Inhibition of non-genomic responses to oestrogen in the rat uterus by testosterone propionate

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Summary. Testosterone propionate (50 mg/kg), administered together with oestradiol, inhibited the oestrogen-induced uterine eosinophilia, deep endometrial oedema and the increase in uterine wet weight, 6 h after treatment. The same dose of the androgen decreased the number of eosinophils in the blood and increased their degranulation, explaining the effect of testosterone in the uterus. The high doses of the androgen used were in the range of the doses reported by others to block selectively the oestrogen-induced increase in uterine peroxidase content but not other responses to oestrogen or the cytosolic oestrogen receptor translocation to the nucleus. The dissociation by high doses of testosterone of the oestrogen-induced uterine eosinophilia, wet weight increase and oedema from other responses to oestrogen in the absence of any measurable effect of testosterone upon cytosolic-nuclear oestrogen receptors supports the idea that uterine eosinophilia and oedema are oestrogenic responses regulated by mechanisms different from those of the genomic responses, and is in agreement with the hypothesis of the mediation of uterine oedema by eosinophils.

Introduction

The dissociation of the responses to oestrogen into separate groups of responses that can be selectively stimulated, inhibited or completely blocked has suggested the existence of multiple and independent mechanisms of oestrogen action in the rat uterus (Tchernitchin, 1983). The first group of responses (oestrogen-induced increases in RNA and protein synthesis, the increase in the content of some specific enzymes and biochemical, morphological and functional differentiation of target cells) are believed to be mediated by the cytosolic-nuclear oestrogen receptor system through hormone-receptor interactions with the genome (Jensen & De Sombre, 1972). These responses can be blocked by actinomycin D (Ui & Mueller, 1963; Hamilton, 1964; Means & Hamilton, 1966; Tchernitchin & Galand, 1982); therefore, they can be considered as the genomic responses to oestrogen stimulation (Tchernitchin & Galand, 1982). The second group of responses (e.g. uterine eosinophilia, oedema, increase in vascular permeability, release of histamine) is independent of uterine genome (Tchernitchin & Galand, 1982) and may be mediated by the uterine eosinophil leucocytes (Tchernitchin, 1972, 1979, 1983). Tchernitchin & Galand (1982) suggest that the eosinophils migrate from the blood to the uterus by a non-genomic mechanism which is dependent on oestrogen levels in the blood but not in the uterus (Tchernitchin & Galand, 1983) and is possibly mediated by oestrogen receptors in the eosinophils (Tchernitchin & Tchernitchin, 1976; Tchernitchin, 1983). It was suggested that the eosinophils release into the uterine stroma various agents and enzymes that may play a role in oestrogen action (Tchernitchin, 1983). The hypothesis of the role of uterine eosinophils in some non-genomic responses to oestrogen stimulation in the uterus is supported by the following findings (see Tchernitchin, 1983; Tchernitchin *et al.*, 1984, for

review). (1) Actinomycin D does not interfere with oestrogen-induced uterine eosinophilia and oedema under conditions blocking the genomic responses to oestrogen. (2) All conditions so far studied that counteract the oestrogen-induced migration of eosinophils to uterine extravascular space (the glucocorticoid-induced blood eosinopenia, the decrease in blood eosinophil levels by other eosinopenic hormones, the interference with eosinophil mobility under a treatment with colchicine and the young age blood eosinopenia) depress or completely block the oestrogen-induced uterine eosinophilia, oedema, increase in vascular permeability and implantation, but do not depress the genomic responses to oestrogen. (3) The oestrogen-induced uterine oedema correlates with the number of eosinophils in the uterus in all experimental conditions so far studied, independently from the extent of the genomic responses to oestrogen. (4) The strong potency of oestriol to induce uterine eosinophilia, oedema, increase in vascular permeability and histamine release, but not the genomic response to oestrogen, correlates with the high affinity that oestriol exhibits for oestrogen receptors in the eosinophils only. (5) Uterine oedema develops under all conditions so far studied inducing uterine eosinophilia in the absence of oestrogen stimulation. (6) Agents decreasing oestrogen binding by the eosinophil receptors but not by the cytosolic-nuclear receptors selectively counteract oestrogen-induced uterine eosinophilia and oedema. (7) Uterine eosinophilia and oedema depend on oestrogen levels in the blood but not in the uterus, while the genomic responses depend on oestrogen levels in the uterus itself.

