Effect of Tamoxifen on Cholesterol Synthesis in HepG2 Cells and Cultured Rat Hepatocytes

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The objective of this study was to investigate the mechanisms by which tamoxifen modifies cholesterol metabolism in cellular models of liver metabolism, HepG2 cells and rat hepatocytes. The effect of tamoxifen on cholesterol and triglyceride-palmitate synthesis was measured using isotopomer spectral analysis (ISA) and gas chromatography–mass spectrometry (GC-MS) and compared with the effects of progesterone, estradiol, the antiestrogen ICI 182,780, and an oxysterol, 25-hydroxycholesterol (25OHC). Cholesterol synthesis in cells incubated in the presence of either [1-^{13}C]acetate, [U-^{13}C]glucose, or [4,5-^{13}C]mevalonate for 48 hours was reduced in the presence of 10 μmol/L tamoxifen and 12.4 μmol/L 25OHC in both HepG2 cells and rat hepatocytes. The ISA methodology allowed a clear distinction between effects on synthesis and effects on precursor enrichment, and indicated that these compounds did not affect enrichment of the precursors of squalene. Progesterone was effective in both cell types at 30 μmol/L and only in HepG2 cells at 10 μmol/L. Estradiol and ICI 182,780 at 10 μmol/L did not inhibit cholesterol synthesis. None of the compounds altered the synthesis of triglyceride-palmitate in either cell type. Treatment of cells with tamoxifen produced accumulation of three sterol precursors of cholesterol, zymosterol, desmosterol, and Δ^8 cholestenol. This pattern of precursors indicates inhibition of $\Delta^{24,25}$ reduction in addition to the previously described inhibition of Δ^8 isomerase. We conclude that tamoxifen is an effective inhibitor of the conversion of lanosterol to cholesterol in cellular models at concentrations comparable to those present in the plasma of tamoxifen-treated individuals. Our findings indicate that this mechanism may contribute to the effect of tamoxifen in reducing plasma cholesterol in humans.

TAMOXIFEN, a compound with antiestrogenic activity, is an adjuvant therapy in the treatment of postmenopausal patients with estrogen receptor–positive breast cancer. Tamoxifen is not considered a true antiestrogen: it produces a mixture of antiestrogenic and estrogenic effects and has been shown to be effective against estrogen receptor–negative breast tumors.[1] Because tamoxifen possess antiestrogenic activity, concerns were raised that administration of tamoxifen would adversely affect the cardiovascular health of women because estrogen is known to be a protective agent against the development of atherosclerosis. However, many studies show that tamoxifen treatment has some beneficial side effects for the cardiovascular health of breast cancer patients. It reduces low-density lipoprotein (LDL)-cholesterol after administration to postmenopausal breast cancer patients for 3 months,[2] 2 years,[3,4] and 5 years.[5] Tamoxifen may also increase high-density lipoprotein-cholesterol levels,[6,7] but the effect is variable.[6,8] One negative effect of tamoxifen is an increase of triglyceride levels.[2-5,7] The mechanism responsible for these effects on lipid homeostasis are not known, but it is postulated that tamoxifen is an estrogen receptor agonist in the liver, because the effects on blood lipid and lipoprotein profiles are similar to the effects in postmenopausal women receiving estrogen replacement therapy.[2] Several other biochemical effects of tamoxifen have been identified that may also contribute to improved cardiovascular health: tamoxifen is an antioxidant and may play a role in decreasing plasma levels of oxidized LDL-cholesterol,[9] and it decreases plasma levels of homocysteine, an amino acid that is correlated with cardiovascular disease.[10]

A direct effect of tamoxifen on the biosynthesis of cholesterol has been suggested by Gylling et al.[11] They found reductions in LDL-cholesterol in patients treated with tamoxifen and toremifene for 12 months. In addition, serum levels of Δ^8 cholestenol, a cholesterol precursor, were increased approximately 50-fold in these patients. The investigators hypothesized that tamoxifen inhibits the conversion of Δ^8 cholestenol to lathosterol, leading to downregulation of cholesterol synthesis. In vitro studies investigating the effects of tamoxifen on ^{14}C acetate incorporation into cholesterol in breast cancer cell lines also indicated that hydroxytamoxifen directly inhibited cholesterol synthesis.[12] The investigators found evidence that tamoxifen produced an increase in methyl sterols and suggested that antiestrogens inhibit a lanosterol dimethylation step in the cholesterol synthesis pathway.

The aim of the present study was to examine the hypothesis that tamoxifen inhibits cholesterol synthesis by direct action in hepatic cells and to explore possible mechanisms for this effect. Hepatic cell models (cultured rat hepatocytes and HepG2 cells) were selected to evaluate the hypothesis that tamoxifen decreases total plasma cholesterol via a direct mechanism on hepatic cholesterol synthesis. Five compounds were investigated: 25-hydroxycholesterol (25OHC), estradiol, progesterone, tamoxifen, and the antiestrogen ICI 182,780. 25OHC was included because the mechanism of action of this oxysterol on hepatic cholesterol synthesis is well known. It inhibits the cleavage of sterol regulatory element binding protein 1 and 2 (SREBP1 and SREBP2), which, upon cleavage, enter the nucleus and activate genes coding for the LDL receptor and enzymes of the cholesterol synthesis pathway, including hepatic hydroxymethyl glutaryl coenzyme (HMG CoA) reductase and HMG CoA synthetase.[13] ICI 182,780 was selected because, as a pure antiestrogen,[14] it would detect the actions of tamoxifen identical to this class of compounds. Progesterone was included because it inhibits cholesterol synthesis in intestinal epithelial

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cells[15] and Chinese hamster ovary (CHO) cells,[16] producing elevated levels of cholesterol precursors. In addition, progesterone blocks cholesterol translocation from lysosomes.[17] This inhibitory action of progesterone does not appear to involve the progesterone receptor mechanism, because progestin receptor agonists and antagonists do not affect this inhibition. Recent evidence suggests that progesterone inhibits cholesterol synthesis by binding to P-glycoprotein[18] and interfering with sterol trafficking.[19]

Stable isotopes enriched in ^{13}C were used as precursors for biosynthesis, and isotopomer spectral analysis (ISA) was used to analyze the pattern of labeling. The ISA technique provides a distinction between the fractional contribution of precursors to the compartment used for lipogenesis and the fraction of molecules synthesized in a specific period.

