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Estrogen Metabolism in Microsomal, Cell, and Tissue Preparations of Kidney and Liver From Syrian Hamsters

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The estrogen-treated golden Syrian hamster has been used as an experimental model for estrogen-induced and estrogen-dependent cancers, but pathways to neoplastic transformation remain unknown in this animal. Metabolism of estrogens to activated or reactive compounds, followed by subsequent oxidative damage to the target tissue, remains a potential step in the tumorigenic process. In this study, the extent of estrogen metabolism is compared in three different *in vitro* preparations from untreated and estrogen-treated Syrian hamsters, primary kidney cell cultures, microsomal preparations, and freshly prepared tissue kidney slices. In primary kidney cell cultures, the amount of catechol estrogens decreased upon increasing estrogen (DES) treatment period, and completely disappeared after about 6 months treatment. This decrease is not a result of formation of less amounts of catechol estrogens, but rather reflects the presence of the enzyme systems to further metabolize any formed catechol estrogens, since the amount of catechol estrogens formed, as detected by ^3H_2O release, is unchanged. The polar metabolites a, b and c increased with estrogen treatment, and metabolite c appeared only after DES treatment. The appearance of polar metabolite c only in kidney preparations from DES-treated animals implies that it may serve as a marker of cellular transformation. Estriol and estrone were detected, but were not affected by DES treatment, while no methoxyestrogens were isolated. Studies of estradiol metabolism in microsomal preparations showed a very low rate of metabolism, compared to the primary kidney cell cultures. In contrast, estrogen metabolism was extensive in kidney slices from untreated hamsters, with only approx. 30% of the substrate estradiol remaining unmetabolized after 6 h of incubation. While no catechol estrogens were detected, a small quantity of estriol, and a large amount of estrone and methoxyestrogens were isolated. The polar metabolite a was the main polar metabolite detected, with very little of metabolite b and no metabolite c. In kidney slices from 4 month DES-treated hamsters, a much higher amount of polar metabolites was detected, and metabolite c appeared after 6 h incubation. Mass spectrometric analysis and HPLC data of metabolite c indicate that this metabolite is 15α-hydroxyestradiol. This metabolite may serve as a biomarker for changes occurring in the hamster kidney cells under continuous estrogen exposure. Finally, formation of water soluble conjugates was demonstrated in both kidney slices and liver slices from Syrian hamsters, with glucuronide, sulfate and thioether conjugates of estrone and estradiol and glucuronides of catechol estrogens detected. The extent of estrogen metabolism observed in the kidney preparations from estrogen-treated hamsters includes increases in the formation of 15α-hydroxyestradiol and other polar metabolites. Furthermore, investigations of estrogen metabolism in kidney tissue slices demonstrate lowered conjugative pathways important for secondary metabolism. The decreased steroid conjugation in the estrogen-treated hamster kidney results in greater availability of estrogen metabolites for participation in the formation of potentially damaging reactive species and enhanced oxidative stress in the kidney.

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INTRODUCTION

The golden Syrian hamster has been used as an experimental model for estrogen-induced and estrogen-dependent cancers [1–3]. Spontaneous renal adenocarcinoma in golden Syrian hamsters are extremely rare; however, upon treatment with estrogens such as estradiol or diethylstilbesterol (DES) for 250 days, these tumors develop in all animals [4]. A wide variety of estrogenic compounds can give rise to tumors, while inactive steroids such as 17α-estradiol do not [4]. Intact males developed renal adenocarcinoma when treated with estrogens, while females had to be ovariectomized to develop these tumors [5], since the circulating progesterone in females reversed the effect of estrogens. Antiestrogens were able to prevent tumor formation when administered with estrogens [6].

Early investigations of estrogen-induced renal carcinoma attributed the tumorigenic property of estrogens solely to its hormonal effects, i.e. binding to its receptor, leading to a cascade of events resulting in cell growth and proliferation. While this is a very important aspect of the process, additional investigations have suggested a role of metabolism of estrogens in cancer development. Administration of modulators or inhibitors of estrogen metabolism resulted in prevention of renal adenocarcinoma [7, 8]. The presence of DNA modifications and formation of endogenous adducts following estrogen treatment of the hamsters were demonstrated [9, 10], and have been postulated to arise from activated metabolites and/or free radicals formed during metabolic pathways.

The pathways to neoplastic transformation remain unknown in this animal model of hormonal carcinogenesis and may involve numerous critical events during tumorigenesis [11], and require additional paracrine and/or autocrine factors [12]. Metabolism of estrogens to activated or reactive compounds, followed by subsequent oxidative damage to the target tissue, remains a potential step in the tumorigenic process. In this study, the extent of estrogen metabolism is compared in three different *in vitro* preparations from Syrian hamsters, primary kidney cell cultures, microsomal preparations, and freshly prepared tissue kidney slices.

EXPERIMENTAL

Materials and methods

Golden Syrian hamsters, 80–120 g, were purchased from Harland Industries, Inc. (Cumberland, IN). Castrated males were maintained under constant temperature and humidity, with a 14 h light–10 h dark cycle. DES-pellets obtained from Copley Pharmaceuticals (Boston, MA) contained 20 mg/pellet and were implanted subpannicularly in the shoulder region under ether anaesthesia. DES-pellets were replaced every 3 months. [2-^{3}H]17β-estradiol (18.1–25.3 Ci/mmol) and [6,7-^{3}H]17β-estradiol (49 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [4-^{3}H]17β-estradiol (39.7 Ci/mmol) was previously synthesized in our laboratory. Steroids were purchased from Steraloids (Wilton, NH), chemicals for protein determination from BioRad Laboratory (Rockville, NY), $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase and bovine serum albumin from Sigma Chemical Co. (St Louis, MO), HPLC grade solvents from Burdick and Jackson (Mcgaw, IL). Amberlite XAD-2 ion exchange resins (20–60 mesh) and all other chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). Culture medium RPMI-1640 and trypsin were obtained from Gibco (Grand Island, NY). Fetal calf serum (Hyclone Laboratories, Logan, UT) was heat inactivated for 60 min at 60°C before use. HPLC was conducted on a Beckman System Gold using a reverse phase column (Altex Ultrasphere ODS, 5 micron, 4.6 mm i.d. × 25 cm) equipped with a Model 757 absorbance UV detector (Applied Biosystems) and a Beckman Model 171 radioisotope detector using Beckman Readyflow III. Radioactive samples were dissolved in BudgetSolve from RPI (Mount Prospect, IL) and counted on a Beckman LS6800 liquid scintillation counter. Protein concentrations were determined using Bradford assay [13]. Mass spectrometry was performed at the OSU Campus Chemical Instrumentation Center on a VG 70-250S mass spectrometer.

