Hydroperoxides as Inactivators of Aromatase: 10β-Hydroperoxy-4-estrene-3,17-dione, Crystal Structure and Inactivation Characteristics†

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ABSTRACT: The crystal structure of 10β-hydroperoxy-4-estrene-3,17-dione (10β-OOH) was determined, and its inhibition of human placental aromatase was investigated. In the absence of added NADPH, 10β-OOH caused a time-dependent loss of aromatase activity (e.g., 50% loss after 90 s with 2.16 μM 10β-OOH). Protection against this loss of activity was provided when a substrate, androstenedione, was included in the incubation. Centrifugation and resuspension of the 10β-OOH-treated microsomes in fresh buffer failed to restore the activity, but partial recovery could be effected by dithiothreitol. Experiments to detect destruction of aromatase protoheme were done but were inconclusive. In the presence of NADPH, 10β-OOH did not cause a time-dependent loss of activity but was instead a competitive inhibitor (K_i = 330 nM) of androstenedione (K_m = 21 nM) aromatization. The added NADPH was not utilized for the aromatization of 10β-OOH to estrogens, and enhanced reduction of 10β-OOH to 10β-hydroxy-4-estrene-3,17-dione could not be detected. In addition, microsomes alone were incapable of using 10β-OOH to support the aromatization of androstenedione. Cumene hydroperoxide and H_2O_2 were also investigated as inactivators of aromatase. Losses of activity comparable to those found for 10β-OOH could only be observed at 500–1000-fold higher concentrations of these agents, and no protection was provided by either androstenedione or NADPH. Extensive destruction of microsomal protoheme was found with these nonsteroidal agents.

The aromatization of androstenedione (**1**) to estrone (**4**) by the aromatase enzyme system of human placental microsomes has been shown to require 3 equiv of both NADPH and O_2 (Thompson & Siiteri, 1974a) and to proceed through the reaction sequence (**1** → **2a** → **3**[1] → **4**) shown in Figure 1 (Holland, 1981). Several lines of evidence substantiate the hypothesis that the protein components of this aromatase enzyme system consist of NADPH-cytochrome *c* reductase (Thompson & Siiteri, 1974b; Osawa et al., 1981) and a unique cytochrome P-450 (Osawa et al., 1982).

Because of our interest in irreversible inhibitors of estrogen biosynthesis (Covey & Hood, 1982a) and the fact that liver microsomal cytochrome P-450 (P-450_{LM})[2] can be inhibited by organic hydroperoxides (Nordblom et al., 1976), it was of interest to know if placental P-450_{AROM} could be inactivated by organic hydroperoxides. Three such agents were evaluated. The steroid 10β-OOH (**2b**; Figure 1) is a steric mimic of the aromatase intermediate **2a** and was found under certain conditions to be an active-site-directed inhibitor of the enzyme. The compounds H_2O_2 and CHP are structurally dissimilar to aromatase intermediates or products and were found to be nonspecific inhibitors of the enzyme.

As mentioned, the interactions of P-450_{LM} with organic hydroperoxides have been studied extensively (O'Brien, 1978; White & Coon, 1980). Characterization of these interactions has demonstrated the following: (a) inactivation of P-450_{LM} by organic hydroperoxides, in the absence of NADPH and substrate, correlates with destruction of P-450_{LM} protoheme; (b) reduction of the organic hydroperoxide to the corresponding alcohol by P-450_{LM} can occur in the presence of NADPH; (c) P-450_{LM} can utilize an organic hydroperoxide in the absence of NADPH for substrate oxidation. We have addressed each of these types of interactions between 10β-OOH and P-450_{AROM}.

Experimental Procedures

Materials. The 4-[1,2-^{3}H(N)]androstene-3,17-dione (46.1 Ci/mmol) and 4-[4-^{14}C]androstene-3,17-dione (55.75 mCi/mmol) were purchased from New England Nuclear. Liquid scintillation counting was done in Budget Solve from Research Products International. The NADPH, NADP, catalase (bovine liver, thymol free, 176 000 units/mg), dithiothreitol, and mannitol were from Sigma. Silica gel G without gypsum precoated plastic TLC sheets were from Sybron/Brinkmann. The CHP was purchased from ICN Pharmaceuticals and was purified by vacuum distillation (Armstrong et al., 1950). The 10β-OOH was prepared according to the literature method (Siegmann & DeWinter, 1970). It was chromatographically pure and had the reported physical and spectroscopic properties. Protein determinations were done with a Bio-Rad Protein Assay Kit I purchased from Bio-Rad Laboratories. Baker Extraction System and columns were purchased from J. T. Baker Chemical Co., Inc., and GC columns were obtained from Supelco Inc.

Crystallographic Studies. Crystals of 10β-OOH are orthorhombic with cell dimensions of a = 9.640 (2) Å, b =

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[1] The actual enzyme-bound form of intermediate **3** may be a *gem*-diol at carbon 19 rather than the aldehyde shown.

[2] Abbreviations: P-450_{LM}, liver microsomal cytochrome P-450; P-450_{AROM}, aromatase cytochrome P-450; 10β-OOH, 10β-hydroperoxy-4-estrene-3,17-dione; CHP, cumene hydroperoxide; 10β-OH, 10β-hydroxy-4-estrene-3,17-dione; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GC–MS, gas chromatography–mass spectrometry; FID–GC, flame ionization detector–gas chromatography; Est. (figures), estrogen; EDTA, ethylenediaminetetraacetic acid.

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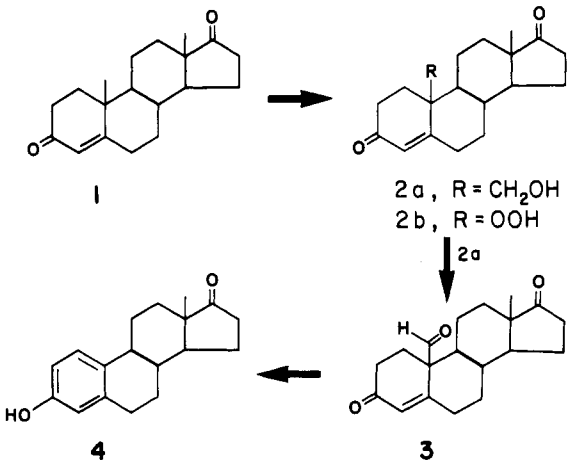

FIGURE 1: Aromatase reaction sequence. The steroid 10β-OOH (**2b**) is a steric mimic of the intermediate C-19 alcohol (**2a**).

20.099 (4) Å, and c = 8.216 (1) Å and have space group $P2_12_12_1$ with four molecules per unit cell. Three-dimensional X-ray diffraction data were measured from an equidimensional crystal (0.15 × 0.15 × 0.15 mm) by using an automated four-circle diffractometer utilizing monochromated Cu Kα radiation. The $\theta - 2\theta$ variable scan rate technique was used to a maximum value of sin (θ/λ) of 0.602 Å^{-1}. The scan rate was varied from 1.0 to 29.3 deg/min depending on the reflection intensity. A total of 1717 independent reflections were measured, and 1695 were above the threshold of $I = 0$. Lorentz and polarization corrections were applied to the intensities to obtain structure amplitudes. No absorption correction was applied [μ(Cu K) = 6.32 cm^{-1}].

