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A Novel Nonhepatic Hydroxycholesterol 7α-Hydroxylase That Is Markedly Stimulated by Interleukin-1β

CHARACTERIZATION IN THE IMMATURE RAT OVARY*

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During studies on the regulation of rat ovarian steroidogenic enzymes by interleukin-1β (IL-1β), we observed substantial metabolism of 25-hydroxycholesterol to two unusual polar products. This unexpected effect was observed both in isolated granulosa cells and in whole ovarian dispersates and was also induced by tumor necrosis factor α, but not by insulin-like growth factor I or follicle-stimulating hormone. The effect was dependent on time and the dose of IL-1β and was blocked by an IL-1 receptor antagonist. The formation of the polar metabolites was inhibited by ketoconazole and trilostane, but not by aminoglutethimide. Subsequent purification of these novel metabolites and analysis by gas chromatography/mass spectrometry, NMR, and high performance liquid chromatography revealed them to be related 7α-hydroxylated hydroxycholesterols (cholest-4-ene-7α,25-diol-3-one and cholest-5-ene-3β,7α,25-triol). IL-1β-stimulated ovarian 7α-hydroxylase activity (3–10 pmol/min/mg of cellular protein) was nearly 4-fold that of control levels using 25-hydroxycholesterol as substrate. Activities at or below control levels were observed when IL-1β-treated cell sonicates were boiled or assayed in the presence of NADH (rather than NADPH), indicating that involvement of a nonenzymatic process was unlikely. IL-1β-stimulated 7α-hydroxylase activity was inhibited to basal levels by a 10-fold excess of unlabeled 25- or 27-hydroxycholesterol, but not by cholesterol, pregnenolone, progesterone, testosterone, or dehydroepiandrosterone, suggesting that ovarian 7α-hydroxylase is specific for hydroxycholesterols. Furthermore, when IL-1β-treated ovarian cultures were incubated with radiolabeled cholesterol or testosterone, no 7α-hydroxylated products were observed. We were also unable to detect any mRNA transcripts for liver cholesterol 7α-hydroxylase in IL-1β-stimulated ovarian cultures. This study describes an ovarian hydroxycholesterol 7α-hydroxylase that differs from liver cholesterol 7α-hydroxylase and from other nonhepatic progestin/androgen 7α-hydroxylases. The novel finding of the regulation of a 7α-hydroxylase by IL-1β (and tumor necrosis factor α) suggests a unique role for cytokines in the regulation of cholesterol metabolism in the ovary and possibly other tissues.

The 7α-hydroxylation of steroids has potentially widespread biological significance. At least three categories of steroid 7α-hydroxylases may be defined. By far the best understood is cholesterol 7α-hydroxylase (cholesterol 7α-monooxygenase, EC 1.14.13.17), a putative liver-specific (1) microsomal enzyme (2) and member of the cytochrome P450 gene family that catalyzes the rate-limiting step of bile acid biosynthesis (3). As such, cholesterol 7α-hydroxylase plays a critical dual role as the primary enzyme promoting both cholesterol catabolism and bile acid formation. A second category of 7α-hydroxylases includes several recently described nonhepatic enzymes that act on side chain-cleaved steroids. A dehydroepiandrosterone/pregnenolone 7α-hydroxylase has been reported in brain and other tissues (4, 5) and in adipose stromal cells (6, 7), and a testosterone 7α-hydroxylase in testis, lung, and kidney (8, 9). The functional significance of these 7α-hydroxylated steroids in nonhepatic tissues has not yet been established. The metabolites may regulate the availability of their biologically active precursors or could have biological activity themselves. For example, Morfin and Courchay (5) have suggested that 7α-hydroxylated metabolites of pregnenolone and dehydroepiandrosterone may regulate the immune response in mice.

The existence of a third class of steroid 7α-hydroxylases that preferentially metabolize hydroxycholesterols is controversial. It is well established that the liver is able to use hydroxycholesterols as well as cholesterol as substrates for bile acid biosynthesis (3, 10, 11). Recently, it has been suggested that this activity involves a hepatic hydroxycholesterol 7α-hydroxylase that is different from cholesterol 7α-hydroxylase (12–14). Using porcine liver microsomes, Toll *et al.* (14) were able to separate 25- and 27-hydroxycholesterol 7α-hydroxylase from cholesterol 7α-hydroxylase activity. Oxysterols such as 25- and 27-hydroxycholesterols (as distinct from cholesterol itself) act via a putative receptor (*cf.* Ref. 15) as potent regulators of cholesterol homeostasis, inhibiting 3-hydroxy-3-methylglutaryl-CoA re-

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ductase activity and LDL[1] receptor levels *in vitro* (16–18). Oxysterols also affect cell growth and viability presumably via their inhibitory effects on cholesterol availability (19, 20). Given the regulatory importance of hydroxysterols, their further metabolism to 7α-hydroxylated products is of considerable interest. Apart from serving as intermediates in bile acid synthesis, 7α-hydroxylated hydroxysterols may themselves regulate cholesterol homeostasis. Dueland *et al.* (21) have made the intriguing proposal that 7α-hydroxylase may indirectly induce the LDL receptor gene through inactivation of oxysterol inhibition.

Both 25- and 27-hydroxycholesterols circulate in the blood (22, 23), and sterol 27-hydroxylase[2] mRNA is widely distributed (25). Both activity and mRNA for 27-hydroxylase have been detected in human and rat ovary (26, 27). Rat luteal cells can metabolize 25-hydroxycholesterol (28). 27-Hydroxycholesterol has been shown to inhibit human ovarian cell sterol synthesis (27).

In this work, we describe a novel ovarian 7α-hydroxylase with apparent substrate specificity for 25- and 27-hydroxycholesterols, thus differentiating it from liver cholesterol 7α-hydroxylase and from nonhepatic 7α-hydroxylases, which catalyze C_{21} and C_{19} steroids. We further describe the dramatic enhancement of ovarian 7α-hydroxylase activity by interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), cytokines that have multiple biological effects in the ovary (29–37). The physiologic significance of cytokine-induced 7α-hydroxylated hydroxycholesterols is unknown at present. However, these studies suggest a possible role for cytokines in the regulation of cholesterol homeostasis in the ovary and perhaps other tissues.

MATERIALS AND METHODS

Reagents and Hormones—McCoy's 5A medium (modified, without serum) was obtained from Life Technologies, Inc. Ovine follicle-stimulating hormone (FSH) (NIH-FSH-17; biological potency equal to 20 units/mg) was the generous gift of the National Pituitary Agency, Pituitary Hormone Distribution Program, NIADDK, National Institutes of Health. NADH and NADPH were from Boehringer Mannheim. Ketoconazole and aminoglutethimide were from Sigma. Trilostane was from Sterling-Winthrop Research Institute (Rensselaer, NY).

2-Hydroxypropyl-β-cyclodextran (2-HPBCD) was obtained from Pharmatec, Inc. (Alachua, FL) and as a gift from George Reed (American Maize Products Co., Hammond, IN). Recombinant human IL-1β (2×10^7 units/mg) was generously provided by Drs. Errol B. DeSouza and C. E. Newton (DuPont Merck Pharmaceutical Co.) Recombinantly expressed, naturally occurring human IL-1 receptor antagonist (38) was generously provided by Dr. Daniel E. Tracey (The Upjohn Co.). Recombinant human TNF-α was generously provided by Dr. H. M. Shepard (Genentech, South San Francisco, CA). Recombinant human insulin-like growth factor I (IGF-I) was from Bachem California (Torrance, CA).

Authentic cholest-5-ene-3β,27-diol (27-hydroxycholesterol) and cholest-4-ene-7α,25-diol-3-one (7α,25-dihydroxycholestenone) were the generous gifts of Dr. Norman Javitt (New York University, New York). Cholest-5-ene-3β,25-diol (25-hydroxycholesterol) and other steroids were obtained from Steraloids, Inc. (Wilton, NH). 25-[26,27-^{3}H]Hydroxycholesterol (83 Ci/mmol), [1,2-^{3}H]cholesterol (51.7 Ci/mmol), [1β,2β-^{3}H]testosterone (42.5 Ci/mmol), and 25-[16,17-^{3}H]hydroxycholesterol (custom preparation) were obtained from DuPont NEN.