Tissue preparations

Kidney and liver microsomes were prepared by differential centrifugation [14]. Hamster kidneys and liver were homogenized in 0.05 M Tris–HCl buffer, pH 7.4, at 4°C. The homogenized solution was then centrifuged at 10,000 *g* for 20 min, and the supernatant layer was then centrifuged at 105,000 *g* for 1 h. The resulting pellets were suspended in Tris–HCl buffer, pH 7.4, then centrifuged at 105,000 *g* for another hour to obtain the microsomal pellets, which were either used fresh or stored at −70°C. Primary kidney cell cultures were prepared as previously described [15]. For the preparation of kidney and liver slices, liver and kidneys were removed and placed in PBS buffer. Using a sharp blade, the tissues were cut longitudinally into 0.5–1 mm thick slices and then used fresh.

Estrogen metabolism in cell cultures, microsomes and tissue slices

Cells used for estrogen metabolism studies were incubated for 24 h with 100 nM 2.5 μCi [6,7-^{3}H]estradiol, and the media and the cells were separated and stored at −20°C [15].

For microsomal preparations, 100 nM, 800,000 dpm of [6,7-^{3}H]estradiol/flask in 100 μl propylene glycol were incubated at 37°C for varying periods of time (15, 30, 60, 120 and 180 min) with 0.8 mM $NADP^+$, 2 mM glucose-6-phosphate, 2.5 units of glucose-6-phosphate dehydrogenase, and 2 ml of hamster liver or kidney

microsomes in 0.1 M Tris–HCl buffer, pH 7.4 with gentle shaking, and the reaction stopped by the addition of 1 ml ethyl acetate and stored at −20°C.

Tissue slices were incubated in 5 ml RPMI-1640 media and varying concentrations of estradiol (10 nM–10 μM, 1 μCi [6,7-^{3}H]estradiol) at 37°C for 0, 3 and 6 h under a 95% oxygen, 5% carbon dioxide atmosphere with gentle shaking. The reaction was then stopped by the removal of the slice, and both the supernatant layer and the slices were stored at −20°C.

To characterize the extent of estrogen metabolism, the supernatant layer from different incubations was thawed, 10 g of ammonium sulfate were added to precipitate the proteins, and then centrifuged at 4°C for 20 min. The supernatant solution was separated from the protein pellet, the latter washed three times with equal volumes of ethyl acetate, and the washings added to the water layer which was further extracted three more times with equal volumes of ethyl acetate. The organic layers were combined, dried using sodium sulfate, then evaporated to dryness under a stream of nitrogen. The precipitate was dissolved in 200 μl of 75% methanol in water for subsequent analysis.

HPLC analysis of estrogen metabolites

Separation of the organic-extractable metabolites was performed by reverse phase HPLC according to the method of Brueggemeier *et al.* [15, 16], using a Beckman system gold HPLC. Aliquots (20–30 μl) were separated on a Beckman Ultrasphere ODS column (5 μm, 4.6 mm × 25 cm) using a gradient of 30–50% methanol in water over 35 min (to separate the more polar metabolites), followed by another gradient of 50–60% methanol in water over 35 min (to separate the less polar metabolites), at a flow rate of 1 ml/min. The eluent was monitored with a UV detector at 254 nm, then combined with 3 ml of Ready flow III scintillation cocktail and the radioactive metabolites detected by a Beckman model 171 radioisotope detector. Authentic steroids were coinjected with the samples and detected by a UV detector.

Mass spectrometry of estrogen metabolites

Freshly prepared liver slices were incubated with different concentrations of estradiol (1, 5, 10 μM) in 5 ml RPMI-1640 at 37°C for 6 h. The reaction was stopped by the removal of the slices from the incubation mixture and the addition of 1 ml ethyl acetate, 1 ml 5% ascorbic acid, and authentic catechol estrogens. The proteins were precipitated using ammonium sulfate, and the resulting protein pellets were washed three times with equal volumes of ethyl acetate. The washings were added to the aqueous layer which was extracted three more times with equal volumes of ethyl acetate and the organic layers combined, dried with sodium sulfate, evaporated to dryness and redissolved in 75% methanol in water. To monitor the experiment, a sample containing 2.5 μM [6,7-^{3}H]estradiol was incubated under the same conditions. The metabolites were then separated on reverse phase HPLC and fractions collected. Each fraction was dried down on a Savant speedvac concentrator and redissolved in 100 μl 75% methanol in water. The mass spectroscopy of metabolites was determined by both electron ionization and desorption ionization.

Characterization of water-soluble metabolites

To investigate the nature of the water-soluble metabolites, the presence of glucuronides, sulfates and thioethers was examined. All procedures were adapted from Ball *et al.* [17], with some modifications. For the presence of glucuronides, 0.2 mg β-glucuronidase enzyme [Sigma (EC 3.2.1.31) type L-III] in 100 mM phosphate buffer, pH 3.8 (to inhibit sulfatase activity), was added to 1 ml of the sample. For the presence of sulfate conjugates, 1 mg aryl sulfatase [Sigma (EC 3.1.6.1) type H-1], 20 mM D-glucaro-1,4-lactone (to inhibit glucuronidase activity) in 100 mM acetate buffer, pH 5 was added to 1 ml of the sample. The reaction mixture in both cases was then incubated for 24 h at room temperature with gentle agitation, the reaction stopped by the addition of 1 ml ethyl acetate, and the water layer extracted three times with equal volumes of ethyl acetate. The organic layers were combined, dried using sodium sulfate and aliquots (0.5 ml) of both the organic and water layers were combined with 4.5 ml of scintillation cocktail and counted on an LS6800 liquid scintillation counter. To determine the presence of thioethers, 1 ml of the sample was combined with 25 mg Raney nickel in 10% acetic acid in water, incubated for 24 h at 4°C under nitrogen with gentle agitation (to keep the Raney nickel suspended in the solution). The solution was then extracted three times with equal volumes of ethyl acetate and an aliquot (0.5 ml) of both the organic and water layers was counted as described above. Finally, the organic-extractable fraction from each experiment was evaporated to dryness, redissolved in 75% methanol in water and injected on reverse phase HPLC, as previously described to identify the conjugated metabolites.

