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PEROXIDASE-MEDIATED OXIDATION, A POSSIBLE PATHWAY FOR METABOLIC ACTIVATION OF DIETHYLSTILBESTROL

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Summary: In vitro oxidation of diethylstilbestrol (DES) by peroxidase preparations from horse radish or mouse uterus in the presence of hydrogen peroxide yields ß-dienestrol, which is also a major in vivo metabolite of DES in several mammalian species. The oxidation reaction appears to involve reactive intermediates, presumably the semiquinone and quinone of DES, since nonextractable binding to salmon sperm deoxyribonucleic acid and bovine serum albumin was found. The peroxidase-catalyzed oxidation of DES to reactive metabolites in estrogen target organs may be related to the organ toxicity of this synthetic estrogen.

The synthetic estrogen diethylstilbestrol (DES) exerts numerous toxic effects both in adult and perinatal animals (1) and has recently been assigned transplacental toxicity in humans (2-4). The mechanisms underlying these effects are at present unknown. Formation of reactive metabolites has been suggested to be involved, and extensive oxidative biotransformation of DES affecting both the aromatic and aliphatic part of the molecule was demonstrated in several mammalian species including man and nonhuman primates (5-8). More recently (9), oxidative metabolites of DES were also found in fetal and neonatal mice, which are susceptible to DES toxi-

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city (10-12). Oxidative metabolism of DES, on the other hand, has been shown to give rise to irreversible binding to DNA (13) and proteins (14,15) in vitro.

Among the metabolites of DES invariably found in vivo are ß-dienestrol, one of the three possible geometrical isomers of dienestrol, and its ω-hydroxy-derivative (7-9). The mechanism of dienestrol formation is as yet unknown. A stilbene oxide was initially proposed as an intermediate (5), but when DES epoxide was administered, no metabolism to ß-dienestrol could be demonstrated (9). As an alternative mechanism, oxidation of DES to the corresponding quinone was proposed (9), which can tautomerize to dienestrol (16).

In this paper, we report that the oxidation of DES to ß-dienestrol can be mediated by peroxidase from horse radish (HRP) and from mouse uterus (MUP) in the presence of H_2O_2. The intermediates of this reaction appear to strongly interact with nucleic acids and proteins. The reactivity of the metabolic intermediates of the peroxidase-mediated oxidation, together with the presence and inducibility of this enzyme in estrogen target organs may be relevant for the organ toxicity of DES.

MATERIALS AND METHODS

[^{14}C]DES (spec. radioact. 58 mCi/mmole) was purchased from the Radiochemical Centre Amersham (England) and found by radio-GLC (7) to be of 99 % purity, containing 85 % of the trans- and 15 % of the cis-isomer. Unlabeled DES and ß-dienestrol were obtained as previously reported (7). Horse radish peroxidase, salmon sperm DNA and bovin serum albumin were purchased from Serva (Heidelberg, West Germany), sephadex G-100 from Pharmacia (Uppsala, Sweden). Mouse uterus peroxidase (MUP) from estradiol-17ß-induced NMRI mice was prepared and measured according to Lyttle and DeSombre (17).

Incubations with horse radish peroxidase (HRP) were carried out with 50 nmole [^{14}C]DES, 10 µg HRP, 70 nmole H_2O_2

and 10 mg DNA or albumin in 5 ml 0.1 M phosphate buffer pH 7.4 at 37°C for 2 hrs, followed by extraction with 2 x 5 ml diethylether. For incubations with MUP, 5 ml of 20 mM tris/Cl buffer pH 7.2 containing 0.25 mM 2.4-dichlorophenol (18) and 0.5 ml of the MUP preparation (17), which corresponded to 1/5 of an uterus, was used as incubation medium.

DNA was recovered from the incubation mixtures after extraction with diethylether and Kirby's phenol reagent (19) by precipitation with ethanol as described by Blackburn et al.(13). Columns with sephadex G-100 were eluted at 4 °C with 10 mM phosphate buffer pH 7.4 containing 0.02 % sodium azide. DNA was measured through its OD 260, albumin according to Lowry et al.(20). Radioactivity was determined by liquid scintillation counting (Packard 3390) after dissolving the macromolecules in Soluene 350 (Packard). The analysis of ether extracts was carried out by radio-GLC and GLC-mass spectrometry as previously described (7).