Animals and Cell Culture—Immature (25–28-day-old) Sprague-Dawley female rats, obtained from Zivic-Miller Laboratories, Inc. (Zelienopole, PA), were killed by CO_2 asphyxiation. Granulosa cells were obtained by follicular puncture as described previously (35). Whole ovarian dispersates were prepared as described previously (29). Cells were plated ($2.5–5 \times 10^5$ viable cells/ml) into 35 × 10-mm tissue culture dishes (for cellular radiolabeling studies) or into loosely capped 12 × 75-mm polystyrene tubes (for enzyme assays) containing 1 ml of serum-free McCoy's 5A medium with or without IL-1β (50 ng/ml) or other agents, as indicated. Cell cultures were maintained for 72 h at 37 °C under a water-saturated atmosphere of 95% air and 5% CO_2. At the conclusion of the incubation period, the media (and/or cells) were collected prior to further processing as described below.

Solubilization of Steroids—Since cholesterol and hydroxycholesterols are relatively insoluble in aqueous solution, these compounds (and the other steroids used) were solubilized using 2-HPBCD, an agent that provides stable effective solubilization of steroids (39). Stock solutions (5 mM) were prepared in 45% (w/v) 2-HPBCD (39), stored at 4 °C, and diluted for use as indicated. In some earlier studies, 25-hydroxycholesterol was dissolved in a 100% methanol stock and diluted into tissue culture medium containing 0.05% (w/v) 2-HPBCD. This method of solubilization gave identical results for studies using 25-hydroxycholesterol as a substrate, but was ineffective when cholesterol was the substrate.

Cellular Radiolabeling Studies—Following the 72-h preincubation and treatment period, cell culture media were discarded, and the cells were washed with fresh media (1 ml). Thereafter, fresh media (1 ml, 0.03–0.05% 2-HPBCD) containing 10,000–30,000 cpm 25-[^{3}H]hydroxycholesterol, [^{3}H]cholesterol, or other labeled substrates (0.2–10 μM, as noted) were added along with the same treatment protocol used during the 72-h preincubation period. After an additional 24 h, media were collected and subjected to HPLC as described below. Initial cellular radiolabeling studies on the polar metabolites of 25-hydroxycholesterol used 25-[16,17-^{3}H]hydroxycholesterol as substrate. Later, we determined that both 25-[16,17-^{3}H]hydroxycholesterol and 25-[26,27-^{3}H]hydroxycholesterol gave similar results (quantitatively and qualitatively) when used as substrates to examine 7α-hydroxylase activity. Subsequently, the latter compound was used whenever possible since supplies of the commercially unavailable 25-[16,17-^{3}H]hydroxycholesterol were limited. However, if cholesterol side chain cleavage activity is present (*e.g.* when cells are stimulated by gonadotropin), only 25-[16,17-^{3}H]hydroxycholesterol can be used as substrate since the labeled side chain is cleaved from 25-[26,27-^{3}H]hydroxycholesterol.

7α-Hydroxylase Assay—Following a 72-h incubation and treatment period, cell cultures were centrifuged at low speed for 2 min, and the media supernatants were removed. The cells were then washed (with centrifugation) by the addition of 1 ml of assay buffer, after which the supernatant was removed, and 0.4 ml of assay buffer was added. Cells were then sonicated (5 s, setting 5) using the microtip of a cell sonicator (Vibracell, Sonics & Materials, Inc., Danbury, CT). 7α-Hydroxylase activity was determined in the freshly prepared cell sonicates by the conversion of 25-[^{3}H]hydroxycholesterol to [^{3}H]cholest-5-ene-3β,7α,25-triol (7α,25-dihydroxycholesterol, X2). Assay conditions did not permit oxidation of the substrate 3β-hydroxy group to form cholest-4-ene-7α,25-diol-3-one (7α,25-dihydroxycholestenone, X1). The assay was performed as follows. A sufficient quantity of 25-[^{3}H]hydroxycholesterol for an entire assay was evaporated to dryness and reconstituted in an appropriate quantity of unlabeled 25-hydroxycholesterol dissolved in 0.4% 2-HPBCD. 20 μl of this substrate preparation (final assay concentration of 20,000–30,000 cpm, 0.2 μM substrate; 0.01–0.03% 2-HPBCD) was placed in each tube. (For competition studies, 10 μl of unlabeled substrate dissolved in 1.8% 2-HPBCD was also added.) Additionally, each assay tube (final volume of 1 ml) contained pH 7.4 assay buffer (50 mM potassium phosphate, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 M sucrose) and cell sonicate from 1×10^6 cells. NADPH (final concentration of 0.5 mM) was added to start the reaction, which proceeded at 37 °C for 3 h. The reaction was stopped by the addition of 1 N NaOH (100 μl). Substrate and product were separated and quantified by HPLC as described below. Enzyme activity is expressed as picomoles of product (7α,25-dihydroxycholesterol, X2) formed per hour/1×10^6 cells.

HPLC—Steroids present in tissue culture media or enzyme assay incubates were extracted twice in a 3-fold volume of ethyl acetate and evaporated to dryness. The residual steroids were redissolved in solvent and separated by HPLC, as described previously (40), on a 10-μm LiChrosorb Diol column (EM Reagents, Gibbstown, NJ) with the use of a Waters HPLC system.

Radioactivity was detected on line by a Flo-One/Beta detector (Packard Instruments), and absorbance at 240 nm was simultaneously monitored by a flow-through spectrophotometer (Lambda-Max, Waters). The column was calibrated with more than 35 steroids (40), including most of those expected to be found in the ovary. The column tends to separate steroids based on the number of their hydroxyl groups, with less polar steroids eluting first.

Purification of Novel 25-Hydroxycholesterol Metabolites (X1 and

[1] The abbreviations used are: LDL, low density lipoprotein; IL, interleukin; TNF-α, tumor necrosis factor α; FSH, follicle-stimulating hormone; 2-HPBCD, 2-hydroxypropyl-β-cyclodextran; IGF-I, insulin-like growth factor I; HPLC, high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; RPL 19, rat ribosomal protein L19; ANOVA, analysis of variance.

[2] The sterol 27-hydroxylase is elsewhere referred to as 26-hydroxylase. In this paper, we designate the enzyme as 27-hydroxylase, which identifies both 27-hydroxylase and 26-hydroxylase for reasons described previously (24). Similarly, herein, 27-hydroxysterol designates both 27-hydroxysterol and 26-hydroxysterol.

X2)—Whole ovarian dispersates were plated (1.5×10^6 cells/3 ml) in 60 × 50-mm culture dishes and incubated for 72 h in the presence of IL-1β (50 ng/ml). Media were then removed, and fresh media (3 ml) containing IL-1β (50 ng/ml), 25-hydroxycholesterol (10 μM), and 2-HPBCD (0.05%) were added. After 24 h, media steroids were extracted, pooled, and subjected to HPLC. Parallel cultures containing radiolabeled substrate were processed identically to monitor completion of the reaction and to delineate elution times of the metabolites. The effluent volumes containing the unknown metabolite peaks were collected and subjected to GC/MS or NMR as described below. Under these conditions, ~1 μg each of X1 and X2 could be obtained per 1.5×10^6 cells. ~1–2 or 100–200 μg of steroid was required for identification by GC/MS or NMR, respectively.

GC/MS—Steroids were derivatized to form trimethylsilyl and methyloxime-trimethylsilyl derivatives according to previously published methods (41). When excess reagents had been removed, the final solution was in cyclohexane (the solvent used for injection into the GC/MS instrument). GC/MS was carried out on a Hewlett-Packard MSD 5970 instrument, housing a 15-m DB1 nonpolar capillary column (J & W Scientific, Folsom, CA). The samples were introduced by syringe injection with cold trapping at 60 °C. After 3 min, the temperature was rapidly increased to the 220 °C starting temperature of the program. Thereafter, the temperature was increased linearly by 3 °C/min to the final temperature of 330 °C. Mass spectra were obtained by continuous repetitive scanning over the 100–700 mass range.