Subcellular distribution of metabolites in tissue slices

All steps were carried out at 0–4°C. Slices were homogenized in 10 ml 0.1 M Tris–HCl buffer, pH 7.4, and the homogenate was then centrifuged at 10,000 *g* for 20 min, and the supernatant layer was then centrifuged at 105,000 *g* for 1 h. The precipitate from the 10,000 *g* fraction and the microsomal pellets from the 105,000 *g* centrifugation were homogenized in 10 ml 0.1 M Tris–HCl buffer, pH 7.4. Aliquots (1 ml) of each fraction (10,000 *g* fraction, cytoplasmic fraction and the microsomal fraction) were counted using scintillation cocktail and an LS6800 counter. Each solution was then subjected to protein precipitation and ethyl acetate extraction, as described above. Aliquots of each

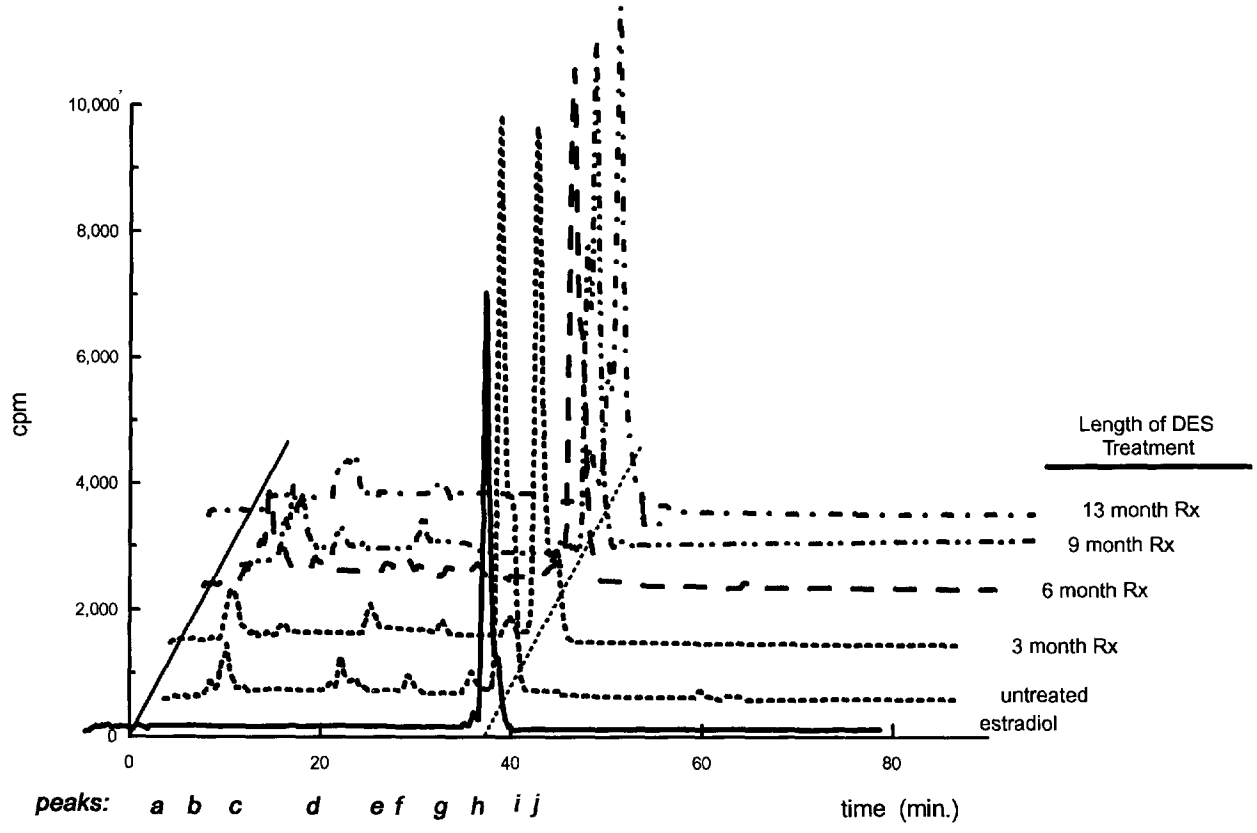

Fig. 1. Reverse-phase HPLC radiochromatograms of organic extractable metabolites of estradiol from kidney cell cultures of untreated and DES-treated hamsters for 3–13 months. Metabolites are polar metabolites (peak a, b and c), estriol (peak d), catechol estrogens (peak e and f), estrone (peak g), estradiol (peak h) and methoxyestrogens (peak i and j).

fraction (protein pellets, aqueous layer and organic layer) were counted for radioactivity, and then the organic layers were evaporated to dryness and redissolved in 75% methanol in water and injected on reverse phase HPLC to detect the metabolites formed.

Radiometric assays of enzyme activities for estrogen 2-hydroxylase and 4-hydroxylase

To determine the kinetic parameters of the enzyme estrogen 2-hydroxylase in liver and kidney microsomal

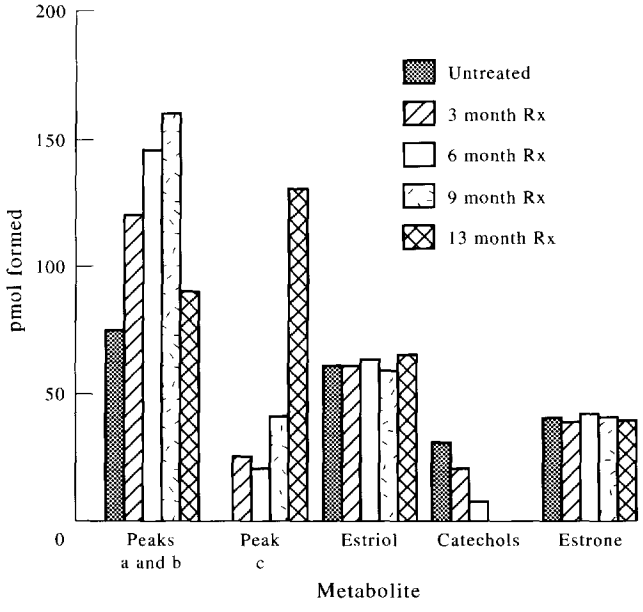

Fig. 2. The effect of DES treatment of Syrian hamsters on the amount of the major estradiol metabolites formed in kidney cell cultures.

