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INFLUENCE OF ESTROGENS ON PEROXIDASE ACTIVITY IN THE SYRIAN HAMSTER LIVER, KIDNEY, AND RENAL ADENOCARCINOMA

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Abstract—Only very low levels of peroxidase activity were detected in castrated male hamster kidneys [1.0 ± 0.8 (S.E.) units/g protein], and chronic estrogen administration, either diethylstilbestrol (DES) or 17β-estradiol, for 1–5 months did not result in any appreciable increase in this activity. In contrast, hamster liver peroxidase activity was initially 10- to 20-fold higher than kidney levels, and chronic estrogen treatment for similar periods resulted in up to a 9-fold elevation in the activity of this enzyme. Moreover, the level of liver peroxidase activity in both intact and in 5α-dihydrotestosterone-treated castrate hamsters was 2-fold higher than castrate-untreated values. Pure renal carcinoma induced after 9 months of estrogen treatment exhibited peroxidase values similar to those found in hamster livers [124 ± 27 (S.E.) units/g protein] following estrogen treatment. When administered concomitantly with DES, tamoxifen significantly reduced the elevated levels of liver peroxidase activity observed after 2 months of DES treatment alone. A high affinity ($K_A = 0.10 \times 10^9\ M^{-1}$) estrogen receptor was found in liver cytosols of DES-treated hamsters which had increased slightly from untreated castrate levels.

Estrogens have been linked to carcinogenesis in a variety of animals including man [1, 2], but their mechanism of action in this regard remains obscure. Recent work has indicated that oxidative metabolism of synthetic as well as natural estrogens can produce a number of electrophiles capable of interacting covalently with cellular macromolecules [3–7]. One pathway leading to potentially reactive diethylstilbestrol (DES) metabolites involves peroxidase [8], an enzyme also suspected in the activation of polycyclic aromatic hydrocarbons to potent carcinogens [9, 10]. This is of special significance since estrogens, including DES, are known to induce peroxidase activity in target tissues like uterus and vagina [11]. The fact that estrogen-related tumors are often found in such organs suggests the possibility of a causal relationship between peroxidase and carcinogenesis.

One of the most sensitive tissues with regard to estrogen carcinogenicity is the hamster kidney [12]. Although not considered a typical target organ, it contains an estrogen receptor sedimenting as 4S and 8S binding components [13, 14]. Furthermore, estrogen treatment induces a sizeable increase in progesterone receptor concentration in this tissue [15, 16]. These results indicate responsiveness to estrogenic hormones and suggest the possibility of peroxidase induction in this organ where it could then serve to activate estrogens to possible proximate or ultimate carcinogens. Alternatively, activation to a proximate carcinogen could occur in the liver with the resulting reactive molecule finding its way to the kidney via the blood. In view of this, we have examined the effect of estrogen treatment on hamster liver and kidney peroxidase as well as in the estrogen-induced and -dependent renal carcinoma. These data are correlated with the level of estrogen receptor found in these livers.

MATERIALS AND METHODS

Chemicals and reagents. [2,4,6,7-^{3}H]-17β-Estradiol (105 Ci/mmole) was obtained from the New England Nuclear Corp. (Boston, MA). Nonradioactive progesterone was purchased from Calbiochem (La Jolla, CA). Other radioinert steroids, DES, Trizma base, Norit A, Dextran-80, dithiothreitol, sodium molybdate, and guaiacol were supplied by the Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide was obtained from Fisher Scientific (Chicago, IL). Ultrapure sucrose (RNase-free) was obtained from Schwartz/Mann (Spring Valley, NY). Tamoxifen [*trans*-1-(*p*-β-dimethylaminoethoxyphenyl)-]-2-diphenylbut-1-ene] was provided by Imperial Chemical Industries (Wilmington, DE). Reagents used for protein determination of the peroxidase samples were purchased from Bio-Rad Laboratories (Richmond, CA).