NMR—For both purified X1 and X2, the entire sample was dissolved in 0.5 ml of $CDCl_3$ (99.96% D; Goss Scientific Instruments Ltd., Ingatestone, Essex, United Kingdom). The solution was filtered using a small square of tissue over the tip of the pipette used to transfer the solution to the NMR tube (Wilmad 535). Spectra were run on a Bruker AMX2–600 spectrometer (University of London Intercollegiate Research Service at Queen Mary and Westfield College, London). Chemical shifts were measured with reference to residual $CHCl_3$ (7.26 ppm) at a temperature of 303 K. In addition to the ^1H spectrum, a double quantum filtered COSY spectrum (42) and an ω_1-decoupled COSY (43) were run to assist with identification and spectral assignment. Signal templates (44) were used to identify as many signals as possible in the ^1H spectrum. Two-dimensional methods (double quantum filtered COSY and ω_1-decoupled COSY) were then used to find the position of coupled signals that overlap in the one-dimensional spectrum. In this way, it was possible to unambiguously assign all of the ring protons and most of the side chain protons (cholestane side chain proton signals have yet to be fully determined; *cf.* Ref. 44).

Liquid Hybridization/RNase Protection Assay—The rat cholesterol 7α-hydroxylase cDNA (1) was a gift from Dr. David W. Russell (University of Texas Southwestern Medical Center, Dallas, TX). For the RNase protection assays, the plasmid containing the cDNA insert was linearized with *Ase*I and transcribed with T7 RNA polymerase (Promega) in the presence of [α-^{32}P]UTP to yield a 409-nucleotide riboprobe, which, upon hybridization, was projected to generate a 346-nucleotide protected fragment. For an internal standard to ensure comparable RNA loadings, a probe for constitutively expressed rat ribosomal protein L19 (RPL19) (45) was generated by reverse transcription of 1 μg of RNA followed by amplification via the polymerase chain reaction. The polymerase chain reaction product was then cloned into the pCR1000 vector (Invitrogen, San Diego, CA), verified by DNA sequencing, digested with *Fok*I, and transcribed with T7 RNA polymerase to generate a 214-nucleotide probe capable of protecting a 153-nucleotide segment. After transcription, the riboprobes were gel-purified. The size differences of the protected fragments generated from the riboprobes for 7α-hydroxylase and RPL19 allow the simultaneous detection of these transcripts on the same gel. RNA of cultured cells or tissue was extracted with RNazol-B (Tel Test, Friendswood, TX) according to the manufacturer's protocol. Liquid hybridization/RNase protection assays were carried out as described previously (46).

Data Analysis—Data are presented as the mean ± S.E. of replicate experiments (n noted in legends), each performed in duplicate. Statistical significance ($p < 0.05$) by ANOVA analysis (Fisher's protected least significant difference) or by two-tailed unpaired t test (for -fold changes) was calculated using Statview 512+ for MacIntosh (Brain Power, Inc., Calabasas, CA).

RESULTS

IL-1β-stimulated Metabolism of 25-Hydroxycholesterol to Novel Polar Metabolites in the Rat Ovary—During studies on the IL-1β-mediated regulation of cytochrome $P450_{CSCC}$ in cultures of isolated rat granulosa cells, we observed that the addition of IL-1β to the cultures promoted a remarkable metabolism of the 25-hydroxycholesterol substrate to a major and a minor polar metabolite. As shown in Fig. 1, granulosa cells were initially cultured for 72 h in the absence or presence of IL-1β (50 ng/ml), with and without a dose of FSH that maximally stimulates cytochrome $P450_{CSCC}$ activity (2 milliunits/ml). Thereafter, cells were washed and incubated with 25-[^{3}H]hydroxycholesterol (0.2 μM) for an additional 24 h. FSH-pretreated cells metabolized the substrate to the cytochrome $P450_{CSCC}$ products, pregnenolone, progesterone, and 20α-dihydroprogesterone, as expected. However, the addition of IL-1β induced the nearly complete loss of substrate, coincident with the appearance of a major (X1) and a minor (X2) metabolite at 15.7 and 20.4 min, respectively, as detected by HPLC. This result was highly reproducible. IL-1β promoted a 4.1 ± 0.5-fold increase ($p < 0.005$) in X1 plus X2 above control levels (n = six separate experiments). The effect was dependent on the dose of IL-1β (Fig. 1, *inset*), with a maximum production of X1 and X2 occurring at 25–50 ng/ml IL-1β. We have previously described a similar dose range for other IL-1β actions in the ovary (29–33).

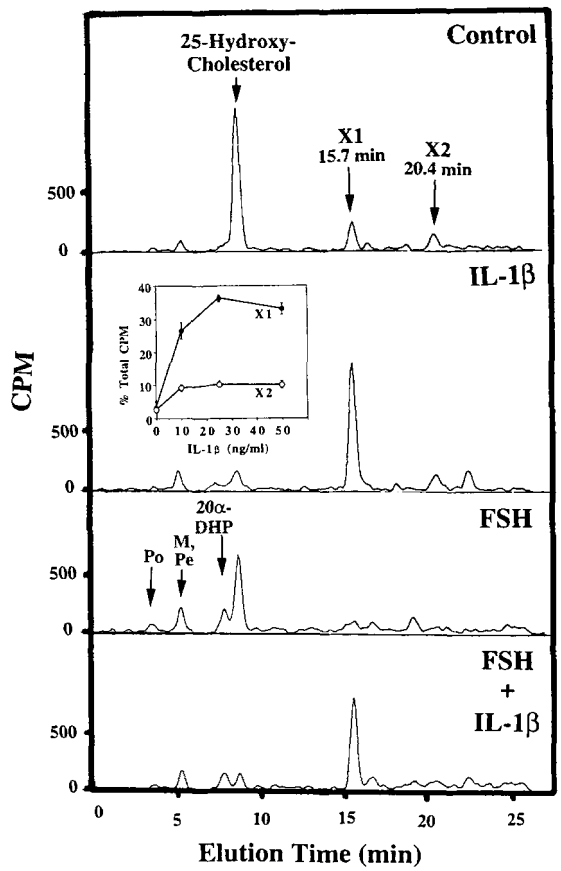

FIG. 1. **Polar hydroxycholesterol metabolites in granulosa cells: stimulation by IL-1β.** Granulosa cells (5×10^5 cells/ml) were initially cultured for 72 h in the absence or presence of IL-1β (50 ng/ml), with and without FSH (2 milliunits/ml). Cells were then washed, and 25-[^{3}H]hydroxycholesterol (0.2 μM) plus the same treatment protocols were added for an additional 24 h. Media steroids were extracted and analyzed by HPLC as described under "Materials and Methods." Standards are indicated by *arrows* as follows: *Po*, progesterone; *Pe*, pregnenolone; *M*, 25-hydroxycholestenone; *20α-DHP*, 20α-dihydroprogesterone. X1 and X2 are polar metabolites eluting at 15.7 and 20.4 min, respectively. Chromatograms from the same representative experiment are shown. The *inset* in the *second panel* shows the IL-1β dose-dependent formation of X1 and X2 (mean ± S.E., n = four values from two separate experiments).

The IL-1β-induced metabolism of 25-hydroxycholesterol to polar metabolites was also apparent in comparable experiments using whole ovarian dispersates (Fig. 2). Significant quantities of X1 accumulated in the absence of exogenous IL-1β

