ followed by a 2.5 h incubation at 22℃. Simultaneous incubation with 300-fold molar excess of DES was used to determine nonspecific binding of [^{3}H]estradiol-17β. After the incubation and two washes with TED buffer, bound radioactivity was extracted with 1 ml absolute ethanol. Then, 0.5 ml aliquots were decanted and radioactivity was counted in Rackbeta 1214/1219 liquid scintillation counter (LKB Wallac). The differences between total counts and counts representing nonspecific binding were regarded as specific binding. Unless otherwise stated, all procedures were carried out at 4℃. Protein contents were estimated by the method of Lowry et al. (1951).

Analyses of results

Data were analysed by the Scatchard method (Scatchard 1949) to determinate the equilibrium dissociation constant (K_d) and total binding sites (B_{max}) using the non-linear regression data analysis software ENZFITTER, version 1.05 (CGA, © Robin J. Leatherbarrow 1987). Effects of ACT exposure were analysed by nonparametric 2-sided 2-sample Wilcoxon's test for individual points of saturation curve (fmol/mg protein) and by *t*-test for B_{max}, K_d values and relative uterus weight values with statistical significance determined at the level of $p < 0.001$.

Results

After a 6-day exposure of rats to ACT, a slight increase of the relative uterus weights was found between control and exposed UFR groups, although statistically not significant (control UFR 0.125 ± 0.035 g, exposed UFR 0.139 ± 0.035 g; control VFR 0.119 ± 0.028 g, exposed VFR 0.120 ± 0.026 g).

Saturation analyses (Figs 1, 2) were the basis to estimate binding parameters of uterine ERs: binding site density (B_{max}) and equilibrium dissociation constant (Kd). Transformations of saturation binding data (Scatchard plots) were linear suggesting that the ligand interacted with a single population of ERs with a single affinity for the ligand. The estimates of the binding parameters of ERs in the cytosol and the nuclear uterine fractions from exposed UFR and VFR groups and their corresponding controls are given in Tab. 1. The average B_{max} values (from three independent experiments) in the nuclear fraction for the control UFR group were

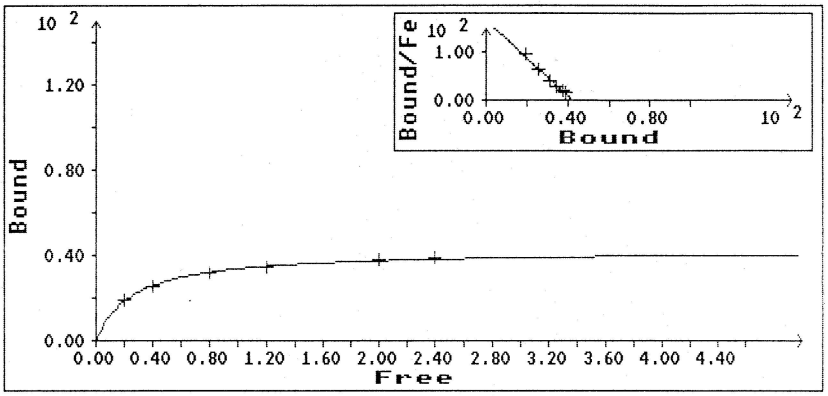

Figure 1. Saturation analysis of specific [^{3}H]estradiol binding in nuclear fraction of the uteri in control groups of uniparous female rats. Inserts show the corresponding Scatchard plots. Points represent the means of duplicate samples from three independent experiments. Data processed by the program Enzfitter. Bound (fmol/mg protein), free (nmol/l).