In addition to the above two groups of responses, other oestrogenic effects have been suggested as being mediated by prostaglandins, cyclic AMP, membrane receptors, and/or cytoplasmic and nuclear type II binding proteins (see Tchernitchin, 1983, for review).

A prolonged (3-day) treatment with high doses of testosterone dissociated the responses to oestrogen in the uterus; this treatment selectively blocked the oestrogen-induced increase in uterine peroxidase content without interfering with the oestrogen-induced increase in glucose 6-phosphate dehydrogenase, the depletion of the cytosolic oestrogen receptors and the accumulation of the oestrogen receptors in the nucleus (Jellinck, Affleck & Newcombe, 1983). Since it is accepted that virtually all of the oestrogen-induced increase in peroxidase content in the uterus corresponds to the enzyme carried by the eosinophils migrating from the blood to the uterus, and only a minor amount corresponds to the endogenous peroxidase synthesized by uterine cells (King, Allen & De Sombre, 1981), it seemed likely that the reported effect of testosterone should correspond to a blockage of oestrogen-induced migration of eosinophils to the uterus. To check for this possibility and to investigate the mechanisms involved in this interaction, we studied the effects of a short-term testosterone treatment on oestrogen-induced accumulation of eosinophils in the uterus and on blood eosinophil levels and degranulation *in vivo* and *in vitro*. Considering that any condition interfering with eosinophil migration to the uterus should modify the oestrogenic responses dependent on eosinophils, we also investigated oestrogen-induced uterine oedema (water imbibition).

Materials and Methods

Hormones. Oestradiol-17 β (Sigma, St Louis, MO, U.S.A.) was dissolved in absolute ethanol (500 μ g/ml) and was diluted 1:9 in saline (9 g NaCl/l) immediately before injection. Testosterone propionate (Merck, Darmstadt, West Germany) was dissolved in propylene glycol (10 mg/ml) and used for injection undiluted.

Effect of testosterone propionate on non-genomic responses to oestradiol in the uterus. Immature 21-day-old female Sprague-Dawley rats, 40–50 g body weight, were injected with testosterone propionate (50 mg/kg) or the vehicle. Immediately thereafter, the animals were injected with oestradiol-17 β (300 μ g/kg) or the vehicle. Both injections were done under ether anaesthesia into the jugular vein. At 6 h after injection, the animals were killed and the uterine horns were excised for

wet weight determination, fixed in 10% neutral formalin and processed for further histological studies.

The following responses to oestrogen stimulation in the uterus were studied: (a) uterine wet weight, (b) total number of eosinophils in the uterus including the mesometrium, (c) total number of eosinophils located in uterine endometrium plus myometrium, and (d) oedema in the deep endometrial stroma, measured as the increase in the reciprocal value of cell density (RVCD) in deep endometrium. Since the different tissues of the uterine horns may reflect different characteristics, uterine eosinophil quantification and deep endometrial cell density evaluation were performed in several uterine cross-sections obtained at various levels of the organ.

The eosinophils were counted and classified according to their distribution within the different histological layers of the uterus (endometrium, myometrium and mesometrium). The total number of eosinophils in the uterus or in endometrium plus myometrium only was estimated by multiplying the average number of eosinophils per uterine cross-section by a previously calculated appropriate factor (300) which takes into account the thickness of the section, and by the total length of the fixed uterine horn expressed in mm (Tchernitchin, Roorijck, Tchernitchin, Vandenhende & Galand, 1974).

The cell density quantification was performed in several areas chosen at random in the deep endometrial stroma. The number of cell nuclei was determined in a standard area of $707\ \mu\text{m}^2$ by a circle marked in the ocular piece of the microscope (at $\times 1000$). The reciprocal number of the value obtained for cell density (RVCD) was calculated by dividing the area in which cells were counted by the number of cells in that area; this value corresponds to the intracellular plus adjacent extracellular area related to one cell, and its increase reflects the oestrogen-induced oedematous reaction of deep endometrium (water imbibition) (Grunert & Tchernitchin, 1982).

The oestrogen-induced increases in uterine wet weight and in deep endometrial RVCD were expressed as a percentage of the control value; uterine eosinophilia was expressed in total numbers.