Animals and treatment. Adult intact and castrate male Syrian golden hamsters and immature male rats were obtained from either Harlan Sprague–Dawley, Indianapolis, IN, or Charles River Lakeview hamster (LAK:LVG) Colony, Wilmington, MA. All hamsters weighed between 80 and 95 g (50–55 days old). Hamsters were castrated 2 weeks prior to treatment or sacrifice. Pellets (20 mg) of the pure hormone were prepared without binder and implanted subcutaneously in the shoulder region as previously described [17]. In one series of experiments, groups of hamsters bearing DES pellets were

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killed at intervals up to 5 months. To maintain constant DES levels in these animals, pellets were reimplanted at 3 months. In a second series, groups of hamsters were implanted with the following compounds 2 months prior to analysis: (1) DES alone, (2) 17β-estradiol, (3) 5α-dihydrotestosterone (5α-DHT) alone, (4) DES and 5α-DHT, (5) tamoxifen alone, and (6) DES and tamoxifen. Since it has been found that absorption rates of antiestrogen are more rapid than that of the hormone [14, 15], 30-mg pellets of tamoxifen were implanted monthly. In animals used for receptor analyses, pellets were removed 63–68 hr before sacrifice to clear endogenous hormone.

Preparation of enzyme extract. Peroxidase activity was extracted according to the method of Lyttle and DeSombre [18]. Following treatment for the periods indicated, etherized animals were exsanguinated via the inferior vena cava and the tissues were removed, dissected free of connective tissue, and placed immediately on ice. The chilled tissues (500–600 mg) were then blotted on filter paper, weighed, and homogenized for 15 sec in 10 ml of cold 10 mM Tris–HCl, pH 7.2, using a polytron homogenizer (setting 6; Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 39,000 *g* for 30 min (4°), and the supernatant fraction which was devoid of peroxidase activity was discarded. The pellet was rehomogenized in the Tris–HCl buffer containing 500 mM $CaCl_2$ (200 mg original tissue wt/ml) and recentrifuged for 30 min at 39,000 *g* whereupon the supernatant fraction was collected for assay.

Enzyme assay. Peroxidase was assayed according to a modification of the guaiacol method described by Himmelhoch *et al.* [19]. The 3-ml reaction mixture contained 13 mM guaiacol and 0.15 mM hydrogen peroxide made up in the Tris–$CaCl_2$ extraction buffer. The extract at equivalent protein concentrations (10 μl for liver and kidney, 200 μl for tumor) was added, and the reaction was followed at 25° for the first 2 min during which time the response was linear. The reaction was monitored at 470 nm with a Gilford model 222 conversion of a Beckman model DU single-beam spectrophotometer coupled to a Hewlett–Packard strip-chart recorder. An enzyme unit is defined as the amount of enzyme required to produce an increase of one absorbance unit per min under these conditions. Protein was determined using the Coomassie blue procedure [20] with a bovine gamma globulin standard.

Cytosol receptor analyses. Livers used for receptor analyses were perfused *in situ* with cold isotonic saline in TED (0.01 M Tris–HCl, 0.0015 M EDTA, 0.001 M dithiothreitol, pH 8.0) buffer containing 20 mM sodium molybdate before excision. Excised perfused livers were washed, minced, and homogenized in 4 vol. of TED buffer (1 g:4 ml) containing 20 mM sodium molybdate and 5% glycerol. Following centrifugation of the liver homogenates at 100,000 *g* for 1 hr in a Spinco L2-65B ultracentrifuge, the cytosol fractions were filtered through a millipore (Millex, 0.22 μM) to obtain clear supernatant fluids as previously described [13, 14]. Final pH of the cytosols was 7.4. Liver cytosol fractions (0.5 to 1.0 ml) were incubated with 5–10 nM tritiated estradiol alone or in combination with radioinert DES for 90 min. Competition was carried out at 200-fold excess non-labeled DES concentration. Following the incubation period, appropriate amounts of each cytosol sample were assayed for radioactivity and then treated with dextran–charcoal (DC) for 1 hr as indicated elsewhere [13, 14]. Liver estrogen receptor concentrations in all groups were calculated from plots varying protein concentrations versus specific hormone receptor binding containing at least four data points.

Protein concentrations of the cytosols were measured by the method of Lowry *et al.* [21] with appropriate corrections for the presence of molybdate, using bovine serum albumin as a standard.

Scatchard analyses [22] for estrogen receptor in the liver were determined in cytosols at 0.5 to 20 nM concentrations of radiolabeled estradiol after overnight incubation. In parallel incubations, 200-fold excess nonlabeled DES was added at each concentration to correct for nonspecific binding.