A trial structure was obtained by direct methods (Germain et al., 1971) and was refined by full-matrix least-squares methods (Carrell, 1969) first with isotropic temperature factors for the non-hydrogen atoms and then with anisotropic temperature factors. All of the hydrogen atoms were located on the difference Fourier map, and further least-squares refinement was carried out on all atoms (C and O, anisotropic; H, isotropic). The final conventional R value was 0.040, and the weighted R value was 0.053. The quantity minimized was $\sum w||F_{obsd}| - F_{calcd}|^2$ where $w = 1/\delta^2(F)$. Values of $\sigma(F)$ were determined from the relation $\sigma(F) = (F/2)[[\sigma^2(I)/I^2] + \delta^2]^{1/2}$ where $\delta = 0.021$ and represents the instrumental uncertainty as determined from intensities of four periodically scanned check reflections. The atomic scattering factors for oxygen, carbon, and hydrogen are found in the literature (Cromer & Mann, 1968; Stewart et al., 1965). The final atomic parameters are given in Table I (for additional information, see paragraph at end of paper regarding supplementary material).

Enzyme Inhibition Studies. Human placental microsomes were prepared by our previously described method (Covey et al., 1981). The specific activities of the preparations used in these experiments ranged from 190 to 311 pmol min^{-1} (mg of protein)$^{-1}$. Standard incubations, done in a shaking water bath at 37 °C in air, contained 100 mM KCl, 10 mM potassium phosphate buffer, pH 7.8, 1 mM EDTA, 100 μM NADPH (omitted in appropriate controls), propylene glycol (1 drop/0.5 mL), and microsomes (0.055–0.083 mg of protein/0.5 mL). Some incubations contained mannitol (60 mM) or NADP (100 μM).

Time course experiments began with the addition to standard incubations (3.0 mL) of inhibitors dissolved in ethanol (0.01 mL/0.5 mL incubation volume). At various times aliquots (0.5 mL) were removed, added to assay tubes containing 4-[1,2-^{3}H(N)]androstene-3,17-dione (0.5 nmol present as a predried film), and measured for remaining enzyme activity (Thompson & Siiteri, 1974a). Five minutes later the assay reactions were stopped by the addition of chloroform (5.0 mL) and vortexing ~40 s. Then, after centrifugation at 1470g for 5 min, aliquots (0.10 mL) were removed from the water phase and added to scintillation cocktail for determination of ^3H_2O production. Control experiments demonstrated that (1) no further significant loss of activity occurred while rates of product formation were measured, (2) complete aromatization of the radiolabeled substrate released 60% of the tritium into water; and (3) the assay was linear over the 5-min period in either the presence or absence of inhibitor. Further discussions regarding our method of performing this assay can be found elsewhere (Covey & Hood, 1982b).

In experiments with H_2O_2 the excess present in each aliquot taken for assay was destroyed by the inclusion of catalase (0.115 mg/0.5 mL) in the assay. The catalase itself had no inhibitory effect on the enzyme.

Competition experiments performed in standard incubations (0.5 mL) contained 4-[1,2-^{3}H(N)]androstene-3,17-dione (six different concentrations ranging from 0.01 to 1.0 μM) alone

Table I: Final Atomic Parameters and Estimated Standard Deviations (in Parentheses)[a]

atom	x	y	z	U(iso)
C(1)	0.14685 (23)	0.35076 (11)	0.40373 (22)	0.0498 (10)
C(2)	0.02370 (22)	0.31206 (12)	0.47100 (25)	0.0525 (11)
C(3)	−0.09029 (22)	0.30118 (9)	0.35002 (25)	0.0478 (10)
C(4)	−0.05479 (22)	0.30381 (10)	0.17683 (25)	0.0458 (10)
C(5)	0.07405 (20)	0.31328 (8)	0.12130 (22)	0.0390 (8)
C(6)	0.10718 (22)	0.30266 (11)	−0.05619 (23)	0.0468 (10)
C(7)	0.19529 (23)	0.35887 (11)	−0.12650 (24)	0.0510 (10)
C(8)	0.32250 (20)	0.37297 (9)	−0.02325 (21)	0.0398 (9)
C(9)	0.27803 (20)	0.39032 (9)	0.15304 (23)	0.0387 (9)
C(10)	0.19457 (20)	0.33184 (9)	0.23095 (21)	0.0390 (9)
C(11)	0.39817 (24)	0.41446 (12)	0.26120 (24)	0.0535 (11)
C(12)	0.48464 (24)	0.47033 (11)	0.18117 (30)	0.0535 (12)
C(13)	0.53353 (21)	0.44824 (9)	0.01395 (26)	0.0436 (10)
C(14)	0.40660 (22)	0.43087 (10)	−0.09133 (23)	0.0428 (9)
C(15)	0.46344 (27)	0.42625 (14)	−0.26498 (28)	0.0643 (13)
C(16)	0.57117 (27)	0.48332 (14)	−0.27100 (33)	0.0677 (14)
C(17)	0.60077 (23)	0.50110 (11)	−0.09498 (33)	0.0545 (12)
C(18)	0.64222 (24)	0.39204 (11)	0.02724 (34)	0.0597 (13)
O(3)	−0.20732 (17)	0.28615 (9)	0.39251 (20)	0.0627 (9)
O(17)	0.66472 (20)	0.54913 (8)	−0.04908 (26)	0.0764 (12)
O(19a)	0.29389 (14)	0.27805 (6)	0.23249 (17)	0.0479 (7)
O(19b)	0.22455 (17)	0.21722 (7)	0.28541 (19)	0.0570 (8)
H(1)	0.1207 (22)	0.3972 (11)	0.3882 (27)	0.058 (6)
H(1′)	0.2188 (22)	0.3495 (11)	0.4794 (28)	0.067 (6)
H(2)	−0.0150 (23)	0.3360 (12)	0.5816 (30)	0.073 (7)
H(2′)	0.0445 (22)	0.2665 (12)	0.5301 (34)	0.075 (6)
H(4)	−0.1237 (22)	0.2930 (11)	0.0879 (25)	0.057 (6)
H(6)	0.0295 (21)	0.2966 (11)	−0.1031 (28)	0.079 (6)
H(6′)	0.1583 (23)	0.2597 (12)	−0.0589 (23)	0.042 (6)
H(7)	0.1389 (22)	0.3994 (11)	−0.1280 (29)	0.053 (6)
H(7′)	0.2219 (22)	0.3469 (11)	−0.2462 (27)	0.071 (6)
H(8)	0.3816 (20)	0.3308 (10)	−0.0229 (25)	0.029 (5)
H(9)	0.2090 (21)	0.4309 (11)	0.1522 (29)	0.036 (6)
H(11)	0.4590 (22)	0.3762 (13)	0.2889 (28)	0.054 (6)
H(11′)	0.3580 (24)	0.4304 (12)	0.3641 (30)	0.060 (6)
H(12)	0.5588 (24)	0.4765 (12)	0.2542 (31)	0.063 (7)
H(12′)	0.4202 (24)	0.5131 (13)	0.1697 (31)	0.071 (6)
H(14)	0.3446 (24)	0.4693 (11)	−0.0822 (28)	0.044 (6)
H(15)	0.4036 (25)	0.4267 (12)	−0.3521 (33)	0.113 (7)
H(15′)	0.5143 (25)	0.3793 (14)	−0.2802 (31)	0.076 (7)
H(16)	0.5345 (27)	0.5190 (14)	−0.322 (4)	0.082 (8)
H(16′)	0.6506 (29)	0.4649 (13)	−0.331 (4)	0.111 (8)
H(18)	0.7326 (25)	0.4095 (13)	0.0923 (32)	0.066 (7)
H(18′)	0.5943 (26)	0.3531 (13)	0.0745 (29)	0.074 (7)
H(18″)	0.6653 (27)	0.3775 (14)	−0.0715 (30)	0.105 (8)
H(19b)	0.2566 (23)	0.2197 (12)	0.3727 (30)	0.100 (6)

[a] The coordinates are listed as fractions of the cell edges. Temperature factors U have the units angstroms. For the non-hydrogen atoms, the U(iso) represents the average of U(11), U(22), and U(33).

or with inhibitors dissolved in ethanol (0.01 mL). Addition of microsomes and NADPH to the incubation initiated the assay reaction. Reactions were stopped by the addition of chloroform (5.0 mL), and the ^3H_2O content was determined as previously described. Concentrations of 10β-OOH evaluated were 0.5 and 2.0 μM and of 10β-OH were 1.0 and 5.0 μM. Kinetic results were analyzed by Lineweaver–Burk plots (Lineweaver & Burk, 1934). Regression lines for these kinetic data were drawn according to a least-squares fit.