MATERIALS AND METHODS

Cell Culture and Lipid Isolation

HepG2 human hepatoma cells were cultured as described in Kelleher et al.[20] Before the experiments, cells were subcultured in six-well plates, 60-mm or 100-mm dishes, until confluency. Rat hepatocytes were prepared according to the method of Barry and Friend[21] from male Sprague-Dawley rats (150 to 300 g). Hepatocytes were washed twice in Dulbecco's modified Eagle's medium (DMEM) containing Pen-Strep (100 μg/mL; BioFluids, Rockville, MD). They were then plated on 60-mm dishes coated with collagen (5 $\mu g/cm^2$) in DMEM supplemented with fetal calf serum ([FCS] 10%), dexamethasone (0.4 μg/mL), and insulin (10 μg/mL). After 4 to 8 hours, the media were changed to DMEM supplemented with 10% lipid-free controlled processed serum supplement (CPSR-1; Sigma, St Louis, MO) and insulin. Metabolism experiments were started the next day. Experimental media for both types of cells consisted of serum-free DMEM supplemented with 10% CPSR-1, Pen-Strep (100 μg/mL), glucose (25 mmol/L for HepG2 and 10 mmol/L for hepatocytes), sodium bicarbonate (45 mmol/L), glutamine (4 mmol/L), and [1-^{13}C]acetate (10 mmol/L). Tamoxifen, 25OHC, estradiol, progesterone (Sigma), and the antiestrogen ICI 182,780 (gift from Zeneca Pharmaceuticals, Macclesfield, UK) were prepared in 100% ethanol and added to the experimental medium as 1/1,000 of the medium volume. Control wells or dishes contained an equivalent amount of ethanol (17.4 mmol/L). ^{13}C-substrates (with specific ^{13}C abundance) were [1-^{13}C]acetate (99%), [U-^{13}C]palmitate (98%), [U-^{13}C]glucose (90%) (Cambridge Isotope Laboratories, Andover, MA), and [4,5-^{13}C]mevalonate (99%) (MSD Isotopes, Montreal, Quebec, Canada). The labeling pattern and abundance information were provided by the manufacturers. To avoid substrate depletion during the ^{13}C-substrate incubation period, the media were changed every 24 hours for HepG2 cells and every 48 hours for the less densely plated rat hepatocytes. Experiments were terminated by washing the cells free of media twice with ice-cold bovine serum albumin (2 mg/mL) in phosphate-buffered saline (PBS) and once with ice-cold PBS. Hexane/isopropanol (3:2 vol/vol, 4 to 8 mL) and internal standards dissolved in hexane/isopropanol (5α-cholest-7-en-3β-ol and triheptadecanoin) were added directly to the dishes or wells. After 30 minutes, the hexane/isopropanol extract was removed into screw-capped glass tubes. A second 4-mL aliquot of hexane/isopropanol was applied to the cells and combined with the first extract. The hexane/isopropanol extracts were washed with 1 to 2 mL sodium sulfate solution (1 g/15 mL). The top layer (hexane) was dried under N_2, reconstituted in hexane, and applied to 20 × 20-cm silica gel GHL plates (Altech, Deerfield, IL). The plates were developed in hexane:ethyl ether:acetic acid (80:20:1 vol/vol/vol). Cholesterol and triglyceride bands were extracted and derivatized as described previously.[20,22] Triglycerides were *trans*-esterified to fatty acid methyl esters using boron trifluoride in methanol (BF3/methanol, 14%; Altech), and cholesterol was converted to trimethylsilyl (TMS) esters using bis-TMS-trifluoroacetamide (BSTFA; Pierce, Rockford, IL).

Sterol cholesterol synthesis intermediates were isolated from total lipid extracts by saponification of the dried extract for 3 hours at 80°C in the presence of 1 mL 10-mol/L NaOH, 75 μL acetone, and 75 μL benzene. This procedure yielded a mixture of fatty acid salts and sterols derived from free sterols and sterol esters. Sterols were isolated from this mixture by hexane extraction that excluded the fatty acid salts. The sterols were then converted to their TMS esters.

