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IRREVERSIBLE BINDING OF ETHYNYL-ESTRADIOL METABOLITES TO PROTEIN AND NUCLEIC ACIDS AS CATALYZED BY RAT LIVER MICROSOMES AND MUSHROOM TYROSINASE

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SUMMARY

17α-Ethynyl-estradiol is bound irreversibly to protein by the catalytic action of rat liver microsomes and also by mushroom tyrosinase. Whereas the binding reaction in the tyrosinase system could be inhibited by cysteine, cysteine derivatives, lysine and analogous, the microsomal binding reaction was not affected by lysine and amines, but was markedly inhibited by cysteine and its derivatives.

Concordantly, an irreversible binding of ethynyl-estradiol metabolites to poly-lysine could only be achieved with tyrosinase, not with rat liver microsomes.

The observations support the concept of different reactive intermediates involved in the binding of estrogens to protein by both enzymic systems examined.

Since estrogen o-quinones are known to be formed from estrogen phenoles, the ability of 17β-hydroxy-4,10(1)-estradiene-2,3-dione to react non-enzymatically with cysteine, lysine and related compounds was examined and found in agreement with the postulate of estrogen o-quinones being the intermediates involved in the tyrosinase-catalyzed protein binding of estrogens.

Ethynyl-estradiol was not bound irreversibly to DNA and RNA by rat liver microsomes, while tyrosinase could catalyze this type of reaction. This behaviour is seen as a result of the dissimilarity in the active intermediates formed from ethynyl-estradiol by microsomes and tyrosinase.

INTRODUCTION

The serious question whether estrogens, particularly as constituents of contraceptive formulations, possibly exert carcinogenic effects is still a matter of discussion. Although a number of clinical and theoretical investigators have denied the possibility of a carcinogenic action of estrogens[1–4, 43], some others are more cautious and believe that it will take much more time to decide definitely whether estrogens possess tumour-inducing abilities or not[5–8].

Since most of the chemical carcinogens known so far can be bound covalently to proteins and/or nucleic acids[9–13], it would be of significance to examine the modalities for an irreversible attachment of estrogens to these macromolecules.

It is well established that rat liver microsomes are able to catalyze an irreversible binding of estrone and estradiol to the microsomal protein[14–20]. In a previous report[20] we extended these observations to 17α-ethynyl-estradiol, the main estrogenic component used in contraceptive formulations, and demonstrated that 17α-ethynyl-estradiol is also irreversibly bound to albumin, when this is added to the microsomal incubation system.

The binding of estrogens to proteins requires a hydroxylation reaction at C-2 and a further oxidative step to the o-quinone or semiquinone[19, 23, 24]. However, it is not known whether these reactive intermediates are able to conjugate to nucleic acids. This is the reason why we aimed at examining the ability of estrogens to bind irreversibly to nucleic acids. Because of its significance in contraceptive treatment the studies were carried out with 17α-ethynyl-estradiol.

Two different procedures for the "bioactivation" of ethynyl-estradiol have been employed: first by rat liver microsomes and then by the action of phenol oxidase ("tyrosinase", EC 1.10.3.1). This latter enzyme was chosen for comparison, because its mechanism of action with estrogens is known, leading to the formation of the o-quinone estrogen derivative[21–23].

EXPERIMENTAL

Materials

[6,7,^{3}H-]-Ethynyl-estradiol and 2-hydroxy-estradiol were generously donated by Schering AG, Berlin.

Phenoloxidase ("tyrosinase", EC 1.10.3.1), prepared from mushrooms (S.A. 500 U/mg) was obtained from P-L Biochemicals, Inc., Milwaukee, Wis.

L-Lysine, L-cysteine, DL-methionine, ethylenediamine, phenylenediamine and mercaptoethanol were commercial products of Merck, Darmstadt.

DNA (from herring sperm) and poly-L-lysine (mol. wt. 100,000) were obtained from Serva, Heidelberg, RNA (from yeast) and glutathione from Boehringer, Mannheim, L-cysteine-methyl ester hydrochloride from Fluka AG, Buchs, Switzerland, N-acetyl-L-cysteine from Schuchardt, München, and bovine serum albumin from Behringwerke AG, Marburg.

Animals

Male Wistar rats of 200–250 g were used as liver donors for microsomal preparations. In order to induce the microsomal mixed function oxidase they were pretreated with phenobarbital, applying a single i.p. dose of 80 mg/kg, followed by a 5-day treatment with 0·1% phenobarbital in the drinking water. One day prior to sacrifice the rats were starved.