Experiments done to investigate the reversibility of the inhibition produced by 10β-OOH, CHP, and H_2O_2 used initial standard incubations (5.0 mL) containing microsomes with or without added NADPH. Addition of 10β-OOH, CHP, and H_2O_2 in ethanol (0.10 mL) yielded final concentrations of 2.0 μM, 1.0 mM, and 2.1 mM, respectively. Controls received ethanol (0.10 mL) only. Sixty minutes after the addition of inhibitors, aliquots (0.5 mL) were removed and assayed for remaining activity as described earlier. Of the remaining incubation volumes approximately 4.0 mL was spun at 100000*g* for 60 min at 4 °C. The supernatants were discarded while the pellets were resuspended in the standard buffer (3.0 mL). Portions (0.5 mL each) were removed for the assay of aromatase activity as described earlier and for protein determination. The remaining resuspended microsomes (2.0 mL) were spun again at 100000*g* for 60 min at 4 °C. Again, the supernatants were discarded while the pellets were resuspended in buffer (1.0 mL), and portions (0.5 mL each) were taken for aromatase assay and protein determination.

Measurement of NADPH. To measure the loss of NADPH (1.0 μM) in standard microsomal incubations (12.0 mL, 0.057 mg of protein/0.5 mL) containing either 10β-OOH (2.0 μM) or 10β-OH (2.0 μM), aliquots (1.0 mL) were removed at various times and added to 1.0 N NaOH (0.03 mL). Control experiments determined that this amount of NaOH was sufficient to stop further loss of NADPH. These timed aliquots were then read fluorometrically against a quinine standard according to the methods of Lowry and Passonneau (Lowry & Passonneau, 1972).

HPLC Chromatographic Analysis of 10β-OOH Conversion to 10β-OH. To determine if conversion of 10β-OOH to 10β-OH occurred in the microsomal incubations, standard incubations (5.0 mL) with NADPH (1.0 μM) or without NADPH or with heat-inactivated microsomes (5 min in boiling water) were exposed to 10β-OOH (2.0 μM) for 6 min and then extracted with 1.0 mL of chloroform followed by 2.0 mL of chloroform. The chloroform extracts were combined and blown dry under nitrogen, and the residue was redissolved in 0.05 mL of ethanol (containing 100 μM 4-androstene-3,6,17-trione as an internal standard). A portion (0.025 mL) of this was injected onto an HPLC (Waters Associates Model 6000 with U6K injector) and eluted at 2 mL/min with acetonitrile/water (30:70) from a Waters Associates RCM100 C_{18} 10-μm reverse-phase cartridge. Steroids were monitored at 240 nm on a Water Associates Model 450 variable wavelength detector, and retention times were 10.4 min for 10β-OOH, 8.2 min for 10β-OH, and 20.7 min for 4-androstene-3,6,17-trione. Control experiments showed that 10β-OOH conversion to 10β-OH did not occur in standard buffer incubations (minus microsomes) with or without NADPH or on the HPLC column. However, ~5–15% conversion did occur as a result of extracting and recovering 10β-OOH from the buffer.

TLC Chromatographic Analysis of Androstenedione Utilization. To demonstrate if 10β-OOH could support hydroxylation of androstenedione, standard incubations (0.5 mL) contained 4.5 nmol of 4-[4-^{14}C]androstene-3,17-dione, microsomes, and 10β-OOH (0.1, 1.0, 10, or 30 μM) dissolved in ethanol (0.01 mL). With the exception of one control, none of the incubations contained NADPH. Incubations continued at 37 °C for 60 min prior to addition of chloroform (5.0 mL) for steroid recovery. The chloroform extract was removed by pipet and dried under a stream of nitrogen, and the residue was taken up in chloroform (0.05 mL) for chromatographic separation on a precoated plastic thin-layer chromatography sheet (20 × 20 cm) of silica gel G (250 μm in thickness) without gypsum. The sheet was run in 2:1 ethyl acetate/cyclohexane with 1 drop of acetic acid for each 3.0 mL of solvent and then allowed to air dry overnight. The sheet was put in a cassette with X-ray film at room temperature for 5 days, and the film was developed. Quantitation was achieved by cutting and counting those regions of the TLC sheets shown by the film to contain radioactivity.

Spectrophotometric Determination of P-450 and Protoheme. Measurements were performed on a Beckman DU-8 microprocessor-controlled UV–visible spectrophotometer. Cytochrome P-450 content was measured according to the method of Juchau et al. (1974) and yielded values of 0.026–0.058 nmol of P-450/mg of microsomal protein. Protoheme content of the microsomes was 0.38 nmol/mg of protein as determined by the method of Paul et al. (1953).

In monitoring the loss of protoheme content in conjunction with loss of aromatase activity, standard initial incubations (1.0 mL) contained microsomes (1.10 mg of protein) and either 10β-OOH (10 μM), CHP (1.0 mM), H_2O_2 (2.0 mM), or ethanol (0.02 mL). Five minutes after the addition of hydroperoxide to the incubations, 0.05- and 0.40-mL aliquots were removed and added to either buffer (0.45 mL) with 4-[1,2-^{3}H(N)]androstene-3,17-dione or 0.2 N NaOH in 40% pyridine (0.40 mL) for assay of remaining aromatase activity and protoheme content, respectively.

Gas Chromatography–Mass Spectrometry Methods. Microsomal metabolites of 10β-OOH and 10β-OH were isolated and analyzed in the following manner. Standard incubations (3.0 mL) containing microsomes (2.27 mg of protein/mL) and NADPH (10 mM) were heated for 60 min at 37 °C in a shaking water bath after the addition of steroid (100 μM) in ethanol (0.03 mL). Reactions were terminated by applying them to 3-mL C_{18} Baker extraction columns which had been preconditioned with methanol (2 mL) and 10 mM phosphate buffer (2 mL). After application of the incubate, the column was rinsed with 10 mM phosphate buffer (2 mL). A final methanol/water (90:10) rinse (2 mL) eluted the steroids and was dried under N_2. The residue was taken up in 50 μL of acetone and analyzed by FID–GC on a Hewlett-Packard 5830A. The following were the instrument conditions: N_2 carrier 40 mL/min; 6 ft × 2 mm i.d. glass column with 1% SP2401 on 80/100 mesh Gaschrom Q; injector 275 °C; detector 275 °C; column 235 °C. GC–MS studies were performed on a Finnigan 3200 using the same column, and the instrument conditions were the following: He 25 mL/min; separator 240 °C; column 225 °C; injector 250 °C; ionizing energy 70 eV.

