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Effects of nafoxidine and oestriol on the oestradiol-induced activation of rat liver tryptophan oxygenase and tyrosine aminotransferase and increase in uterine weight

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The effects of oestrogens or other sex hormones on metabolic functions of the liver have been reviewed extensively [1–3], but less is known about the influence of antioestrogens on the liver. A few reports have appeared on the effects of non-steroidal antioestrogens; clomiphene citrate has been shown to decrease oestrogen-dependent histidase activity in rat liver [4] and RNA and protein content in mouse liver [5], while Cl-628 (α-(4-pyrrolidinoethoxy)phenyl-4-methoxy-α-nitro-stilbene) and nafoxidine blocked oestradiol-induced phosphoprotein and very low-density lipoprotein synthesis in the avian species [6–8]. Tamoxifen effectively blocks the oestrogen-induced decrease of hepatic 3β-hydroxy steroid dehydrogenase activity in rats [9].

The role of oestriol as an antioestrogen is controversial. It has been known for a long time that injection of oestriol can antagonize stimulation of uterine growth by oestradiol in the rat [10–12]. Recently, however, it has been shown [13] that if oestriol is continuously present, it is a potent oestrogen without antagonistic properties. Until now, no reports on the possible antagonistic effect of oestriol on oestradiol-induced changes in metabolic functions of the liver have appeared. The aim of the present study was to examine whether nafoxidine (a non-steroidal antioestrogen) or oestriol could antagonize metabolic changes in rat liver induced by oestradiol. The effects of these compounds on two oestrogen-inducible liver enzymes-tryptophan oxygenase and tyrosine aminotransferase [14] in ovariectomized adult rats were studied. In order to compare the effects on the liver and on the uterus, uterine weights were also determined.

Female Cpb:WU, spf-bred rats (TNO, Zeist, The Netherlands), weighing 150–175 g, were allowed to acclimatize for 5 days before ovariectomy. The rats were housed two per cage and kept at a room temperature of 22–24° and a relative humidity of 55 per cent with a light-dark cycle of 14 hr light–10 hr darkness. Intact or ovariectomized rats were provided with Hope Farm RMH-B pellets and tap water *ad lib*. The experiments were performed according to the randomized block design. Each block represented a day and each experiment consisted of 4 blocks. For each treatment group 8 rats were used. Vaginal smears confirmed complete absence of oestrus 1 week after ovariectomy. Treatments were always started 1 week after surgery and continued for 1 week, unless indicated otherwise. All compounds were administered daily at 09.00 and at 16.00 When two compounds were administered s.c., each one was administered at a separate injection site. Subcutaneously administered compounds were dissolved in arachis oil and orally administered compounds were suspended in a 0.5 per cent gelatin–5 per cent mannitol solution. Placebo treated animals were given the vehicles only. The last treatment was given at 16.00 on the day before autopsy. All animals were killed by decapitation between 09.00 and 10.00.

Liver T–O activity (tryptophan oxygenase; L-tryptophan:oxygen 2,3 -oxidoreductase decyclizing, E.C. 1.13.11.11) was measured on the day of autopsy, using the single wavelength spectrometric assay of Symanski and Bennett [15]. The cytosols, prepared by centrifugation at 105,000 *g*, were preincubated with 9.2 μM hematin at 37° for 20 min [16].

Enzyme activities are expressed as μmoles formylkynurenine plus kynurenine (FK + K) formed·g liver (wet wt)$^{-1}$·hr^{-1}.

Liver TAT (tyrosine aminotransferase; L-tyrosine:2-oxogluterate aminotransferase, E.C. 2.6.1.5) activities were determined according to Diamondstone [17], using 0.1 M potassium phosphate buffer (pH 7.6) [18]. Prior to the TAT assay the cytosols were kept for 28–30 hr at 4° to prevent enzymatic oxidation of *p*-hydroxypyruvic acid [17]. Enzyme activities are expressed as μmoles pHBA (*p*-hydroxybenzaldehyde) formed.g liver (wet wt)$^{-1}$.hr^{-1}. Statistical calculations were performed by analysis of variance for randomized block experiments. The difference in relation to placebo (P) is indicated as

$$\frac{\text{mean treatment group}}{\text{mean placebo group}} \times 100.$$

The difference in relation to oestradiol is indicated as

$$\frac{\text{mean treatment group}}{\text{mean oestradiol group}} \times 100.$$

A result is significantly different from placebo or oestradiol (at 5 per cent level) if the 95 per cent confidence limits do not include the value 100.

Dose-response curves for the stimulation of T–O and TAT activities are shown in Fig. 1. The increase in T–O and TAT activities was more pronounced after 2 weeks treatment with oestradiol than after 1 week. Continuation of the treatment for 3 or 4 weeks did not further increase enzyme activities. Both enzymes were stimulated by about 50 per cent of the maximal response at a dose of 0.15 μg twice daily for 1 week. This dose was selected for testing the effects of the oestrogen antagonists.