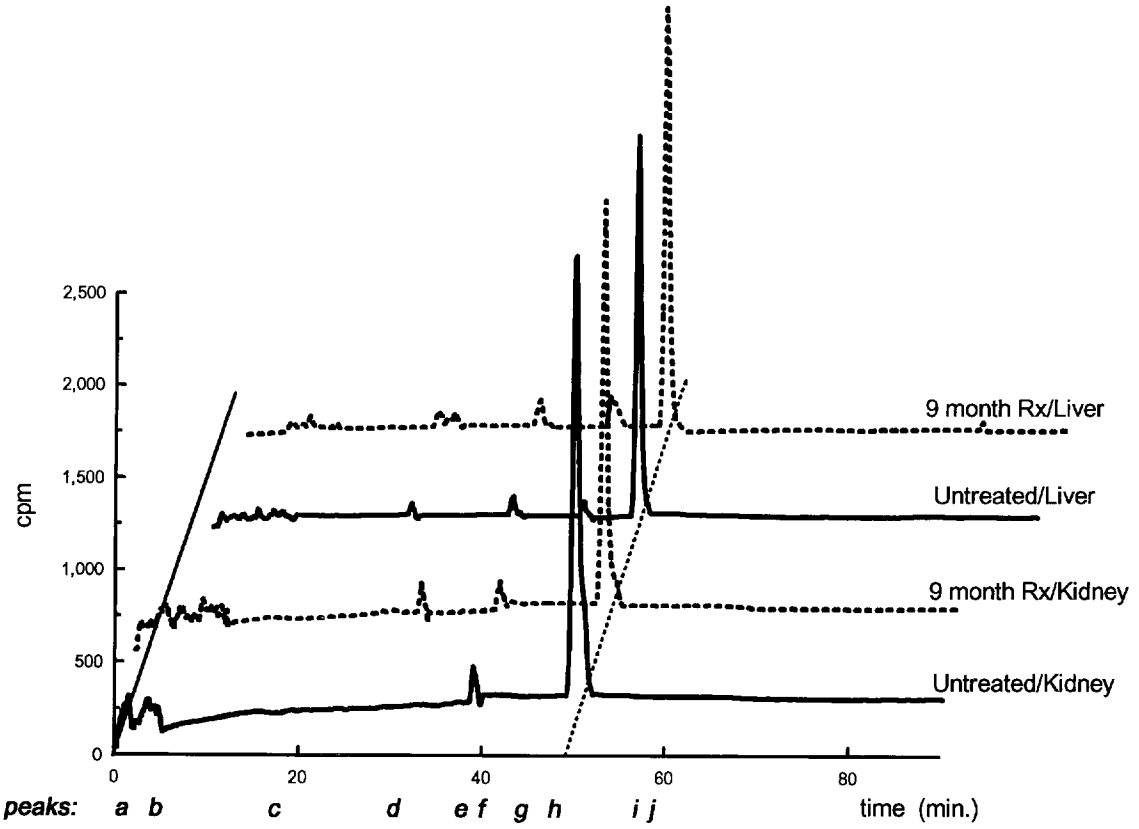

Fig. 3. Reverse-phase HPLC radiochromatograms of organic extractable metabolites of estradiol from kidney and liver microsomes of untreated and 9 months DES-treated hamsters. Metabolites are polar metabolites (peak a, b and c), estriol (peak d), catechol estrogens (peak e and f), estrone (peak g), estradiol (peak h) and methoxyestrogens (peak i and j).

preparation, the studies were carried out under initial velocity conditions, and measured by the ^3H_2O release assay as previously described [18, 19]. Similarly, the estrogen 4-hydroxylase activities were determined by measuring the release of ^3H_2O from [4-^{3}H]estradiol. Various concentrations of estradiol (0.5–10 μM, approx. 250,000 dpm of [4-^{3}H]estradiol/flask) in 100 μl propylene glycol were incubated at 37°C for 15 min with 0.8 mM $NADP^+$, 2 mM glucose-6-phosphate, 2.5 units of glucose-6-phosphate dehydrogenase (as cofactors for the enzymatic reaction) and 2 ml of hamster liver or kidney microsomes in 0.1 M Tris–HCl buffer, pH 7.4. The assay was performed in triplicate, with blank samples incubated with boiled microsomes and/or in the absence of cofactors. The incubations were stopped by the addition of 2 ml 2 N HCl and the samples frozen overnight, then later thawed and the released ^3H_2O was separated from the steroids using a non-ionic XAD-2 column (1.2 × 6 cm) with water as the eluent to give a final volume of approx. 20 ml. One ml of each sample was mixed with 4 ml scintillation cocktail and counted in an LS6800 liquid scintillation counter.

RESULTS

Estrogen metabolism in primary kidney cell cultures

The estradiol metabolic profiles in the primary kidney cell cultures of untreated hamsters were compared to those of hamsters treated with DES for different periods of time, and are shown in Fig. 1. The metabolites were separated by reverse phase HPLC, using a gradient of 30–75% methanol in water over 50 min, and detected by a Beckman model 171 radioisotope detector. In primary kidney cell cultures, the isolation of catechol estrogens (peaks **e** and **f**) decreased upon increasing DES treatment period, and completely disappeared after about 6 months treatment. This decrease is not a result of formation of less amounts of catechol estrogens, but rather reflects the presence of the enzyme systems to further metabolize any formed catechol estrogens, since the amount of catechol estrogens formed as detected by ^3H_2O release is unchanged [15]. The polar metabolites **a**, **b** and **c** increased with DES treatment, and metabolite **c** appeared only after DES treatment. The appearance of polar metabolite **c** only in kidney preparations from DES-treated animals implies that it may serve as a marker of cellular