Results

Crystal Structure of 10β-OOH. The structure of 10β-OOH determined in this study is shown in Figure 2. The bond lengths and interbond angles for the four rings of the 4-androstene nucleus of 10β-OOH are comparable to the bond lengths and angles reported for 4-androstene-3,17-dione (Busetta et al., 1972) and for 19-hydroxy-4-androstene-3,17-dione (Duax & Osawa, 1980). The bond lengths in the

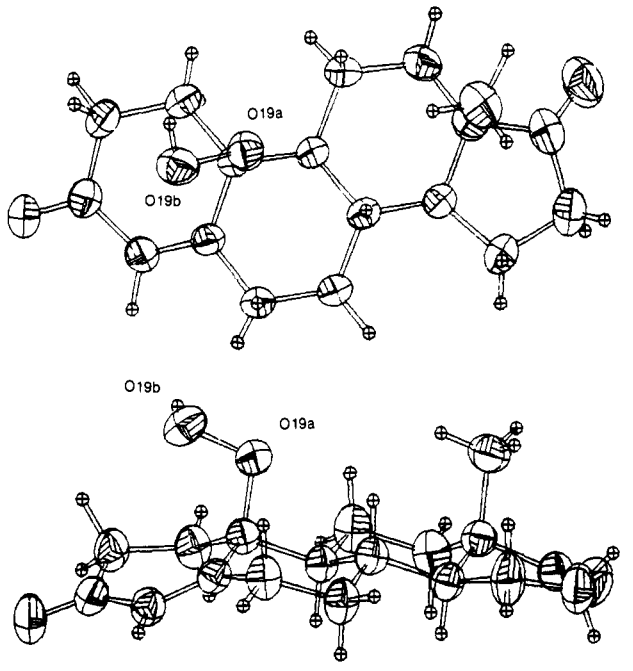

FIGURE 2: Two views of 10β-OOH. The thermal ellipsoids are drawn at 50% probability (Johnson, 1965). The diagrams were drawn by using the CHEMGRAF program system (Davies, 1983).

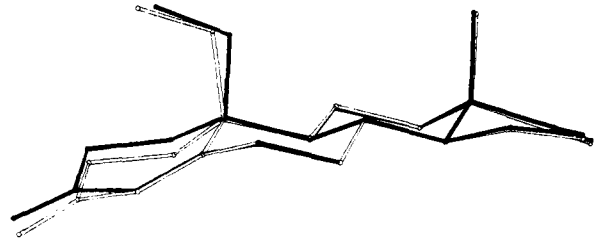

FIGURE 3: Diagram showing the superposition of the structure of 19-hydroxy-4-androstene-3,17-dione and 10β-OOH. The open circles and bonds represent the 19-hydroxy compound while the shaded circles and bonds represent the 10β-hydroperoxy compound. This diagram was produced by using the program VIEW (Carrell, 1976).

10β-hydroperoxyl group are C(10)–O(19a) = 1.444 (3) Å and O(19a)–O(19b) = 1.460 (2) Å. The bond angle C(10)–O-(19a)–O(19b) is 109.0 (1) Å. A comparison of the structure of this molecule with the structure of 19-hydroxy-4-androstene-3,17-dione is illustrated in Figure 3. This figure shows that O(19b) of the 10β-hydroperoxyl group is oriented over the A ring of the steroid nucleus in a manner virtually identical with the 19-hydroxyl group in 19-hydroxy-4-androstene-3,17-dione. The superposition of the two molecules demonstrates that the conformation of the B, C, and D rings is virtually the same for both molecules and that the A ring of the 19-hydroxy compound is bent in the direction of the α-face of the molecule relative to the 10β-hydroperoxy compound. On the other hand, the 10β-hydroperoxyl group is bent in the direction of the B ring. The difference in the conformations of the two molecules is apparently due to different modes of relieving steric strain introduced by the presence of the terminal –OH group.

Time-Dependent Loss of Aromatase Activity by 10β-OOH in the Absence of NADPH. Incubation of microsomes with 10β-OOH in the absence of NADPH resulted in an accelerated time-dependent loss of aromatase activity relative to control (Figure 4, upper panel). Both the rate of loss of activity and the maximum activity lost were dependent on the 10β-OOH concentration. At the highest concentration tested (2.16 μM), ~90% loss of initial activity occurred within 35 min.

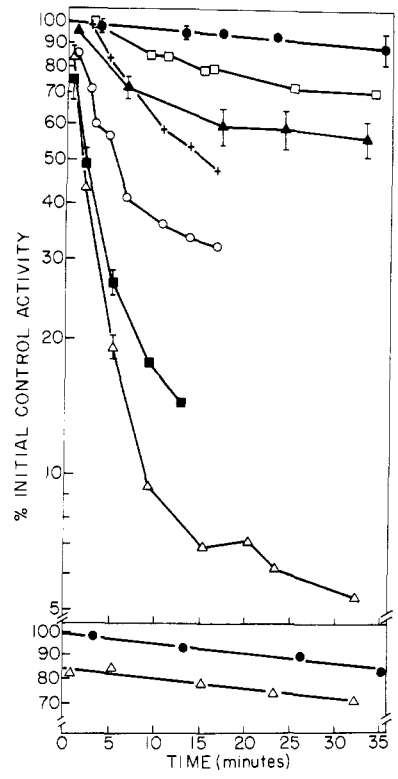

FIGURE 4: Time courses for the decrease in aromatase activity by 10β-OOH in the absence (upper panel) or presence (lower panel) of NADPH. (Upper panel) Control (●), 100% activity = 75.4 pmol of estrogen (5 min)$^{-1}$ (0.083 mg of protein)$^{-1}$; 10β-OOH, 0.18 μM (□); 0.27 μM (▲); 0.54 μM (○); 1.08 μM (■); 2.16 μM (△); 10β-OOH, 1.08 μM, and androstenedione, 0.5 μM (+). Duplicate experiments were run at each concentration, but assays for remaining activity were not always taken at identical times. When duplicate experiments were assayed at identical times, the data shown are the average and the range. (Lower panel). Control (●), 100% activity = 76.8 pmol of estrogen (5 min)$^{-1}$ (0.083 mg of protein)$^{-1}$; 10β-OOH, 2.16 μM, and NADPH, 100 μM (△). Points on each line are from a single experiment.

Evidence that the loss of aromatase activity caused by 10β-OOH was active site directed was provided by a protection experiment. Androstenedione (0.5 μM) greatly decreased the rate at which 1.08 μM 10β-OOH inactivated microsomal aromatase, so that, at 10 min, 60% activity rather than 17% activity remained.

Protection was not provided by mannitol (60 mM) which was used as a trap for hydroxy radicals (data not shown). Finally, no protection was provided by NADP (100 μM) in the incubations. This last result contrasts markedly with the result found when NADPH was present in the incubations (see below).

Competitive Inhibition of Aromatase by 10β-OOH and 10β-OH in the Presence of NADPH. The time-dependent loss of enzyme activity observed when 2.16 μM 10β-OOH was incubated with microsomes in the presence of 100 μM NADPH was unchanged from that observed for the microsomes and NADPH control (Figure 4, lower panel). Thus, instead of the potent enzyme inactivation caused by 10β-OOH alone, only time-independent inhibition (i.e., ~15% reduction in activity relative to control at all times) was observed. Competition experiments done with NADPH and androstenedione as substrate demonstrated that 10β-OOH behaved as a competitive inhibitor under these conditions (Figure 5, upper panel). The K_m and K_i values were determined to be 21 nM and 330 nM for androstenedione and 10β-OOH, respectively.

