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Localization of diethylstilbestrol metabolites in the mouse genital tract

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Diethylstilbestrol (DES*), a potent estrogen and reported transplacental carcinogen [2], is biotransformed to a variety of metabolites in several animal species and also in humans [3]. The oxidative metabolism of DES affects its hormonal activity [4] and may also be of importance for its carcinogenicity, as it has been proposed that DES is bioactivated to reactive intermediates in analogy to chemical carcinogens [5, 6]. Of particular interest in this respect is the peroxidase-catalysed metabolism of DES, which leads to Z,Z-dienestrol (Z,Z-DIES) via a reactive semiquinone and quinone [7]. Peroxidative activity has been demonstrated in tissues depending on estrogens for growth, and these enzymes can be induced by estrogens [8]. Recently, it has been shown that the mouse fetal genital tract, a known *in vivo* target organ for the hormonal and carcinogenic activity of DES, can metabolize DES to Z,Z-DIES when maintained in organ culture [9]. However, it is unknown whether Z,Z-DIES or other oxidative DES metabolites are formed in the mouse genital tract under *in vivo* conditions. Therefore, the present communication reports on a study of the metabolites of DES in the mouse genital tract *in situ*.

Materials and methods

Monoethyl-2-^{14}C-DES (sp. act. 56 mCi/mmole, The Radiochemical Centre, Amersham, U.K.) consisted of 66.9% E-isomer and 33.1% Z-isomer with no impurities detectable at a level of 0.2% by radio-HPLC and radio-TLC. Reference compounds E-DES and its monoglucuronide and monosulphate, Z-DES, 1-HO-E-DES, 4′-O-CH_3-E-DES, Z,Z-DIES and 1-HO-Z,Z-DIES [9–11] were characterized by mass spectrometry and their retention times in HPLC and GLC as previously described [3, 12].

Female CD-1 mice (Charles River France S.A., Cleon, France) were at the age of 6 weeks. Ovariectomy was carried out under anesthesia with Nembutal (Abbot AG, Ingelheim, F.R.G.) 5 days prior to the experiments. For estrogen pretreatment, DES (40 μg/kg b.w.) was injected s.c. as a solution in tricaprylin (Carl Roth OHG, Karlsruhe, F.R.G.) daily for 3 days prior to the experiment. Controls received tricaprylin only. All animals were injected i.v. (tail vein) with ^{14}C-DES dissolved in propan-1,2-diol (1 μg/μl) at a dose of 1.6 μg/g b.w.

The animals were sacrificed by decapitation and the vagina, cervix, and uterus removed. Half of the tissue from each animal was used for the extraction of DES metabolites, the other half to determine peroxidase activity. Metabolites were extracted from the tissue homogenate by precipitation with ethanol and successive washing of the precipitate with ethanol, diethyl ether/ethanol 3:1 (v/v), and diethyl ether as previously described [5, 7]. The residue of the combined extracts was dissolved in 40 μl ethanol for HPLC analysis. Non-extractable radioactivity bound to the sediment was determined by liquid scintillation counting after combusting an aliquot of the air-dried precipitate in a Packard 306 Sample Oxidizer. Peroxidase activity was solubilized from the tissues and determined as described by Lyttle and DeSombre [8], using guaiacol as the substrate.

The tissue extracts containing the metabolites were separated by reverse-phase HPLC as reported previously [12]. The retention times were 5–8 min for conjugated metabolites, 15.8 min for 1-HO-Z,Z-DIES, 18.3 min for 1-HO-E-DES, 23.2 min for E-DES, 24.6 min for Z,Z-DIES, 26.4 min for Z-DES, and 29.1 min for 4′-O-CH_3-E-DES. The column eluate was collected in 0.4 ml fractions and an aliquot of each fraction used for radioactivity measurement in a Packard Tricarb 3390 liquid scintillation counter with automatic external standard (Packard Instruments, Frankfurt, F.R.G.).

Results

In order to identify the DES metabolites present in the uterus of the intact, estrogen-induced mouse *in situ* and to determine their concentrations at different time points, a single dose of ^{14}C-DES was i.v. injected into 6-week-old intact mice pretreated with unlabelled DES for 4 days. After 15, 30 and 60 min, the animals were sacrificed, the uteri removed, and DES and its metabolites extracted. The highest amount of extractable uterine radioactivity was found after 15 min (Table 1). The amount of non-extractable radioactivity did not exceed 4 pmole/g wet weight and did not differ significantly between animals sacrificed at different time points.