Sucrose density gradient analyses. Liver fractions of cytosol (0.4 ml) were layered on 4.6 ml linear 5–20% sucrose gradients prepared in TED buffer, pH 7.4, containing 20 mM molybdate using a Buchler gradient former.

The liver samples applied were centrifuged for 17 hr at 39,000 rpm in a Spinco SW50.1 rotor at 3°. Gradient tubes were pierced with a 20-gauge needle and 9 drop fractions were collected in scintillation vials. Details of the radioactivity measurements have been amply described earlier [14–16]. Samples were counted at 5° in a Packard Tri-Carb model 3375 liquid scintillation spectrometer with a counting efficiency of about 43% for tritium.

Sedimentation coefficients were determined by the method of Martin and Ames [23] using both radiolabeled and unlabeled standards [13–16].

RESULTS

The effect of chronic DES treatment on liver and kidney peroxidase activity in the castrate male hamster is summarized in Table 1. The activity found in the untreated kidney was extremely low, occurring right around the minimal detectable level of the assay, and did not appreciably increase in response to DES regardless of the duration of treatment. In contrast, liver peroxidase activity was initially about 10- to 20-fold greater than kidney levels and responded to DES with a 9-fold increase after 5 months of treatment compared to untreated liver values. Comparable increases in liver peroxidase activity were also evident following continuous 17β-estradiol treatment for 5 months (Table 1). Pure renal carcinoma taken from kidneys of animals that had been treated with DES in excess of 9 months exhibited peroxidase values similar to those of liver at 124 ± 27 (S.E.) units/g protein, indicating a dramatic rise in peroxidase activity compared to levels found in either untreated or estrogen-primed kidneys. For comparative purposes, uteri of four immature rats (25 days old) injected with 100 μg of 17β-estradiol and killed 24 hr later were analyzed for peroxidase activity. The peroxidase activity in these uteri was considerably greater than that found in the

Table 1. Effect of diethylstilbestrol (DES) on peroxidase activity in Syrian hamster liver, kidney, and renal carcinoma

Duration of DES treatment (months)	Peroxidase activity* (units/g protein)	
	Liver	Kidney
0	20 ± 4 (23)	1.0 ± 0.8 (12)
1	88 ± 16† (12)	1.4 ± 0.7 (9)
2	108 ± 9† (9)	4.3 ± 2.8 (6)
3	113 ± 21† (12)	1.2 ± 0.8 (9)
4	119 ± 25‡ (12)	5.2 ± 2.6 (12)
5	186 ± 49‡ (8)	3.1 ± 1.8 (14)
5§	181 ± 32† (4)	
9–11		124.0 ± 27.0‖ (6)

* Values are expressed as the means ± S.E.M. Numbers in parentheses indicate number of individual determinations.
† $P < 0.001$ versus untreated control value.
‡ $P < 0.005$ versus untreated control value.
§ Animals were treated with 17β-estradiol instead of DES.
‖ Value for pure renal carcinoma.

hamster tissues examined, yielding a mean value of 6540 units/g protein which is comparable to values previously reported for this tissue [18]. It was initially thought that at least a portion of the activity seen in the liver might be due to the presence of hemoglobin since this is claimed to have weak peroxidatic activity [24]. However, no appreciable peroxidase activity was found in the supernatant fraction from the first 39,000 *g* centrifugation which contains most of the hemoglobin. Furthermore, no difference in activity was seen when livers were perfused with isotonic saline until blanched. Preliminary studies also indicated that the level of DES in the blood at the time of sacrifice did not influence peroxidase levels since no differences were observed between animals in which the pellet was left implanted up to the time of sacrifice and those in which it had been removed 63–68 hr earlier. Boiling the sample for 1 min eliminated all peroxidase activity.

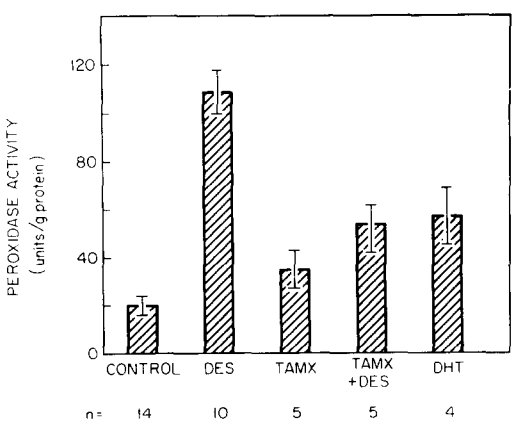

Fig. 1. Liver peroxidase levels in hamsters treated for 2 months with the compounds indicated (means ± S.E.). N equals the number of animals in each group. A unit of enzyme activity is defined as the amount of enzyme required to produce an increase of 1 absorbance unit per min at 470 nm at 25°. Abbreviations: DES, diethylstilbestrol; DHT, 5α-dihydrotestosterone; and TAMX, tamoxifen.

