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EFFECT OF CHRONIC ESTROGEN TREATMENT OF SYRIAN HAMSTERS ON MICROSOMAL ENZYMES MEDIATING FORMATION OF CATECHOLESTROGENS AND THEIR REDOX CYCLING: IMPLICATIONS FOR CARCINOGENESIS

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Summary—Estrogens have previously been shown to induce DNA damage in Syrian hamster kidney, a target organ of estrogen-induced cancer. The biochemical mechanism of DNA adduction has been postulated to involve free radicals generated by redox cycling of estrogens. As part of an examination of this postulate, we measured the effect of chronic estrogen treatment of hamsters on renal microsomal enzymes mediating catechol estrogen formation and free radical generation by redox cycling of catechol estrogens. In addition, the activities of the same enzymes were assayed in liver in which tumors do not develop under these conditions. At saturating substrate concentration, 2- and 4-hydroxyestradiol were formed in approximately equal amounts (26 and 28 pmol/mg protein/min, respectively), which is 1–2 orders of magnitude higher than reported previously. Estradiol treatment for 2 months decreased 2-hydroxylase activity per mg protein by 75% and 4-hydroxylase activity by 25%. Hepatic 2- and 4-hydroxylase activities were 1256 and 250 pmol/mg protein/min, respectively. Estrogen treatment decreased both activities by 40–60%. Basal peroxidatic activity of cytochrome *P*-450, the enzyme which oxidizes estrogen hydroquinones to quinones in the redox cycle, was 2.5-fold higher in liver than in kidney and did not change with estrogen treatment. However, when normalized for specific content of cytochrome *P*-450 the enzyme activity in kidney was 2.5-fold higher than in liver and increased further by 2–3-fold with chronic estrogen treatment. The activity of cytochrome *P*-450 reductase, which reduces quinones to hydroquinones in the estrogen redox cycle, was 6-fold higher in liver than in kidney of both control and estrogen-treated animals. When normalized for cytochrome *P*-450, the activity of this enzyme was similar in liver and kidney, but over 4-fold higher in kidney than liver after estrogen treatment. Basal concentrations of superoxide, a product of redox cycling, were 2-fold higher in liver than in kidney. Estrogen treatment did not affect this parameter in liver, but increased it in kidney by 40%. These data provide evidence for a preferential preservation of enzymes involved in estrogen activation.

INTRODUCTION

Active radical formation is known to result in damage to DNA [1, 2] which, in turn, has been proposed to lead to tumor formation [3]. Studies of estrogen-induced renal cancer in hamsters support the postulation that the carcinogenic action of estrogens depends on their conversion to catechol metabolites [4, 5], a potential source of such damaging active radicals [6, 7]. Hamsters treated for 7–9 months with estrogen consistently develop cancer in the renal cortex [10]; microsomes from hamster kidney cortex convert estrogens to their catechol metabolites [5].

Catechol estrogens (CE), like stilbene estrogens and their corresponding quinones, have been shown by electron spin resonance techniques to form semiquinones [8, 9], and superoxide radicals are generated when the stilbene quinone DES Q is incubated with microsomes and NADPH [7]. Both stilbene and catechol estrogens undergo redox cycling [6], a mechanism for the generation of active radicals. This process is evidenced by the oxidation of these estrogens to their respective quinones in the presence of microsomes and organic hydroperoxide, and the reduction

Abbreviations: (E)-DES, *trans*-diethylstilbestrol or (E)-diethylstilbestrol; DES Q, diethylstilbestrol-4′,4″-quinone; CHP, cumene hydroperoxide; DIES, (Z,Z)-dienestrol; (Z)-DES, *cis*-diethylstilbestrol or (Z)-diethylstilbestrol; E2, 17β-estradiol; CE, catechol estrogen; 2-OH-E2, 2-hydroxyestradiol; 4-OH-E2, 4-hydroxyestradiol.

of quinones to a mixture of (E)- and (Z)-DES or CEs, respectively, in the presence of microsomes and NADPH. The extent of the oxidizing and reducing potential of microsomal enzymes may be estimated from amounts of (Z)-DES, a marker of reduction and of DIES, the spontaneous rearrangement product of DES Q, the marker product of oxidation.