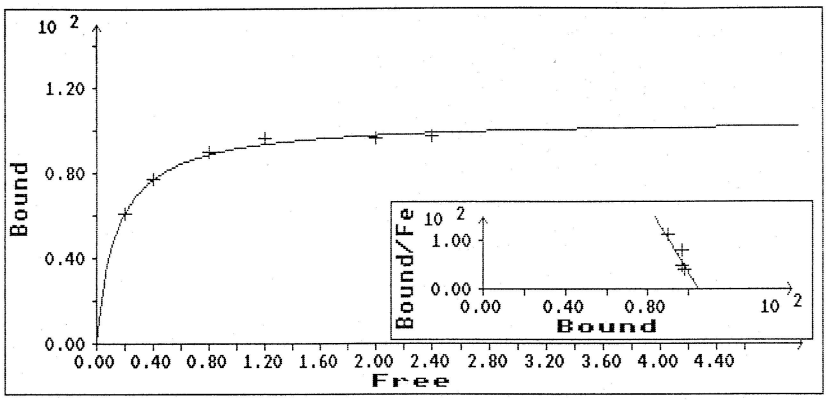

Figure 2. Saturation analysis of specific [^{3}H]estradiol binding in nuclear fraction of the uteri in acetochlor exposed groups of uniparous female rats. Inserts show the corresponding Scatchard plots. Points represent the means of duplicate samples from three independent experiments. Data processed by the program Enzfitter. Bound (fmol/mg protein), free (nmol/l).

significantly different ($p < 0.001$) from those for the control VFR group. The same result was obtained when comparing individual points on the saturation curves (fmol/mg protein) (Fig. 3). Values representing estrogen bound to the receptors in nuclear extracts for the control VFR group ($B_{max} = 154.375 \pm 21.462$) were

Table 1. Effect of acetochlor on estrogen receptors in rat uteri

Group N°	Group	Stage	B_{max} (fmol/mg protein)	K_d (nmol/l)	
	Cytosol fraction				
1	Control	UFR	206.799 ± 63.631	0.439 ± 0.057	$n = 10$
2	Acetochlor	UFR	244.493 ± 52.706	0.541 ± 0.019	$n = 10$
3	Control	VFR	231.214 ± 73.751	0.426 ± 0.036	$n = 10$
4	Acetochlor	VFR	153.411 ± 14.792	0.426 ± 0.194	$n = 10$
	Nuclear fraction				
5	Control	UFR	43.634 ± 9.516	0.296 ± 0.173	$n = 10$
6	Acetochlor	UFR	123.234 ± 5.666***	0.160 ± 0.009	$n = 10$
7	Control	VFR	$154.375 \pm 21.462^{\#\#\#}$	0.374 ± 0.169	$n = 10$
8	Acetochlor	VFR	104.348 ± 11.830	0.169 ± 0.069	$n = 10$

***Significant difference in estrogen receptor density (B_{max} values) between control and acetochlor groups, group 5 vs. group 6; $p < 0.001$. $^{\#\#\#}$ Significant difference in estrogen receptor density between control groups, group 5 vs. group 7; $p < 0.001$. Values represent mean ± S.E. of three independent experiments. UFR – uniparous female rats; VFR – virgin female rats. n = number of animals in group

statistically higher in comparison with the values for the control UFR group (B_{max} = 43.634 ± 9.516). Cytoplasmic ER binding activities for the control VFR group vs. control UFR group were comparable (Tab. 1).

Our results indicate that ACT may interfere with ligand binding to the high-affinity, limited-capacity uterine ERs. Significant differences ($p < 0.001$) in the estrogen binding activity (B_{max} and individual points on the saturation curve) between control and ACT exposed groups of UFR were found for nuclear uterine ERs (Tab. 1; Fig. 3). Exposure to ACT in the presence of endogenous estradiol elicited remarkable nuclear accumulation of ERs (B_{max} = 123.234 ± 5.666 for the exposed UFR group vs. B_{max} = 43.634 ± 9.516 for the control UFR group). This event in the nuclear fraction was not accompanied with any expressive changes in the cytoplasmic uterine fraction of UFR. No significant differences in binding activity of cytoplasmic and nuclear ERs were detected between control and exposed VFR groups after ACT treatment (Tab. 1), although there was a tendency to a reduced ER binding capacity in both uterine fractions in exposed groups. Analyses of the data by Scatchard plots gave equilibrium dissociation constants (K_d values, Tab. 1) which were not changed to a significant extent after ACT exposure either in UFR or in VFR groups.