Gas Chromatography–Mass Spectrometry Analysis

Lipid derivatives were injected (1 μL) into a Hewlett-Packard model 5890 (series II) gas chromatograph connected to a mass selective detector (Hewlett-Packard model 5971, Palo Alto, CA) and equipped with either a DB-5ms (30 m × 0.32 ID × 0.25 mm) or DB-XLB (30 m × 0.25 ID × 0.25 mm) capillary column. Helium flow was maintained at 0.8 mL/min via electronic pressure control. For cholesterol analysis, the temperature of the injection port was 340°C and the transfer line was 310°C. The temperature program for the column was as follows: initial temperature of 200°C for 2 minutes, and then an increase to 300°C at 25°C/min maintained for 14 minutes. For triglyceride analysis, the injection port temperature was 280°C and the transfer line was 280°C. The temperature program for the column was the following: initial temperature of 130°C for 3 minutes, and then an increase to 280°C at 20°C/min maintained for 5 minutes. For cholesterol, data were collected on a major fragment of the TMS-cholesterol ester (mass 368, M + 0) using selected ion monitoring (SIM). Twenty ions were monitored, M-1 through M + 18. For fatty acid methyl esters, data were collected on the molecular ions (myristate, 242; palmitate, 270; stearate, 298). Between 15 and 20 ions were collected for the fatty acid methyl esters of myristate, palmitate, and stearate. The mass of cholesterol and triglyceride-palmitate and the relative abundance of cholesterol and sterol cholesterol synthesis intermediates were measured by GC-MS.

The lipid mass was quantified using a standard curve comparing the signal of cholesterol or triglyceride-palmitate against the appropriate internal standard. The relative abundance of cholesterol and sterol intermediates was measured by comparing the area of the individual chromatographic peaks. For experiments in which the purpose was to detect sterol intermediates, TMS derivatives were analyzed by GC-MS using the same program described for ^{13}C-TMS cholesterol esters, except that the mass spectrometer was operated in the scan mode to observe the fragmentation pattern of TMS esters. Cholesterol isomers were identified by the presence of the molecular ion *m/z* 458 and by comparison of fragmentation patterns described in Brooks et al[23] and Gerst et al.[24]

ISA

The mass isotopomer distribution of cholesterol and other lipids isolated from cells following incubation with ^{13}C-labeled precursors was analyzed using SIM data. Parameters describing the flux of ^{13}C-labeled precursors, acetate or mevalonate, to steroids were estimated from these data using a nonlinear regression ISA procedure described previously.[20,22] For this study, a model allowing ^{13}C precursors to enter the sterol biosynthetic pathway as either acetate or mevalonate was used (Fig 1). ISA solves simultaneously for two key parameters of the biosynthesis. The precursor dilution parameter (D or F) indicates the fractional contribution of a ^{13}C-enriched compound to the precursor pool for biosynthesis. The product dilution parameter g(t) represents the fraction of total synthesized product present at the time of analysis (t), that resulted from de novo synthesis. For these studies, the product is cellular free cholesterol or triglyceride palmitate. Each parameter has a maximum value of 1, indicating that 100% of the flux

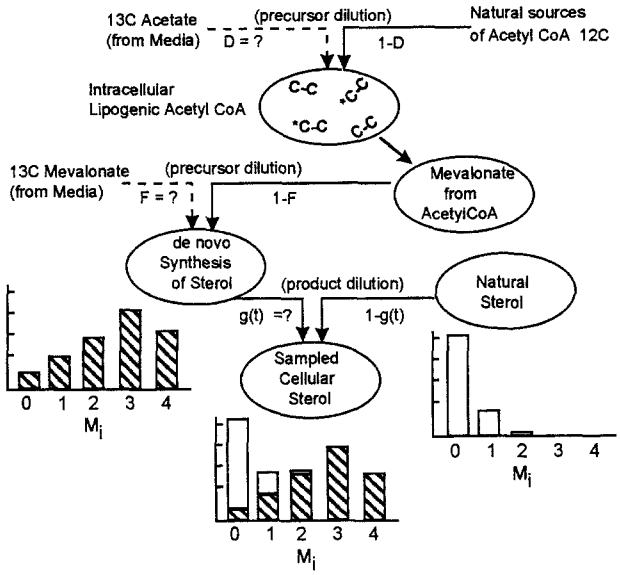

Fig 1. ISA model of sterol synthesis from ^{13}C-labeled precursors. ^{13}C acetate or ^{13}C mevalonate enter the biosynthetic pathway (---). A precursor dilution parameter, D for acetate and F for mevalonate, represents the fractional contribution of ^{13}C- enriched precursor to the biosynthetic precursor pool. Using either precursor, the mass isotopomer distribution of newly synthesized sterols is enriched in ^{13}C (▨). Sterol isolated from cells is a mixture of newly synthesized sterol and "natural" sterol with a mass isotopomer distribution characteristic of the natural abundance of ^{13}C. ISA uses g(t) for the time-dependent parameter indicating the fraction of sampled sterol derived from de novo synthesis.

was from the ^{13}C-enriched source. Small values for D or F significantly less than 1 indicate that the ^{13}C-labeled compound added to the medium is not an effective precursor for lipogenesis. To identify the source of ^{13}C-labeled material for the precursor dilution parameters, subscripts (eg, $D_{(acetate)}$) are used. The product dilution parameter g(t) normally increases over time. A value for g(t) close to 1 indicates that at time t, the entire compartment of cholesterol or triglyceride-palmitate is derived from newly synthesized lipid. To obtain the amount of a newly synthesized product, the g(t) value is multiplied by the amount of the product.