In the present study we examined the effect of treatment of hamsters with E2 for 2 months on the following key biochemical parameters of free radical formation by kidney microsomes: (a) the rates of conversion of E2 to its catechol metabolites, the substrates for redox cycling; (b) the specific content of cytochrome *P*-450, the peroxidatic activity of cytochrome *P*-450 and cytochrome *P*-450 reductase, enzymes which catalyze redox cycling; and (c) superoxide free radical generation. Microsomes prepared from liver, which is not a target for estrogen-induced cancer in this species, were assayed in parallel. A preferential preservation of those functions of cytochrome *P*-450 which are involved in estrogen activation were identified.

MATERIALS AND METHODS

Chemicals

(E)-DES, CHP, NADPH, E2, cholesterol, cytochrome c from horse heart, and L-ascorbic acid were purchased from Sigma Chemical Co., St Louis, Mo. (Z)-DES was a gift of Dr P. Murphy, Eli Lilly and Co., Indianapolis, Ind. DES Q and DIES were prepared as previously described [12]. The nature and sources of chemicals and radioactive steroids used as substrates and tracers in the estrogen hydroxylase assay have been provided previously [13–15].

Instrumentation

u.v. Spectra were recorded on a Hewlett Packard, Model 8452A diode array spectrophotometer. HPLC analyses were carried out using a Waters Associates (Milford, Mass.) instrument consisting of 2 solvent delivery systems, Model 6000A and Model M45, a Model 660 solvent programmer, and a Model 440 detector (fixed wavelength at 254 nm). The assay conditions for stilbene estrogens have been described previously [6].

Animals and tissue preparation

Male Syrian hamsters were purchased from Harlan Sprague–Dawley, Houston, Tex., at 8–10 weeks of age (approx. 100 g). Laboratory rodent chow and water were available *ad libitum*. A group of 15 hamsters were implanted s.c. with 1 E2 pellet (25 mg E2 containing 10% cholesterol) as described previously [4]. Fifteen animals remained untreated. After 2 months the animals were killed by decapitation and the livers and kidneys were excised immediately. The kidneys were separated into cortex and medulla in ice-cold 0.25 M sucrose containing 10 mM EDTA. Microsomes were prepared from kidney cortex and liver by differential centrifugation according to the method of Dignam and Strobel[16].

Enzyme activity assays

Cytochrome *P*-450 specific content in hamster liver and kidney cortex was measured by CO difference spectra [17], cytochrome *P*-450 reductase activity by the method of Dignam and Strobel[16], peroxidatic activity of cytochrome *P*-450 by the method of Hrycay and O'Brien[18]. The generation of superoxide radicals catalyzed by liver and kidney cortex microsomes was assayed by the method of Kuthan *et al.*[19] based on the reduction of partially succinoylated ferricytochrome c by superoxide anion.

Redox cycling by product isolation assay

Microsomal oxidation of DES to DES Q. The oxidation of (E)-DES to DIES with hamster liver microsomes (0.36 nmol cytochrome *P*-450/ml final conc.) and CHP or NADPH were carried out as described previously [6]. Incubations with kidney cortex microsomes (0.18 nmol cytochrome *P*-450/ml final concn) were performed by the same method except that the reaction time was increased to 1 h and the cofactor concentrations to 1.8 mM CHP. The stilbene estrogens were extracted from incubation mixtures and analyzed as described previously [6].

Microsomal reduction of DES Q to DES. The reduction of DES Q was assayed as described previously [6] with the following modifications: microsomal protein (0.36 nmol liver cytochrome *P*-450/ml and 0.18 nmol kidney cortex cytochrome *P*-450/ml final conc., respectively) was used instead of purified cytochrome *P*-450 reductase. Cofactor concentrations were 5 mM NADPH (reaction time: 30 min) when using liver microsomes, 10 mM NADPH (reaction time: 1 h) when using kidney microsomes.

Catechol estrogen assay. The assay procedure and its validation have been described in detail previously [13–15]. Briefly, 50 μg microsomal protein was incubated in 150 μl Tris–HCl/Hepes buffer, pH 7.4, containing 10 mM ascorbic acid, [6,7-^{3}H]E2 and 5 mM NADPH. The incubations were carried out at 30°C and in the presence of 10 mM ascorbic acid, strategies previously shown to protect CEs from oxidative degradation without significantly affecting enzyme activity [13–15]. Enzyme activity was arrested after 10 min of incubation by addition of 0.25 M perchloric acid. The CEs were adsorbed from the aqueous medium onto neutral alumina to separate them from residual precursor and non-catecholic metabolites. The CEs eluted from the neutral alumina with HCl were separated from each other by thin-layer chromatography. Procedural losses were corrected for by use of internal ^{14}C-labeled tracer. Blank values, obtained with heat-denatured tissue, were subtracted.