Microsomal incubations

Microsomes were prepared as described by Remmer *et al.*[25]. Microsomal incubations with [6,7,^{3}H-]-ethynyl-estradiol as substrate (1·23 μmol per 5 ml incubation), NADPH-regenerating system and bovine serum albumin (100 mg) were performed as already described previously[20].

After the incubation microsomes and albumin-containing supernatant were separated by ultracentrifugation. The amount of radiolabelled substrate irreversibly bound to albumin was estimated after charcoal absorption of the loosely attached steroid. The microsomal pellet was extracted exhaustively with organic solvents to remove the reversibly bound radioactivity. The detailed procedure and its reliability has been presented in a previous report[20]. In further experiments the albumin has been substituted by DNA, RNA and poly-L-lysine.

Tyrosinase incubations

Tyrosinase is known to catalyze two types of reactions[26]: First it can act as a phenol hydroxylase, giving the o-diphenol: phenol + NAD(P)H + H^+ + O_2 → catechol + $NAD(P)^+$ + H_2O. Secondly, the o-diphenol can be further oxidized to the o-quinone. In this step the enzyme acts as a o-diphenol-oxidase: (a) catechol + $NAD(P)^+$ → o-quinone + NAD(P)H + H^+ (in presence of $NAD(P)^+$ as hydrogen acceptor) or (b) catechol + 1/2 O_2 → o-quinone + H_2O. Using a high excess of NAD(P)H, significant amounts of estrogen catechols can be isolated from an incubation of tyrosinase with estrogens[27].

In a first series of experiments we found that tyrosinase was also capable of catalyzing the irreversible binding of estrogens to albumin, the optimal amount of which was 1 mg (500 U) per 5 ml incubation, when 1 mg NADPH was used. The following experiments were done under these conditions.

For the incubation the substrate (24 μg = 80·97 nmol [6,7,^{3}H-]-ethynyl-estradiol) was given to the incubation tube in ethanolic solution and the solvent evaporated. After addition of 50 mg albumin and 2 ml 0·1 M Tris–HCl buffer, pH 7·4, it was preincubated for 15 min to achieve the dissolution of the substrate. Then additional 3 ml of buffer were added with tyrosinase (1 mg), NADPH (1 mg) and inhibitor (SH- or NH_2- compound) and the incubation was performed for 1 h at 37 C. For incubations with DNA, RNA and poly-lysine albumin was substituted by these compounds.

After incubation 1 ml was taken for determination of the total radioactivity present by liquid scintillation counting with 0·2 ml hyamine® hydroxide in 10 ml Bray's solution[28]. For determination of the radioactivity irreversibly bound to the macromolecules 2 ml of incubation were shaken with 2 ml charcoal suspension (1% norit A + 0·01% dextran in 0·1 M Tris–HCl buffer pH 7·4) for 1 h. After centrifuging the charcoal 2 ml supernatant (corresponding to 1 ml of the original incubation) were counted with 0·2 ml hyamine® hydroxide in 18 ml Bray's solution[28].

Binding of estrogen o-quinone to SH- and NH_2- compounds

The capability of some NH_2- and SH- compounds to bind estrogen o-quinone in a non-enzymatic reaction was investigated by using 17β-hydroxy-estra-4,10[1]-diene-2,3-dione (estradiol-o-quinone), which was prepared according to Gelbke and Knuppen[29]: 2-Hydroxy-estradiol was oxidized in 50% acetic acid with $NaJO_4$ to give the o-quinone, which was extracted with chloroform and washed with aq. $NaHCO_3$ and water. The quinone content of the solution was determined by iodometric titration[29].

0·7 ml of 2·68 mM quinone in chloroform was given to 0·7 ml 1/15 M phosphate buffer, pH 7·4, containing 0·185 M of the SH- or NH_2- compounds listed in Table 3. The reaction was performed in "Eppendorf" micro tubes, which were shaken for 1 h at room temperature.

After the incubation 0·5 ml of the chloroform phase was taken for iodometric determination of the quinone, which was not bound by the reagent. According to Gelbke and Knuppen[29] 0·25 ml glacial acetic acid was added, nitrogen was bubbled through the solution and some crystals of potassium iodide were added. After waiting 1 min and giving 2 ml starch solution the mixture was titrated with 1/200 N sodium thiosulfate.

