Factors Affecting Anti-Estrogen Binding Site Concentration in Rat Liver (43256)

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Abstract. **It is known that synthetic anti-estrogens such as tamoxifen bind to specific high affinity anti-estrogen binding sites (AEBS), which are distinct from estrogen receptors. These binding sites are widely distributed in animal and human tissues, the highest concentrations being found in the liver. The physiological role of these intracellular binding sites, which are located predominantly in the microsomal fraction, is currently unknown, as is the nature and identity of their endogenous ligands. In an attempt to gain information which may provide clues to the possible physiological role of these binding sites, studies were carried out to determine whether the concentration of these binding sites in rat liver was affected by a number of physiological variables. The results of these studies indicated that in the rat (i) liver AEBS increased progressively with age; (ii) liver AEBS concentration tended to be higher among females than males after 100 days of age; (iii) there was no significant variation in liver AEBS level with different phases of the estrous cycle; (iv) liver AEBS level was not significantly affected by castration in both males and females or by estradiol replacement in castrated females; (v) liver AEBS concentration increased significantly with increases in ambient temperature; (vi) there was no clearly detectable alteration in liver AEBS levels with changes in the light:dark cycle; (vii) starvation for 24, 48, and 72 hr increased liver AEBS by approximately 1.5-, 3-, and 2-fold, respectively, while refeeding decreased its level; and (viii) liver AEBS was not affected by increasing dietary fat content from 0.5% to 20% (w/w), but was increased modestly by the addition of cholesterol (2% w/w) to the diet. These observations identify several physiological variables which are associated with changes in liver AEBS concentration and suggest possible avenues for future studies to define the physiological role of these binding sites.** [P.S.E.B.M. 1991, Vol 197]

Since the anti-estrogen binding site (AEBS) was first identified about 10 years ago as a distinct entity by Sutherland and his colleagues (1), considerable information has accumulated regarding its physicochemical properties, its ligand specificity, and its tissue and subcellular distribution (2, 3). It is now known that the AEBS is a protein which binds synthetic anti-estrogens such as tamoxifen and clomiphene with high affinity, but which, unlike the estrogen receptor, has no binding affinity for estrogens. The AEBS also binds several other classes of compounds, including phenothiazines, bibenzyl and stibene derivatives, cyclophenyl analogues, diphenylmethane derivatives, histamine antagonists, and miscellaneous other compounds with high affinity (4–7). The AEBS is ubiquitously distributed in animal and human tissues (2, 8, 9) and is located predominantly in the microsomal fraction of tissue homogenates (2, 9). The highest concentrations are found in the liver (2). It has not yet been purified to homogeneity, but it is known to be protein in nature since its binding activity is readily destroyed by proteases (10). Estimates of its molecular mass have been quite variable, ranging from 80 to 700 kDa in different studies (2, 10–12).

Several major questions about the AEBS remain unanswered. There is currently no information about its possible physiological role and the identity of its endogenous ligand. In an attempt to obtain information which might provide clues about its function, we have attempted to identify physiological variables which might affect the concentration of AEBS in the liver of the rat. The studies reported here focused on the liver

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Received October 19, 1990. [P.S.E.B.M. 1991, Vol 197]
Accepted March 11, 1991.

0037-9727/91/1973-0279$3.00/0
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because this organ displays, by far, the highest concentration of AEBS among animal tissues, and it was surmised that useful information about the possible physiological function of AEBS might be more readily obtained by studying variations of AEBS concentration in this organ than by examining other tissues.

Materials and Methods

Animals. Sprague-Dawley rats obtained from our local breeding facility were used for these studies. For the study on age and sex-related variations in liver AEBS, male and female rats of age 11–367 days were examined, whereas for all other studies rats aged 90–130 days were used. Unless otherwise stated, the animals were housed at room temperature (28–29°C), given rat chow and water *ad libitum*, and exposed to 12 hr of light and 12 hr of darkness, with the light cycle starting at 0600 hr.

Chemicals. [*N-methyl*-^{3}H]Tamoxifen (sp act 87 Ci/mmol or 3.2 TBq/mmol) was obtained from Amersham International (Buckinghamshire, England) and stored in ethanol at −20°C protected from light. Tamoxifen, diethylstilbestrol, estradiol 3-benzoate, L-methionine, cholesterol, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and were obtained from conventional sources.