Enzyme activities are expressed both in terms of mg microsomal protein and nmol cytochrome *P*-450.

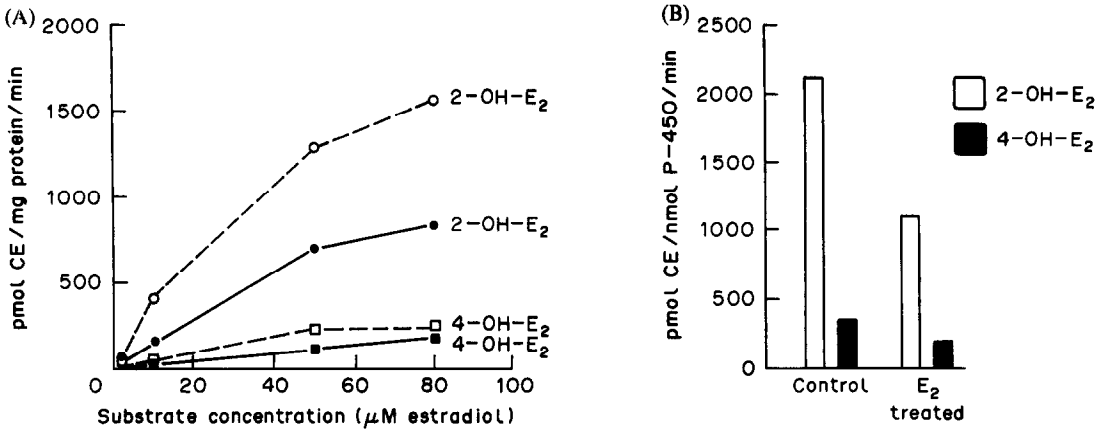

Fig. 1. Rates of NADPH-dependent 2- and 4-hydroxylations (round and square symbols, respectively) of E2 by microsomes isolated from liver of male Syrian hamsters treated for 2 months with E2 (solid lines, closed symbols) and of normal controls (broken lines, open symbols). Values are expressed as pmol CEs/mg protein/min (Panel A, left) or as pmol/nmol *P*-450/min (Panel B, right). The incubations were carried out as described previously using 50 μg microsomal protein in a total volume of 150 μl incubation medium (0.05 M Tris, 0.05 M HEPES buffers at pH 7.4, 10 mM ascorbic acid, 5 mM NADPH, and 0.5 μCi [6,7-^{3}H]estradiol substrate at the concentrations indicated) for 10 min at 30°C. The catechol estrogens were isolated by chromatography on alumina columns and separated by thin-layer chromatography. Samples were assayed in duplicate and values obtained for boiled tissue blanks were subtracted.

This allows for a more valid comparison of enzyme activities in kidney vs liver, since the microsomes prepared from these organs differ greatly in their composition. Microsomes from kidney contain a large amount of mitochondrial and villous proteins [20].

RESULTS

Catechol estrogen formation

Hamster liver. In incubations of microsomes from hamster liver, 2-hydroxylation predominated over 4-hydroxylation. The ratio of 2- and 4-OH-E2 formation at saturating substrate concentration was 6:1 (Fig. 1A). Estrogen treatment for 2 months lowered both 2- *and* 4-hydroxylation equally (approximately 48–63 and 38–57% lower, respectively, depending on substrate concentration). Values for 2- and 4-hydroxylation when normalized for nmol cytochrome *P*-450 were similarly reduced, since cytochrome *P*-450 specific content of hepatic microsomes was not affected by 2 months of estrogen treatment (Fig. 1B).

Hamster kidney. In incubations of renal microsomes from control hamsters, 2- and 4-OH-E2 were formed in similar amounts (Fig. 2A). Ratios of 2- and 4-hydroxylation ranged from 0.8 to 1.5, depending on substrate concentration. As in liver, CE formation after 2 months of E2 treatment was lower than in age-matched controls. However, in contrast to