Since we also wished to address in this study the enzymatic conversion of 10β-OOH to 10β-OH, we examined the kinetic

Table II: Aromatase Activity of Microsomes from 60-min Incubations with Hydroperoxides

	control[a]		10β-OOH (2 μM)		H_2O_2 (2.1 mM)	CHP (1.0 mM)
	+	-	+	-	-	-
recovered microsomes	100[b]	88.9	80.5	3.1	26.7	6.2
first resuspended pellet	116 (0.038)[c]	119 (0.038)	90.3 (0.042)	13.6 (0.047)	28.1 (0.038)	14.1 (0.035)
second resuspended pellet	90.9 (0.062)	93.4 (0.062)	80.1 (0.070)	7.1 (0.071)	18.1 (0.060)	11.1 (0.061)

[a] Incubations were performed with (+) or without (-) the addition of NADPH (100 μM). Results are from a single experiment. [b] Activities are expressed as percent of activity measured in microsomes incubated with NADPH for 60 min [100% = 57.6 pmol of estrogen (5 min)$^{-1}$ (0.055 mg of protein)$^{-1}$]. [c] Numbers in parentheses indicate actual protein concentration (mg/0.5 mL) in the assay.

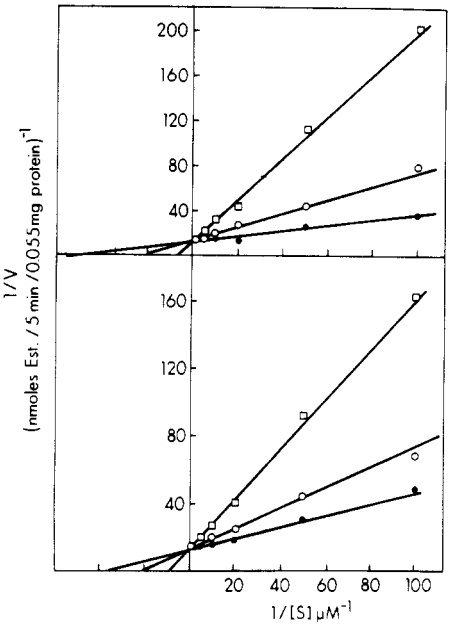

FIGURE 5: Competitive inhibition of androstenedione aromatization by 10β-OOH (upper panel) and 10β-OH (lower panel). (Upper panel). No inhibitor (●); 10β-OOH, 2.0 (□) and 0.5 μM (○). (Lower panel). No inhibitor (●); 10β-OH, 5.0 (□) and 1.0 μM (○).

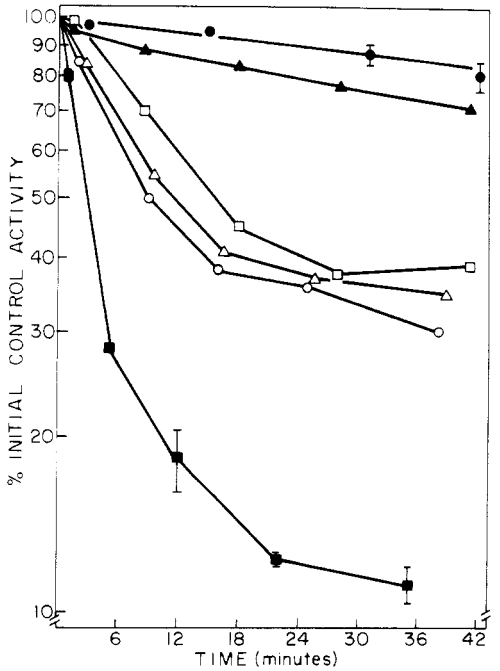

FIGURE 6: Time course for the decrease in aromatase activity by H_2O_2. Control (●), 100% activity = 57.6 pmol of estrogen (5 min)$^{-1}$ (0.057 mg of protein)$^{-1}$; H_2O_2, 2.0 μM (▲), 500 μM (○), and 2.0 mM (■); androstenedione, 0.5 μM, and H_2O_2, 500 μM (□); NADPH, 1.0 mM, and H_2O_2, 500 μM (△). Points on each line are from a single experiment except for those done with 2.0 mM H_2O_2 which are the averages and ranges of duplicate experiments.

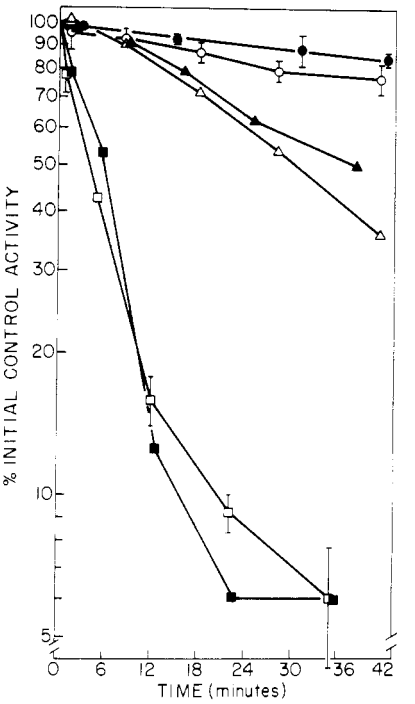

FIGURE 7: Time course for the decrease in aromatase activity by CHP. Control (●), 100% activity = 59.6 pmol of estrogen (5 min)$^{-1}$ (0.057 mg of protein)$^{-1}$; CHP, 2.0 μM (○), 100 μM (△), and 1.0 mM (□); androstenedione, 1.0 μM, and CHP, 1.0 mM (■); NADPH, 200 μM, and CHP, 100 μM (▲). Points are from a single experiment except for those done with 2.0 μM and 1.0 mM CHP which are the average and ranges of duplicate experiments.

characteristics of the latter compound. In the absence of NADPH no time-dependent loss of activity relative to control was observed (data not shown). Competition experiments (Figure 5, lower panel) showed that 10β-OH was also a competitive inhibitor of androstenedione aromatization. The K_m and K_i values determined were 28 nM and 1.5 μM for androstenedione and 10β-OH, respectively. Thus, 10β-OOH binding was approximately 5-fold stronger than 10β-OH binding to microsomal aromatase.

Time-Dependent Loss of Aromatase Activity by H_2O_2 and CHP. To observe inactivation rates and maximal losses of activity similar to those caused by 2.16 μM 10β-OOH, concentrations of 2.0 mM H_2O_2 (Figure 6) and 1.0 mM CHP (Figure 7) were required. In addition, there were two other major differences in the inactivation caused by these agents vs. that caused by 10β-OOH. The presence of NADPH did not abolish the inactivation caused by either H_2O_2 or CHP, and androstenedione alone did not protect aromatase against inactivation by these agents.[3]

[3] Although neither NADPH nor androstenedione alone will protect aromatase against inactivation by CHP, the combination of these substrates does provide adequate protection during the 5-min assay period. The reason for this phenomenon is unclear.

Table III: Reactivation of Aromatase by Dithiothreitol

preincubation[a] treatment	% initial activity[b] -DTT	+DTT[c]
10β-OOH (2.0 μM)	15.6	35.6
H_2O_2 (1.0 mM)	25.9	80.0
CHP (1.0 mM)	10.6	42.5

[a] Microsomes (0.057 mg of protein/0.5 mL) were incubated for 22 min with either H_2O_2 or CHP or incubated for 9 min with 10β-OOH in the absence of NADPH according to the procedure given under Experimental Procedures. Results are from a single experiment. [b] Activities are expressed as percent of time matched control activities. [c] Aliquots taken for assay were exposed to 10 mM dithiothreitol during the 5-min assay period.