Effect of testosterone propionate on blood eosinophils. Adult female Sprague-Dawley rats, 230–310 g body weight, were ovariectomized 10 days before the experiment. The animals were injected with testosterone propionate (50 mg/kg) or the vehicle. The injection was done under ether anaesthesia into the jugular vein. The blood samples were obtained under ether anaesthesia from the tail of each rat before testosterone propionate or vehicle injection and 6 h after treatment.

The blood samples were collected in small glass vials containing a 5% (w/v) EDTA solution in distilled water (blood:EDTA dilution 9:1, v/v) and incubated for 60 min at 18°C . Aliquants were taken immediately after blood sample collection, at 30 and at 60 min of incubation for a dilution 1:10 (v/v) with freshly prepared eosin stain solution (1 ml 1% eosin yellow stock solution in absolute ethanol diluted in 19 ml distilled water and 1 ml acetone) and subsequent quantification in a Neubauer chamber. The eosinophils were counted and classified as degranulated or non-degranulated according to their histological characteristics (Arroyo, Barrera, Grunert, Reyes & Tchernitchin, 1983). Degranulated eosinophils were those that appeared with a marked decrease in the number of eosin-stained cytoplasmic granules or that had lost almost all granules.

The changes in blood eosinophil levels 6 h after treatment were expressed, for each rat, as a percentage of the values before treatment. The increase in eosinophil degranulation 6 h after treatment was expressed, for each rat, as an increase in the degranulation percentage (measured at the time of blood sample collection) over the values before treatment. Because under some conditions (e.g. 6 h after hormone treatment) there was an increased percentage of degranulated eosinophils at the time of blood sample collection, the *in-vitro* eosinophil degranulation had to be evaluated by considering the eosinophils that were not degranulated at the time of blood sample collection only; the values of eosinophil degranulation *in vitro* were expressed as the additional eosinophil degranulation *in vitro* (Arroyo *et al.*, 1983) at 30 and 60 min of incubation, and were calculated as the percentage degranulation of eosinophils at the end of the 30 or 60 min incubation minus the percentage degranulation at the time of blood sample collection, divided by the

percentage of non-degranulated eosinophils at the time of blood sample collection and multiplied by 100. The determination of the number of eosinophils per microlitre of blood at 30 and 60 min of incubation (in addition to the values at the time of blood sample collection) was to check that there was not a significant loss of eosinophils due to complete degranulation during the incubation; this would have invalidated the results on eosinophil degranulation percentage *in vitro*.

Statistics. In the studies of the effect of testosterone propionate on responses to oestrogen stimulation in the uterus, a Student's *t* test or a Behrens–Fisher test was applied for the comparisons of oestradiol-treated to oestradiol + testosterone propionate-treated and of testosterone propionate-treated to oestradiol + testosterone propionate-treated animals. Two-tailed tests were applied in all comparisons. Student's *t* test was used in all comparisons when variances were not significantly different. Variances were assumed to be heterogeneous when the quotient between both sample variances was significant at the level of $P < 0.01$ (F distribution) and then the Behrens–Fisher test was used. Since multiple comparisons were done *a posteriori*, data were also subjected to the Least Significant Difference *a-posteriori* test.

In the studies of the effect of testosterone propionate on blood eosinophils, Student's *t* test was applied for the comparison of testosterone-injected animals to vehicle-injected rats. This was possible since no heterogeneity between variances was detected at the level of $P < 0.01$ (F distribution) in any comparison. A one-tailed Student's *t* test was applied for the comparison on the increase in degranulation percentage *in vivo* because it was expected *a priori* that testosterone propionate treatment could not decrease the degranulation percentage of eosinophils; two-tailed Student's *t* tests were applied for all other comparisons. A one-way analysis of variance (ANOVA) was used for the comparison of blood eosinophil levels during the incubation *in vitro*.

Results

Effect of testosterone propionate on non-genomic responses to oestrogen in the uterus

Table 1 shows that treatment of immature rats with oestradiol-17 β (300 $\mu\text{g/kg}$) induces an increase in the uterine wet weight, in the number of eosinophils in the uterus (including or

Table 1. Effect of testosterone propionate on non-genomic responses to oestrogen in the uterus of immature rats