Table 1 shows that s.c. administered nafoxidine, at a 100-fold (third column) but not at a 10-fold (second column) excess relative to oestradiol blocked the oestradiol-induced increase in T–O activity. TAT activity was also decreased at a 100-fold excess of nafoxidine, but the effect was not statistically significant. Uterine weights were significantly decreased compared to the group treated with oestradiol alone, but remained about 200 per cent above placebo level. Orally administered nafoxidine (fourth column) blocked the oestradiol effects on T–O and TAT. Uterine weights were reduced to the level reached with nafoxidine alone.

Nafoxidine alone, administered either s.c. or orally, partially stimulated uterine weights at doses which antagonized the oestradiol effects, but it had no effect on T–O or TAT activities.

The effectiveness of oestriol as an oestradiol antagonist was tested after s.c. administration (Fig. 2) or oral administration (Fig. 3) of oestriol. Figure 2 shows the dose-dependent antioestrogenic effect of oestriol. At a 100-fold excess compared to oestradiol [Fig. 2(c)], oestriol blocked the oestradiol-induced activation of both T-O and TAT; uterine weights were slightly reduced but remained higher than placebo levels. At a 1000-fold excess of oestriol with respect to oestradiol [Fig. 2(d)], however, no antagonistic effect of the former was observed and enzyme activities and uterine weights were stimulated to the same extent or slightly more than with oestriol alone in the same dose [Fig. 2(f)].

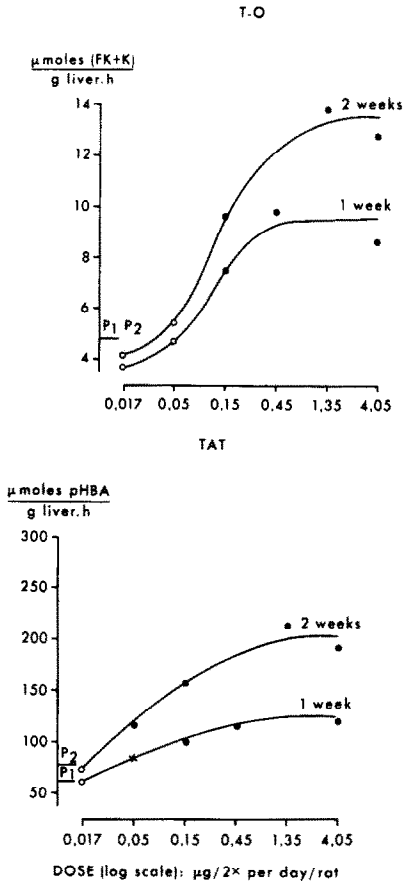

Fig. 1. Dose–response curves for liver T–O and TAT activities in ovariectomized rats after s.c. treatment with oestradiol for 1 and 2 weeks. P_1 = placebo after 1 week; P_2 = placebo after 2 weeks. Differences in relation to placebo are indicated by different points on the curves as follows: ○, not significant with respect to placebo; × – $P < 0.05$; ● – $P < 0.001$. Doses indicated on the abscissa were given twice daily.

Oestriol alone, at doses which were antioestrogenic, did not stimulate enzyme activities [cf. Fig. 2(e) as against (c)]. These results show that s.c. administered oestriol was about 1000 times weaker than oestradiol with regard to enzyme activation, although it cannot be completely excluded that the enzyme activation was due to a trace amount of oestradiol present in the oestriol preparation.

Also after oral administration oestriol counteracted the oestradiol-induced increase in T–O and TAT activities (Fig. 3). A minimal dose of orally administered oestriol between 30 and 62.5 μg twice daily [Figs. 3(a) and (b)] was needed to counteract the oestradiol stimulation of the liver enzymes. As opposed to the effect of s.c. administered oestriol, however, oral administration of oestriol did not counteract the increase in uterine weights caused by oestradiol. Oestriol alone given orally, at a dose which resulted in a two-fold increase in uterine weight, did not stimulate the liver enzymes.

The above results demonstrate that subcutaneous administration of nafoxidine in at least 100-fold excess with respect to oestradiol blocks oestradiol-induced enzyme activation in rat liver. It has been reported that nafoxidine in 20-fold excess with respect to oestradiol did not inhibit the oestradiol-induced decrease in hepatic microsomal 3β-hydroxysteroid dehydrogenase activity in the rat [9].

Table 1.