Evidence for Irreversibility in the Inactivation Caused by 10β-OOH, H_2O_2, and CHP. Microsomes were incubated for 1 h with each compound and then pelleted by centrifugation. The supernatants were discarded, and the microsomes were resuspended and assayed in fresh buffer. The microsomes were again pelleted, resuspended and assayed in fresh buffer. The results are summarized in Table II. For 10β-OOH (2.0 μM) in the presence of NADPH (100 μM), 20% loss of activity was observed after the 1-h incubation, and this activity was not recovered after two centrifugation and resuspension steps. By contrast, 97% loss of activity by 10β-OOH (2.0 μM) was observed when the NADPH was omitted in the 1-h incubation, and only 4% of the activity was recovered after the centrifugation and resuspension steps. Since the presence of NADPH had no effect on the aromatase inactivation caused by H_2O_2 and CHP, centrifugation experiments were not done with added NADPH for these agents. In the absence of NADPH, the H_2O_2 (2.1 mM) caused a 73% loss of activity, and CHP (1.0 mM) caused a 94% loss of activity. No activity was recovered after the centrifugation and resuspension steps for H_2O_2, while only 5% activity was recovered in the case of CHP.

Partial Reactivation of Aromatase by Dithiothreitol. Dithiothreitol rapidly reduces 10β-OOH and CHP to their corresponding alcohols, and in our preliminary time-dependent inactivation studies (Covey et al., 1983) it was added to reduce CHP (and thus terminate its actions) prior to the assay step. Dithiothreitol was not added prior to assay in the present studies because we found that it would partially reverse the inactivation caused not only by CHP but also by 10β-OOH and H_2O_2 as well (Table III). The effect of dithiothreitol was immediate, and no further reactivation occurred when microsomes inactivated with 10β-OOH were allowed to stand for extended times prior to assay (reactivations of H_2O_2- and CHP-inactivated microsomes in the presence of dithiothreitol for extended periods of time were not examined). The partial reversal of inactivation by dithiothreitol suggests that sulfhydryl oxidation is one event occurring during inactivation by these hydroperoxides.

Destruction of Microsomal Protoheme by 10β-OOH, H_2O_2, and CHP. Microsomes were treated for 5 min with each agent, and aliquots were removed for the determination of aromatase activity and protoheme content (Figure 8). No correlation could be demonstrated between the two measurements. Destruction of protoheme (25% destruction was estimated to be minimum loss which could be reliably measured) could not be established for 10β-OOH even when only 45% of the activity remained. Conversely, no protoheme could be measured in the H_2O_2- and CHP-treated microsomes even though the remaining aromatase activity measured was 78% and 90%, respectively. We attribute the failure to correlate the two parameters measured to sensitivity problems resultant from the low microsomal content of cytochrome P-450_{AROM}.

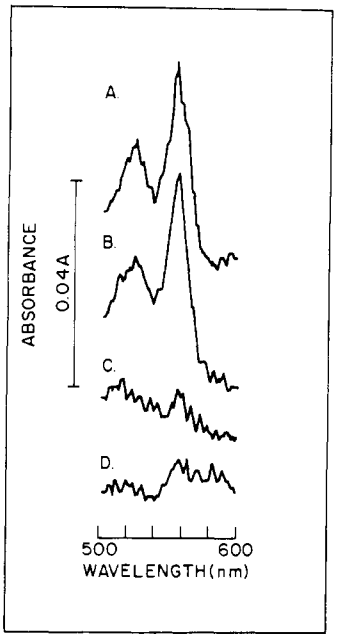

FIGURE 8: Pyridine hemochromogen content of microsomes. Protoheme content of placental microsomes following a 5-min incubation with each hydroperoxide in the absence of NADPH was measured as pyridine hemochromogen. Aromatase activity was also measured after the 5-min incubation. (A) Control, activity = 100% [55.7 pmol of estrogen (5 min)$^{-1}$ (0.055 mg of protein)$^{-1}$]; (B) 10β-OOH (10 μM), activity = 45%; (C) CHP (1.0 mM), activity = 78%; (D) H_2O_2 (2.0 mM), activity = 90%.

This issue is addressed further under Discussion. These results do, however, further demonstrate the nonspecific destructive effects of H_2O_2 and CHP.

Investigations on the Protective Effect of NADPH against 10β-OOH-Induced Aromatase Inactivation. The earlier results (Figure 4) demonstrating the protective effect of NADPH were obtained with 2.16 μM 10β-OOH and 100 μM NADPH. Experiments (not shown) in which the 10β-OOH concentration was 2.0 μM and the NADPH concentration lowered showed that complete protection was provided by 5.0 μM NADPH. At 1.0 μM NADPH, an ~6-min lag was observed before inactivation by 10β-OOH commenced. At 0.1 μM NADPH, no lag was apparent. These preliminary experiments suggested that NADPH was consumed during the incubation and raised the possibility that NADPH protection was due to the NADPH-dependent reduction of 10β-OOH to 10β-OH by aromatase. The experiments described below were designed to address this issue.

Aliquots were withdrawn over a 14-min period from incubations containing microsomes, 1.0 μM NADPH, and either 2.0 μM 10β-OOH or 2.0 μM 10β-OH for fluorometric determination of remaining NADPH concentration and enzymatic determination of remaining aromatase activity (Figure 9). In the control incubation (no steroid added) there was a gradual loss of NADPH, but no loss of enzyme activity. In the 10β-OH incubation there was an accelerated loss of NADPH which fell to a constant level after ~9 min, but no loss of enzyme activity. In the 10β-OOH incubation there was an even greater rate of NADPH loss, and the same constant remaining fluorescence that was observed in the 10β-OH incubation was achieved in ~6 min. The loss of enzyme activity commenced at ~6 min of incubation when the NADPH was exhausted. The fact that the NADPH was rapidly consumed in the 10β-OH as well as the 10β-OOH incubation raised the possibility that NADPH was being consumed in the reduction of the 17-keto group of each steroid by an NADP(H)-dependent 17β-hydroxysteroid dehydrogenase. This was indeed

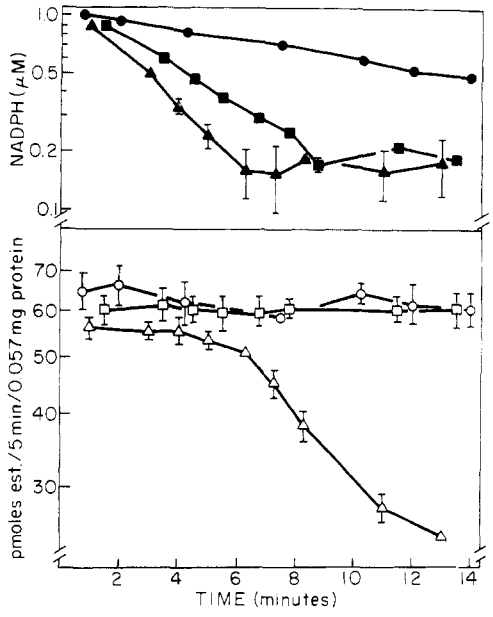

FIGURE 9: Time course for the loss of NADPH (upper panel) and its temporal relationship to loss of aromatase activity (lower panel) caused by 10β-OOH and 10β-OH. (Upper panel). NADPH content in microsomal incubations was determined by its fluorescence as described under Experimental Procedures. Control (●); 10β-OH, 2.0 μM (■); 10β-OOH, 2.0 μM (▲). Each point is from duplicate experiments; the data shown are the average and the range. The fluorescence of the microsomes alone corresponded to 45 ± 5 divisions on the fluorometer. The fluorescence of 1.0 μM NADPH increased the reading an additional 45.5 divisions. (Lower panel). Aromatase activity as a function of remaining NADPH. Control (○); 10β-OH, 2.0 μM (□); 10β-OOH, 2.0 μM (△). Each point is from duplicate experiments; the data shown are the average and the range.

the case, and experiments demonstrating this will be discussed later in this section.