When implanted simultaneously with DES, the antiestrogen tamoxifen significantly reduced the elevated liver peroxidase level seen after 2 months of DES treatment alone ($P < 0.005$). However, the activity in the DES + tamoxifen group was still higher than that in untreated controls ($P < 0.005$) (Fig. 1). Tamoxifen given alone did not produce any significant changes in liver peroxidase levels compared to untreated castrate controls. Although peroxidase activity was significantly elevated following 2 months of 5α-DHT treatment ($P < 0.005$), it was only half the level induced following estrogen treatment. Consistent with this finding is that liver peroxidase activity in intact male hamsters [43.0 ± 5.0 (S.E.) units/g protein, N = 5] is 2-fold greater than castrate levels. Although the amount of kidney peroxidase activity was higher in intact hamsters [13.0 ± 2.0 (S.E.) units/g protein, N = 5] than in either castrate or DES-treated castrate hamsters (range 1–5 units/g protein), this difference does not appear to be appreciable. A lesser reduction in liver peroxidase activity was obtained when animals were treated concomitantly with DES + 5α-DHT [94.0 ± 48.7 (S.E.) units/g protein, N = 5]. Additional studies, however, are needed to determine more precisely the effect of concomitant androgen treatment on estrogen-induced peroxidase activity. Since kidney peroxidase activity was barely detectable and showed no elevation in response to DES (Table 1), it was not assayed in these animals.

To further support the estrogen responsiveness of the hamster liver, a high affinity ($K_A = 0.10 \times 10^9$ M^{-1}) estrogen receptor was found in DES-treated animals (Fig. 2). The presence of molybdate had only a slight stabilizing effect on the receptor. The liver estrogen receptor in DES-primed animals sedimented as an 8S moiety following centrifugation in low salt gradients, as indicated by its specific binding shown in Fig. 3. Specificity of the liver estrogen receptor was also ascertained by the minimal inhibition found when either radioinert cortisol, progesterone, triamcinolone, or methyltrienolone (R1881) were used at 100-fold excess concentrations. Only a small portion of the substantial 4S binding

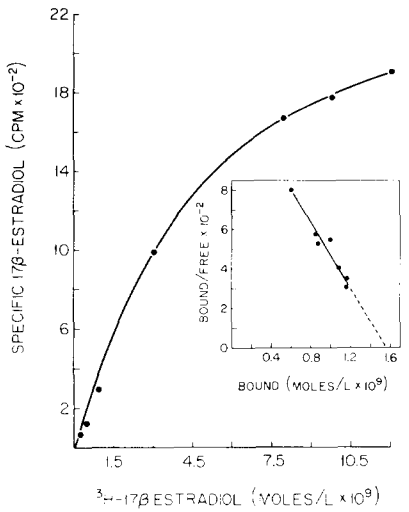

Fig. 2. Specific binding of [^{3}H]-17β-estradiol at 4° in liver cytosols obtained from 4.0 month DES-treated hamsters. The concentration of the tritiated 17β-estradiol varied from 0.5 to 1.2 nM at a cytosol protein concentration of 20 mg/ml. The insert illustrates a Scatchard plot for 4.0 month DES-treated hamster liver. Nonspecific binding was subtracted from these data using radioinert DES at 200-fold excess.