Preparation of 20,000*g* Supernatant from Rat Liver. Livers were removed from the rats under diethyl ether anesthesia and were homogenized in 12 vol of ice-cold buffer (10 m*M* Tris-Cl, 1.5 m*M* EDTA, and 10% (v/v) glycerol (pH 7.5), at 4°C) using an Ultra-Turrax homogenizer (medium speed for 30 sec). The homogenate was centrifuged at 20,000*g* for 30 min at 4°C and the supernatant was stored at −70°C in suitable aliquots.

Ligand-Binding Assays and Saturation Analysis. Ligand-binding assays and saturation analysis were carried out as described previously (13). In brief, the 20,000*g* liver supernatant was diluted with homogenizing buffer to a protein concentration of about 0.5–1 mg/ml, and incubated with varying concentrations of [^{3}H]tamoxifen (0.2–7.0 n*M*) in the absence or presence of excess unlabeled tamoxifen (final concentration 500 n*M*) in a final volume of 600 μl. Diethylstibestrol was added to all tubes at a concentration of 1 μ*M* to eliminate possible binding to estrogen receptors. After 16–20 hr of incubation at 4°C, the assay was terminated by the addition of dextran-coated charcoal, as described previously (13).

Dietary Composition Studies. Female rats aged 110–120 days were fed semisynthetic diets containing varying amounts of corn oil (14) or cholesterol (15). In the study examining the effect of variations in corn oil content, the low-fat diet mixture contained (w/w) 0.5% corn oil, 18% casein, 72% dextrose, 4% Phillips-Hart salt mix, and 5% Cellu flour. The high-fat mixture contained (w/w) 20% corn oil, 23% casein, 46% dextrose, 5% Phillips-Hart salt mix, and 5% Cellu flour. In addition, both diets contained, in each kilogram of diet mixture, the following nutrients: 100 mg of vitamin A, 110 mg of DL-tocopherol acetate, 30 mg of vitamin K, 7.5 mg of thiamine, 7.5 mg of pyridoxine, 0.3 mg of biotin, 1.5 g of choline chloride, 150 mg of inositol, 15 mg of riboflavin, 75 mg of nicotinic acid, 88 mg of calcium pantothenate, and 1.5 mg of folic acid. For the studies involving varying amounts of cholesterol, either 1.2 g or 21.2 g of cholesterol were added to each kilogram of dietary mixture which contained the following ingredients: 270 g of casein, 200 g of starch, 207 g of glucose, 50 g of cellulose, 180 g of beef tallow, 20 g of safflower oil, 50 g of Phillips-Hart mineral mix, 2.75 g of choline, 6.25 g of L-methionine, 20,000 IU of vitamin A, 2,000 IU of vitamin D, 100 mg of vitamin E, 5 mg of menadione, 5 mg of thiamine, 8 mg of riboflavin, 40 mg of pyridoxine, 40 mg of niacin, 40 mg of pantothenic acid, 2,000 mg of choline, 100 mg of *myo*-inositol, 100 mg of *p*-aminobenzoic acid, 0.4 mg of biotin, 2 mg of folic acid, and 30 mg of vitamin B_{12}. Rats were sacrificed at the times indicated and their livers were removed for analysis.

Other Procedures. Protein concentration was determined by the method of Lowry *et al.* (16). Staging of the estrous cycle was carried out according to established procedure (17). For statistical analysis, an unpaired Student's *t* test was used to compare AEBS levels between two groups, while one-way analysis of variance was used to compare mean values of three or more groups. Other procedures were carried out as described in the appropriate figure legends and tables.

Results

Figure 1 shows the effect of age and sex on liver AEBS concentrations. In both sexes, there was a progressive increase in liver AEBS concentration with increasing age, the increase being seen up to 72 days in males and to about 1 year among females. Among the

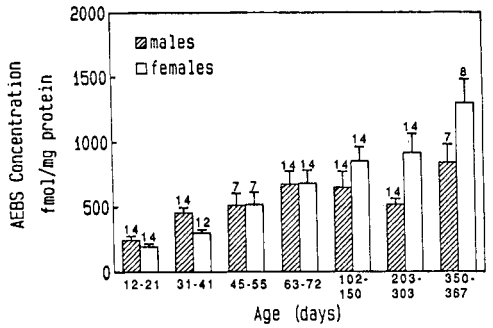

Figure 1. Variation of rat liver AEBS concentration with age and sex. The values shown represent mean ± SE of AEBS levels, and the values above each bar indicate the number of animals in each group.