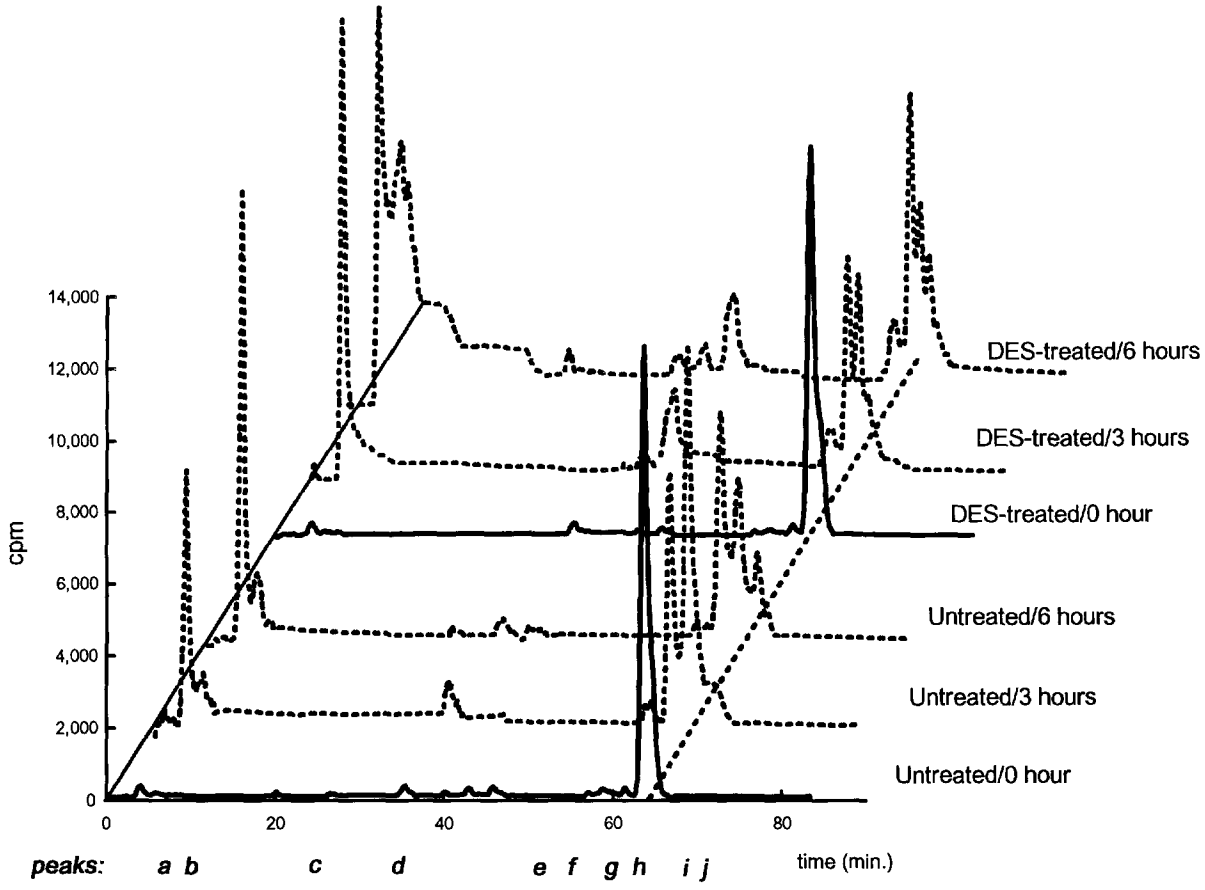

Fig. 4. Reverse-phase HPLC radiochromatograms of organic extractable metabolites of estradiol from kidney tissue slices from untreated and DES-treated hamsters for 4 months. Metabolites are polar metabolites (peak a, b and c), estriol (peak d), catechol estrogens (peak e and f), estrone (peak g), estradiol (peak h) and methoxyestrogens (peak i and j).

transformation. Estriol (peak **d**) and estrone (peak **g**) were detected, but were not affected by DES treatment, while no methoxyestrogens (peaks **i** and **j**) were isolated. A summary of these results is presented in Fig. 2. Metabolism of estradiol was also compared in kidney cell cultures from untreated hamsters using [6,7-^{3}H]estradiol and [4-^{14}C]estradiol as substrates to detect formation of metabolites which would release the tritium at positions 6 and 7. The metabolic profiles from these cell cultures were similar and no additional peaks appeared.

Estrogen metabolism in microsomal preparations

Studies of estradiol metabolism in microsomal preparations showed a very low rate of metabolism, compared to the primary kidney cell cultures. In kidney microsomes, metabolite **c** appeared only in preparations from DES-treated animals, and the overall polar metabolites were of higher quantities in these preparations. No estrone, catechol estrogens or methoxyestrogens were detected, while estriol was unaffected by DES treatment (Fig. 3). In liver microsomes, the same results were obtained, except that metabolite **c** appeared in preparations from untreated animals, and increased by DES treatment, and low amounts of catechol estrogens were detected as well (Fig. 3).

Estrogen metabolism in tissue slices

In these studies, the HPLC elution gradient was modified to improve the separation of polar metabolites. The gradient used was a biphasic one; from 30 to 50% methanol in water over 35 min, then 50–60% methanol in water over another 35 min.

In kidney slices from untreated hamsters, approx. 30% of the substrate estradiol remained unmetabolized after 6 h of incubation. The polar metabolite **a** was the main polar metabolite detected, with very little of metabolite **b** and no metabolite **c**. While no catechol estrogens were detected, a small quantity of estriol, and a large amount of estrone and methoxyestrogens were isolated. In kidney slices from 4 month DES-treated hamsters, a much higher amount of polar metabolites was detected, and metabolite **c** appeared after 6 h incubation (Fig. 4).

In liver slices, the rate of metabolism was even higher, with only about 10–15% of the substrate remaining after 6 h of incubation. The polar

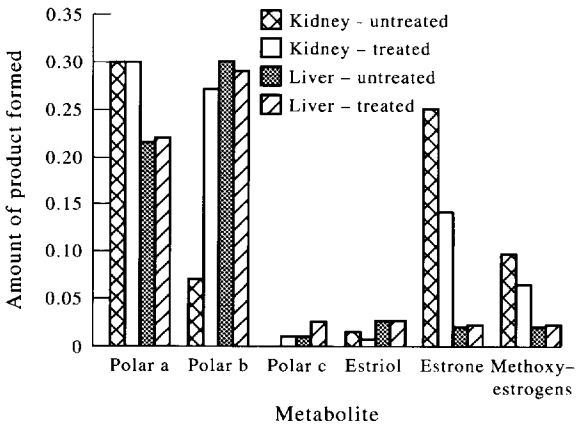

Fig. 5. The amount of estradiol metabolites formed in kidney and liver slices from untreated and 4-months DES-treated animals. Slices were incubated for 6 h with 1 μM estradiol.

metabolites **a**, **b** and **c** were detected in slices from untreated animals. These polar metabolites showed a quantitative increase in slices from DES-treated animals, especially metabolite **c**, while amounts of estrone and methoxyestrogens decreased slightly. Comparison of the amounts of the major metabolites formed in both tissue slices from untreated and DES-treated animals is shown in Fig. 5. These results confirm the findings from experiments in cell cultures and microsomal preparations, but the results also provide a more complete picture of estrogen metabolism in the Syrian hamsters.