Protein and LDL-Cholesterol Determinations

Cell protein remaining on the dishes after hexane/isopropanol extraction was dissolved in 0.1N NaOH, and protein levels were measured using the Bradford assay (Coomassie Plus assay; Pierce). Total cellular LDL-cholesterol association was measured using a fluorescent probe (Molecular Probes, Eugene, OR), 3,3'-dioctadecylindocarbocyanine-LDL (DI-LDL), according to the method of Stephan and Yurachek.[25] Human LDL-cholesterol was prepared from the plasma of normal individuals by sequential ultracentrifugation and then labeled with Dil. HepG2 cells were plated in 24-well plates and subjected to the experimental conditions for 48 hours. At the end of the incubation period, the media were removed and the cells were incubated for 2 hours in the presence of 60 μg/mL Dil-LDL at 37°C to measure total cellular association of LDL-cholesterol. To terminate the incubation, the plates were placed in ice, and the cells were rinsed with ice-cold PBS and then subjected to isopropanol extraction to remove total associated DI-LDL. Controls with an excess of unlabeled LDL in addition to the DI-LDL were included to determine nonspecific binding. Fluorescence was determined using a Perkin-Elmer model LS-50B spectrofluorometer with excitation and emission wavelengths set at 520 and 578 nm, respectively.

Statistical Analysis

Differences between mean values were analyzed by ANOVA using the SAS program (SAS Institute, Cary, NC).

RESULTS

ISA of cholesterol synthesized in the presence of ^{13}C-enriched substrates produced a good fit of the model to the data as indicated by sample data shown in Fig 2. Precursor and product dilution parameters (D, F, and g(48 h)) were calculated as already described. The five test compounds, 25OHC, estradiol, progesterone, tamoxifen, and ICI 182,780, were added to cells incubated in the presence of [1,2-^{13}C]acetate, and ISA was performed on isolated lipid fractions (Figs 3 and 4). For both rat hepatocytes and HepG2 hepatoma cells, the test compounds

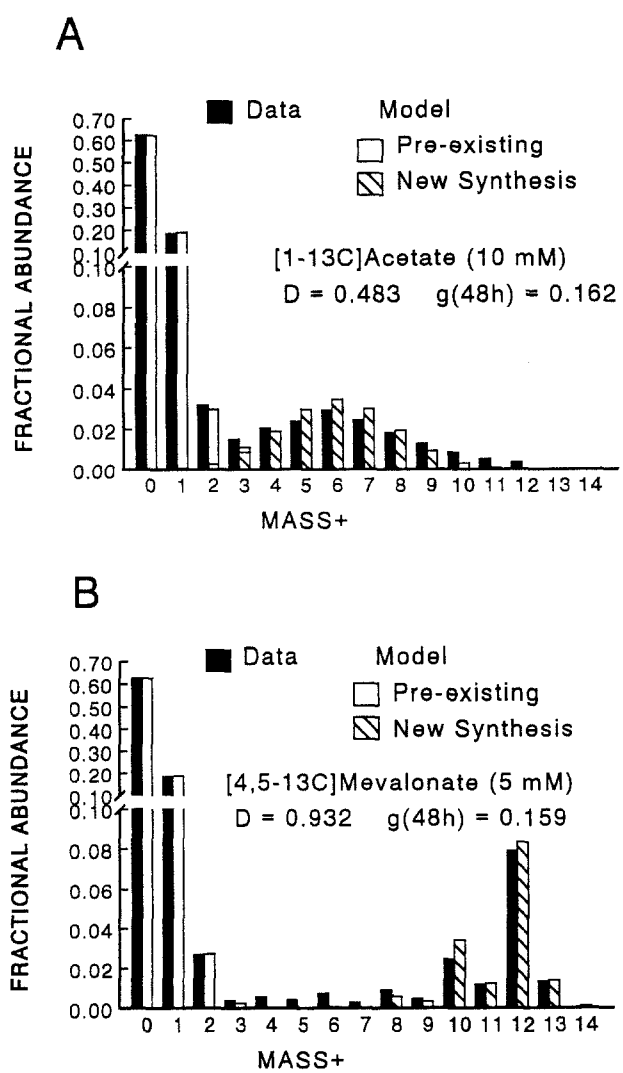

Fig 2. Sample isotopomer spectra obtained from HepG2 cells incubated for 48 hours in either 10 mmol/L [1-^{13}C]acetate (A) or 5 mmol/L [4,5-^{13}C]mevalonate (B). ISA was used to determine precursor dilution parameters, D for acetate and F for mevalonate, and g (48h) values. The model solution plotted at right represents the best-fit solution using the indicated parameter values.