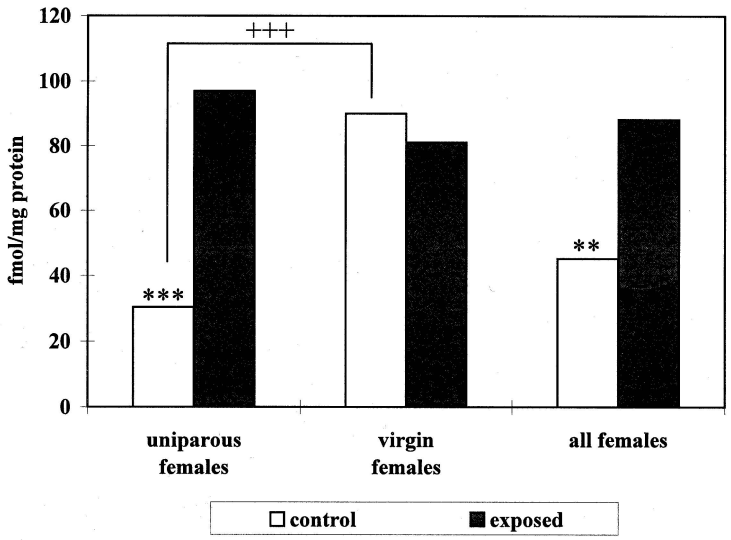

Figure 3. Nuclear estrogen receptor binding activity in the uterus of non-ovarectomised VFR and UFR after ACT treatment and the corresponding values for the control groups. Each bar represents the median of individual points on the saturation curve (fmol/mg protein) from three independent experiments with duplicates. (+++) indicates significant difference between control UFR and control VFR ($p < 0.001$). (***) indicates significant difference between control UFR and exposed UFR ($p < 0.001$). (**) indicates a significant difference between control female rats (UFR and VFR) and ACT exposed female rats (UFR and VFR) in both stages of the reproductive life cycle ($p < 0.01$).

Discussion

Because of the known role of absorption, metabolism, distribution, and excretion of chemicals on the manifestation of their estrogenic activity (Shelby et al. 1996) and metabolic deficiency of current *in vitro* estrogenicity assays (Gray Jr. et al. 1997; Odum et al. 1997) we used a saturation binding assay in the rat uteri after *in vivo* exposure to the xenobiotic. To include the exposure history of animals we used non-ovarectomised female rats at two different stages of reproductive life cycle: 1. virgin female rats and 2. uniparous female rats after weaning of the youngs.

Uterine and vaginal changes during the estrous cycle are the response to two ovarian hormones, particularly estrogen, which, in turn, reflects the altered hypothalamic activity and changes in gonadotropin secretion (Lapolt et al. 1986; Lerner et al. 1990). In relation to fluctuations of estrogen concentrations in plasma, total intracellular content and distribution of ERs vary in the estrogen-target tissues. Diestrus interval of the estrous cycle is known to persist in suckling mothers and the recurrence of the regular 4–5 day estrous cycle (metestrus, diestrus, proestrus, estrus) in the vaginal smear can take place from three to twelve days

after the removal of the youngs (Long and Evans 1922). In the uterus of mothers, total ER concentrations return to the normal levels at quiescence after the termination of the 3-week lactation period (Leung et al. 1976). Nuclear receptor content in cycling adult rats varies (independently of the constant cytosol ERs concentrations) reaching a maximum value during proestrus when the nuclear receptor levels are 5-fold higher than during estrus and metestrus/early diestrus (Thrower et al. 1977). A dramatic increase of nuclear ERs content can also be observed along with an increase of the uterus weight in aging virgin rats (from 50 to 81 days of age), when the average amount of nuclear ERs in the animals reaches 48% of the total receptor content during proestrus interval with the highest ER concentrations at all (Thrower et al. 1977). This might correspond to the control VFR group in our experiments with significantly higher levels of nuclear uterine ERs when compared to the control UFR group. On the other hand, an adequate decrease of the nuclear ER levels can be expected in UFR since the serum levels of estradiol on day 20 after parturition are significantly lower in lactating animals than in nonlactating animals (Goméz et al. 1977). To take into consideration the facts mentioned above we assume that the significantly lower level of estrogen binding to nuclear ERs in control UFR in comparison to the control VFR might be a consequence of the period of previous pregnancy and puerperium. Serum estradiol levels are reduced in such animals (Goméz et al. 1977) and subsequently, a reduction of nuclear ERs levels occurs. Based on these results we can conclude that the major difference in the nuclear estrogen receptors between the two control VFR and UFR groups might be explained by their different stages of reproductive life cycle.