To address the question of whether or not any of the NADPH loss observed in the 10β-OOH incubation was in part associated with 10β-OH production, steroids were recovered from 6-min (the time at which NADPH loss stops) incubations which contained 2.0 μM 10β-OOH with or without 1.0 μM NADPH and analyzed by HPLC. Heat-inactivated microsomes incubated with 2.0 μM 10β-OOH served as a control for nonenzymatic conversion of 10β-OOH to 10β-OH. The results from one of four sets of experiments (similar results were found in each case) are shown in Figure 10. From each incubation ~60% of the 10β-OOH was recovered. Inspection of the elution patterns shows that approximately the same ratio of 10β-OH to 10β-OOH was obtained with active and heat-inactivated microsomes. There was one additional substance present in the products recovered from incubations done with active microsomes, but its appearance was not dependent on NADPH addition. The structure and relevance of this additional compound to these aromatase investigations were not determined. Additionally, no significant enhanced conversion of 10β-OOH to 10β-OH could be attributed to the presence of NADPH in the incubation with 10β-OOH. Thus, these experiments failed to provide any evidence to substantiate the hypothesis that aromatase can use NADPH to support the conversion of 10β-OOH to 10β-OH and that the protection afforded by NADPH resulted from this conversion.

Experiments were also performed to demonstrate that the 10β-OH- and 10β-OOH-dependent NADPH consumption found in the experiments summarized in Figure 9 was not due to aromatization of these steroids. Incubations of 10β-OH and 10β-OOH (100 μM each) were carried out with microsomes (2.27 mg of protein/mL) and NADPH (10 mM) at 37 °C

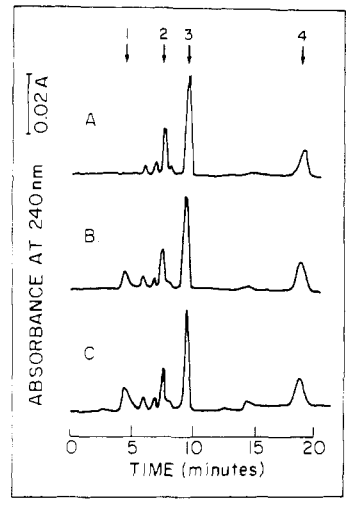

FIGURE 10: HPLC analysis of 6-min incubations of 10β-OOH (2.0 μM). Steroids were recovered from the incubations by extraction as reported under Experimental Procedures. The numbered peaks are (1) unidentified material, (2) 10β-OH, (3) 10β-OOH, and (4) 4-androstene-3,6,17-trione (internal standard). Unnumbered peaks are those present in incubations of 10β-OOH with buffer only. Incubations were done with (A) heat-inactivated microsomes, (B) microsomes without added NADPH, and (C) microsomes with NADPH, 1.0 μM.

for 1 h. The steroids were isolated and analyzed by GC–MS (see Experimental Procedures). In neither case was it possible to detect the formation of either estrone or estradiol. Aromatization of androstenedione was readily detected under these conditions.

It was possible, however, to demonstrate the presence of an NADP(H)-dependent 17β-hydroxysteroid dehydrogenase in the microsomes. Thus, GC analysis showed that 18% of the original 10β-OH recovered from its incubation with microsomes and NADPH was recovered as 10β,17β-dihydroxy-4-estren-3-one. This structural assignment was established by identical GC retention times and MS fragmentation patterns upon comparison with authentic standard prepared from 10β-OH by sodium borohydride reduction. Reduction of the 17-keto group of 10β-OH could not be detected in microsomal incubations from which NADPH had been omitted.

Since there was considerable non-NADPH-dependent conversion of 10β-OOH to 10β-OH in these incubations and since 10β-OOH decomposed under the conditions used for GC analysis, it was not possible to prove the direct action of the NADP(H)-dependent 17β-hydroxysteroid dehydrogenase on 10β-OOH. However, 10β-OH and 10β,17β-dihydroxy-4-estren-3-one were both detected by GC–MS from the microsomal incubations of 10β-OOH done in the presence of NADPH, whereas only 10β-OH was detected when NADPH was omitted.

Evidence That 10β-OOH Cannot Support the Aromatization of Androstenedione. Microsomes, 4-[4-^{14}C]androstene-3,17-dione and 10β-OOH (0.1–30 μM) were incubated at 37 °C for 1 h. Control incubations contained microsomes and 4-[4-^{14}C]androstene-3,17-dione with or without NADPH. The steroids were isolated by chloroform extraction, separated by thin-layer chromatography, and detected by autoradiography (see Experimental Procedures). The results are shown in Figure 11. The autoradiograph was heavily exposed so that even trace impurities (~0.05% determined by cutting and counting the chromatogram region of interest) present in the 4-[4-^{14}C]androstene-3,17-dione were detectable. Aromatization intermediates and products were detectable only in the control (lane 1) which contained microsomes and NADPH. All incubations that contained 10β-OOH (lanes 3–6) were

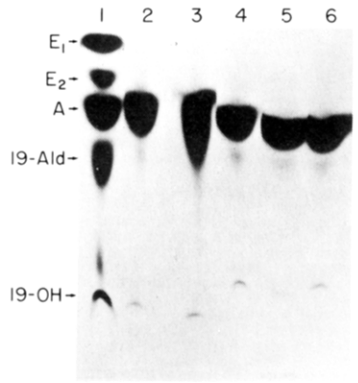

FIGURE 11: TLC analysis of 4-[4-^{14}C]androstene-3,17-dione aromatization. Microsomes were incubated with 4-[4-^{14}C]androstene-3,17-dione either in the presence (lane 1) or absence (lane 2) of NADPH or with 10β-OOH (30, 10, 1.0, and 0.1 μM, lanes 3–6, respectively) in the absence of NADPH. The steroids were isolated and chromatographed as described under Experimental Procedures. E_1, estrone; E_2, estradiol; A, androstenedione; 19-Ald, 3,17-dioxo-androst-4-en-19-al; 19-OH, 19-hydroxy-4-androstene-3,17-dione.

identical with the control which contained microsomes without added NADPH (lane 2) in that >99% of the total cpm present ran as 4-[4-^{14}C]androstene-3,17-dione. Under these conditions, 10β-OOH could not support the conversion of androstenedione to any aromatization intermediates or products.

Discussion

In the absence of added NADPH, 10β-OOH was found to be a potent active-site-directed inactivator of the microsomal aromatase from human placenta. Although the inactivation rates observed did not follow pseudo-first-order kinetics, probably due to the instability of 10β-OOH in the incubations, it was still possible to achieve ~95% inactivation with only 2.16 μM 10β-OOH. The protection afforded by androstenedione against this inactivation supports the involvement of the aromatase active site in the action of 10β-OOH. This is in contrast to H_2O_2 and CHP, compounds without structural resemblance to aromatase substrates or intermediates, which had to be used at 500–1000-fold higher concentrations to give comparable inactivation rates and whose action could not be blocked by androstenedione.