younger rats (age up to 72 days), no consistent sex difference was observed. Among these younger rats, the mean liver AEBS concentration among males averaged 464 ± 42 (SE) fmol/mg protein, whereas the average among females was 451 ± 57 (SE) fmol/mg protein; the difference was not statistically significant ($P = 0.85$). Among rats older than 102 days, however, female rats appeared to have higher liver AEBS levels. For these older rats, aged 102–367 days, the AEBS levels observed among males and females were, respectively, 636–61 (SE) and 966 ± 84 (SE) fmol/mg protein, the difference being statistically significant ($P = 0.002$). It is worth pointing out that the divergence between the sexes was most obvious among the oldest rats studied and did not appear until about 100 days of age, some 2 months after the time of pubertal development. There was no significant difference in the binding affinities or K_d values of AEBS in the different age and sex groups studied (results not shown).

Figure 2 shows the concentrations of liver AEBS in adult female rats at different stages of the estrous cycle. No significant change was observed during the reproductive cycle in the concentration of liver AEBS ($P = 0.37$ by one-way analysis of variance). There was also no detectable cyclical change in the binding affinity of AEBS during the estrous cycle. The K_d values observed for proestrus, estrus, metestrus, and diestrus were, respectively, 0.53 ± 0.04, 0.69 ± 0.1, 0.62 ± 0.1, and 0.69 ± 0.06 nM (mean ± SE).

Table I shows the effect of castration and estrogen replacement in female rats and the effect of orchiectomy in male rats. Ovariectomy tended to decrease liver AEBS concentration and estrogen replacement tended to restore its level, but the changes observed did not reach statistical significance ($P = 0.14$). Among male rats, orchiectomy had no discernible effect on liver AEBS concentration ($P = 0.55$). The K_d values were also not significantly altered by castration or hormone replacement.

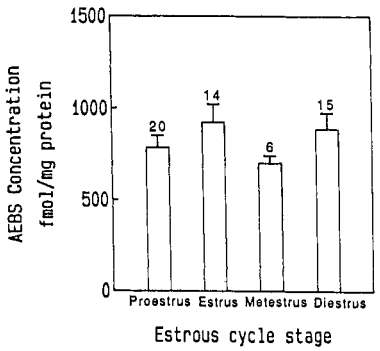

Figure 2. Liver AEBS concentrations during the estrous cycle. The stages of the estrous cycle were determined by examination of the vaginal smear following established criteria (17). The values indicate mean ± SE of AEBS concentrations. The values above each bar indicate the number of animals in each group.

Table II shows the effects of changing the light:dark cycle and the ambient temperature on liver AEBS concentration in adult female rats. Liver AEBS levels did not change significantly when rats were exposed to different photoperiods. The levels observed with 12 hr of light and 12 hr of darkness, and 24 hr of light or 24 hr of darkness were not significantly different. In contrast, an increase in ambient temperature was clearly associated with an increase in liver AEBS concentration. After being housed at 21°C, 29°C, and 34°C for 10 days, adult female rats showed liver AEBS levels of 281 ± 47 (SE), 516 ± 55 (SE), and 829 ± 90 (SE) fmol/mg protein, respectively. Compared with the concentration observed at 21°C, the increases seen at 29°C and 34°C were statistically significant ($P < 0.05$ and $P < 0.001$, respectively).

Figure 3 shows the effect of starvation on liver AEBS concentration. Food deprivation clearly caused a significant rise in liver AEBS levels. The AEBS levels observed after 24, 48, and 72 hr of fasting were, respectively, 158%, 309%, and 190% that of control, all changes being statistically significant ($P < 0.05$, $P < 0.001$, and $P < 0.05$, respectively). When animals that had been starved previously for 72 hr were fed, there was a prompt fall in liver AEBS concentration which could be observed as early as 6 hr after the initiation for refeeding. At both 6 and 24 hr after the initiation of refeeding, liver AEBS levels were not significantly different from those of rats that had been given free access to food throughout the experiment. At 24 hr after refeeding, the AEBS level was significantly lower than that seen after 48 or 72 hr of starvation ($P < 0.05$). There was no significant change in the binding affinity of liver AEBS in the feeding experiments (result not shown).