Kinetics of estrogen 2-hydroxylase and 4-hydroxylase

The amounts of different metabolites produced by kidney and liver slices from untreated animals were calculated and represented an average of three experiments. From triplicate incubations at 5 different concentrations of estradiol (10 nM–10 μM), the kinetic parameters of the enzyme(s) that catalyze the formation of these metabolites were calculated using a weighted regression computer program [20], and are reported in Table 1. Enzymes catalyzing the formation of metabolites **b** and **c** were not determined in kidney slices due to the very small quantities produced. The apparent K_m values for the formation of estrone and methoxyestrogens were similar in liver and kidney, but the enzyme system that catalyzes the formation of polar metabolite **a** had an apparent K_m that was 6-fold lower in kidney than in liver. The apparent V_{max} for formation of estrone and methoxyestrogens was 100-fold lower in kidney compared to liver, while for the formation of polar metabolite **a** was less than 10-fold lower. These results indicate that the enzyme system responsible for the formation of polar metabolite **a** is much more active than other estrogen metabolism enzymes in the kidney and may thus account for the rapid conversion of any formed catechol estrogens to the more polar metabolites.

Previously in our laboratory, the enzyme estrogen 2-hydroxylase activity in liver and kidney microsomes from Syrian hamster was characterized using the ^3H_2O assay, and the reported apparent K_m for kidney microsomes was 6.43 ± 3.22 μM with an apparent V_{max} of 0.051 ± 0.016 nmol/min/mg, and in the liver microsomes, an apparent K_m of 2.86 ± 1.40 μM and an apparent V_{max} of 0.129 ± 0.015 nmol/min/mg [14, 15]. These studies were extended to determine the same parameters for the estrogen 4-hydroxylase enzymatic activity, using the ^3H_2O assay and Cleland's weighted regression computer program. The apparent K_m for this enzyme in freshly prepared liver microsomes is 11.42 ± 2.1 μM, with an apparent V_{max} of 0.017 ± 0.002 nmol/min/mg, while in kidney microsomes the apparent K_m was 13.9 ± 7.1 μM and the apparent V_{max} of 0.061 ± 0.016 nmol/min/mg. Comparison of these values along with values obtained from frozen microsomes showed that while the apparent V_{max} for estrogen 2-hydroxylase in the liver is almost 10-fold higher than that of estrogen 4-hydroxylase, in the kidney the apparent V_{max} of these enzymes are equal. This agrees with reports that the amount of 2-hydroxy and 4-hydroxyestrogens formed are equal in the kidneys, while in the liver the 2-hydroxylated product is dominant. When comparing results obtained from fresh and frozen preparations, the apparent V_{max} is about 10-fold higher in fresh preparations. This is probably due to the partial degradation of the enzyme upon freezing. The apparent K_m values were different

Table 1. Values of apparent $K_m \pm SE$ in μM and apparent $V_{max} \pm SE$ in pmol/mg/min of enzymes catalyzing the formation of the major metabolites in liver and kidney slices from untreated hamsters ($n = 3$)

	Apparent $K_m \pm$ SE		Apparent $V_{max} \pm$ SE	
Metabolite	Liver slices	Kidney slices	Liver slices	Kidney slices
Estrone	5.40 ± 3.50	7.43 ± 1.61	1.30 ± 0.39	0.0102 ± 0.0012
Estriol	10.97 ± 3.80	ND	0.58 ± 0.11	ND
2-Methoxyestrone	10.61 ± 4.10	7.20 ± 0.49	0.34 ± 0.08	0.0042 ± 0.0001
Polar peak **a**	17.37 ± 6.40	2.81 ± 0.93	0.02 ± 0.005	0.0031 ± 0.0003
Polar peak **b**	4.69 ± 2.00	ND	0.01 ± 0.001	ND
Polar peak **c** (15α-hydroxy-estradiol)	9.12 ± 9.90	ND	0.01 ± 0.005	ND

ND, not determined.

Table 2. Percent of water soluble metabolites formed in liver and kidney slices from untreated hamsters. Slices were incubated for 3 and 6 h with 1 μM estradiol (n = 3)

	Liver slices		Kidney slices	
Conjugate	Percent of water soluble metabolites	Percent of total metabolites	Percent of water soluble metabolites	Percent of total metabolites
Glucuronides				
3 h	20.57 ± 1.21	3.20 ± 0.009	29.99 ± 2.25	0.23 ± 0.00010
6 h	27.07 ± 1.63	4.75 ± 0.062	32.77 ± 0.88	0.33 ± 0.00004
Sulfates				
3 h	12.76 ± 1.32	1.99 ± 0.01	22.11 ± 0.70	0.17 ± 0.00004
6 h	23.06 ± 2.88	3.96 ± 0.11	25.75 ± 1.02	0.26 ± 0.005
Thioethers				
3 h	19.60 ± 1.58	3.06 ± 0.012	31.69 ± 0.42	0.25 ± 0.0002
6 h	22.20 ± 4.38	3.81 ± 0.170	32.91 ± 0.92	0.33 ± 0.0004

in fresh and frozen preparations, as well, but the standard errors were high. Dannan *et al.* reported the presence of at least eleven different estrogen 2/4-hydroxylase isozymes in rat liver [21]. The reason for the differences in apparent K_m values between fresh and frozen preparations and the high standard errors obtained may be due to the presence of different isozymes in hamster kidney and liver responsible for the hydroxylation and/or differences in isozyme degradation upon freezing.