Treatment	Uterine wet wt (% of control)	Eosinophils in the uterus (including the mesometrium)	Eosinophils in the uterine endometrium and myometrium	Deep endometrial oedema as RVCD (% of control)
Control	100.0 $\pm$ 3.40 (23)	218 $\pm$ 55 (21)	87 $\pm$ 35 (21)	100.00 $\pm$ 1.63 (19)
Oestradiol (300 $\mu\text{g/kg}$)	190.64 $\pm$ 6.05 (17) ^a	5109 $\pm$ 1101 (17) ^a	3269 $\pm$ 794 (17) ^a	264.46 $\pm$ 18.86 (9) ^a
Testosterone propionate (50 mg/kg)	102.77 $\pm$ 4.78 (5)	258 $\pm$ 151 (8)	100 $\pm$ 100 (8)	117.01 $\pm$ 5.46 (6)
Testosterone propionate + oestradiol	142.93 $\pm$ 4.02 (7) ^{c,d,f,g}	967 $\pm$ 242 (13) ^{b,e,g}	301 $\pm$ 165 (13) ^{e,g}	174.25 $\pm$ 4.99 (7) ^{c,d,e,g}

The rats were killed 6 h after the intravenous administration of the hormone(s) and/or vehicle(s). Not all characteristics were measured in every animal.

Values are mean $\pm$ s.e.m. for the number of rats indicated in parentheses.

Absolute values in controls: uterine wet weight (both uterine horns) = 29.6 mg, deep endometrial oedema as RVCD = 46.39 μm^2 .

^a $P < 0.001$ compared with controls (LSD test); ^b $2P < 0.05$; ^c $2P < 0.001$ compared with testosterone propionate-treated rats (Student's *t* test); ^d $P < 0.001$ compared with testosterone propionate-treated rats (LSD test); ^e $2P < 0.01$; ^f $2P < 0.001$ compared with oestradiol-treated rats (Behrens–Fisher test); ^g $P < 0.001$ compared with oestradiol-treated rats (LSD test).

excluding the mesometrium) and in the deep endometrial RVCD (interpreted as oedema) 6 h after its administration. Treatment with testosterone propionate alone (50 mg/kg) did not significantly modify any of these values 6 h after its administration. Testosterone propionate, administered together with oestradiol, inhibited, but not completely, the oestrogen-induced accumulation of eosinophils in the uterus (including its mesometrium), the uterine wet weight increase and the deep endometrial oedema, 6 h after treatment, and almost completely blocked the oestrogen-induced increase in the number of endometrial plus myometrial eosinophils.

Effect of testosterone propionate on blood eosinophils

Table 2 shows that treatment of adult ovariectomized rats with testosterone propionate (50 mg/kg) resulted in a decrease in the number of eosinophils in the blood and an increase in the degranulation percentage of the eosinophils *in vivo*, 6 h after treatment. The rate of eosinophil degranulation *in vitro*, measured as the additional degranulation percentage *in vitro* at 30 and 60 min of incubation (the degranulation percentage of those eosinophils that were not degranulated at the time of blood sample collection), was also increased 6 h after treatment of the rats with the androgen.

Table 2. Effect of testosterone propionate on blood eosinophils in adult ovariectomized rats

	Experimental condition	
	At 6 h after vehicle injection (control)	At 6 h after treatment with testosterone propionate (50 mg/kg)
Eosinophils per mm ³ of blood (% of values before treatment)	95.76 ± 4.47 (8)	31.96 ± 9.01 (7) ^c
Degranulation percentage <i>in vivo</i> (increase over the values before treatment)	3.09 ± 1.60 (8)	15.77 ± 8.46 (5) ^a
Additional eosinophil degranulation <i>in vitro</i> at 30 min of incubation	19.71 ± 4.01 (8)	71.80 ± 12.90 (5) ^c
Additional eosinophil degranulation <i>in vitro</i> at 60 min of incubation	43.14 ± 2.66 (8)	82.36 ± 10.88 (5) ^b

Blood samples were taken immediately before and 6 h after the administration of the hormone or vehicle.

Values are mean ± s.e.m. for the number of rats indicated in parentheses.

Absolute values in controls before injection: blood eosinophil levels = 208.34/mm³ blood, eosinophil degranulation *in vivo* = 9.48%.

^a $P < 0.05$ compared with control (one-tailed Student's *t* test); ^b $2P < 0.01$; ^c $2P < 0.001$ compared with control (two-tailed Student's *t* test).