Table III shows the effect of altering the dietary content of fat and cholesterol on liver AEBS concentration. Increasing the corn oil content from 0.5% to 20% (w/w) did not significantly alter AEBS concentration in the liver after 14 days ($P > 0.05$). In contrast, an increase of cholesterol content from 0.12% to 2.12% (w/w) led to a 40% rise in liver AEBS concentration, which was modest but significant ($P < 0.01$). There was no significant difference in the weight gain of animals given the different diets, indicating that the changes observed were not likely to be secondary to differences in the amount of food ingested during the experimental period.

Discussion

The studies reported here represent an attempt to identify some physiological variables that might affect the concentration of AEBS in the liver of the rat. There is currently very little information on the factors regulating AEBS levels in the liver or, indeed, in any other tissue. To the authors' knowledge, there are only three

Table I. Effect of Castration and Hormone Replacement on Liver AEBS Concentration in Adult Rats[a]

Treatment group	No. of rats	AEBS (fmol/mg protein)	K_d (n*M*)
Female rats			
Sham-operated	8	457 ± 56 (100)	0.35 ± 0.05
Ovariectomized	7	325 ± 42[b] (71)	0.40 ± 0.04[b]
Ovariectomized and E_2-treated	7	604 ± 172[b] (132)	0.59 ± 0.14[b]
Male rats			
Sham-operated	8	442 ± 50 (100)	0.62 ± 0.06
Orchiectomized	8	401 ± 46[b] (91)	0.55 ± 0.04[b]

[a] Adult rats, 120–130 days old, were sham-operated or castrated. Ovariectomized rats were given daily subcutaneous injections of 3.75 μg estradiol 3-benzoate (E_2) in 0.2 ml of corn oil or vehicle alone for 2 weeks, after which the animals were sacrificed and their livers were removed for analysis. Male rats were orchiectomized or sham-operated and sacrificed 2 weeks later. The AEBS values shown represent mean ± SE. K_d values are expressed as mean ± SE. The figures in parentheses indicate mean AEBS concentrations as a percentage of that observed in control animals.
[b] Compared with the sham-operated group, the differences observed are not statistically significant ($P > 0.05$).

Table II. Effect of Changes in Light:Dark Cycle and Ambient Temperature on Liver AEBS[a]

Environmental condition	No. of animals	AEBS (fmol/mg protein)	*P*
Photoperiod			
12 hr light:12 hr darkness	7	833 ± 159	
24 hr light	8	803 ± 116	> 0.05
24 hr darkness	8	700 ± 69	> 0.05
Ambient temperature			
21°C	6	281 ± 47	
29°C	7	516 ± 55	< 0.05
34°C	7	829 ± 90	< 0.001

[a] Adult female rats, aged 110–120 days, were exposed to different photoperiods for 7 days or to different ambient temperatures for 10 days before sacrifice. The liver AEBS concentrations were expressed as mean ± SE.
[b] Compared with the first group in each experiment.

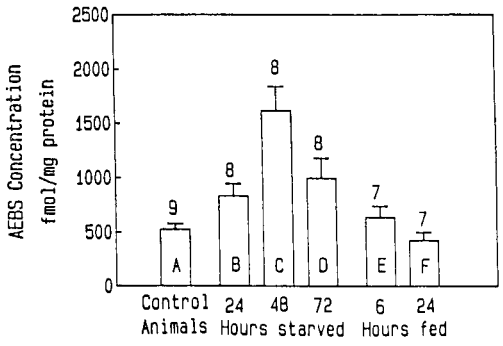

Figure 3. Effect of starvation and refeeding on rat liver AEBS concentration. Control rats (A) were given food *ad libitum*. Groups B, C, and D were starved for 24, 48, and 72 hr, respectively. Groups E and F were initially starved for 72 hr and then given free access to rat chow for 6 and 24 hr, respectively, before sacrifice. All animals received water *ad libitum* throughout the experimental period. The values represent mean ± SE of AEBS levels and the values above each bar indicate the number of animals in each group.