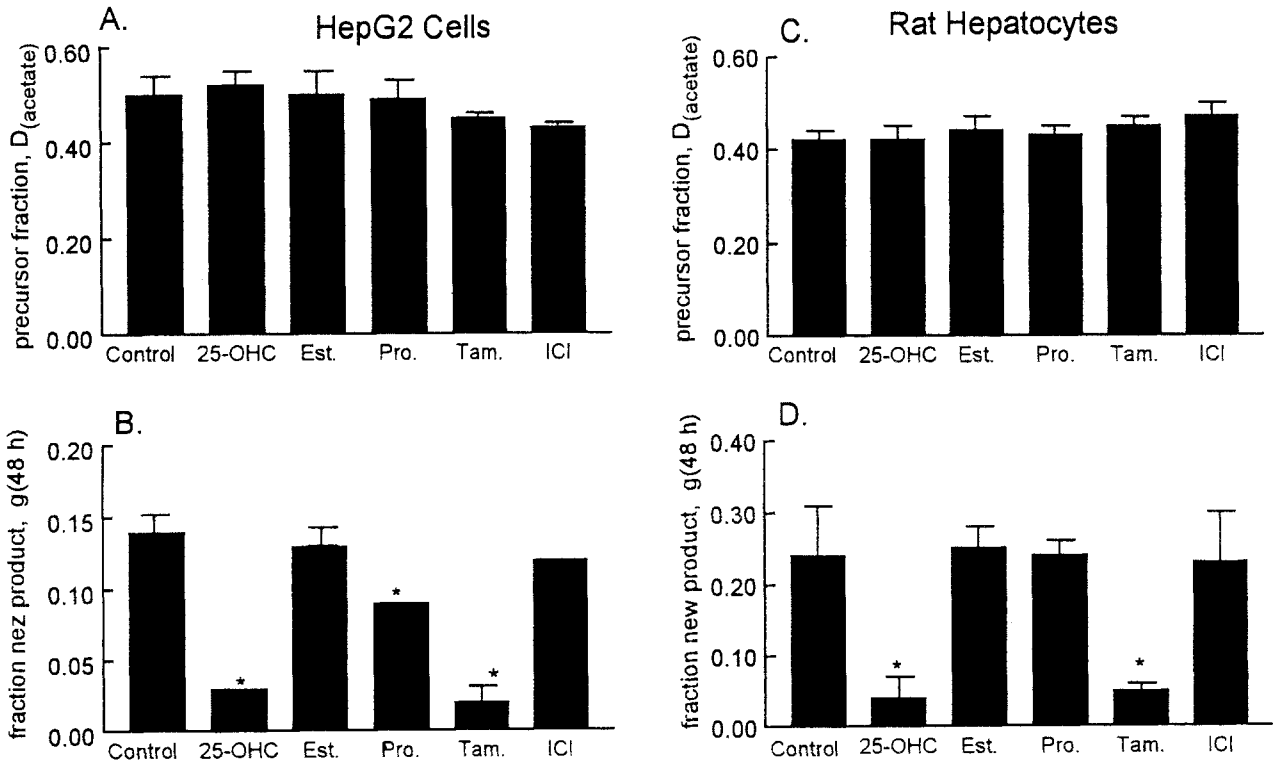

Fig 3. D and g(48 h) values for cholesterol synthesis in HepG2 cells and rat hepatocytes incubated for 48 hours in DMEM containing 25 mmol/L glucose (or 10 mmol/L glucose for rat hepatocytes), 10 mmol/L [1-^{13}C]acetate, 4 mmol/L glutamine, 10% CPSR, and additions as noted. The concentration of estradiol, progesterone, tamoxifen, ICI 182,780 was 10 μmol/L and 25OHC was 12.4 μmol/L. Significant difference *v* control, $P \leq .05$ by ANOVA and Student-Newman-Keuls (mean ± SE, n = 5).

produced no significant effects on the precursor dilution parameter $D_{(acetate)}$ (Fig 3). This indicates that the test compounds had no effect on the fractional contribution of ^{13}C acetate to the steroid biosynthesis precursor pool (Figs 3 and 4, A and C). In contrast, the fraction of new cholesterol synthesized in 48 hours, g(48 h), was significantly affected by the addition of 25OHC and tamoxifen (Fig 3B and D). Progesterone 10 μmol/L moderately inhibited cholesterol synthesis in HepG2 cells, but was ineffective in rat hepatocytes.

The studies described in Fig 3 were performed in a lipid-free medium containing the serum substitute CPSR. To determine if the effects observed were limited to this medium, experiments were performed comparing lipid-free media with media containing 10% FCS. Addition of FCS moderately inhibited cholesterol synthesis, as shown by comparing g(48 h) values for control cells in the two media. However, the inhibitory effects of 25OHC and tamoxifen were apparent in both media (Fig 4B). The similarity between $D_{(acetate)}$ values for cholesterol and triglyceride-palmitate synthesis (Fig 4A and C) is consistent with the fact that a common acetyl CoA pool is the precursor for the biosynthesis of steroids and fatty acids. The synthesis of triglyceride-palmitate was not affected by any of the test compounds (Fig 4C and D), demonstrating that the effects observed were specific for the steroidogenic pathway. Triglyceride synthesis from fatty acids supplied in the medium was also investigated to determine if the test compounds affected this pathway for triglyceride synthesis. [U-^{13}C]palmitate (0.3 mmol/L) added to the CPSR medium as an albumin complex was readily incorporated into cellular triglycerides and accounted for 70% of triglyceride-palmitate after 48 hours. This flux was not altered by addition of any of the test compounds at the concentrations used for data presented in Figs 3 and 4 (data not shown).

Although both tamoxifen and progesterone were observed to inhibit cholesterol synthesis in HepG2 cells, tamoxifen was effective at lower concentrations than progesterone (Fig 5). Tamoxifen inhibited cholesterol synthesis by 50% at 2 μmol/L, whereas progesterone requires 15 μmol/L to achieve this level of inhibition. To determine whether the mechanism of action of tamoxifen and progesterone was similar to that of 25OHC, the effect of these compounds on cholesterol turnover was investigated. ISA allocates the total cellular free cholesterol to an amount contributed by new synthesis (g(t) × total) and preexisting cholesterol synthesis ((1 − g(t)) × total free cholesterol), as shown in Fig 6. This analysis showed that 25OHC produced a significant decrease in the amount of preexisting cholesterol. In contrast, three compounds, 25OHC, progesterone, and tamoxifen, decreased the de novo synthesis of cholesterol (Fig 4B). Since cholesterol was not included in the medium, the decreased preexisting cholesterol found with 25OHC is likely due to an increased turnover of cholesterol. These data indicate that 25OHC was unique among the tested compounds in decreasing cellular free cholesterol both by decreasing the synthesis and by increasing the turnover of preexisting cholesterol.