RESULTS

When [^{14}C]DES was incubated with HRP in the presence of H_2O_2 and either DNA or albumin, a considerable portion of water soluble radioactivity was formed which could not be extracted with ether (Table 1). Omission of H_2O_2, enzyme, or macromolecule from the incubation led to a considerable decrease in water soluble radioactivity (Table 1).

When the DNA was precipitated from the ether extracted aqueous phases, approximately half the radioactivity remained associated with the DNA from the incubations containing HRP and H_2O_2 (Table 2). The specific radioactivity of this adduct was not altered by repeated precipitation (Table 2). Moreover, gel filtration on sephadex G-100 showed a coincidence of radioactivity and DNA, as measured by absorbance at 260 nm; no radioactivity was lost from the adduct during dialysis against distilled water for 3 days.

Precipitation of the DNA from the control experiments, where H_2O_2 or HRP had been omitted (Table 1), led to a virtually unlabeled DNA (containing < 0.002 nmol DES / mg DNA).

Table 1

FORMATION OF WATER SOLUBLE RADIOACTIVITY FROM [^{14}C]DES IN AN IN-VITRO SYSTEM CONTAINING HRP, H_2O_2 AND DNA OR ALBUMIN

System[a]	Macro-molecule	Per cent radioactivity[b] in Water	Ether
Complete	DNA	42.6 ± 5.3 (10)[c]	57.4
H_2O_2 omitted	DNA	5.4 ± 3.6 (4)	94.6
HRP omitted	DNA	8.8 ± 1.2 (2)	91.2
Complete	albumin	66.1 ± 4.9 (4)	33.9
Complete	None	13.0 ± 3.2 (4)	87.0

[a]Incubations were as described in Methods.

[b]Calculation based on recovered radioactivity. Recovery was 85-95 %. Values represent mean ± S.D. where appropriate.

[c]Number of experiments.

Table 2

DES/DNA RATIO OF ADDUCT OBTAINED FROM INCUBATION OF [^{14}C]DES WITH HRP/H_2O_2 AND DNA

Purification step	DES/DNA ratio[a]
Prior to 1st precipitation	1.83
After 1st precipitation	0.97
After 2nd precipitation	1.03
After 3rd precipitation	1.07

[a]nmole DES per mg DNA. DES was measured by its radioactivity, DNA by its absorbance at 260 nm.

Similar results were obtained when the ether extracted aqueous phases (Table 1) were extracted with Kirby's reagent. Thus, only half the radioactivity could be extracted from the water phase of the incubations with DNA and HRP/H_2O_2, and the

total binding is reduced the absolute amount of diol epoxide adduct formed is not much increased under these conditions. The proportion of adduct 1 is increased by use of 3-methylcholanthrene-stimulated instead of control microsomes as expected from HPLC analysis of benzo(a)pyrene metabolite pattern (18).

While the increase in peak 1 shown in Fig. 1a is representative for the 3 experiments performed this is not the case for the reduction in peak 3 and 4. Peak 3 is partially due to the K-region epoxide adduct (14), and induction of epoxide hydratase by ethoxyquin might be expected to decrease its formation. However, Tab. 2 reveals that the slight decrease observed was statistically not significant. Small amounts of adduct 3 if any are found when microsomes from 3-methylcholanthrene-treated animals are used (Tab. 3).

Peak 4 has been shown to contain secondary metabolites of benzo(a)pyrene phenols (17, 14). This peak was extremely variable when control animals were used for microsome preparation (Tab. 2); statistically, no influence of ethoxyquin feeding was observed. However, in the experiments with induced monooxygenase a more consistent view was obtained. Peak 4 was the main peak with 3-methylcholanthrene microsomes as the activating system but its proportion was reduced by ethoxyquin feeding; about equal peak heights were observed for adduct 1 (diol epoxides) and adduct 4 (phenol oxides) after treatment with ethoxyquin plus 3-methylcholanthrene (Fig. 1b; Tab. 3). In view of decreased total binding this means a marked reduction of the amount of metabolites present in the form of adduct 4.