The extractable radioactivity was analyzed by radio-HPLC (Table 1). Besides small amounts of glucuronide or sulphate conjugates, which were not further identified, two metabolites of DES were present in the uteri as identified by cochromatography with authentic reference compounds: Z,Z-DIES and 4′-O-CH_3-DES. Both metabolites were found in the same relative amounts (approximately 9% and 5%, respectively) at all time points studied.

In a control experiment, ^{14}C-DES (200 pmole) was added to a freshly obtained mouse uterus prior to homogenization, and the tissue processed as usual. Traces of Z,Z-DIES (1.5%) but no other metabolites (limit of detection 0.05%)

* Nomenclature of DES and metabolites according to the system of Metzler and McLachlan [1].

were found in the extract by HPLC analysis. Macromolecular binding was less than 0.05%.

In order to evaluate whether there was a correlation between the amounts of Z,Z-DIES formed and the activity of peroxidase in the genital tract, ovariectomized mice, either DES-pretreated or not pretreated, were i.v. injected with ^{14}C-DES and the vagina, cervix, and uterus removed after 15 min. The wet weight of the tissues of the DES-pretreated animals was considerably higher as compared to the tissues of the control animals: vagina twofold, cervix sevenfold, and uterus fivefold (Table 2). Peroxidase activity of the tissues was also significantly increased by DES-pretreatment: vagina threefold, uterus tenfold. The amount of extractable radioactivity in each tissue, however, did not always correlate with the increase in wet weight or peroxidase activity (Table 2). For example, radioactivity in the vagina was the same for the pretreated animals as for the controls. In the cervix, pretreated mice had more radioactivity than untreated ones, but the increase in radioactivity was lower than in wet weight. Only in the uterus did the increase in radioactivity correspond to the increase in wet weight.

The following DES metabolites were identified by their retention times in HPLC in all three sections of the mouse genital tract: conjugates, Z,Z-DIES, and 4′-O-CH$_3$-DES. Surprisingly, however, the relative amounts of DES and Z,Z-DIES were the same in all tissues and in untreated and DES-treated animals (Table 2). On the contrary, 4′-O-CH$_3$-DES was found only in DES-pretreated mice, with higher amounts in the cervix and uterus than in the vagina.

Non-extractable radioactivity was less than 1% of the total radioactivity of the tissues and did not differ significantly between different tissues and between pretreated and control animals (data not shown).

Discussion

Data on *in vivo* metabolites of DES in target tissues for its hormonal and carcinogenic effect are scarce. Shah and McLachlan [13] have shown that, after maternal administration of ^{3}H-DES to pregnant mice, radioactivity reaches

Table 1. Metabolites of DES extractable from the uteri of mice after i.v. injection of ^{14}C-DES*

	Time after dosing (min)		
	15	30	60
Uterine wet weight (mg)	220 ± 25	190 ± 30	180 ± 28
Extractable uterine radioactivity†	178 ± 46	86 ± 51	49 ± 25
Metabolites‡			
Conjugates§	13 ± 4	9 ± 6	18 ± 14
E-DES	35 ± 7	41 ± 15	46 ± 9
Z-DES	36 ± 1	35 ± 2	26 ± 4
Z,Z-DIES	10 ± 3	10 ± 5	6 ± 1
4′-O-CH$_3$-DES	6 ± 2	6 ± 4	4 ± 1

* Data represent mean value and standard deviation from 4 animals, each pretreated with 40 μg/kg DES for 4 days. Dose of ^{14}C-CES was 200 nmole per animal.

† Expressed in pmole per total tissue.

‡ Expressed as percent of extractable radioactivity.

§ Coeluting with DES-monoglucuronide and DES-monosulphate, which did not separate under the HPLC conditions used.

Table 2. Amounts of extractable DES metabolites and peroxidase activity in different sections of the female genital tract of ovariectomized mice 15 min after i.v. injection of ^{14}C-DES*

	Vagina		Cervix		Uterus	
	Control	Pretreated	Control	Pretreated	Control	Pretreated
Tissue wet weight (mg)	26 ± 5	62 ± 11	18 ± 4	130 ± 22	50 ± 9	254 ± 34
Peroxidase activity†	43 ± 26	137 ± 64	83 ± 66	870 ± 412	37 ± 31	387 ± 175
Extractable radioactivity‡	67 ± 45	52 ± 43	49 ± 41	142 ± 104	43 ± 26	198 ± 175
Metabolites§						
Conjugates‖	2 ± 1	8 ± 6	15 ± 12	4 ± 2	6 ± 3	9 ± 7
E-DES	31 ± 10	30 ± 7	33 ± 8	34 ± 10	41 ± 12	47 ± 13
Z-DES	46 ± 12	41 ± 8	36 ± 8	46 ± 8	41 ± 6	31 ± 9
Z,Z-DIES	11 ± 5	12 ± 3	9 ± 3	7 ± 3	8 ± 4	6 ± 3
4′-O-CH$_3$-DES	n.d.¶	1 ± 0.7	n.d.	3 ± 2	n.d.	4 ± 1.5

* Data represent mean value and standard deviation from 6 pretreated (4 days with DES) and 4 not pretreated ovariectomized six-week-old mice. Dose of ^{14}C-DES was 200 nmole per animal.