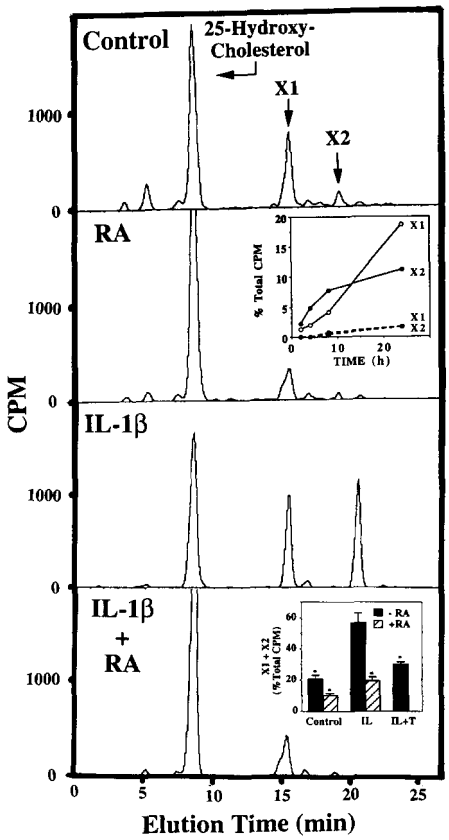

FIG. 2. **IL-1β-stimulated polar hydroxycholesterol metabolites in whole ovarian dispersates: inhibition by receptor antagonist.** Whole ovarian dispersates (2.5×10^5 cells/ml) were initially cultured for 72 h in the absence or presence of IL-1β (50 ng/ml), IL-1 receptor antagonist (*RA*; 5 μg/ml), or trilostane (3×10^{-5} M). Cells were then washed, and 25-[^{3}H]hydroxycholesterol (10 μM) plus the same treatment protocols were added for an additional 24 h. Media steroids were extracted and analyzed by HPLC. Steroid elution times are indicated by *arrows* as described for Fig. 1. Chromatograms from the same representative experiment are shown. The *inset* in the *second panel* shows the time-dependent formation of X1 and X2 in control (*broken lines*) and IL-1β-treated (*solid lines*; mean ± difference, n = two values from one experiment) cells. The *inset* in the *bottom panel* shows the accumulation of X1 plus X2 in control, IL-1β-treated, and IL-1β + trilostane (*IL+T*)-treated cultures in the absence (*solid bars*) and presence (*hatched bars*) of receptor antagonist. Results are the mean ± S.E. or difference of two to five separate experiments. Significance by ANOVA analysis, relative to IL-1β in the absence of receptor antagonist, is indicated (*).

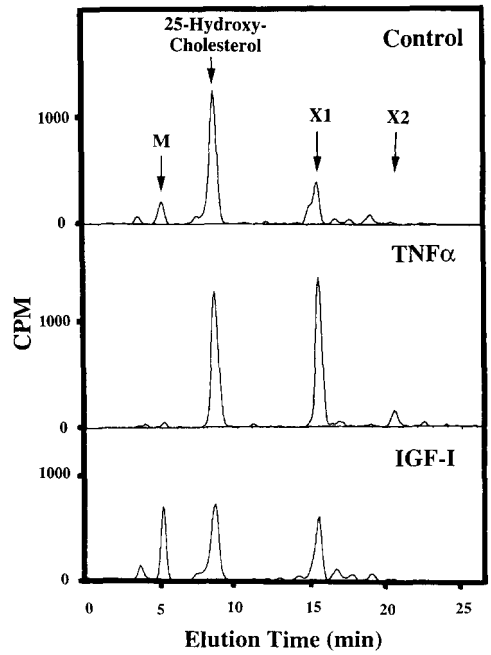

FIG. 3. **Polar hydroxycholesterol metabolites in whole ovarian dispersates: peptide specificity.** Whole ovarian dispersates were cultured and analyzed as described for Fig. 2, except in the absence or presence of TNF-α (10 ng/ml) or IGF-I (50 ng/ml). The doses used were maximally effective in modulating ovarian steroidogenesis (not shown). Results were replicated in an additional experiment. In three other similar experiments (not shown) using granulosa cells rather than whole ovarian dispersates, TNF-α stimulated accumulation of X1 and X2. *M*, 25-hydroxycholestenone.

(Fig. 2, *top panel*). The formation of X1 and X2 by control cells was reduced (Fig. 2, *second panel*) by the concomitant application of a naturally occurring IL-1 receptor antagonist (38), suggesting that the formation of the polar metabolites in control cells could be related to endogenous production of IL-1β. We have observed similar evidence of receptor-mediated, endogenous IL-1β activity for other end points of IL-1β activity in whole ovarian dispersates (*e.g.* nitrite and prostaglandin production; *cf.* Ref. 33). The accumulation of both X1 and X2 was markedly increased above control levels (2.9 ± 0.5-fold, $p < 0.05$, $n = 4$) by the addition of IL-1β (Fig. 2, *third panel*). IL-1β-induced formation of the X2 metabolite was consistently more prominent in whole ovarian dispersates compared with granulosa cells. The accumulation of X1 and X2 was time-dependent in both whole ovarian dispersates (Fig. 2, *second panel, inset*) and granulosa cells (not shown). The addition of receptor antagonist to cells that were exogenously stimulated by IL-1β dramatically inhibited the formation of the polar products (Fig. 2, *bottom panel*), suggesting that the hydroxycholesterol metabolizing action of IL-1β is mediated via its receptor. Data from replicate experiments are summarized in Fig. 2 (*bottom panel, inset*), demonstrating that the addition of receptor antagonist promotes a 51% ($p < 0.05$) and a 66% ($p < 0.005$) reduction in the formation of X1 and X2 by control and IL-1β-stimulated cells, respectively.

The formation of X1 and X2 by IL-1β was also inhibited (47%, $p < 0.05$) when ovarian cells were cultured in the presence of trilostane (Fig. 2, *bottom panel, inset*), an agent that reportedly inhibits 3β-hydroxysteroid dehydrogenase/isomerase activity (47). Furthermore, a $UV_{240\,nm}$ peak (characteristic of Δ^4-3-oxosteroids) was always coincident with formation of the radiolabeled X1 metabolite in both granulosa cells and whole ovarian dispersates. Taken together, these data suggest that the formation of X1 involves, in part, oxidation of the C-3 moiety of 25-hydroxycholesterol by the abundant 3β-hydroxysteroid dehydrogenase/isomerase activity present in these ovarian cells. A 3-oxo moiety on X1 was later confirmed as described below (*cf.* Figs. 5 and 7). However, inhibition of X2 formation by trilostane is not explained by trilostane inhibition of 3β-hydroxysteroid dehydrogenase. Trilostane inhibition of both X1 and X2 is suggestive of a more generalized inhibition, perhaps of the 7α-hydroxylase activity we report herein.

Characterization of Polar Hydroxycholesterol Metabolites in the Rat Ovary—To determine whether the formation of X1 and X2 is specifically induced by IL-1β, whole ovarian dispersates were cultured in the absence or presence of other known ovarian paracrine/autocrine factors. Like IL-1β, TNF-α is a cytokine that we have shown to regulate ovarian steroidogenesis (34–37). We have also reported that IGF-I modulates ovarian steroidogenesis (48). As shown in Fig. 3, TNF-α acted like IL-1β in promoting the formation of X1 from 25-hydroxycholesterol in whole ovarian dispersates. Similar results were obtained with granulosa cell cultures (not shown). In contrast, IGF-I, like FSH (Fig. 1), did not induce the formation of these polar metabolites above control levels. IGF-I was able to enhance the

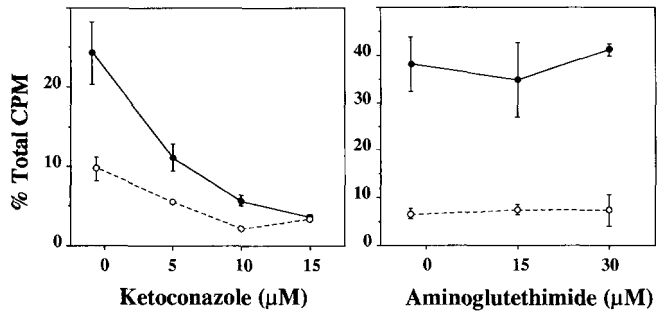

FIG. 4. **Formation of polar hydroxycholesterol metabolites: effect of P450 inhibitors.** Granulosa cells (5×10^5 cells/ml) were initially cultured for 72 h in the presence of IL-1β (50 ng/ml), with and without ketoconazole (5–15 μM) or aminoglutethimide (15 and 30 μM). Cells were then washed, and 25-[^{3}H]hydroxycholesterol (0.2 μM) plus the same treatment protocols were added for an additional 24 h. Accumulation of X1 (*solid lines*) and X2 (*broken lines*) was determined by HPLC analysis. Data are the mean ± S.E. or difference (n = two to five separate experiments).

formation of a metabolite that coelutes with 25-hydroxycholestenone (Fig. 3, *M*).