Peak 2 appears unaltered by ethoxyquin in Fig. 1a and Tab. 2. This peak has been postulated to contain secondary benzo(a)pyrene quinone metabolites (14). Its proportion but not the absolute amounts formed seem to increase after treatment with ethoxyquin plus 3-methylcholanthrene instead of 3-methylcholanthrene only (Tab. 3).

DISCUSSION

Induction of epoxide hydratase without concomitant induction of monooxygenase is difficult to obtain (19). A limiting role of this enzyme for the formation of

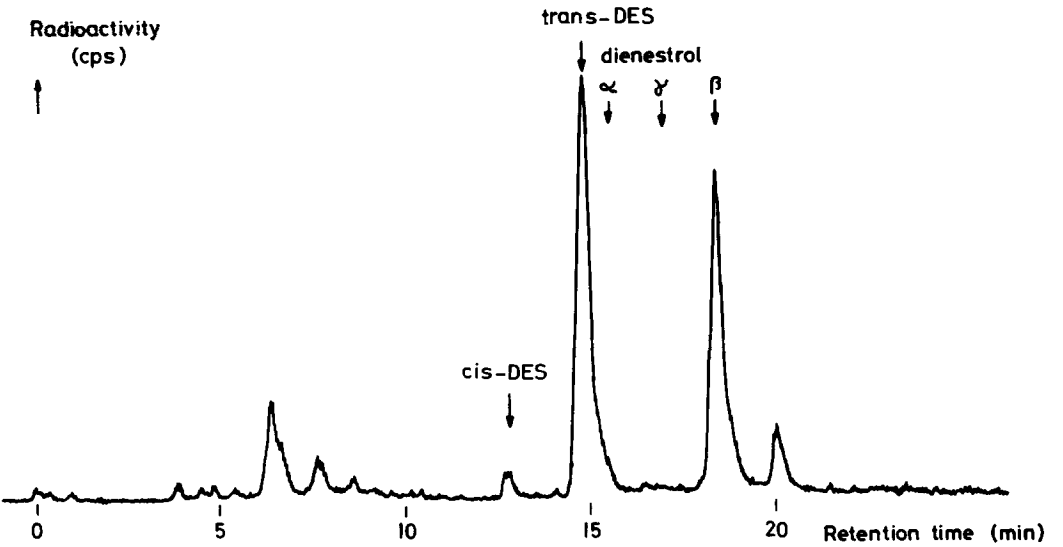

Fig. 2. Radio gas chromatogram of the ether extract after incubation of [^{14}C]DES with HRP and H_2O_2.

The ether extracts of the complete incubation systems (Table 1) were analyzed by radio-GLC (Fig. 2). At least six peaks were found, and three of them were identified as cis- and trans-DES and ß-dienestrol by cochromatography with authentic reference compounds. By using a larger scale incubation mixture, ß-dienestrol could also be identified with GLC-mass spectrometry.

When [^{14}C]DES was incubated with mouse uterus peroxidase and H_2O_2, a similar although less pronounced binding to DNA and albumin was observed. Thus, after extraction with ether, 10-12 % of the ^{14}C-label was found in the aqueous phase of the incubation with DNA, and half of this radioactivity did not dissociate from the DNA upon repeated precipitation with ethanol or gel filtration. This adduct obtained from the MUP-mediated binding reaction had a DES/DNA ratio of 0.25 nmole DES per mg DNA as compared to 1.0 for the HRP-catalyzed re-

action (Table 2). Similarly, when albumin was incubated with [^{14}C]DES and MUP/H_2O_2, 6 % of the radioactivity was found in the adduct after gel filtration on sephadex G-100.

When the ether extracts of the MUP incubations were analyzed by radio-GLC, ß-dienestrol was found to account for 30-35 % of the radioactivity. The chromatogram looked very similar to that given in Fig. 2, except that the peaks eluted before cis-DES were even smaller.

The peroxidase activity of MUP and HRP was measured by the guaiacol assay (17) and found to be at least 15 fold higher for HRP under the conditions used in our incubation experiments.