Differences among the compounds were also apparent when the syntheses of cholesterol using [1-^{13}C]acetate (10 mmol/L)

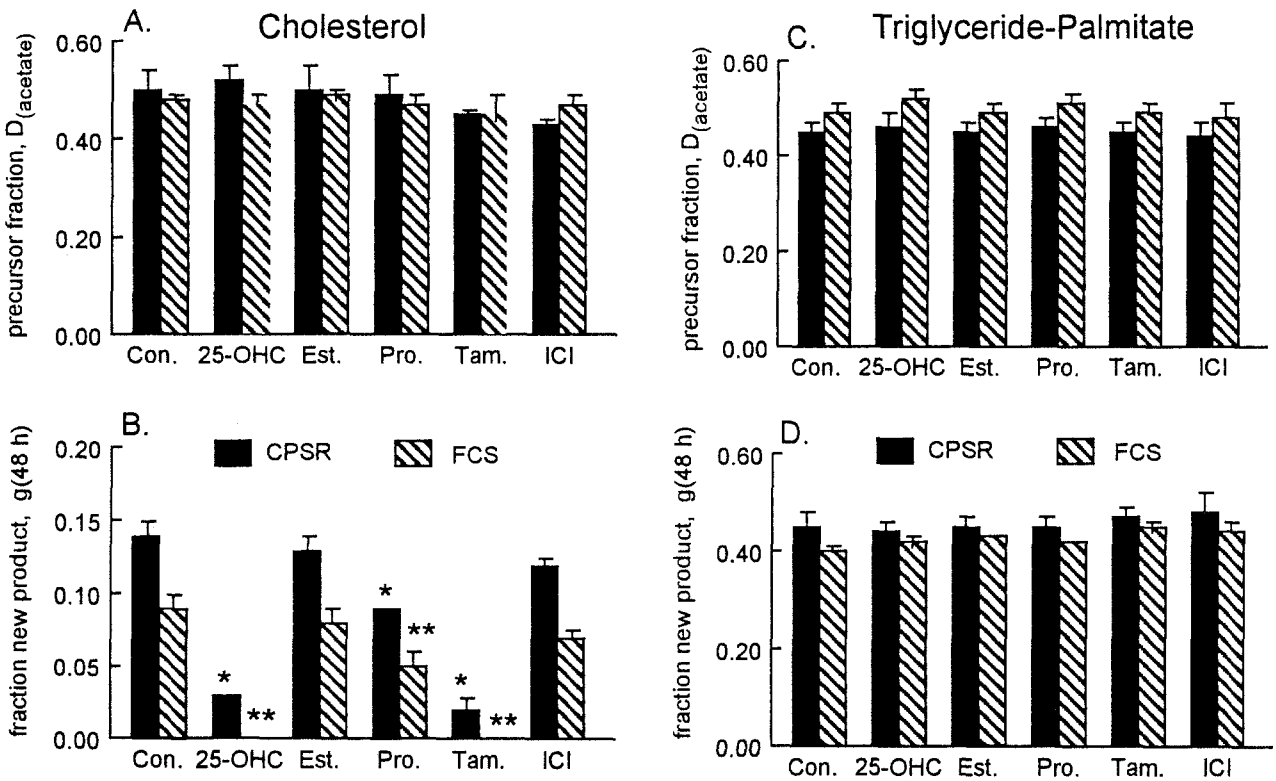

Fig 4. Effect of FCS and hormone and drug additions on cholesterol and triglyceride synthesis in HepG2 cells. Hormone and drug concentrations and incubation conditions were identical to those in Fig 3. Significant difference *v* control for CPRS* and FCS media, $P \leq .05$ by ANOVA and Student-Neuman-Keuls (mean ± SE, n = 5 for control and n = 3 for FCS).**

and [4,5-^{13}C]mevalonate (5 or 10 mmol/L) were compared (Fig 7). When glucose, acetate, or palmitate was used as the labeled precursor, each of these compounds contributed no more than 50% of the acetyl CoA precursor used for biosynthesis. However, when ^{13}C-labeled mevalonate was used, the labeling pattern indicated that mevalonate added to the medium contributed 93% to 98% of mevalonate molecules used for biosynthesis. Thus, mevalonate added to the medium almost completely replaced the flux from unlabeled precursors to the lipogenic mevalonate pool (Fig 7A). The contribution of mevalonate from the medium is also shown in Fig 2, where [4,5-^{13}C]mevalonate yields largely M + 12 cholesterol as the newly synthesized peak. M + 12 corresponds to a newly synthesized cholesterol molecule where all six mevalonate molecules used for the biosynthesis were derived from [4,5-^{13}C]mevalonate supplied in the medium. (Mevalonate carbons 4 and 5 are retained at each step of the biosynthesis). None of the test compounds produced any change in the precursor dilution, as illustrated for tamoxifen in Fig 7A. In contrast, g(48 h) values were affected by changes in the composition of the media produced by adding an external source of mevalonate (Fig 7B). These data indicate that 25OHC allows greater synthesis of cholesterol from mevalonate than from acetate, as expected for a compound known to block HMG CoA reductase. Progesterone and tamoxifen do not share this effect, indicating that they have a mechanism of action not identical to that of 25OHC and are not dependent on blocking steps prior to mevalonate for their inhibitory action.