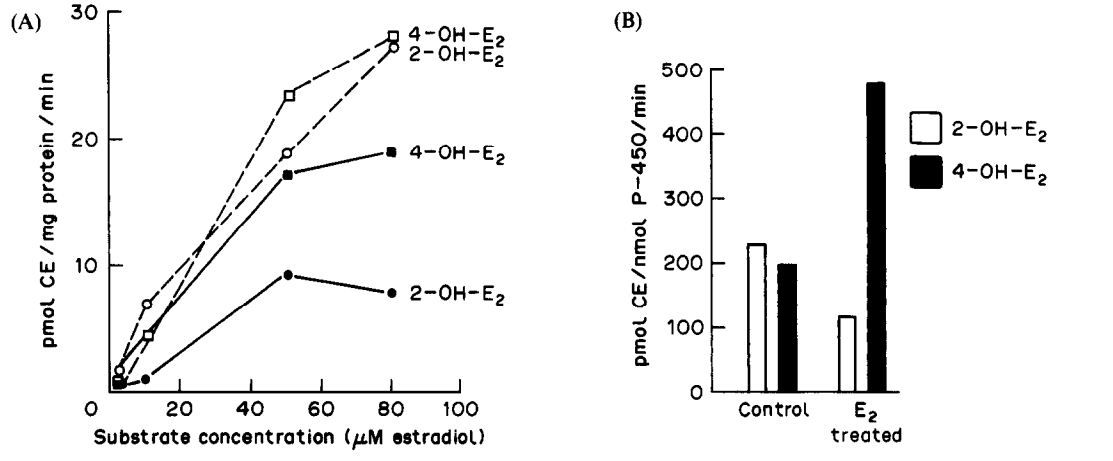

Fig. 2. Rates of NADPH-dependent 2- and 4-hydroxylations (round and square symbols, respectively) of E2 by microsomes isolated from kidney cortex of male Syrian hamsters treated for 2 months with E2 (solid lines, closed symbols) and of normal controls (broken lines, open symbols). Values are expressed as pmol CEs/mg protein/min (Panel A, left) or as pmol/nmol *P*-450/min (Panel B, right). Reactions were carried out as described in the caption for Fig. 1 and in the Materials and Methods section except that the incubations were done at pH 8.0.

Table 1. Activities of enzymes mediating redox cycling of estrogen. Activities in liver and kidney cortex microsomes of untreated or E2-treated male Syrian hamsters

Source of microsomes	Cytochrome *P*-450 reductase (pmol/mg protein/min)	(pmol/nmol *P*-450/min)	Cytochrome *P*-450 content (nmol/mg protein)	Microsomal peroxidase (pmol/mg protein/min)	(pmol/nmol *P*-450/min)
Liver					
Control	190 ± 25	257	0.74 ± 0.15	94 ± 11	127
E2-treated	150 ± 17	197	0.76 ± 0.23	105 ± 15	138
(% change)	(−21%)	(−23%)	(+3%)	(+12%)	(+9%)
Kidney					
Control	31 ± 15	258	0.12 ± 0.03	38 ± 5	317
E2-treated	34 ± 8	850	0.04 ± 0.01*	41 ± 4	1025
(% change)	(+9%)	(+229%)	(−67%)	(+8%)	(+223%)

The preparation of microsomes and measurement of cytochrome *P*-450 reductase activity has been previously described [16]. The cytochrome *P*-450 content was assayed by the method of Omura and Sato [17]. Microsomal peroxidatic activity of cytochrome *P*-450 was determined by the method of Hrycay and O'Brien [18]. Values given are means of 10–12 determinations/microsomal sample ± SD. The statistical significance was evaluated by Student's *t*-test. $*P < 0.001$.

liver, 4-hydroxylation was affected much less than 2-hydroxylation resulting in 4-OH-E2 as the predominant product of aromatic ring hydroxylation (ratio of 2-OH-E2/4-OH-E2 was 0.2–0.5, depending on substrate concentration).

When expressed in pmol CE/nmol cytochrome *P*-450/min (Fig. 2B), 2-hydroxylation remained unchanged after 2 months of E2 treatment, whereas 4-hydroxylation was twice that of age-matched controls (Fig. 2B).

Enzymes involved in redox cycling

Because catechol estrogens have been shown to undergo redox cycling, this process was investigated by measuring the peroxidatic activity of cytochrome *P*-450 and cytochrome *P*-450 reductase, and the specific content of cytochrome *P*-450 of microsomes. Peroxidatic activity of cytochrome *P*-450 was also assayed by its ability to oxidize DES and cytochrome *P*-450 reductase by its ability to reduce DES Q.

Hamster liver. Cytochrome *P*-450 specific content of liver microsomes from hamsters treated for 2 months with E2 was not significantly different from that of controls (Table 1). There was only a modest decline (21%) in cytochrome *P*-450 reductase activity after 2 months of treatment with E2, whether the values were expressed in terms of mg microsomal protein or normalized for cytochrome *P*-450 content of microsomes.