Discussion

The results show that testosterone propionate, given with oestradiol to immature rats, inhibits the oestrogen-induced uterine eosinophilia and two responses to oestrogen that have been suggested (Tchernitchin, 1972, 1979, 1983) to be mediated by eosinophils: the increase in uterine wet weight and the deep endometrial oedematous response, 6 h after hormone treatment. The dose of androgen used in the present study is in the range of the doses shown by Jellinck *et al.* (1983) to block selectively the oestrogen-induced increase in uterine peroxidase content without interfering with the oestrogen-induced increase in glucose 6-phosphate dehydrogenase activity, the depletion of the oestrogen receptors from the uterine cytosol and oestrogen receptor accumulation in the nucleus. Considering the previous reports that virtually all oestrogen-induced uterine peroxidase corresponds to the enzyme carried by the eosinophils from the blood to the uterus (King *et al.*, 1981), we suggest that our findings confirm the possibility that the interference by testosterone with the increase in uterine peroxidase is due to a blockage of eosinophil migration from the blood to the uterus.

The lack of interaction by testosterone with the cytosolic or nuclear oestrogen receptors reported by Jellinck *et al.* (1983) is not in agreement with previous reports of a weak binding of testosterone by the cytosolic oestrogen receptors (Garcia & Rochefort, 1979), of a testosterone-induced translocation of the cytosolic oestrogen receptors to the nucleus (Rochefort, Lignon & Capony, 1972) and of an inhibition by testosterone of the cytosolic oestrogen receptor replenishment (Bhakoo & Katzenellenbogen, 1977). The results reported by Jellinck *et al.* (1983) may be possibly explained by the increased dissociation of the weakly bound testosterone from the oestrogen receptors (Rochefort, Capony & Garcia, 1979) that would favour, in the presence of the high doses of oestrogen used, the formation of oestradiol-oestrogen receptor complexes. However, independently from the way of interaction of testosterone with the cytosolic-nuclear oestrogen receptors, the dissociation by testosterone of the different oestrogenic responses (Jellinck *et al.*, 1983) and the selective action of testosterone upon some uterine cell types but not others (Gonzalez-Didi, Komisaruk & Beyer, 1972; Yudaev & Pokrovskij, 1972) suggest that testosterone may selectively interfere with one of the mechanisms of oestrogen action and that there may exist differences amongst cytosolic-nuclear oestrogen receptors in the different uterine cell-types. In this context, the present findings confirm the dissociation of oestrogen-induced uterine eosinophilia and oedema from other responses to oestrogen in the absence of any measurable effect upon cytosolic or nuclear oestrogen receptors, support the idea that uterine eosinophilia and oedema are oestrogenic responses regulated by mechanisms different from those of the genomic responses, and are in agreement with the hypothesis of the mediation of uterine oedema by eosinophils.

It has been previously shown that any eosinopenic condition, such as treatment with glucocorticoids (Tchernitchin, Rooryck, Tchernitchin, Vandenhende & Galand, 1975), insulin (Steinsapir, Rojas, Alarcón & Tchernitchin, 1982a) or thyroid hormones (Steinsapir, Rojas, Mena & Tchernitchin, 1982b), that would limit eosinophil availability for migration to the uterus, inhibit eosinophil accumulation in the uterus after oestrogen administration and depress the responses that have been proposed as being mediated by the eosinophils. Our finding of a decrease in the number of eosinophils in the blood by the androgen may explain the limitation of eosinophil migration to the uterus and the selective blockage of the eosinophil-mediated responses. Further, the increased eosinophil degranulation *in vivo* and *in vitro*, which would leave smaller amounts of enzymes in the migrating eosinophils, would explain the counteraction by testosterone of the increase in uterine peroxidase (Jellinck *et al.*, 1983) and of the eosinophil-mediated responses (present results).

Jellinck & Newcombe (1983) reported that the antiandrogen flutamide abolishes the inhibitory action of testosterone on uterine peroxidase, and suggested that the inhibitory effect is mediated by specific androgen receptors. To corroborate the mediation of this effect by specific androgen receptors it is necessary to confirm the development of blood eosinopenia at lower doses of testosterone. Although it is not clear whether the eosinopenia and the resulting antioestrogenic effect of the androgen is mediated directly by testosterone or indirectly via hypersecretion of another hormone, it is possible to speculate that the target organ for this inhibitory action of the androgen is not the uterus. Further studies are necessary to elucidate the mechanisms involved in the eosinopenic action of high doses of testosterone and to identify the target organ(s) or cell-types for this action of the androgen.

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