Tamoxifen and progesterone were compared with 25OHC for activity in the SREBP pathway, a well-described mechanism for

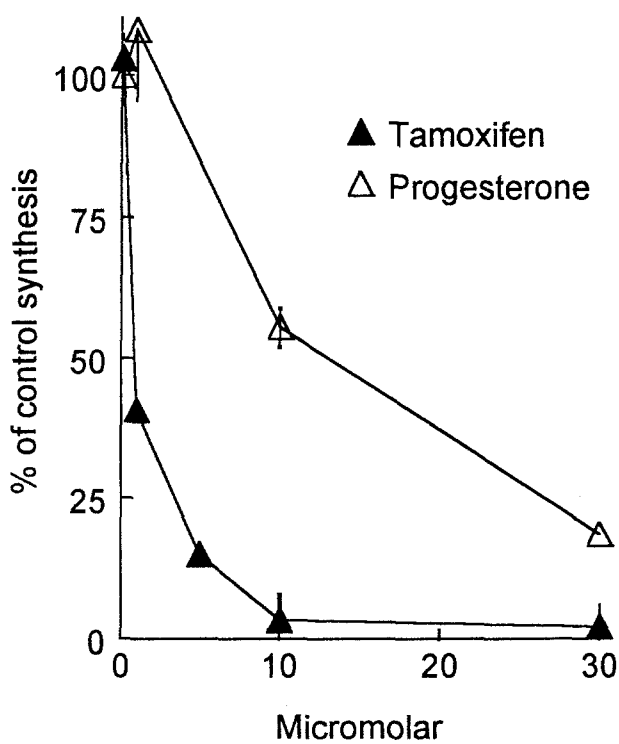

Fig 5. Cholesterol synthesis in HepG2 cells (percent of control) as a function of tamoxifen and progesterone concentrations. Error bars represent standard deviations of triplicates. Cells were incubated as noted in Fig 3.

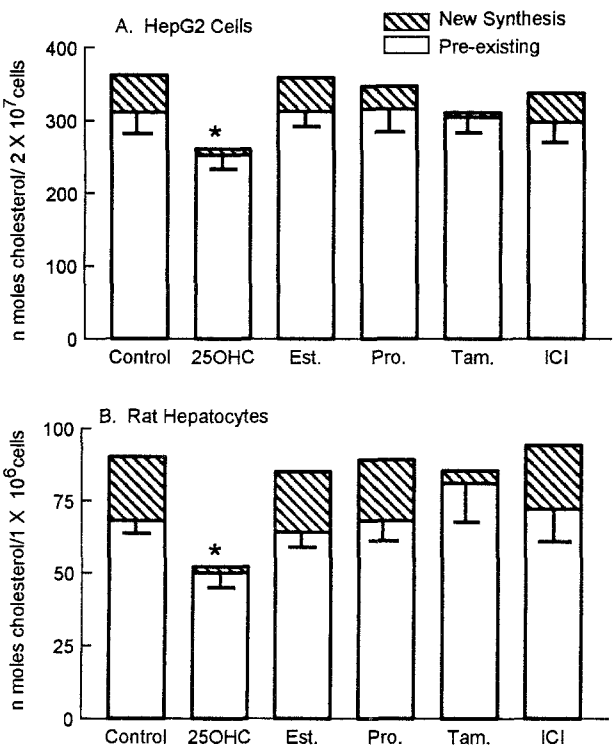

Fig 6. Effect of steroids and antiestrogens on preexisting and total free cholesterol in hepatic cell models. Total free cholesterol estimated by GC-MS was divided into the portion newly synthesized during the 48-hour experimental period (g (48 h) × total free cholesterol and the portion derived from preexisting cholesterol (1-g(48 h) × total free cholesterol. Cells were incubated as noted in Fig 3. Differences between preexisting cholesterol were compared (n ≥ 5). *Significant difference *v* control, $P \leq .05$.

inhibition of cholesterol synthesis by oxysterols. The effects of these compounds on the LDL receptor population of HepG2 cells was investigated using a Dil-LDL binding assay (Fig 8). As expected, 25OHC caused a large reduction in the apparent number of receptors, consistent with the mechanism of action involving the SREBP and decreased synthesis of the LDL receptor. Tamoxifen and progesterone at the concentrations used in this study were less effective in this assay. Since the SREBP mechanism leads to decreases in both the LDL receptor and HMG CoA reductase synthesis, these findings support the data in Fig 7B suggesting that tamoxifen does not act by the SREBP mechanism described for oxysterols.

The effects of tamoxifen and progesterone on the cholesterol synthesis pathway beyond the formation of lanosterol were investigated by identifying sterol intermediates that accumulate in a 72-hour incubation using GC-MS (Fig 9). Sterols with the Δ^{24} double bond in the side chain were identified as deriving from lanosterol and were differentiated from sterols without the double bond in the side chain identified as deriving from dihydrolanosterol. The two types of sterols were distinguished by a difference in mass: sterols derived from dihydrolanosterol are 2 mass units heavier than those derived from lanosterol. Under control conditions, sterols other than cholesterol occur in low amounts. (Numbers in brackets correspond to compounds labeled in Fig 9A.) Three intermediates identified in control samples were monomethyl and dimethyl sterols derived from dihydrolanosterol [5 and 7] and a second monomethyl sterol derived from lanosterol [6]. Addition of progesterone resulted in the accumulation of two sterols not observed in the control incubation (Fig 9C). These compounds were identified as desmosterol [3] and a dimethyl sterol [8], both derivatives of lanosterol. Other sterols present were a monomethyl sterol [6], a