To determine whether the ovarian metabolism of 25-hydroxycholesterol is sensitive to P450 enzyme inhibitors, granulosa cells were cultured in the absence or presence of IL-1β with and without ketoconazole (5–15 μM), a well-known inhibitor of cholesterol hydroxylases and other P450 enzymes (17, 49–51), or with aminoglutethimide (15–30 μM), a potent inhibitor of the $P450_{CSCC}$ enzyme (52, 53). As shown in Fig. 4, IL-1β-dependent formation of X1 and X2 was inhibited by ketoconazole in a dose-dependent manner, with nearly complete inhibition at the 15 μM dose. Simultaneous assessment of cell viability by tetrazolium dye reduction (54) indicated that ketoconazole was not toxic to these cells at the doses used (not shown). In contrast to the inhibitory action of ketoconazole, aminoglutethimide had no effect on the formation of X1 and X2. In a parallel experiment (not shown), FSH-dependent $P450_{CSCC}$ activity was inhibited to control levels by aminoglutethimide (15 μM), as expected. These data demonstrate that the ovarian enzyme that catalyzes the formation of X1 and X2 is ketoconazole-sensitive and aminoglutethimide-insensitive.

Identification of IL-1β-stimulated Polar Metabolites by GC/MS and HPLC—X1 and X2 were isolated from ovarian cultures as described under "Materials and Methods," and their methyloxime and/or trimethylsilyl derivatives were analyzed by GC/MS as shown in Fig. 5 (*top panel*) and Fig. 6 (*top section* of comparison spectrum), respectively. These data indicate that X1 and X2 are noncleaved metabolites of 25-hydroxycholesterol since both compounds demonstrated a prominent m/z ion at 131, which identifies the 25-hydroxylated side chain (55).

The spectrum for the methyloxime-trimethylsilyl derivative of X1 (Fig. 5, *top panel*) was characterized by a molecular ion at m/z 589 and ions formed by loss of the oxime (m/z 558 (M − 31)) and trimethylsilyl (m/z 468 (M − 31 − 90); m/z 378 (M − 31 − 90 − 90)) groups, among others. These data indicate a cholestenediolone structure with an underivatized molecular mass of 416 Da.

The spectrum for the trimethylsilyl derivative of X2 (Fig. 6, *top section* of comparison spectrum) demonstrated it to be a cholestenetriol with a derivatized molecular mass of 634 Da to include three trimethylsilyl groups. The underivatized molecular mass would be 418 Da. The most prominent ion in the spectrum was at m/z 544 (M − 90), although M − 90 − 90 and M − 90 − 90 − 90 ions were also present. The high abundance of the M − 90 ion immediately gave a strong indication of the presence of a 7α-hydroxy moiety since virtually all steroid classes (androstanes, pregnanes, cholestanes) give base peaks

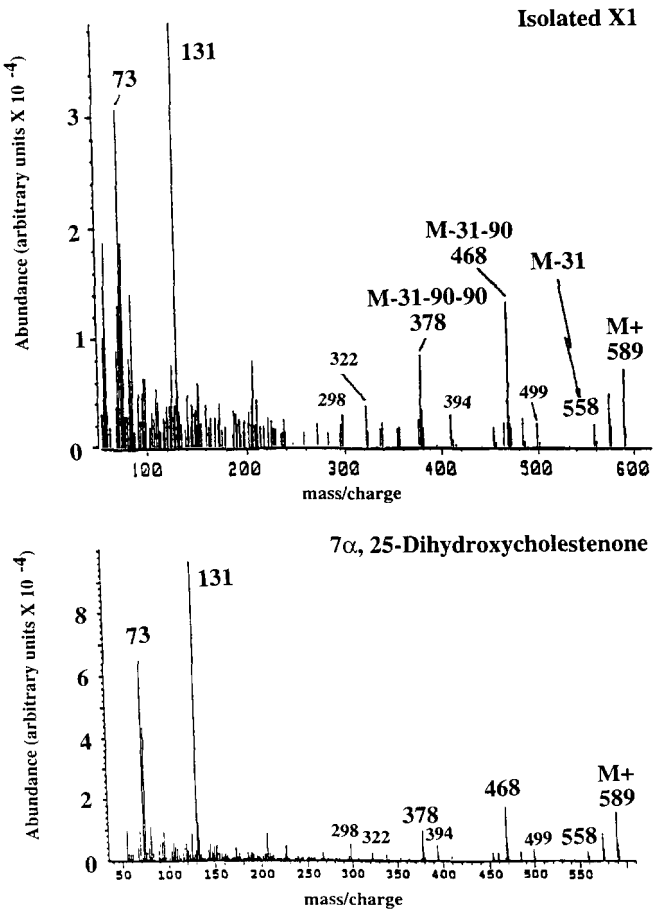

FIG. 5. **Identification of X1 by GC/MS.** X1 was purified from ovarian cultures and analyzed by GC/MS. Representative mass spectra for the methyloxime-trimethylsilyl derivatives of isolated X1 (*top panel*) and authentic cholest-4-ene-7α,25-diol-3-one (7α,25-dihydroxycholestenone) (*bottom panel*) are shown. The molecular ion (M^+) is indicated at m/z 589, and removal of methyloxime and trimethylsilyl groups is noted by 31- and 90-mass unit losses, respectively. The m/z 131 ion is composed of the terminal three side chain carbons with the derivatized C_{25} hydroxyl group.

at M − 90 for trimethylsilyl derivatives of 7α-hydroxy compounds. Furthermore, comparison with standards available at the time of analysis indicated that the additional hydroxyl group on X2 was not at C-23, C-24, or C-26.

Sodium borohydride reduction of X1 gave two compounds following GC with similar spectra to X2, but slightly shorter and longer retention times, respectively. These almost certainly represented reduction of the carbonyl group to α- and β-hydrogens. The comparison spectra for reduced X1 (Fig. 6, *bottom section*, inverted) and X2 (*top section*) show that the only difference is the presence of an ion at m/z 196 in the spectrum for reduced X1. This ion is very distinctive for trimethylsilyl derivatives of steroids with 3,7α-dihydroxy-4-ene structures (56). The presence of a 4-ene group in reduced X1 strongly indicated that the moiety prior to reduction was a 3-carbonyl. The lack of the m/z 196 ion in X2 with an otherwise identical spectrum to reduced X1 indicated that X2 had a 5-ene group.

Subsequent analysis by NMR (see below) definitively identified X1 as cholest-4-ene-7α,25-diol-3-one (7α,25-dihydroxycholestenone). As shown, isolated X1 has an identical spectrum to that of authentic 7α,25-dihydroxycholestenone (Fig. 5, *bottom panel*). Furthermore, the X1 metabolite of 25-[^{3}H]hydroxycholesterol from IL-1β-stimulated whole ovarian dispersates (Fig. 7, *bottom panel*, *broken line*) coeluted with authentic 7α,25-dihydroxycholestenone (*top panel*) in our HPLC system. Note

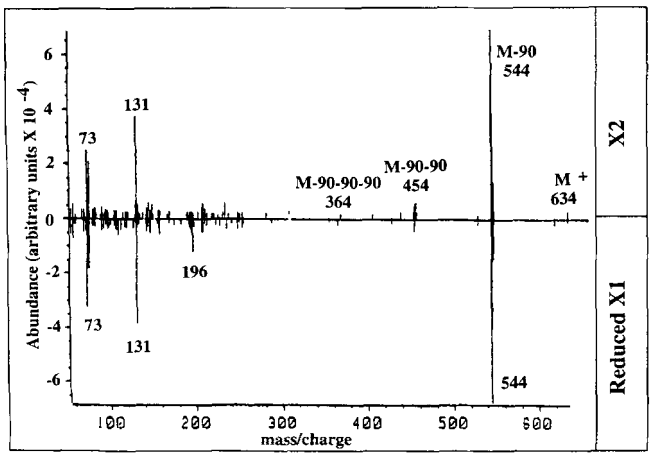

FIG. 6. **Identification of X2 by GC/MS: comparison with reduced X1.** X2 was purified from ovarian cultures and analyzed by GC/MS. The comparison spectrum for the trimethylsilyl derivatives of X2 (*top section*) and $NaBH_4$-reduced X1 (*bottom section*, inverted) is shown. The molecular ion (M^+) is at m/z 634, and the base peak is at m/z 544. The representative spectrum of X2 (*top section*) is identical to that of authentic cholest-5-ene-3β,7α,25triol (7α,25-dihydroxycholesterol; *cf.* Ref. 14). Removal of each trimethylsilyl group is noted by a 90-mass unit loss.