Distribution of estrogen metabolites in kidney and liver slices

The distribution pattern of various metabolites produced from liver and kidney slices were examined. In liver slices, the amount of steroids retained within the slices increased gradually, reaching 40–45% of total steroids after 6 h, and was unaffected by DES treatment. In kidney slices, the amount retained in the slices from DES-treated hamsters was higher after 3 h, but then increased slowly to reach the same levels as in kidney slices from untreated hamsters after 6 h. Within the slices, the distribution to different cellular compartments (nuclear, cytoplasmic and microsomal fractions) in both liver and kidney slices revealed equal amounts of steroids in each fraction after 6 h, although the cytoplasmic fraction contained much higher amounts after 3 h in liver slices. In the kidney slices, very small amounts of water soluble metabolites (about 3–5%) and protein bound steroids (3–4%) were formed, while organic soluble materials were more than 90%. In the liver slices, about 20–25% of the metabolites are water soluble, while 15–20% are bound to proteins. Detection of the metabolites that were retained in the cellular compartments from liver slices showed that while the 10,000 *g* fraction, containing nuclear fragments and large pieces of the plasma membrane, and the microsomal pellets retained mainly estrone and estradiol, metabolites from the cytoplasmic fraction included polar metabolites, estriol, estrone and methoxyestrogens, but no catecholestrogens. Slices from DES-treated animals showed the same patterns as discussed above.

Table 3. Partial listing of peaks identified in electron ionization mass spectroscopy of metabolite **c**, *with their relative intensities and calculated molecular formulas*

Molecular weight	Relative intensity	Molecular formula
288	7	$C_{18}H_{24}O_3$
217	16	$C_{15}H_{21}O_1$
175	89	$C_{12}H_{15}O_1$
149	72	$C_{10}H_{13}O_1$
147	122	$C_{10}H_{11}O_1$

Characterization of water-soluble metabolites

Conjugation of estrogens to water-soluble metabolites occurs via formation of glucuronides, sulfates and thioethers. The presence of these metabolites was detected in liver and kidney slices of Syrian hamsters (Table 2). The amounts of these metabolites were not changed in slices from hamsters treated with DES. Cleavage of glucuronides and sulfates was accomplished using specific enzymes, while thioethers were reduced by Raney nickel to liberate the free steroids. The chemical nature of the liberated metabolites was further examined and separated on reverse phase HPLC. In the liver slices, metabolites that form sulfates and thioethers are mainly estrone and estradiol, while glucuronidated metabolites include catecholestrogens and polar metabolite **a** as well. In the kidney slices, the evaluation of thioether metabolites was not accomplished due to the very low amounts isolated, but sulfates were mainly conjugated to estradiol, and glucuronides resulted from polar metabolite **a**, catecholestrogens, estrone and estradiol.

Metabolite identification

Investigations to identify polar metabolite **c** were initiated. This metabolite appears only after 3 months of DES treatment in kidneys of Syrian hamsters, which coincides with the appearance of other morphological changes such as appearance of dysplastic foci and the increase in area of vascularization [22]. Using electron ionization mass spectrometry, an M^+ peak with a mass

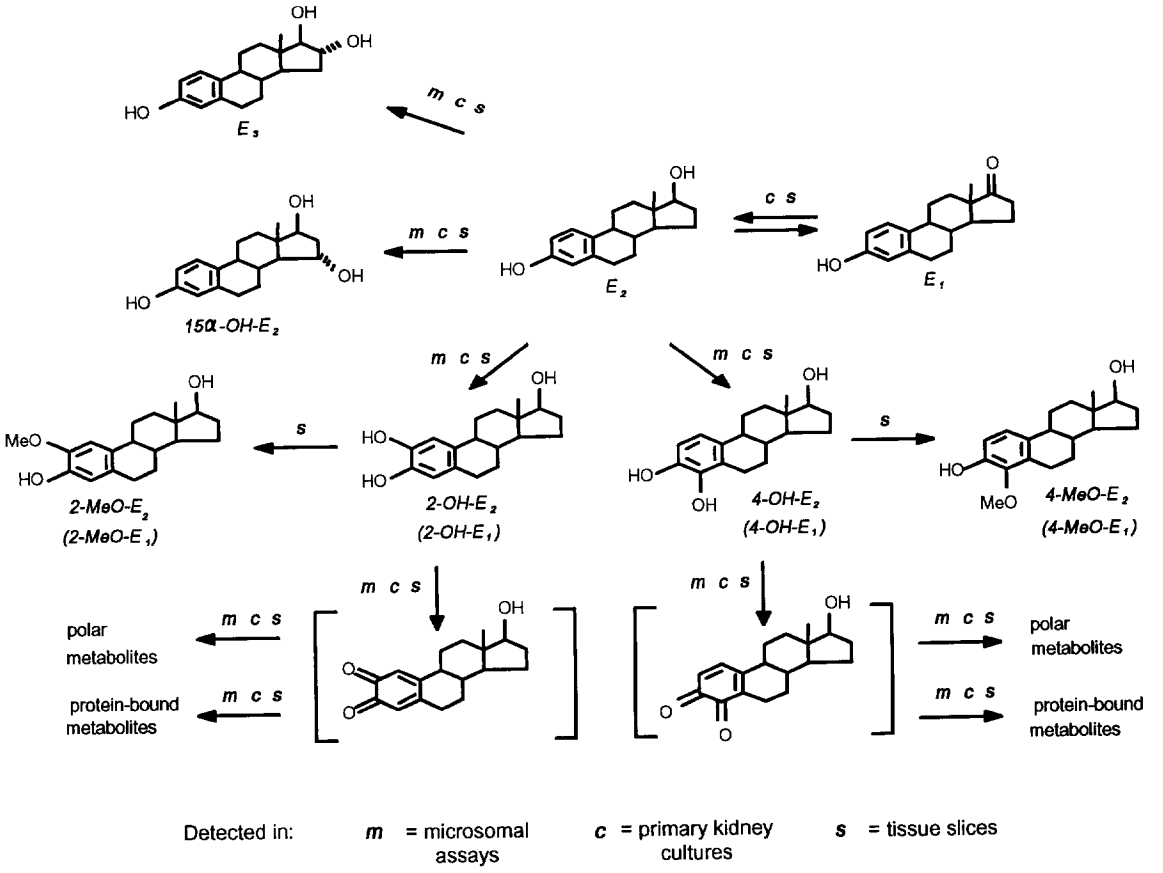

Fig. 6. Metabolic pathways involved in estradiol metabolism in different kidney preparations of Syrian hamsters (m, microsomal preparation; c, primary cell culture; s, tissue slices). Abbreviations include: estradiol, E_2; estrone, E_1; estriol, E_3; 2-hydroxyestradiol, 2-OH-E_2; 4-hydroxyestradiol, 4-OH-E_2; 2-hydroxyestrone, 2-OH-E_1, 4-hydroxyestrone, 4-OH-E_1, 2-methoxyestradiol, 2-MeO-E_2, 4-methoxyestradiol, 4-MeO-E_2, 2-methoxyestrone, 2-MeO-E_1, 4-methoxyestrone, 4-MeO-E_1, 15α-hydroxyestradiol, 15α-OH-E_2.