DISCUSSION

The data obtained so far imply that oxidation of DES in the presence of peroxidase and H_2O_2 leads to one or more compounds which interact with DNA and protein. The chemical nature of the bound metabolite(s) is at present not known. There is evidence, however, that a semiquinone and quinone of DES are involved. A quinone (Fig. 3) has been demonstrated through its UV absorption in a system containing DES, HRP and H_2O_2 (21). DES quinone, which can be obtained by chemical synthesis (16), has been reported to easily tautomerize to ß-dienestrol (16,21). We have repeated the synthesis of DES quinone and found by GLC analysis, under conditions where all isomers of dienestrol are separated (see Fig. 2), that only ß-dienestrol is formed by the tautomerization. Thus, a quinone intermediate could well account for the dienestrol found in the ether extract of the incubations of DES with HRP or MUP (Fig. 2).

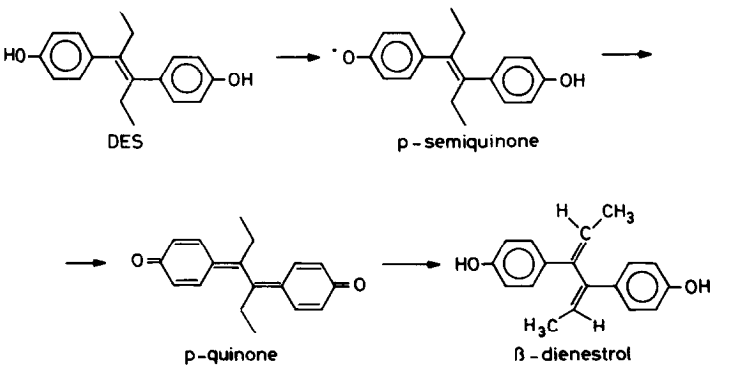

Fig. 3. Peroxidase-mediated oxidation of DES.

Recently, binding to DNA in vitro has been observed by Blackburn et al.(22) when DES is treated with iodine. The authors conclude from kinetic data, "that binding is a result of a net 2-electron oxidation of DES, rather than of its iodination". This oxidation reaction could well be the formation of DES quinone, since ß-dienestrol is a major product found when DES is reacted with iodine under the conditions used by Blackburn (Metzler, unpublished).

These findings make a strong point for the DES quinone and/or the semiquinone (Fig. 3) to be the reactive metabolic intermediates capable of binding to DNA and protein. Since, however, several other products of unknown structure are formed by the peroxidase-mediated oxidation of DES (Fig. 2), the final decision about which compounds are bound must await the structure elucidation of the adducts. The same situation applies for the nature of the chemical bond prevailing in the adducts. Although their stability against gel filtration, precipitation, and solvent extraction are in support of a covalent bonding, definite proof can only be obtained from the structure elucidation of the adducts.

The peroxidase-mediated binding of DES to DNA and proteins may have relevance for the mechanism by which DES exerts its toxicity. Peroxidase activity is preferentially found in tissues depending on estrogens for growth, and can be dramatically induced by estrogens including DES in these cells (17, 23-25). Thus, peroxidase-mediated oxidation of DES and binding to DNA can take place in estrogen target organs, which could explain in part the organotropic toxicity of DES. In support of this hypothesis is a recent finding (29) that the kidneys of male hamsters, which are susceptible organs for the carcinogenic effect of DES (26), have a much higher peroxidase activity than the kidneys of rats and mice.

Moreover, peroxidase-mediated oxidation of DES may play a role in the fetotoxicity of this synthetic estrogen. The target tissues for the carcinogenic and teratogenic effect of DES in the fetus are the Müllerian ducts (3,10,27,28), which represent the embryonic precursor of the upper genital tract of women and should therefore have the genetic information to produce peroxidase. Whether this enzyme can indeed be induced prenatally, is the subject of present investigations. Another question currently under investigation in our laboratories is whether peroxidase can mediate the binding of steroid estrogen catechols to DNA and proteins. The catechols are important circulatory metabolites of steroid estrogens, and their metabolic activation and binding in estrogen target organs could have implications for the toxicity of these compounds, which is a matter of growing concern.

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