Reduction of quinones when measured by stilbene product isolation assay was unaffected by 2 months of E2 treatment (Table 2). Similarly, hepatic microsomal peroxidatic activity of cytochrome *P*-450 expressed per mg protein or normalized for cytochrome *P*-450 content of microsomes was unaffected by estrogen treatment (Tables 1 and 3).

Hamster kidney. Cytochrome *P*-450 specific content of renal microsomes declined 67% after 2 months of estrogen treatment (Table 1). Basal cytochrome *P*-450 reductase activity/mg protein of renal microsomes was about one-sixth that of hepatic microsomes (Table 1) and was unaffected by the estrogen treatment (Tables 1 and 2). However, when expressed per nmol cytochrome *P*-450, the enzyme activity was increased by 230% by estrogen treatment

Table 2. Activities of enzymes mediating redox cycling of estrogen. Stilbene product isolation assay of cytochrome *P*-450 reductase activity in liver and kidney cortex microsomes of untreated and E2-treated male Syrian hamsters

Source of microsomes	(E)-DES	(Z)-DES	DIES
Liver			
Control	11.0	55.3	35.7
E2-treated	8.9	52.1	39.0
Kidney			
Control	8.5	20.5	71.0
E2-treated	10.0	20.0	70.0

Microsomal cytochrome *P*-450 reductase activity was assayed by conversion of DES Q to (Z)-DES as described previously [6]. Amounts of products are expressed as percent of total stilbenes extracted. Values are the mean of 2 experiments. Reactions with liver microsomes were carried out for 0.5 h with 0.39 mg microsomal protein, 5 mM NADPH and 10 μM DES Q. With kidney microsomes, reaction conditions were the same except that 1 mg protein and 10 mM NADPH were used.

Table 3. Activities of enzymes mediating redox cycling of estrogen. Stilbene product isolation assay of peroxidatic activity of cytochrome *P*-450 in liver and kidney cortex microsomes of untreated and E2-treated male Syrian hamsters

Source of microsomes	(E)-DES	(Z)-DES	DIES
Liver			
CHP			
Control	27.5	39.5	33.0
E2-treated	27.7	37.2	35.1
No microsomes	92.0	7.0	<0.1
NADPH			
Control	49.9	45.8	4.3
E2-treated	59.9	36.1	4.0
No microsomes	95.3	4.0	<0.1
Kidney			
CHP			
Control	41.7	48.0	10.3
E2-treated	53.7	34.0	12.3
NADPH			
Control	88.2	11.8	<0.1
E2-treated	80.3	19.7	<0.1

Microsomal peroxidatic activity was assayed by conversion of (E)-DES to DIES as described previously [6]. Amounts of products are expressed as percent of total stilbenes extracted. Values are the mean of 2 experiments. Reactions with liver microsomes were carried out for 0.5 h with 0.39 mg microsomal protein, 0.9 mM CHP or 5 mM NADPH, respectively, and 13.1 μM E-DES except in reactions which did not contain microsomes. Reactions with kidney microsomes were carried out under the same conditions except that 1 mg protein and 10 mM NADPH were used.

Table 4. Stimulation of superoxide radical formation by liver or kidney cortical microsomes of hamsters treated with E2

Source of microsomes	μM DES Q	Reduction of succinoylated ferricytochrome c (nmol/mg protein/min)
Liver		
Control	0	8.0 ± 1.6
E2-exposed	0	6.0 ± 0.8
Control	50	34.0 ± 5.0[a]
E2-exposed	50	31.9 ± 4.8[b]
Kidney		
Control	0	3.4 ± 0.5
E2-exposed	0	6.1 ± 1.1
Control	50	17.0 ± 2.1[c]
E2-exposed	50	24.2 ± 3.5[d,e]

The generation of superoxide radicals was measured as described by Kuthan *et al.* [19] using 0.62 mg microsomal proteins, 0.4 mM NADPH and 50 μM DES Q. Superoxide radical formation was measured by the reduction of succinoylated ferricytochrome c. Values represent mean of 4 measurements ± SD. The statistical significance was evaluated by Student's *t*-test. [a] $P < 0.001$ compared to control liver; [b] $P < 0.001$ compared to E2-exposed liver; [c] $P < 0.03$ compared to control kidney; [d] $P < 0.03$ compared to E2 kidney; [e] $P < 0.05$ compared to control kidney (in the presence of 50 μM DES Q).

