AN IMMUNOCHEMICAL STUDY OF THE HAPTEN FORMED FROM ETHYNYLOESTRADIOL AND GUINEA PIG LIVER MICROSOMES

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1. Introduction

It has been suggested that the diffuse arterial intimal lesions observed at necropsy in young women who had died from thrombosis while taking oral contraceptives were consistent with an immunological mechanism [1]. In support of this a monoclonal IgGλ with specificity for 17α-ethynyloestradiol (EE_2) has been isolated from the plasma of a woman who had suffered a pulmonary artery thrombosis while taking an oral contraceptive containing EE_2 and norethisterone [2]. It is known that EE_2 becomes irreversibly bound to protein when incubated with rat and human microsomes and could, therefore, form immunogenic haptens in vivo [3,4].

A major obstacle in the detection and characterisation of antibodies to drugs is that the chemical structure of the hapten is difficult to predict. It has been suggested that EE_2 is metabolised by sequential oxidations to an electrophilic quinone intermediate which can form covalent bonds with endogeneous macromolecules [3–5]. However, EE_2 may also undergo metabolism at the 17α-ethynyl group with subsequent rearrangement [6] and furthermore, the antibodies isolated [2] had a 50% cross-reaction with oestradiol.

We have investigated the effect of nucleophiles on the irreversible binding of EE_2 to guinea pigs liver microsomes and have examined a protein conjugate of EE_2 using two anti-EE_2 antisera; one which was specific for only the D ring and one which was specific for both the A and D ring. The results are consistent with the irreversible binding of EE_2 being through the A ring probably via a thioether linkage and indicate that the 17α-ethynyl group remains intact.

2. Materials and methods

2.1. *Reagents*

Tritiated 17α-[6,7-^{3}H(N)] ethynyloestradiol was obtained from New England Nuclear, Boston, MA. Unlabelled EE_2 and NADPH were obtained from Sigma. Scintillant (NE 260) was obtained from Nuclear Enterprises, Edinburgh. Antisera raised against ethynyloestradiol-7-(3-thiopropionic acid)–BSA (anti-EE_2-7–BSA) was a gift from Dr E. Cooke of Research Triangle, PO 12194, NC. and antisera were raised against 4-(4-carboxyphenylazo)ethynyloestradiol–BTG (anti-EE_2-4–BTG) as in [7]. General reagents were purchased from BDH and all solvents were redistilled prior to use.

2.2. *Covalent binding studies*

Dunkin and Hartley guinea pigs (360–400 g) were killed by cervical dislocation and their livers immediately excised into ice-cold 1.15% KCl solution (pH 8) washed and roughly chopped over ice with scissors. The chopped liver was then homogenised in ~1 vol. ice-cold 1.15% KCl (pH 8) with a motor driven glass–Teflon homogeniser. Cell debris, nuclei and mitochondria were then removed by centrifugation (12 000 × *g*, 20 min) in an MSE High Speed 18 Centrifuge at 4°C. The resulting supernatant was carefully decanted and centrifuged at 127 000 × *g* for 60 min in a Beckman L-2 centrifuge at 4°C. The

microsomal protein content was determined by the Lowry method [8].

Control incubation mixtures (3 ml) contained liver microsomes (6 mg), tritiated ethynyloestradiol (0.75 μmol; 250 000 dpm) in 25 μl DMSO and NADPH (2 μmol) in 0.2 M phosphate buffer, pH 7.4. Reactions were started by the addition of the NADPH and incubated under air at 37°C for 30 min. For some experiments the microsomes were denatured by heating at 90°C for 10 min and in other experiments the microsomes were presaturated with carbon monoxide for 15 min and the incubation carried out under an atmosphere of carbon monoxide.

The reactions were stopped by pouring the incubation mixture into 3 M trichloroacetic acid (0.8 ml). The precipitated protein was centrifuged at 1000 × *g* for 15 min and the supernatant removed and discarded. The precipitate was resuspended in 0.6 M trichloroacetic acid, centrifuged and the supernatant again discarded. The precipitate was then washed on GF/F filters (Whatman) with 6 × 5 ml methanol–water (4:1, v/v). The protein was then eluted with 0.05 M sodium hydroxide (1 ml) at 50°C for 30 min. The protein content of the eluate was measured by the Lowry method [8], and the radioactive content measured, after neutralisation with acetic acid, by liquid scintillation counting in NE 260.