Table 1. Irreversible protein binding of [6,7,^{3}H-]-ethynyl-estradiol (1·23 μmol/5 ml incubation) to microsomes and albumin (100 mg/5 ml) added to the incubation with NADPH-regenerating system: Inhibition of the binding reaction by SH- and NH_2- compounds. (Microsomes were obtained from livers of male, phenobarbital-pretreated rats)

Inhibitor	nmol Ethynyl-estradiol metabolites irreversibly bound by 1 mg microsomal protein: Bound to 100 mg albumin	Bound to 1 mg microsomal protein	Total bound	% Inhibition
Control, no inhibitor (n = 12)	14·35 ± 0·62	19·65 ± 1·96	34·00 ± 1·95	—
Glutathione 1 mM (n = 4)	4·60 ± 0·50	4·50 ± 1·26	9·10 ± 1·43*	73·2%
N-acetyl-cysteine 1 mM (n = 4)	4·19 ± 0·68	6·30 ± 0·52	10·49 ± 0·66*	67·7%
Cysteine methyl ester 1 mM (n = 4)	7·75 ± 0·37	5·62 ± 0·84	13·37 ± 0·97*	60·7%
Cysteine 1 mM (n = 4)	9·42 ± 0·20	7·35 ± 0·43	16·77 ± 0·51*	50·8%
Methionine 1 mM (n = 4)	14·90 ± 1·01	18·60 ± 1·70	33·50 ± 2·24 ns	—
Lysine 10 mM (n = 4)	11·0 ± 1·06	20·5 ± 2·1	31·5 ± 1·6 ns	—
Ethylenediamine 10 mM (n = 4)	9·4 ± 1·2	21·0 ± 1·9	30·4 ± 3·0 ns	—
Ethanolamine 10 mM (n = 4)	10·9 ± 1·74	21·7 ± 2·41	32·6 ± 2·84 ns	—

Mean values ± S.D. are shown.
* $P < 0·001$ vs control.
ns = not significant vs control.
No binding occurred to DNA, RNA or poly-L-lysine, if albumin was substituted by these compounds.

RESULTS

Irreversible binding of ethynyl-estradiol to macromolecules, as catalyzed by rat liver microsomes

As shown in Table 1, 1 mg of rat liver microsomal protein catalyzes the irreversible binding of 34 nmol ethynyl-estradiol with the microsomes and albumin added to the incubation. This value corresponds closely to our previously published data[20].

It is known that an o-quinone or semiquinone must be involved in the "covalent" protein binding of estrogens[23, 24]. This reaction might proceed *via* SH- or NH_2- groups of proteins. For this reason we started with studying the inhibition of the binding of ethynyl-estradiol to protein by NH_2- and SH- containing compounds. Glutathione, cysteine derivatives, lysine and the model compounds ethylenediamine and ethanolamine were chosen as inhibitors.

The binding reaction was inhibited by every SH-compound examined at 1 mM concentration, but most prominently by glutathione, as it could be expected from the strong nucleophilic properties of this substance.

In contrast, compounds containing NH_2-groups do not interfere with the microsome-catalyzed irreversible protein binding of ethynylestradiol, even when these compounds are used in a concentration of 10 mM. These results strongly suggest that NH_2- groups are not able to bind to the intermediate involved in the microsomal reaction. This is further confirmed by the fact that poly-lysine does not bind the metabolite of ethynyl-estradiol either. Therefore it is very unlikely that the conversion and irreversible binding of ethynyl-estradiol to proteins which is catalyzed by liver microsomes proceed through the formation of Schiff's bonds

Table 2. Irreversible binding of [6,7,^{3}H-]-ethynyl-estradiol to macromolecules (albumin, poly-L-lysine, DNA, RNA) by tyrosinase and inhibition of albumin binding by amines and cysteine derivatives: 5 ml incubations with 81 nmole ethynyl-estradiol, 500 U tyrosinase, 1 mg NADPH and 50 mg albumin (or poly-lysine, DNA, RNA) at 37°C for 1 hr