Treatment dose; μg twice daily	Placebo	Oestradiol s.c. 0.15	Oestradiol s.c. plus naf. s.c. 0.15 plus 1.5	Oestradiol s.c. plus naf. s.c. 0.15 plus 15	Oestradiol s.c. plus naf. p.o. 0.15 plus 125	naf. s.c. 15	naf. p.o. 125
T–O μmoles (FK + K) g liver (wet wt)$^{-1}$hr^{-1}	3.3	4.8	5.9	2.8	2.9	2.0	2.9
Per cent of placebo		146 (117; 182)*	179 (139; 231)*	85 (66; 115)	87 (66; 115)	76 (59; 98)	87 (66; 115)
Per cent of oestradiol			122 (98; 152)	58 (47; 73)†	60 (45; 79)†		
TAT μmoles pHBA g liver (wet wt)$^{-1}$hr^{-1}	75	131	151	111	91	73	86
Per cent of placebo		175 (142; 216)*	201 (158; 255)*	148 (116; 188)*	122 (96; 156)	98 (77; 124)	115 (90; 147)
Per cent of oestradiol			114 (93; 141)	84 (69; 104)	70 (55; 89)†		
Uterine weight (mg)	99	288	297	213	152	153	143
Per cent of placebo		291 (251; 337)*	300 (254; 355)*	215 (182; 255)*	154 (132; 180)*	155 (131; 183)*	145 (124; 170)*
Per cent of oestradiol			103 (89; 119)	74 (64; 86)†	53 (45; 62)†		

All determinations were carried out in duplicate. For each treatment group 8 rats were used.
* significant *increase* compared to placebo treatment.
† significant *decrease* compared to oestradiol treatment.

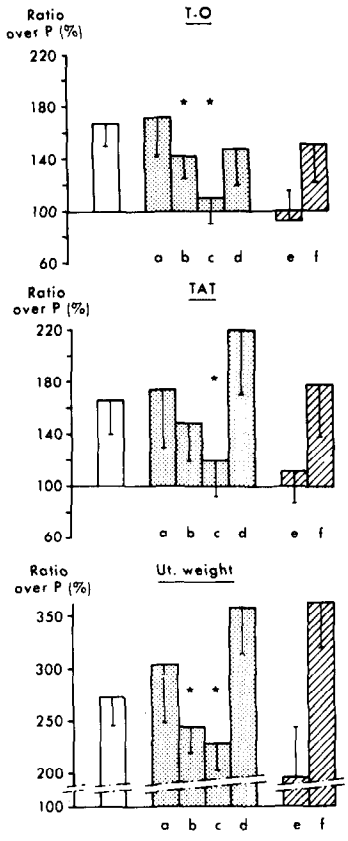

Fig. 2. Inhibition by subcutaneously administered oestriol of oestradiol action on liver enzymes and uterine weight. Duration of treatment: 1 week. □ Oestradiol 0.15 μg twice daily s.c. ▨ Oestradiol 0.15 μg twice daily s.c. plus oestriol: (a) 0.15; (b) 1.5; (c) 15; (d) 150 μg twice daily s.c. ▨ Oestriol: (e) 15; (f) 150 μg twice daily s.c. * significant decrease compared to oestradiol. ⊥ 95 per cent confidence limits.

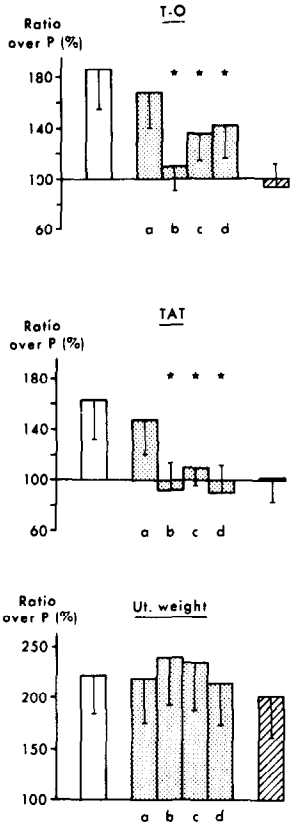

Fig. 3. Inhibition by orally administered oestriol of oestradiol action on liver enzymes. Duration of treatment: 1 week. □ Oestradiol 0.15 μg twice daily s.c. ▨ Oestradiol 0.15 μg twice daily s.c. plus oestriol: (a) 30, (b) 62.5; (c) 125; (d) 250 μg twice daily orally. ▨ Oestriol 125 μg twice daily orally. * significant decrease compared to oestradiol. ⊥ 95 per cent confidence limits.

This failure of nafoxidine to inhibit the effect of oestradiol may have been due to an underdosing of the compound.