Table III. Effect of Changes in Dietary Composition on Liver AEBS Concentration[a]

Experimental diet	No. of animals	AEBS (fmol/mg protein)	*P*
0.5% corn oil	10	509 ± 54	
20% corn oil	10	513 ± 53	> 0.05
0.12% cholesterol	10	564 ± 53	
2.12% cholesterol	10	794 ± 53	< 0.01

[a] Adult female rats, aged 110–120 days, were given diets the composition of which are as detailed in the Materials and Methods section. After 14 days, the rats were sacrificed and their livers were analyzed. The values given represent mean ± SE.
[b] Compared with the first group in each experiment.

studies to date which have examined variations in AEBS levels in individual tissues under different physiological or pathological conditions, and only one of these measured AEBS concentrations in the liver of the rat. Winneker and Clark (18) examined AEBS concen-

trations in the liver and uterus of the rat under a variety of conditions, while Faye *et al.* (19) and Mehta *et al.* (20) reported variations in AEBS concentration in the uterus and mammary gland of the rat, respectively. Winneker and Clark (18) reported that rat liver AEBS concentration increased with age, was higher in females than in males, decreased with ovariectomy, increased with estrogen replacement, and varied cyclically during the estrous cycle. We were able to confirm some, but not all, of these findings. The results described in the present study confirm and extend the observation of Winneker and Clark (18) that rat liver AEBS increases progressively with age. In that study, Sprague-Dawley rats up to 60 days of age were examined. We have included older rats (up to 367 days of age) in the present study and have shown that the progressive increase of liver AEBS continued to 72 days of age among male rats and to 1 year among female rats (Fig. 1). We also confirm that liver AEBS levels tended to be higher in adult female rats than in male rats, but the sex difference was modest and achieved statistical significance only for rats older than 100 days (Fig. 1). However, unlike Winneker and Clark (18), we failed to detect cyclical variations in liver AEBS concentration with different phases of the estrous cycle (Fig. 2). Furthermore, although we observed that liver AEBS tended to fall after ovariectomy and to rise with estrogen replacement (Table I), these changes did not reach statistical significance. It should be noted, however, that estradiol treatment following ovariectomy increased mean liver AEBS level 1.8-fold, a magnitude of change not dissimilar from that observed by Winneker and Clark (18). In their study,the rise was significant ($P < 0.05$), whereas in our study it was not ($P = 0.14$). Methodological differences may account for our failure to demonstrate significant estrogenic control of liver AEBS levels. In this regard, it is worth noting that Sudo *et al.* (9) failed to detect an effect of estrogen treatment on rat uterine AEBS concentration, whereas Winneker and Clark (18), in a longer-term study, showed that estrogen treatment increased uterine AEBS levels. Further studies are clearly needed to clarify the issue of estrogenic control of AEBS levels.

Among the other physiological variables examined, alterations in the light:dark cycle and changes in dietary fat content had no effect on liver AEBS concentration. An increase in ambient temperature, starvation, and a high-cholesterol diet led to an increase in liver AEBS levels, whereas refeeding after a period of starvation reduced liver AEBS.

It is difficult and, given the current state of our knowledge, probably unprofitable to speculate on the physiological significance of these observations. Nevertheless, it is intriguing that dietary manipulations caused significant changes in liver AEBS concentration. In this regard, it is worth noting that the liver, which is the first organ traversed by absorbed nutrients carried by the portal circulation, is also the organ with the highest AEBS concentration in the body. It is possible that AEBS levels fluctuate in response to the absence or presence of one or more food ingredients.

In an attempt to determine whether specific nutrients in the diet affect liver AEBS levels, studies were carried out in which the dietary composition was altered by the addition of fat or cholesterol. These specific nutrients were examined because the endoplasmic reticulum, in which AEBS is found, is intimately involved in their metabolism. The results (Table III) indicate that while changes in fat intake did not alter liver AEBS concentration, an increase in dietary cholesterol appeared to increase liver AEBS levels to a modest extent. This observation is of particular interest because a number of AEBS ligands, such as tamoxifen and 7-ketocholesterol, have been reported to inhibit cholesterol biosynthesis (21, 22), raising the possibility that AEBS may be involved in some way with cholesterol metabolism. Further studies will be required to address this question. The mechanism by which an increase in ambient temperature induces a rise in liver AEBS will also require further exploration. Preliminary data obtained in our laboratory suggest that the rise in liver AEBS seen with an increased ambient temperature is probably not due to reduced food intake or dehydration.

The studies described here have identified several physiological variables that are associated with significant alterations of hepatic AEBS concentration in the rat. Although it is clearly premature to speculate on the biological significance of these observations, we believe that these findings suggest avenues for future studies to delineate possible functions for the AEBS, particularly with respect to nutrient handling and cholesterol metabolism.

This work was supported by the National University of Singapore. The superb secretarial help of Asha Das is gratefully acknowledged.

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