Macromolecular compound	Inhibitor	% Ethynyl-estradiol bound ($\bar{x} \pm$ S.D.) and No. of individual experiments
No compound added (binding only to the tyrosinase protein)	—	7·34 ± 0·91% (n = 7)
Albumin	—	14·9 ± 2·5% (n = 7)
Albumin	1 mM glutathione	2·9 ± 0·2% (n = 4)
Albumin	1 mM N-acetyl-cysteine	2·6 ± 0·9% (n = 4)
Albumin	1 mM cysteine	5·2 ± 0·4% (n = 4)
Albumin	10 mM lysine	8·0 ± 0·7% (n = 4)
Albumin	10 mM ethylenediamine	9·0 ± 0·6% (n = 4)
Albumin	10 mM ethanolamine	10·4 ± 0.5% (n = 4)
Poly-L-lysine	—	16·9 ± 1·1% (n = 4)
DNA	—	26·8 ± 1·7% (n = 4)
RNA	—	21·5 ± 8·1% (n = 4)
Albumin (boiled enzyme)	—	No binding

to the ε-amino group of lysine. Concordantly, microsomes are not able to catalyze an irreversible binding of ethynyl-estradiol to DNA and RNA (Table 1).

Tyrosinase-catalyzed irreversible binding of ethynyl-estradiol to macromolecules

The results with tyrosinase as catalyst are presented in Table 2. Under the reaction conditions employed, 14·9% of the ethynyl-estradiol are irreversibly bound to proteins if albumin is present. If albumin is omitted, even 7·34% are bound to the enzyme protein itself, which is comparable with the portion bound to microsomes in the microsomal system. This sheds some light on the considerable reactivity of the intermediate formed by tyrosinase, which apparently tends to combining with functional groups of proteins immediately after its formation. So 1 mg of tyrosinase protein—the place where the intermediate is formed—binds as much metabolized ethynyl-estradiol as 50 mg albumin in presence of tyrosinase. However, in contrast to our findings with microsomes, SH- as well as NH_2- compounds can inhibit the protein binding in the tyrosinase system. Although the inhibition by cysteine derivatives is much more effective than that by the amines, it is most likely that the reactive intermediate involved in the tyrosinase-catalyzed protein binding of ethynyl-estradiol can also react with NH_2- groups. This behaviour also favors a binding of ethynylestradiol to poly-lysine, which ranges in the same order of magnitude as the binding to albumin. In contrast to our findings with microsomes, tyrosinase was able to catalyze an irreversible binding of ethynyl-estradiol to DNA and RNA, which even exceeded the binding of ethynyl-estradiol to albumin.

These results strongly suggest that the intermediate involved in the tyrosinase reaction is different from that, which binds ethynylestradiol to microsomal proteins.

Non-enzymatic reaction of estrogen-o-quinone with SH- and NH_2-compounds

As it is known estrogen o-quinones are able to react readily in non-enzymatic reaction with amines[23, 29, 30] as well as with sulfhydryl reagents [31–33]. Therefore we compared these two types of reaction in relation to our findings with both enzymic systems.

A microsomal system is regarded as a two-phase system, composed of lipid layers, in which the proteins are embedded with their aqueous surrounding. This is the reason why we used a two-phase system for examining the reactivity of 17β-hydroxy-estra-4,10[1]-diene-2,3-dione (estradiol-o-quinone) with amines and thiols: the lipid-soluble quinone in chloroform solution reacted with cysteine, lysine and analogous which were dissolved in phosphate buffer at physiological pH. The results of this experiment are given in Table 3.

The sulfhydryl compounds examined took up all of the quinone present. Among the amines, the chloroform-soluble phenylenediamine, ethylenediamine and ethanolamine also trapped the quinone completely, but lysine was significantly less reactive than the SH- compounds in this system. However, increasing concentration of lysine resulted in a considerable

Table 3. Non-enzymatic reaction of 17β-hydroxy-estra-4,10(1)-diene-2,3-dione (2·68 mM) in chloroform solution with various NH_2- and SH-compounds in phosphate buffer, pH 7·4. The two-phase system was shaken for 1 h. After reaction the untreated o-quinone was determined by iodometric titration. Values are expressed as % of o-quinone trapped off by the reactants

Reactant	Concentration reactant in the aq. phase	o-Quinone trapped off ($\bar{x} \pm$ S.D.)	Number of individual experiments
Control, no reactant		zero†	6
Phenylenediamine	0·185 M	100%	2
Ethylenediamine	0·185 M	100%	2
Ethanolamine	0·185 M	100%	2
Lysine	0·185 M	22·8 ± 14·0%	4
Lysine	0·925 M	42·9 ± 4·2%*	4
Lysine	1·85 M	76·1 ± 3·1%*	4
Mercaptoethanol	0·185 M	100%	2
Cysteine	0·185 M	100%	2
N-acetyl-cysteine	0·185 M	100%	2
Cysteine-methyl ester	0·185 M	100%	2

* $P < 0{\cdot}001$.