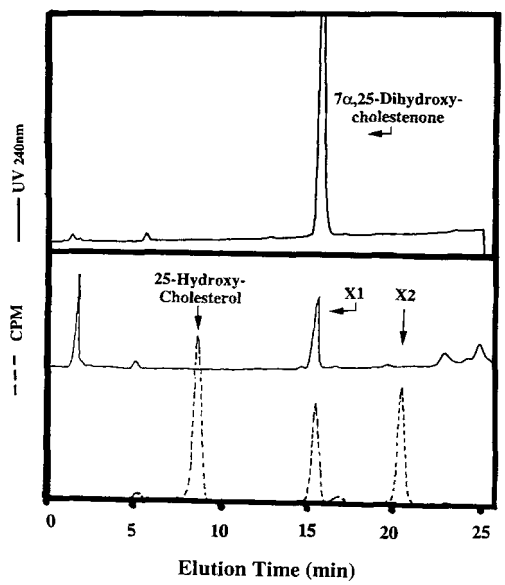

FIG. 7. **Coelution of X1 and authentic 7α,25-dihydroxycholestenone on HPLC.** Authentic 7α,25-dihydroxycholestenone (10 nmol) (*top panel*) and the 25-[^{3}H]hydroxycholesterol metabolites (*bottom panel*) from IL-1β-stimulated whole ovarian dispersates (prepared as described for Fig. 2) were analyzed by HPLC. Elution was simultaneously monitored by absorbance at 240 nm (*solid lines*) and on-line scintillation counting (*broken line*).

that only X1 and 7α,25-dihydroxycholestenone show the $UV_{240\,nm}$ absorbance (*solid lines*) that is typical of Δ^4-3-oxosteroids. NMR analysis (see below) identified both X1 and X2 as specifically 7α (rather than 7β or 7-keto) metabolites. The X2 metabolite was identified by NMR as cholest-5-ene-3β,7α,25-triol (7α,25-dihydroxycholesterol). Isolated X2 has a spectrum (Fig. 6, *top section*) identical to that reported for authentic 7α,25-dihydroxycholesterol (*cf.* Ref. 14). Taken together, these data suggest that X2 is a 7α-hydroxy metabolite of 25-hydroxycholesterol that is additionally oxidized at C-3 to form X1.

Identification of IL-1β-stimulated Novel Ovarian Metabolites as 7α-Hydroxylated Hydroxycholesterols by NMR—To unequivocally determine the structure of X1 and X2, the purified metabolites were analyzed by NMR. On the basis of the spectral

TABLE I
NMR characteristics of X1

Signal	Description
1. 5.81 ppm	A doublet due to 4-H with a splitting of ~2 Hz; typical of a 4-en-3-one structure
2. 3.97 ppm	A broad quartet due to the 7β-proton
3. 2.62 ppm	A quartet of doublets due to the 6β-proton
4. 1.21 ppm	A singlet due to 26/27-H_3
5. 1.19 ppm	A singlet due to 19-H_3. The chemical shift is typical of 19-H_3 in a steroidal 4-en-3-one type structure
6. 0.93 ppm	A doublet due to 21-H_3
7. 0.72 ppm	A singlet due to 18-H_3

assignment described under "Materials and Methods," X1 was identified as cholest-4-ene-7α,25-diol-3-one. This was confirmed by comparison with reference spectra of pregn-4-en-7α-ol-3,20-dione (7α-hydroxyprogesterone) and cholest-5-ene-3β,25-diol (25-hydroxycholesterol). The characteristic signals that are of particular significance are indicated in Table I. Signals 1–3, 5, and 7 corresponded almost exactly to signals in the spectrum of 7α-hydroxyprogesterone (44). Assignment of the remaining ring protons (which overlap in the ^{1}H spectrum) also corresponded closely with 7α-hydroxyprogesterone, with the exception of the D-ring protons, which are affected by the different 17β-side chain. Signals 4 and 6 corresponded closely to signals in the spectrum of 25-hydroxycholesterol, confirming the same side chain structure.

The X2 metabolite was identified by NMR as cholest-5-ene-3β,7α,25-triol. At the time of analysis, a suitable reference compound (*i.e.* one containing the 5-ene-3β,7α-dihydroxy structure) was not available. Assignment was further complicated by the presence of an impurity (~60%), at a similar concentration to the steroid, which was probably some type of carbohydrate. The characteristic signals for this compound are listed in Table II. As with X1, it was possible to assign chemical shifts to all of the ring protons.

The identification of X1 and X2 as specifically 7α-hydroxy is confirmed by the characteristic multiplet pattern and chemical shift (*cf.* Ref. 44) of the 7β-proton in the NMR spectrum. A 7α-proton in a 7β-hydroxy compound has a different pattern and shift. A 7-keto compound would show no 7-proton signal. That the relevant signal in each spectrum is a 7-proton is confirmed by the cross-peaks present in the two-dimensional COSY spectrum.

Characterization of IL-1β-stimulated Ovarian 7α-Hydroxylase Activity—To further examine ovarian 7α-hydroxylase activity, we designed a cell-free enzyme assay that was linear with time (up to 5 h of incubation) and cell number ($0.2–1 \times 10^6$ cells). As shown in Fig. 8, whole ovarian dispersates were initially cultured for 72 h in the absence (*solid bar*) or presence (*hatched bars*) of IL-1β and assayed for 7α-hydroxylase activity using 25-[^{3}H]hydroxycholesterol as substrate. IL-1β promoted a significant ($p < 0.05$) 3.7-fold increase in NADPH-dependent activity above control levels (16.6 ± 3.2 *versus* 4.5 ± 1.4 pmol/h/10^6 cells, respectively; $n = 5$). IL-1β-stimulated ovarian 7α-hydroxylase activity, expressed as the range of activity observed per milligram of cellular protein (3–10 pmol/min/mg), is comparable to activity reported for testosterone 7α-hydroxylase in mature rat testis (~6 pmol/h/mg) (9), cholesterol 7α-hydroxylase in rat liver and hepatocytes (~30 pmol/min/mg) (57), and 25-hydroxycholesterol 7α-hydroxylase in liver microsomes (39–106 pmol/min/mg) (14). Preliminary studies (Fig. 8, *inset*) provided a Michaelis constant (K_m) for IL-1β-stimulated 7α-hydroxylase (0.2 and 0.9 μM in two separate experiments). Activities at or below control levels were observed when IL-1β-treated cell sonicates were boiled or assayed in the presence of NADH, rather than NADPH. This latter finding indicates that the hydroxylase is NADPH-specific, as is typical of P450 en-

TABLE II
NMR characteristics of X2

Signal	Description
1. 5.50 ppm	A doublet of doublets due to 6-H. The signal is simplified relative to the 6-H signal in 25-hydroxycholesterol, due to the loss of coupling to 7α-H.
2. 3.85 ppm	A broad triplet due to 7β-H. Again, the signal is simplified relative to the 7β-H signal in 7α-hydroxyprogesterone, due to the loss of coupling to 7α-H.
3. 3.58 ppm	The multiplet due to 3α-H has virtually the same appearance in 25-hydroxycholesterol.
4. Signals due to 26/27-H_6, 19-H_3, and 18-H_3 are at almost exactly the same chemical shifts as 25-hydroxycholesterol.	