of 288 was identified. The exact mass analysis revealed that the compound is a trihydroxylated derivative of estradiol. The relative intensities and the molecular formulas of the M^+ peak and other peaks obtained from the fragmentation of the parent compound are shown in Table 3. Metabolic **c** did not co-elute with several readily available trihydroxylated derivatives of estradiol, including 2-, 4-, 6α-, 11α-, and 16α-hydroxyestradiol. The fragmentation pattern from electron ionization mass spectrometry and the relative retention time on reverse phase HPLC are consistent with those for 15α-hydroxyestradiol, as reported recently [23, 24].

DISCUSSION

Golden Syrian hamsters develop renal adenocarcinomas upon exposure to estrogens for a prolonged period of time [4]. The tumorigenic process has been postulated to involve two estrogenic effects: the hormonal effects and the carcinogenic toxicity [11]. The hormonal effects arise from receptor binding, followed by a cascade of events leading to an increase in gene expression, DNA synthesis and stimulation of protein production. The carcinogenic toxicity of estrogens is postulated to arise from the formation of reactive metabolites and free radicals, that can bind to proteins and form DNA adducts leading to cellular damage, possibly mutations and tumor formation [25]. Estrogen metabolism was investigated in the kidney of the Syrian hamsters, the site of tumor formation, and compared to the results obtained from the same studies in the liver, the main site of metabolism in the body and an organ that shows no estrogen-dependent tumor formation.

The present studies showed that the amounts of catechol estrogens formed from estradiol in kidney of Syrian hamsters diminished by prolonging DES treatment. This decrease reflected the further metabolism of these catechol estrogens to other highly polar metabolites. Polar metabolites were separated to three compounds **a**, **b** and **c**, which showed a quantitative increase after DES-treatment. While metabolite **a** did not change with estrogen treatment, metabolite **b** showed a significant increase in hamsters treated with DES compared to untreated hamsters. Metabolite **c** was not isolated in untreated hamsters, but appeared in DES-treated hamsters. This metabolite may be important in tumorigenesis, due to its absence in kidneys of untreated hamsters, but was present in livers of these

hamsters. It also appears in kidneys after 3 months of DES treatment, which coincides with other changes that are related to tumor formation such as increase in area of vascularization and appearance of dysplasic foci [22]. Mass spectrometric analysis and HPLC data of metabolite **c** indicates that this metabolite is 15α-hydroxyestradiol. This metabolite may serve as a biomarker for changes occurring in the hamster kidney cells under continuous estrogen exposure. The complete elucidation of the chemical structure of other polar metabolites may shed additional light on their possible roles in tumorigenesis, and whether the metabolites themselves or some reactive intermediates and/or free radicals that may arise during the pathway leading to their formation may bind cellular proteins or form DNA adducts and cause cell damage. Among the other metabolites formed in the kidneys of Syrian hamsters are estriol and other 16-hydroxylated compounds, estrone and methoxyestrogens. The amounts of these metabolites were affected by DES treatment.

The study of the kinetics of the microsomal enzymes that catalyze the formation of these metabolites demonstrated that the apparent V_{max} of these enzymes was higher in liver than in kidney of Syrian hamsters, while the apparent K_m was similar. One exception was the enzyme system catalyzing the formation of metabolite **a**, where it had a 6-fold lower apparent K_m in kidney than in liver. Moreover, while the apparent V_{max} for enzymes catalyzing the formation of estrone and methoxyestrogens was 100-fold lower in kidney than in liver, the apparent V_{max} for the enzyme system responsible for metabolite **a** formation was less than 10-fold lower, indicating that this enzyme system is very active in the kidney compared to other metabolic enzymes.

Estrogen 4-hydroxylase activity catalyzes the conversion of estrogens to the 4-hydroxylated catechol estrogen. The evaluation of the kinetics of this enzyme in the Syrian hamsters revealed that it has a 2-fold higher apparent K_m than estrogen 2-hydroxylase in kidney, with a similar apparent V_{max}. In liver, the apparent K_m of estrogen 4-hydroxylase was abut 4–5-fold higher than estrogen 2-hydroxylase, and the apparent V_{max} was about 10-fold lower. These results agree with other reports that both the 2- and the 4-hydroxylated catechol estrogens are formed in equal amounts in the kidney, but that the 2-hydroxyestrogens are the predominant in the liver of Syrian hamsters [26]. Other reports indicate that the *O*-methylation of 4-hydroxyestrogens is inhibited by 2-hydroxyestrogens [27]. This may lead to the accumulation of this catechol estrogen and its availability to undergo further metabolism and may be the precursor of the damaging reactive compounds.

The formation of water soluble conjugates was demonstrated in both the liver and the kidney of Syrian hamsters. Glucuronide, sulfate and thioether conjugates of estrone and estradiol were detected,and glucuronides of catechol estrogens and metabolite **a** were also isolated. The amount of these metabolites formed in the kidney constituted a very low percentage of total metabolites while higher amounts were formed in the liver, consistent with a higher capacity of liver to conjugate metabolites.

Figure 6 summarizes the metabolic pathways involved in estradiol metabolism in the different preparations from Syrian hamsters. The extent of estrogen metabolism observed in the kidney preparations from estrogen-treated hamsters include increases in the formation of 15α-hydroxyestradiol and other polar metabolites. Furthermore, investigations of estrogen metabolism in kidney tissue slices demonstrate that lowered conjugative pathways are important for secondary metabolism. The decreased steroid conjugation in the estrogen-treated hamster kidney results in greater availability of estrogen metabolites for participation in the formation of potentially damaging reactive species and enhanced oxidative stress in the kidney.

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