† Expressed as milliunits per mg protein; unit defined according to [8].

‡ Expressed as pmole per total tissue.

§ Expressed as percent of extractable radioactivity.

‖ See Table 1.

¶ n.d., not detectable (less than 0.2%).

the fetal genital tract; however, a structural identification of the *in situ* metabolites of DES in the fetal tract has not been possible thus far.

In the present study, we have investigated the nature of the DES metabolites in the genital tract of adult mice. After i.v. injections of ^{14}C-DES into mice pretreated with unlabelled DES, radioactivity reached the mouse uterus very fast (Table 1). The high uterine weight implies an estrogenized uterus and, therefore, high peroxidase activity. In accordance with the expectation, the peroxidative metabolite of DES, Z,Z-DIES, was indeed the main metabolite present in the uterus at all time points studied. No other oxidative metabolite of DES was observed, but a significant amount of a methylation product, 4'-O-CH_3-DES. Therefore, the only pathways in DES metabolism in the adult female mouse genital tract other than conjugation with glucuronic or sulphuric acid appear to be peroxidation and methylation (Fig. 1). This pattern of unconjugated DES metabolites found in the uterus *in situ* strikingly resembles that produced by the fetal genital tract in culture, where also Z,Z-DIES and 4'-O-CH_3-DES were the major metabolites [9].

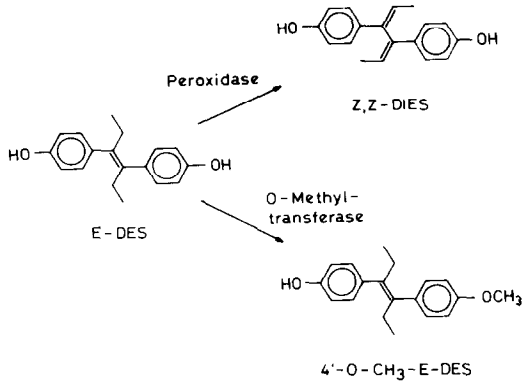

Fig. 1. Pathways of DES metabolism operative in the female mouse genital tract.

Although the peroxidatic oxidation of DES is operative in the mouse genital tract *in vivo*, the question remains as to which peroxidative enzyme(s) catalyse(s) this reaction. An attempt was made to correlate the activity of the estrogen-inducible peroxidases and the amount of Z,Z-DIES found in different sections of the genital tract (Table 2). As expected, marked differences were observed for the tissue wet weight and the peroxidase level between ovariectomized controls and estrogenized animals. Moreover, significant differences were found in the peroxidase activity of the various sections of the tract, the activity being highest in the cervix and lowest in the vagina (Table 2). In a recent study on the distribution of estrogen-inducible peroxidase in rat uterus and vagina, Affleck *et al.* [14] also found that the cervix has about double the peroxidase activity of the uterus. However, when the patterns of DES metabolites present in vagina, cervix, and uterus of control and estrogenized mice were compared in the present study, virtually no difference could be observed for the Z,Z-DIES; this metabolite represents approximately 10% of the extractable radioactivity in every case (Table 2). In contrast, 4'-O-CH_3-DES was only detectable in estrogenized animals in small, but highly significant amounts, which appeared to correlate with the peroxidase activity. This finding implies the presence of an estrogen-inducible phenol-O-methyl transferase in the genital tract.

In conclusion, two metabolites of DES, viz. Z,Z-dienestrol and the monomethyl ether of DES, were identified in the genital tract of the adult female mouse *in situ*. The peroxidative DES metabolite Z,Z-DIES occurred in the vagina, cervix, and uterus of both ovariectomized and estrogen-treated mice, and no correlation could be found between the concentration of Z,Z-DIES in the tissues and the activity of estrogen-inducible peroxidase. On the contrary, the monomethyl ether of DES could only be found in the various sections of the genital tract after estrogen pretreatment. The identification of these *in vivo* metabolites of DES in its target organ for carcinogenicity may further help to understand the mechanism of organ-specific toxicity of this hormonal carcinogen.

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