Incubations which included bovine serum albumin (BSA) were stopped by cooling to 0°C. The microsomes were removed by centrifugation (127 000 × *g* for 60 min) and the supernatant dialysed against running water until no further radioactivity could be removed (4 days).

2.3. *Radioimmunoassay*

Standard radioimmunoassay reagents were prepared as in [9] and cross-reactions were determined using the 50% displacement method [10]. The cross-reaction for haptenic EE_2 was determined using EE_2 irreversibly bound to BSA as this material was soluble in the assay solution.

3. Results and discussion

Similarities between the metabolism of EE_2 in the guinea pig and humans have been shown [4] and the present work with hepatic microsomes shows that the similarity extends to irreversible binding. Table 1 shows that substantial irreversible binding of tritiated EE_2 occurs in incubation mixtures containing guinea pig microsomes and the amount of binding increased with time. Irreversible binding with heat denatured microsomes did not increase with time and is probably due to radiochemical impurities [12]. The reaction required NADPH and was sensitive to carbon monoxide indicating that it involves P450 mixed-function oxygenases [3].

Irreversible binding of EE_2 to guinea pig microsomes could be inhibited by the addition of nucleophiles such as *N*-acetylcysteine, histamine

Table 1
The effect of various inhibitors on the irreversible binding of EE_2 to guinea pig liver microsomes

Conditions	Mean irreversible binding (nmol/mg protein)	% Inhibition
Control (air atmosphere)	6.82 (± 0.81)	–
– NADPH	1.16 (± 0.07)	83
+ CO	2.65 (± 0.21)	61
+ 0.05 mM histamine	6.5 (± 0.76)	3
+ 0.5 mM histamine	5.5 (± 0.44)	19
+ 0.05 mM *N*-acetylcysteine	6.6 (± 0.57)	3
+ 0.5 mM *N*-acetylcysteine	2.03 (± 0.09)	71
+ 0.15 mM BSA	2.89 (± 0.63)	58
Heat-treated microsomes	1.23 (± 0.11)	82

Results are means ± SE of 4 determinations

and BSA which indicates that the reactive intermediate has a significant half-life. The greater reactivity (table 1) of the thiol group in comparison with the amino group is consistent with the reactive intermediate being an *o*-quinone and suggests that EE_2 becomes irreversibly bound via a thio-ether linkage through either the 1- or 4-position.

We have assessed the immunoreactivity of irreversibly bound EE_2 using a BSA–EE_2 conjugate which was soluble in the assay solution; the molar steroid–protein ratio of the conjugate was 1:20. The cross-reactions of various steroids with antisera, anti-EE_2-4–BTG and anti-EE_2-7–BSA are given in table 2 together with the cross-reactions of EE_2 irreversibly bound to BSA. The conjugate had a very low cross-reaction when tested with antiserum anti-EE_2-7–BSA which is specific for the A and D rings of EE_2 but the cross-reaction increased 100-fold with anti-EE_2-4–BTG which is specific for only the D ring of EE_2. The increased immunoreactivity of the conjugate towards anti-EE_2-4–BTG is paralleled by the changes in cross-reactions for mestranol and norethisterone and indicates that the EE_2 becomes attached to BSA through the A ring and that the 17α-ethynyl group remains intact. Presumably the cross-reaction of the conjugate is lower than that of mestranol because of increased chemical modification.

Table 2
Cross-reactions (as %) of various steroids with anti-EE_2-4-BTG and anti-EE_2-7-BSA antisera

	% Cross-reaction of antiserum	
Steroid	Anti-EE_2-4–BTG	Anti-EE_2-7–BSA
Ethynyloestradiol	100	100
Oestradiol	0.69	1.1
Norethisterone	42	0.019
Mestranol	77	0.9
Irreversibly-bound ethynyloestradiol	3.57	0.036

Cross-reactions were determined using the 50% displacement method [10]

In conclusion we have provided further evidence that EE_2 becomes irreversibly bound to protein through the A ring, probably via a thio-ether linkage. The experimental approach outlined here may be useful for the detection of EE_2–protein conjugates in women who are currently taking oral contraceptives.

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