Since organic hydroperoxides have been shown to destroy the protoheme of P-450$_{LM}$ in incubations done in the absence of substrate or NADPH (O'Brien, 1978; White & Coon, 1980), an attempt was made to correlate the loss of aromatase activity by 10β-OOH, H_2O_2, and CHP with the destruction of P-450$_{AROM}$ protoheme. The results were inconclusive because P-450$_{AROM}$ protoheme is such a small fraction of the total microsomal protoheme that its specific destruction could not be detected. An upper limit on the percent of the microsomal protoheme associated with P-450$_{AROM}$ would be 10% since total P-450 protoheme only accounts for 10% of the total microsomal protoheme. Although it was not possible to correlate loss of aromatase activity with destruction of P-450$_{AROM}$ protoheme, the experiments performed did emphasize the greater nonspecific destructive effects of H_2O_2 and CHP relative to the specific effect of 10β-OOH.

Aromatase activity lost on exposure to any of the hydroperoxides used could not be readily reversed by pelleting the microsomes and resuspending them in fresh buffer, suggesting that some chemical modification had occurred. Since aromatase is known to be sensitive to sulfhydryl-modifying reagents (Bellino & Osawa, 1980), chemical modification of critical sulfhydryl groups could be expected to abolish aromatase activity. Indeed, oxidation of a sulfhydryl group(s) to a sulfenic and/or sulfonic acid could be caused by the hydroperoxides. The reactivation found with dithiothreitol would then be attributed to reduction of the sulfenic acid. Reactivation due to reduction of the sulfonic acid would not be expected (Little & O'Brien, 1969).

In the presence of added NADPH, 10β-OOH was no longer an inactivator of aromatase but instead competitively inhibited the aromatization of androstenedione. That this effect of NADPH was due to 10β-OOH metabolism by aromatase could not be inferred from the observation that NADPH was consumed, because microsomal 17β-hydroxysteroid dehydrogenase also utilized NADPH for the production of 10β,17β-dihydroxy-4-estren-3-one. Hence, a search for NADPH-dependent metabolites of 10β-OOH, generated by aromatase, was undertaken. Two possibilities were 10β-OOH aromatization to estrone or reduction to 10β-OH. We found using a GC–MS method that microsomes with added NADPH did not aromatize 10β-OOH. By use of an HPLC method, no enhanced reduction of 10β-OOH to 10β-OH in the presence of NADPH could be detected. Consequently, we were unable to establish that the loss of aromatase inactivation by 10β-OOH in the presence of NADPH resulted from 10β-OOH metabolism by aromatase to these nonreactive steroids.

Finally, aromatase could not use 10β-OOH to support the conversion of androstenedione to either 19-hydroxylated intermediates or estrogen products. Thus, the protection against 10β-OOH inactivation of aromatase by androstenedione is apparently due to competition for binding between the two steroids at the active-site and not to the ability of androstenedione to accept "activated" oxygen which has been transferred from 10β-OOH to the cytochrome.

Hrycay & O'Brien (1972) studied the metabolism of pregnene 17α-hydroperoxides by bovine adrenal microsomes, and Van Lier & Smith (1970) studied the metabolism of 20α-hydroperoxycholesterol by bovine adrenal mitochondria. Although neither group addressed the issue of enzyme inactivation by these hydroperoxides, both groups found that the hydroperoxides were enzymatically converted to other products in the absence of added NADPH, even when the incubations were done under N_2. The pregnene 17α-hydroperoxides were reduced to the corresponding 17α-alcohols and cleaved to androgens (C_{17}–C_{20} desmolase products), while 20α-hydroperoxycholesterol was converted to (22R)-20α,22-dihydroxycholesterol (the second intermediate in the cholesterol side-chain cleavage reaction). Unlike 10β-OOH, however, these hydroperoxides may simulate the substrate/oxygen complex actually present in the active site of the enzymes (i.e., the compounds act simultaneously as substrates and sources of "activated" oxygen and undergo conversion to either the final product or the next reaction intermediate). For the aromatase reaction, the analogous mimic of the substrate/oxygen complex would be 19-hydroperoxy-4-androstene-3,17-dione and not 10β-OOH. Perhaps the 19-hydroperoxide would be converted by aromatase to compound **3** (Figure 1), the next isolable aromatase intermediate. This is not possible for 10β-OOH because there is no 19-carbon atom attached to C-10. However, since 19-norsteroids are known to be aromatized (Ryan, 1959), it was possible that 10β-OOH was a mimic of a substrate/oxygen complex for 19-norsteroids, and it was for this reason that the aromatization of 10β-OOH was investigated. Since we did not detect estrogen formation, we conclude that this is not the case. Our finding is consistent with an earlier study (Townsley et al., 1966) in which it was shown that 10β-OH is not converted to estrone by aromatase.

Although it appears that 10β-OOH cannot simulate the substrate/oxygen complex involved in the aromatization of androstenedione or 19-norsteroids, there is another interesting possibility for this compound that our data do not address. By analogy to the 20α-hydroperoxycholesterol results mentioned above, some microsomal component could bind 10β-OOH and use the peroxide as a source of oxygen for oxygenation of 10β-OOH itself. A candidate for the product of this enzymatic autoxidation of 10β-OOH could be compound **1** of Figure 10. Studies are presently underway to determine (1) the identity of this compound, (2) if it arises from 10β-OOH by the pathway outlined above, and (3) if it has a role in the inactivation of aromatase by 10β-OOH.

These studies have shown that 10β-OOH is an excellent active-site-directed inhibitor of aromatase. The inactivation characteristics of 10β-OOH differ dramatically from those of H_2O_2 and CHP with regard to potency and protection by substrate or NADPH. The questions asked about NADPH oxidation and 10β-OOH reduction, but not definitively answered, should be answerable when highly purified P-450$_{AROM}$ preparations are available, and we believe that 10β-OOH can be a useful tool in future mechanistic studies. Since its irreversible effects are abolished in the presence of NADPH, 10β-OOH would not seem to have much utility as an in vivo inhibitor of estrogen biosynthesis. However, it is interesting to note that the 17β-ethynyl-substituted analogue of 10β-OOH has been prepared, evaluated in vivo, and found to have potent contraceptive properties (Jacob & Morris, 1969; Watnick et al., 1965). The 17α-methyl analogue of androstenedione is aromatized more slowly than androstenedione (Ryan, 1960; Braselton et al., 1974), possibly because the 17α-methyl substituent alters the alignment of the steroid in the aromatase active site. A similar shift in the alignment of the 17α-ethynyl analogue of 10β-OOH at the aromatase active site could eliminate the protective effect of NADPH and explain the potent in vivo effects of this 10β-OOH analogue. Since no reports of aromatase inhibition by this analogue have appeared, its evaluation as an inhibitor of this enzyme would seem to be warranted.

Acknowledgments

We thank Carol Brown and Dr. Oliver Lowry for the fluorometric measurements of NADPH concentration.

Supplementary Material Available

Anisotropic temperature parameters (Table I) and a table of calculated structure factors (Table II) (12 pages). Ordering information is given on any current masthead page.

Registry No. 10β-OOH, 2135-57-1; CHP, 80-15-9; H_2O_2, 7722-84-1; androstenedione, 63-05-8; aromatase, 9039-48-9.

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