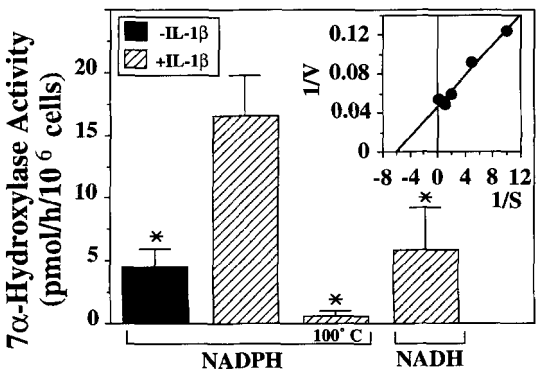

FIG. 8. **IL-1β-stimulated 7α-hydroxylase activity in whole ovarian dispersates.** Whole ovarian dispersates (5×10^5 cells/ml) were cultured for 72 h in the absence (*solid bar*) or presence (*hatched bars*) of IL-1β (50 ng/ml). Cells were then sonicated and assayed for 7α-hydroxylase activity using 25-[^{3}H]hydroxycholesterol (0.2 μM) as substrate. Heat-treated sonicates (100 °C, 10 min) or NADH (0.5 mM)-substituted reactions (rather than NADPH) monitored basal assay activity as indicated (mean ± S.E., n = three to five separate experiments). Significance by ANOVA analysis, relative to maximal activity (IL-1β, standard conditions), is indicated (*). The *inset* is a representative experiment showing a double reciprocal plot of activity (V; picomoles/hour/10^6 cells) for IL-1β-treated cells in the presence of different concentrations of 25-hydroxycholesterol (S; micromolar).

zymes. Importantly, the demonstration of coenzyme specificity and the lack of activity with boiled enzyme preparations illustrates that the 7α-hydroxylation of 25-hydroxycholesterol is not due to autoxidation (58). Furthermore, when 25-[^{3}H]hydroxycholesterol was incubated without cells under conditions identical to those described for Fig. 1, there was no degradation of the substrate to X1 or X2 (not shown). Together, these data demonstrate that the 7α-hydroxylation of 25-hydroxycholesterol in the ovary is enzyme-dependent.

Ovarian 7α-Hydroxylase Is Specific for Hydroxycholesterols—To determine the substrate specificity of ovarian 7α-hydroxylase, we assayed IL-1β-stimulated activity with 25-[^{3}H]hydroxycholesterol (0.2 μM) as substrate in the presence of a 10-fold excess of unlabeled steroids that are known to be substrates for 7α-hydroxylases in other tissues. As shown in Fig. 9, IL-1β-dependent 7α-hydroxylase activity was maximally reduced to below control levels (controls not shown) in the presence of unlabeled 25-hydroxycholesterol (2 μM), as expected. Of particular interest was the complete competition of 7α-hydroxylase activity by 27-hydroxycholesterol and the absence of competition by cholesterol. Thus, ovarian 7α-hydroxylase resembles the newly described liver hydroxycholesterol 7α-hydroxylase (12–14), which is distinct from microsomal cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid biosynthesis (1, 3). Furthermore, none of the C_{21} (pregnenolone, progesterone) or C_{19} (testosterone, dehydroepiandrosterone) steroids, which are 7α-hydroxylated in other nonhepatic tissues (4–9), inhibited ovarian 7α-hydroxylase activity.

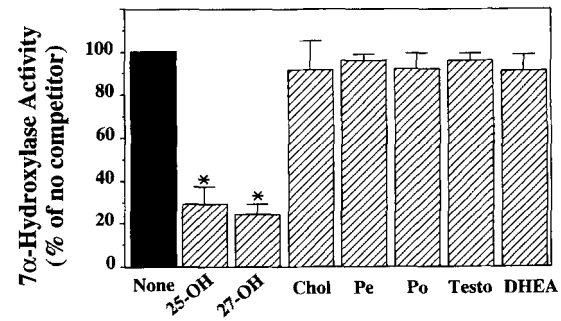

FIG. 9. **IL-1β-stimulated 7α-hydroxylase activity in whole ovarian dispersates: substrate specificity.** Whole ovarian dispersates were cultured in the presence of IL-1β and assayed for 7α-hydroxylase activity as described for Fig. 8. Activity was measured in the absence (*solid bar*) or presence (*hatched bars*) of a 10-fold excess of unlabeled steroids (2 μM), which were potential competitors of the activity determined using 25-[^{3}H]hydroxycholesterol as substrate. Data are normalized relative to activity in the absence of excess steroid (*solid bar*, 100%), and significance by ANOVA analysis relative to this value is indicated (*). *25-OH*, 25-hydroxycholesterol; *27-OH*, 27-hydroxycholesterol; *Chol*, cholesterol; *Pe*, pregnenolone; *Po*, progesterone; *Testo*, testosterone; *DHEA*, dehydroepiandrosterone. Data are the mean ± S.E. or difference from two to three separate experiments, each in duplicate.

To more directly examine the specificity of ovarian 7α-hydroxylase, whole ovarian dispersates were initially cultured for 72 h in the absence or presence of IL-1β, after which cells were labeled with [^{3}H]cholesterol or [^{3}H]testosterone (Fig. 10). No polar metabolites of cholesterol (indicative of 7α-hydroxylase activity) were observed in either the absence (*broken lines*) or presence (*solid lines*) of IL-1β, the chromatograms being qualitatively similar. An increased accumulation of an unidentified, less polar metabolite was consistently observed in IL-1β-treated cells. Importantly, in parallel experiments using FSH-pretreated cultures, we observed substantial metabolism of [^{3}H]cholesterol to $P450_{CSCC}$ products (not shown), demonstrating that solubility/cellular availability of the added cholesterol was not a problem.

As shown in Fig. 10 (*bottom panel*), a variety of [^{3}H]testosterone metabolites were observed in both control (*broken line*) and IL-1β-treated (*solid line*) cells. However, no metabolism to 7α-hydroxytestosterone was apparent. These data directly confirm the conclusion of the enzyme competition assays (Fig. 9), that ovarian 7α-hydroxylase is specific for hydroxycholesterols.

To begin to delineate the substrate specificity of ovarian 7α-hydroxylase at the molecular level, total RNAs from immature female rat liver and from cultured whole ovarian dispersates were probed with rat liver cholesterol 7α-hydroxylase [^{32}P]UTP-labeled riboprobe using a highly sensitive and specific liquid hybridization/RNase protection assay (Fig. 11). A strong signal (protected fragment) of the appropriate size (346 nucleotides) was apparent for the rat liver positive control. In contrast, no cholesterol 7α-hydroxylase mRNA transcripts were apparent for either control or IL-1β-treated ovarian cells in spite of an obvious protected fragment for the normalizing probe (RPL19, 153 nucleotides) in both these lanes. (The band seen in the control ovary lane is undigested RPL19.) Given the sensitivity inherent in the liquid hybridization/RNase protection assay and since we were able to detect mRNA transcripts for ovarian 5α-reductase (not shown; an enzyme of comparable abundance in this system), it seems likely that immature rat ovaries lack liver cholesterol 7α-hydroxylase transcripts.

DISCUSSION

Herein, we describe a novel, cytokine-regulated, nonhepatic hydroxycholesterol 7α-hydroxylase. Activity was initially observed in cultures of rat granulosa cells or whole ovarian dispersates as the IL-1β-dependent, receptor-mediated accumula-

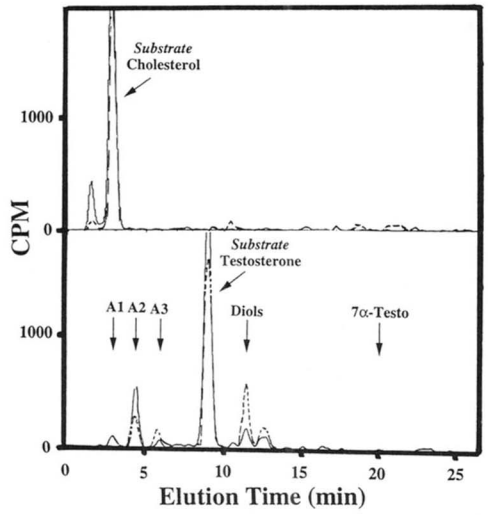

FIG. 10. **IL-1β does not promote ovarian 7α-hydroxylation of cholesterol or testosterone.** Whole ovarian dispersates (2.5×10^5 cells/ml) were initially cultured for 72 h in the absence (*broken lines*) or presence (*solid lines*) of IL-1β (50 ng/ml), after which cells were washed, and either [^{3}H]cholesterol (3 μM; *upper panel*) or [^{3}H]testosterone (3 μM; *lower panel*) was added for an additional 24 h. Media steroids were extracted and analyzed by HPLC. Elution times of standards are indicated by *arrows* as follows: *A1*, androstanedione; *A2*, androstenedione; *A3*, androsterone and dihydrotestosterone; *DHEA*, epiandrosterone; *Diols*, androstenediol, 3β-androstanediol, and 3α-androstanediol; *7α-Testo*, 7α-hydroxytestosterone. Representative chromatograms are shown.