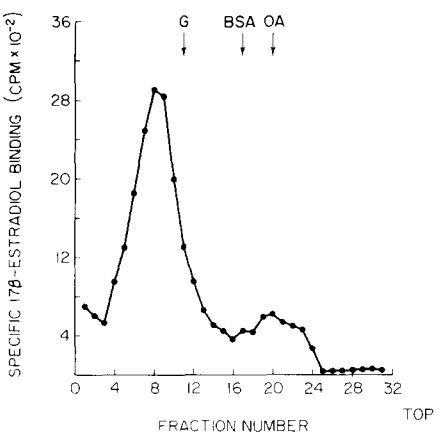

Fig. 3. Sedimentation profile of estradiol receptor in liver cytosol derived from 4.0 month DES-treated hamster liver. Cytosol was incubated *in vitro* with 5 nM [^{3}H]-17β-estradiol at 4° for 90 min and then treated with dextran–charcoal (1 hr). Sucrose gradient profiles represent specific hormone binding and were obtained by subtracting gradient profiles of cytosol containing radiolabeled synthetic hormone in the presence of 100-fold excess corresponding nonlabeled hormone from cytosols containing tritiated hormone alone. Protein concentration of the cytosol was 20 mg/ml. Aliquots of 0.4 ml were layered on 5–20% linear sucrose gradients containing 20 mM sodium molybdate. Bovine serum albumin (BSA), ovalbumin (OA), and γ-globulin (G) were used as sedimentation standards.

(not shown) was specific. Although a slight increase in the level of liver estrogen receptor was found following 4.0 months of DES treatment [3.52 ± 0.78 (S.E.) fmoles/mg protein, N = 7] compared to untreated controls [2.24 ± 0.86 (S.E.) fmoles/mg protein, N = 5], it was not significant.

DISCUSSION

It has been suggested that peroxidase may be responsible for an organ specific bioactivation of DES which then leads to its carcinogenicity [8]. This is a particularly attractive hypothesis in view of the fact that peroxidase is induced by DES in estrogen target organs [11] and is capable of generating DES metabolites that bind to macromolecules [8]. In support of this hypothesis is a recent claim by McLachlan *et al.* [25] that the male hamster kidney, a known target of estrogen carcinogenicity [12], contains significant peroxidase levels compared to rat and mouse kidney and hamster liver, none of which have been shown to be susceptible to estrogen tumorigenesis. However, the results of the current study fail to confirm their findings in regard to the hamster tissues studied since we detected substantial peroxidase activity in liver but only low levels in the kidneys of either untreated intact or castrate hamsters as well as in estrogen-primed animals. We are unable to explain these conflicting results because essentially the same assay procedures were used and the animals were obtained from the same source in both studies. Moreover, while kidney peroxidase activity in intact male hamsters was found to be slightly higher than castrate levels in the present study, it does not account for the much higher values reported in intact animals by these authors. Interestingly, when 5α-DHT was administered alone to castrated hamsters, liver peroxidase activity increased to intact levels. Our castrate liver values do agree quite well with those reported by Keenan *et al.* [26] for ovariectomized rat. It should be noted that, in both the hamster kidney and liver [25] and rat liver [26], brief treatment with estrogen (3–4 days) did not affect peroxidase activity levels in these tissues.

Although the hamster kidney contains an estrogen receptor that is sensitive to estrogens [14, 15], the fact that peroxidase activity was nil initially and was unresponsive to estrogen treatment suggests to us that this enzyme is not involved in the activation of estrogen in the kidney itself. However, ample liver peroxidase does exist and chronic exposure to either DES of 17β-estradiol generates up to a 9-fold increase in this activity. Moreover, concurrent treatment with either the antiestrogen, tamoxifen, or the androgen, 5α-DHT, reduces the effectiveness of DES in terms of liver peroxidase induction, and when these compounds are administered alone they have little or markedly less effect in inducing liver peroxidase activity than DES. These results indicate that the enhanced levels of liver peroxidase activity following estrogen treatment is a specific effect of this hormone rather than a nonspecific action of a foreign compound. The fact that the livers of DES plus α-naphthoflavone-treated hamsters exhibited a cytosolic estrogen receptor which translocates into the nuclear compartment [27], similar to that found

in livers of other species [28–30], strongly indicates that the hamster liver responds to this hormone. Rogan *et al.* [31] have postulated that peroxidase may be involved in 1-electron oxidation of various carcinogens including polycyclic aromatic hydrocarbons to reactive intermediates. The present findings suggest that, if peroxidase activation of estrogens is involved in hamster renal tumorigenesis, the liver is a more likely site for this to occur than the kidney. It is possible that estrogen activation could occur in the liver, and then these metabolites could either accumulate in the kidney, an organ known to be sensitive to estrogens [13–16], or be further metabolized in this organ to form more reactive intermediates.

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