† Addition of poly-L-lysine trapped off 21·6% of the quinone present.

rise in its reaction with quinone. This behaviour of lysine fits with the data obtained from the tyrosinase system, where a tenfold lysine concentration, compared with that of the thiols, had been necessary to evoke an efficient inhibition of the tyrosinase-mediated ethynyl-estradiol-protein binding.

DISCUSSION

The participation of active intermediates in binding reactions has been established for most of the xenobiotics actually known to bind irreversibly to macromolecules such as proteins or nucleic acids in biological systems[10, 11, 13]. The involvement of such metabolites in chemical carcinogenesis has been postulated by Fieser as early as 1938[34]. These intermediates are able to react with electron-rich sites of macromolecules. So the ε-amino group of lysine may be involved in the binding of aminopyrine metabolites[35, 36] and penicillin[37, 39]. The sulfur atom of methionine is attacked by a metabolite of N-acetyl-aminofluorene[38]. Estrogens are thought to bind to the sulfhydryl group of cysteine, in analogy to their coupling with glutathione[32].

The reactive intermediate of the irreversible estrogen–protein binding is formed by a second oxidative step following 2-hydroxylation and may be either the o-quinone or semiquinone[18, 19, 23, 24]. The present data show that *tyrosinase*, which is able to form estrogen-o-quinones[21–23], can catalyze the irreversible binding of estrogens to protein. The o-quinone is known to react with both sulfhydryl groups[31–33] and amino compounds[23, 29, 30], as formulated in Fig. 1.

In the present investigation we demonstrate that the reactivity of lysine to estrogen-o-quinone is considerably less than that of cysteine and cysteine derivatives. However, this reactivity is sufficient to produce an irreversible binding of estrogen to poly-lysine in the tyrosinase system.

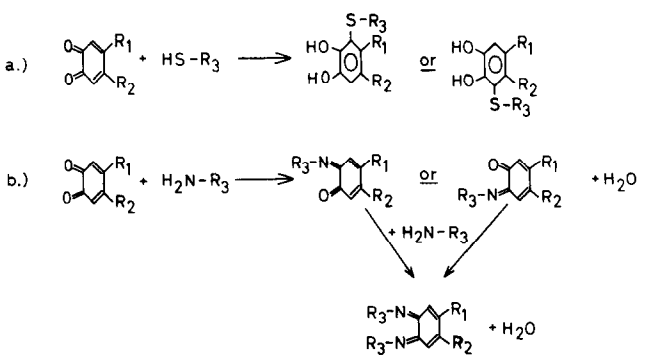

Fig. 1. Mode of reaction of estrogen o-quinone with (a) sulfhydryl compounds and (b) amines. (See ref. 23, 29–33.)

The formation of an o-quinone intermediate favours also an affinity for DNA and RNA, which irreversibly bind even more metabolized ethynyl-estradiol in the tyrosinase incubation than albumin does. It may reasonably be assumed that this kind of binding involves the attachment of quinone to the NH_2- groups of adenine, guanine and/or cytosine.

The microsomal irreversible binding of estrogens to protein must proceed *via* a metabolite different from the o-quinone, because amino groups do not interact with the binding to proteins. This is also established by the lack of reactivity of estrogen metabolites with polylysine in the microsomal system. Our results confirm the concept of Hecker *et al.*[24] that the o-semiquinone is formed from estrogens rather than the o-quinone. The difference in the nature of the reactive intermediates in the microsomal and the tyrosinase enzymic system must be the reason for our findings that ethynyl-estradiol after its metabolism does not bind irreversibly to DNA or RNA with rat liver microsomes.

The formation of covalent bonds of carcinogens to nucleic acids is involved in chemical carcinogenesis, as it has been demonstrated in recent publications[12, 13, 40–42]. The present results that estrogens do not bind to nucleic acids by the catalytic action of liver microsomes contrast to these findings in carcinogenic chemicals. From this point of view it appears to be unlikely that ethynyl-estradiol may act as a chemical carcinogen. This conclusion agrees with the results of clinical investigations in patients under long-term medication with contraceptives, as already initially mentioned.

However, the physiological or a possible pathological relevance of the established irreversible binding of estrogens to proteins still remains to be eludicated.

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