In spite of a possible degradation process of receptors stored in liquid nitrogen (Leake and Habib 1987; Brtko et al. 1993) a significant increase in concentrations of nuclear ERs was observed in three independent experiments after ACT stimulation without corresponding depletion of the cytoplasmic receptor concentrations. This is in agreement with other authors who showed increases in the numbers of nuclear estradiol binding sites in the rat liver, the uterus and the ovary after exogenous steroid administration which did not correlate well with the depletion of cytosol receptor concentrations (Marr et al. 1980; Tamulevicius et al. 1982; Kim and Greenwald 1987). Moreover, evidence has been provided by observations of Kupfer and Bulger (1976) that the insecticide *o, p′* DDT (1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane) can also act as a typical estrogen. They showed that similarly to estradiol (Anderson et al. 1972), *o, p′* DDT administration resulted in an increase of uterine nuclear ER levels. Recently, a number of reports have demonstrated the ability of pesticides to inhibit [^{3}H]-estradiol binding to the cytoplasmic ERs after *in vitro* treatment of the uterine cytosol fraction and their potential to interact with receptor protein in the absence of any of their metabolites. Insecticides methoxychlor, *p, p′* DDT, *p, p′* DDE have been shown to inhibit estradiol binding in a statistically significant extent (Danzo 1997). The chlorotriazine herbicide atrazine present in elevated levels has been shown to inhibit steroid binding to cytoplasmic ERs of the rat uterus under nonequilibrium conditions. These conditions theoretically could exist during certain periods of the

diestrus of the cycling female rat, when estradiol secretion is at an ebb (Eldridge et al. 1994).

A possible explanation of the presented results is that nuclear accumulation of ERs after ACT exposure might be a result of the synergistic effect (Arnold et al. 1996, 1997) of ACT (or its metabolites) and a low serum level of endogenous estradiol as shown in non-ovarectomized uniparous female rats. The other possibility is the presence of additional binding sites on the rat ER molecules, which has been suggested in association with the synergistic effect of combinations of environmental chemicals on the human ERs (Martin et al. 1988). We can not exclude the effect *via* an increase of ERs mRNA, as exogenous estrogens and antiestrogens not only exert their effects by interacting with the ER proteins already present in target tissues, but they have also been shown to alter ER mRNA levels (Sato et al. 1996).

Contrary to UFR group, a decrease of ER concentrations (although not significant) was observed after ACT exposure in both the cytoplasmic and the nuclear fraction in VFR group. Andersen (Andersen et al. 1999) suggested that in conditions of a high endogenous estradiol serum level an estrogenic chemical can compete with endogenous estrogens for binding to ERs and may exhibit antiestrogenic potential. Inhibition of binding to ERs as a result of a denaturation effect (Danzo 1997; Gray Jr. et al. 1997) of ACT is not probable because a low dose of the tested chemical was used and no similar effect was observed in UFR group.

Based on the results presented we can summarise that:

1) Female rats in two stages of the reproductive life cycle (UFR and VFR) show significantly different nuclear uterine ER levels what might be a precondition for different responses to ACT exposure.

2) ACT interferes with estrogen receptor binding activity in rat uteri.

3) Under specific conditions (UFR group) ACT can induce significant accumulation of nuclear ERs in the rat uterus. Through this mechanism high concentrations of ACT could apparently overcome normal regulatory mechanisms, and an uncontrolled action in target cells of the fetus and the adult can occur.

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