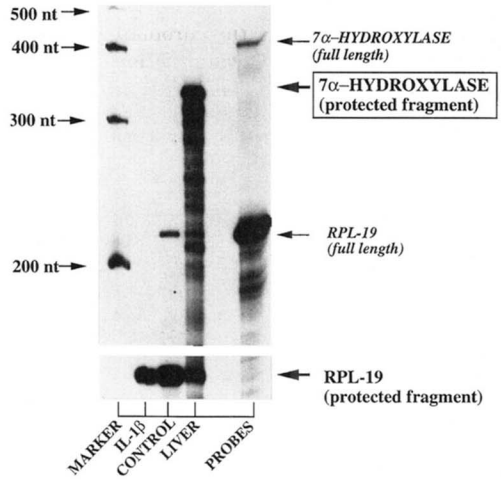

FIG. 11. **Analysis of cholesterol 7α-hydroxylase transcripts in rat liver and ovary.** Whole ovarian dispersates (1.5×10^6 cells/3 ml) were cultured for 72 h in the absence or presence of IL-1β (50 ng/ml). Thereafter, total RNA was prepared from cultured cells (RNA from 1.5×10^6 cells, each lane) and from immature female rat liver (20 ng/lane) and subjected to liquid hybridization/RNase protection assay. Both 7α-hydroxylase and RPL19 (to monitor equality of loading) transcripts were simultaneously probed. Also shown are nucleotide (*nt*) size markers (Ambion Inc., Austin, TX) and the undigested, full-length probes for 7α-hydroxylase and RPL19. Results were replicated in an additional experiment.

tion of two unknown polar metabolites (X1 and X2) of 25-[^{3}H]hydroxycholesterol. X1 and X2 were subsequently identified by GC/MS, NMR, and HPLC as cholest-4-ene-7α,25-diol-3-one (7α,25-dihydroxycholestenone) and cholest-5-ene-3β,7α,25-triol (7α,25-dihydroxycholesterol). Although autoxidation of cholesterol at C-7 (primarily to 7-oxo or 7β-hydroxyl moieties; *cf.* Ref. 58) is well known, it is unlikely that the hydroxycholesterol 7α-hydroxylation described herein is a nonenzymatic event. IL-1β-stimulated ovarian 7α-hydroxylase activity demonstrated all the hallmarks of a typical enzyme reaction. It displayed coenzyme specificity (NADH is not effective), Michaelis-Menten kinetics, and heat instability. Product accumulation was time- and IL-1β dose-dependent and did not occur in the absence of living cells. Furthermore, ovarian 7α-hydroxylase activity was subject to dose-dependent inhibition by ketoconazole, a well-known inhibitor of P450 enzymes (17, 50, 51), at a dose range similar to that reported for inhibition of rat hepatocyte cholesterol 7α-hydroxylase (49). In contrast, ovarian 7α-hydroxylase activity was not inhibited by aminoglutethimide, a potent inhibitor of the $P450_{CSCC}$ enzyme (52, 53).

The ovarian hydroxycholesterol 7α-hydroxylase enzyme differs from hepatic cholesterol 7α-hydroxylase (1–3) since cholesterol does not compete in assays using 25-hydroxycholesterol as substrate and is not metabolized to any polar products and since no mRNA for hepatic cholesterol 7α-hydroxylase can be detected in stimulated or unstimulated ovaries. The ovarian hydroxycholesterol 7α-hydroxylase enzyme also differs from nonhepatic enzymes that act on side chain-cleaved steroids (4–9), based on indirect and direct substrate specificity studies. However, the identity of the physiologically pertinent substrate for ovarian 7α-hydroxylase is unknown at this time. 27- and 25-hydroxycholesterols are good candidates since activity and mRNA for 27-hydroxylase have been detected in human and rat ovary (25, 27), and rat luteal cells can metabolize 25-hydroxycholesterol to reproductive steroids (28). Furthermore, we have detected 27-hydroxylase mRNA in cultured granulosa cells and whole ovarian dispersates from both control and IL-1β-treated cells (not shown). However, no information exists regarding the presence or regulation of such hydroxysteroids in the ovary. It is unclear whether the ovarian 7α-hydroxylase preferentially catalyzes a hydroxycholesterol or a hydroxycholestenone as substrate. Certainly, oxidation of hydroxycholesterols at C-3 by 3β-hydroxysteroid dehydrogenase/isomerase is prominent in the ovary, as has been shown in the adrenal gland (59) and liver (60). Thus, any of a variety of 7α-hydroxylated hydroxycholesterols or hydroxycholestenones may be of biological significance in the ovary. The challenge in identifying a physiologic role for ovarian 7α-hydroxylase will be to determine what are its substrates *in vivo*.

We have previously reported rat ovarian IL-1β gene expression (61) and a wide range of receptor-mediated (62) biological activities for IL-1β in the ovary (29–33). TNF-α modulates rat ovarian steroidogenesis (34–37). Both these cytokines can also induce hydroxysterol 7α-hydroxylase activity in the ovary. This effect is peptide-specific, however, since other ovarian modulatory factors such as FSH and IGF-I (48) are ineffective in this regard. Although this report extends our understanding of the ovarian actions of cytokines, it is perhaps of more interest as a demonstration of the existence of a previously unreported class of 7α-hydroxylases that are regulated by IL-1β and TNF-α. Cholesterol 7α-hydroxylase is primarily regulated at the level of gene transcription via feedback inhibition by bile acids, but is also subject to regulation by diurnal, hormonal, and dietary factors (1, 3, 57, 63). Regulatory mechanisms for liver hydroxycholesterol 7α-hydroxylase or for other nonhepatic 7α-hydroxylases are unknown. Therefore, the potential relevance of cytokines to the regulation of steroid 7α-hydroxylases is of interest and remains to be determined.

A physiologic function for 7α-hydroxycholesterols or 7α-hydroxycholestenones can only be speculated upon at this time. Although hydroxycholesterols are substrates for $P450_{CSCC}$ (Refs. 22 and 28; *cf.* Fig. 1), preliminary studies (not shown) indicate that 7α-hydroxylated hydroxycholesterols are not side chain-cleaved, suggesting that such steroids are not precursors for reproductive steroids. It is possible that 7α-hydroxy metabolites mediate some of the biological actions of cytokines in the ovary or play a role in ovarian cholesterol homeostasis. With

respect to the latter, Dueland *et al.* (21) showed that Chinese hamster ovary cells, expressing hepatic 7α-hydroxylase, are resistant to down-regulation of LDL receptor genes by 25-hydroxycholesterol. These authors propose that 7α-hydroxylase increases the expression of LDL receptor by metabolic inactivation of hydroxycholesterol inhibitors of this gene. This hypothesis also suggests a mechanism by which liver cells (that express 7α-hydroxylase) are resistant to down-regulation of LDL receptor (20). However, the nature of the link between 7α-hydroxylase and cholesterol homeostasis is controversial (14, 64).

Treatments that stimulate macrophages also increase hepatic cholesterol synthesis and mRNA levels for 3-hydroxy-3-methylglutaryl-CoA reductase (65). Macrophage products (*i.e.* cytokines) increase serum cholesterol levels (66). The Dueland hypothesis (20, 21) proposes that cholesterol biosynthesis is stimulated by 7α-hydroxylase-mediated inactivation of oxysterol repressors. Perhaps cytokines enhance cholesterol biosynthesis (*cf.* Ref. 66) by just such a mechanism. The data described herein are consistent with these previous observations and, taken together, predict that cytokines could enhance cholesterol accumulation in the ovary via the inactivation of inhibitory hydroxycholesterols by cytokine-induced 7α-hydroxylation.

Although we may speculate that cytokine-induced 7α-hydroxylation blocks the inhibitory action of oxysterols on cholesterol biosynthesis, thereby enhancing cholesterol availability, the significance of the IL-1β-stimulated 7α-hydroxylation of hydroxysterols in the ovary (or other tissues) remains to be established. In any case, the finding of a nonhepatic C_{27} 7α-hydroxylase that is regulated by IL-1β (and TNF-α) suggests a unique role for cytokines in the metabolism of C_{27} steroids.

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