(Table 1). Values of cytochrome *P*-450 reductase/nmol cytochrome *P*-450 were identical in liver and kidney of control hamsters, but were 4-fold higher in kidney than liver of treated animals.

Superoxide radical formation

Hamster liver. Basal levels of superoxide radical formation by hepatic microsomes from control or estrogen treated hamsters were similar (range 3.4–8.0 nmol reduced ferricytochrome c/mg protein/min) (Table 4). After addition of DES Q the rate of increase in superoxide radical formation by microsomes from control and estrogen treated hamsters were also similar (34.0 and 31.9 nmol/mg protein/min respectively).

Hamster kidney. Basal activities of renal microsomes of control and estrogen treated hamsters were similar and comparable to that of hepatic microsomes (3.4–6.21 nmol/mg protein/min) (Table 4). However, after addition of DES Q the increase in rate of superoxide generation by renal microsomes from E2 treated hamsters was 42% greater than that of microsomes from age matched controls.

DISCUSSION

The values obtained in this study for the formation of CEs, substrates for redox cycling, by both kidney and liver microsomes are much higher than those reported previously [5, 21–23]. The values for kidney estrogen 2-hydroxylase activity were one order of magnitude and those for liver two orders of magnitude higher than those in the literature [5, 21–23]. Moreover, previous studies failed to identify estrogen 4-hydroxylase in the hamster kidney, which is shown in this study to equal the activity of 2-hydroxylase and, following chronic estrogen treatment, even to exceed 2-hydroxylase activity. The low values obtained previously for estrogen 2-hydroxylase activity are attributable to the use of a catechol-*O*-methyltransferase-coupled radioenzymatic assay [24] under conditions which failed to protect fully the CEs generated by CE synthases and which were not optimized for *O*-methylation of CEs [25]. 4-Hydroxylation has been virtually missed when using the radioenzymatic assay because methylation of 4-OH-E2 is markedly inhibited by the presence of 2-OH-E2 [26]. If such inhibition occurs *in vivo*, it implies that 4-hydroxylated estrogens would escape inactivation by catechol-*O*-methyltransferase and remain available as substrates for redox cycling. This condition may exist specifically in the kidney, since it is a tissue in which 2-hydroxylation is accompanied by comparable levels of 4-hydroxylation. This phenomenon is not evident in liver.

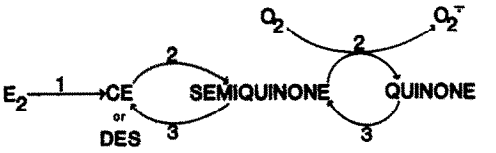

Fig. 3. Free radical generation during microsome-mediated redox cycling of catechol or stilbene estrogens. The enzymes participating in the metabolic conversions are: **1**, estrogen 2- and/or 4-hydroxylase; **2**, peroxidatic activity of cytochrome *P*-450; **3**, cytochrome *P*-450 reductase; the estrogen semiquinone and the superoxide free radicals are proposed to damage DNA and other cell components.

To compare the potential of liver and kidney to promote redox cycling as illustrated in Fig. 3 requires an evaluation of enzyme activities normalized for specific content of cytochrome *P*-450. This takes into account the difference in the composition of microsomes prepared from the 2 tissues [20]. Renal cytochrome *P*-450 reductase activity per nmol cytochrome *P*-450 equalled that of liver in control animals and was 4-fold higher in those treated with estrogen. Renal peroxidatic activity of cytochrome *P*-450 exceeded hepatic values 2.5-fold in control hamsters and 7.5-fold in those treated with E2. Taken together, we take this as evidence for a high potential of hamster kidney to promote redox cycling in particular following estrogen treatment (Fig. 3). The increase in superoxide generation by kidney microsomes of estrogen-treated hamsters is consistent with this interpretation. The high potential for redox cycling in this organ precedes the development of kidney tumors.

We conclude that the susceptibility of hamster kidney to estrogen-induced carcinogenesis may be related to the presence of high levels of estrogen 4-hydroxylation in addition to 2-hydroxylation and the relatively high potential for redox cycling in this organ. In addition to the potential relevance of these findings to estrogen-induced carcinogenesis, the data highlight characteristics of cytochrome *P*-450 metabolism. The differential effect of estrogen treatment on 2- and 4-hydroxylation confirms that these

2 activities are mediated by different forms of cytochrome *P*-450 [27]. The mechanism of estrogen-dependent expression of these forms of cytochrome *P*-450 remains to be determined.

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