The oestrogenic effects of the antioestrogens are somewhat difficult to interpret. On the one hand it has been shown that tamoxifen, like oestrogens, increases the concentration of steroid binding globulins in serum [19]. On the other hand it is clear that both the particular parameter and the route of administration chosen are important factors in determining whether the oestrogenic effect of antioestrogens expresses itself or not [20]. Apparently, under our conditions, T-O and TAT—as opposed to the uterus—are not sensitive to the oestrogenic effects of nafoxidine and oestriol.

The importance of the route of administration appears from the fact that orally administered oestriol (as opposed to s.c.) did not antagonize oestradiol-induced uterine growth. Taking this into account, the finding that oestradiol-induced changes in hepatic metabolism can be inhibited by oestriol may offer a possibility to counteract unwanted side-effects of other oestrogens.

In conclusion, dose–response curves for the stimulation of liver tryptophan oxygenase and tyrosine aminotransferase activities by subcutaneously administered oestradiol in ovariectomized rats were established. The antioestrogenic effects of s.c. or orally administered nafoxidine or oestriol on the oestradiol-induced enzyme activities were studied, and compared with their effect on oestradiol-stimulated uterine growth. It was found that the oestradiol-induced stimulation of the liver enzymes could be blocked by oral or subcutaneously administered nafoxidine or oestriol. Nafoxidine administered by both routes, as well as subcutaneously administered oestriol antagonized oestradiol-induced stimulation of uterine growth, but orally administered oestriol failed to do so.

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Effect of Triton X-100 and Alamethicin on the susceptibility of brain adenylate cyclase to EGTA inhibition*

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Divalent cations are important modulators of hormone-sensitive adenylate cyclase. The presence of a metal ion is required for enzymatic activity since the substrate for the enzyme is a metal–ATP complex. Mg^{2+} · ATP is probably the physiological substrate but Mn^{2+} or Co^{2+} can substitute for Mg^{2+} *in vitro* [1, 2]. In most systems, in fact, the activity measured with Mn^{2+} is higher than that measured with Mg^{2+} [2–8]. In addition to the requirement for metal to complex with ATP, recent evidence suggests that there is a separate, regulatory metal ion-binding site on the enzyme and that binding of a metal ion to this site is required for full adenylate cyclase activity [3–5].

Adenylate cyclase from several regions of the brain is inhibited by EGTA† (0.1 to 0.5 mM), but the enzyme from other tissues is not affected [2, 6–10]. This inhibition by 0.1 to 0.5 mM EGTA is not due to chelation of the metal required to form the substrate metal · ATP complex since it occurs in the presence of 5–12 mM Mg^{2+}. Rather, it is probably due to chelation of a metal ion associated with the enzyme itself. Recent experiments from this laboratory have shown that inhibition of detergent-solubilized brain adenylate cyclase by EGTA can be almost entirely reversed by the addition of Mn^{2+} in excess of EGTA [11]. These experiments suggest that brain adenylate cyclase may have associated manganese ions which are required for activity. Co^{2+} does not reverse EGTA inhibition [11], whereas Ni^{2+}, Zn^{2+} and Fe^{2+} are inhibitors of the enzyme [2]. The EGTA inhibition can also be partially reversed by adding calcium in excess of EGTA [11]. Brostrom *et al.* [8] and Cheung *et al.* [9] have show that the effect of calcium requires the presence of calmodulin, a small protein which mediates the action of calcium in several systems. It is not clear whether Ca^{2+} and Mn^{2+} affect the same population of adenylate cyclase molecules.

Inhibition of brain adenylate cyclase by EGTA has been reported to occur both in detergent-solubilized and in particulate preparations. In the former, the degree of inhibition is between 75 and 100 per cent [2, 10, 11]. The results with particulate enzyme, however, are much more variable, with reports ranging from no inhibition to 70 per cent inhibition [7, 12, 13]. The present studies show that enzyme need not be solubilized for maximum inhibition by EGTA, but that some disruption of the lipid bilayer seems to be required. This suggests that the metal ion required for full brain adenylate cyclase activity may be located in a region that is not accessible to EGTA in the intact membrane.

Mature rats were killed by asphyxiation in CO_2 or by a blow to the head. The brains were immediately removed and chilled, and the cortices were dissected free of white matter. The tissue was homogenized in a Dounce homogenizer with a loose fitting pestle in 0.1 M Tris · Cl (pH 7.6), 0.075 M sucrose, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The unfractionated homogenate was used in some experiments. For others, a membrane-enriched fraction was prepared by discontinuous sucrose density gradient centrifugation as described previously [14]. Homogenates were also prepared in hypotonic buffer, a procedure frequently used to lyse synaptosomes; cortical tissue was homogenized in 2 mM Tris · Cl (pH 7.6), the homogenate was centrifuged at 20,000 *g* for 20 min at 4°, and the pellet was washed twice more and resuspended in the same buffer.

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† Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; and ECTEOLA, epichlorhydrin triethanolamine cellulose.