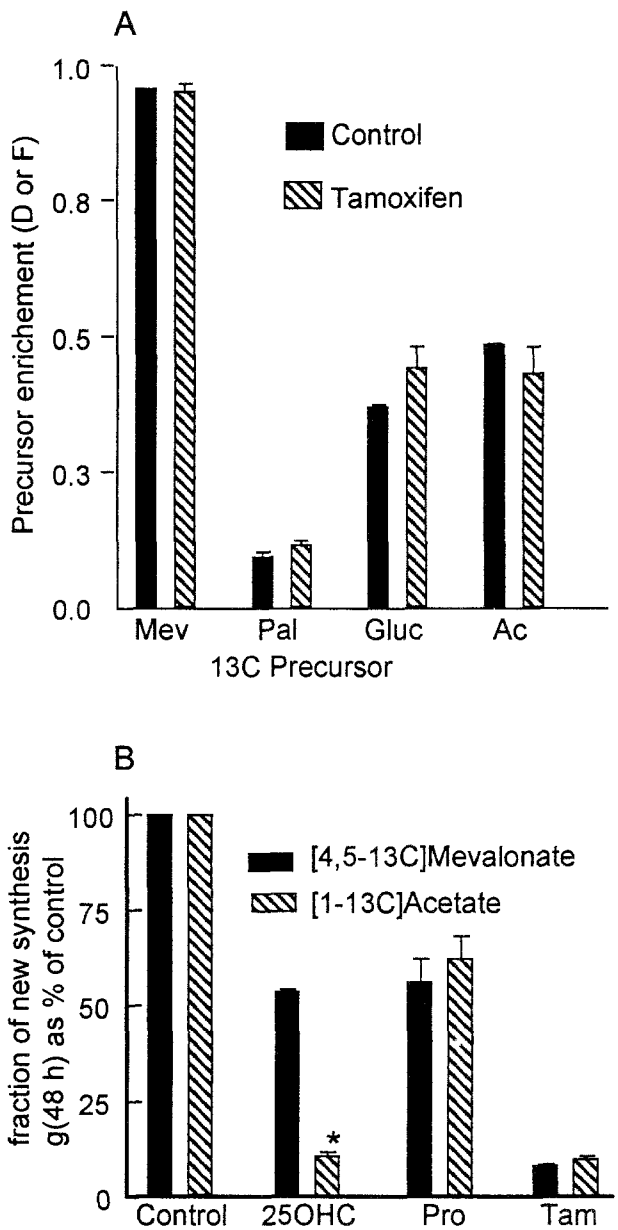

Fig 7. (A) Precursor enrichment (D or F value) for each ^{13}C-enriched precursor used to monitor de novo cholesterol synthesis. For acetate (Ac), palmitate (Pal), and glucose (Gluc), the y-axis indicates the fractional contribution of labeled precursor to the lipogenic acetyl CoA. For mevalonate (Mev), the y-axis represents the fractional contribution of precursor to the cholesterogenic mevalonate pool. Error bars represent standard deviations of triplicates. Cells were incubated as noted in Fig 3. (B) Effect of inhibitors of cholesterol synthesis in media containing either 10 mmol/L ^{13}C acetate or 15 mmol/L ^{13}C mevalonate. To allow a comparison of the effect of treatment on the de novo synthesis of cholesterol, g (48 h) values (0.09 for acetate media and 0.25 for mevalonate media) were normalized to 100% for control conditions. *Significant difference for mevalonate *v* acetate, $P \leq .05$.

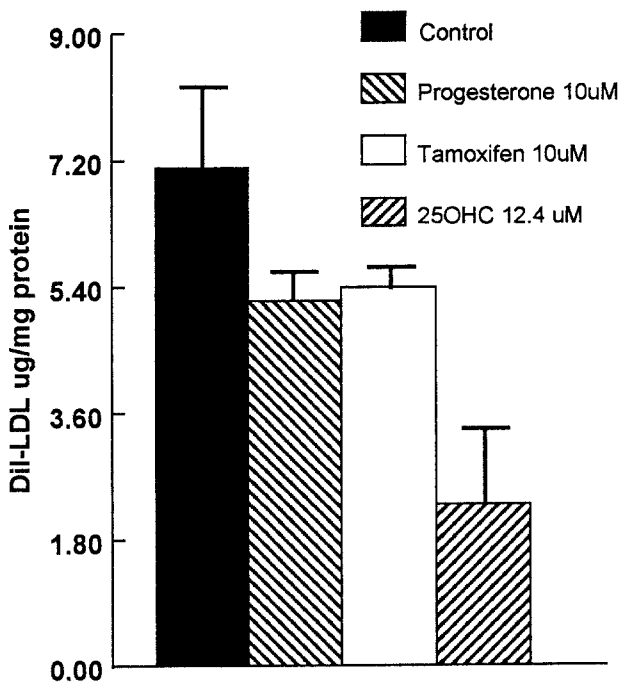

Fig 8. Total cellular LDL-cholesterol association as a function of hormone and drug addition. After standard incubation conditions for 48 hours, cells were washed with PBS and incubated for 2 hours in the presence of 60 μg/mL Dil-LDL. Results are the mean of duplicates; error bars represent the range.

derivative of lanosterol and present in larger amounts relative to the control incubation, and two methylated sterols [5 and 7] derived from dihydrolanosterol and present in smaller amounts relative to the control incubation. Thus, progesterone appears to cause an accumulation of Δ^{24} sterols [3, 6, and 8], consistent with a decrease in Δ^{24} reductase activity.

The pattern of sterol accumulation in the presence of progesterone (Fig 9C) was not identical to that for tamoxifen (Fig 9D). Addition of tamoxifen resulted in the accumulation of five sterol intermediates not found under control conditions. Four of the five sterols were derived from lanosterol [3, 4, 6, and 8], with the exception of Δ^{8} cholestenol [2], an isomer of cholesterol with the double bond between carbons 8 and 9. A sixth sterol was observed, a derivative of dihydrolanosterol [5], present in low amounts relative to the control incubation. The pattern of sterol accumulation produced by tamoxifen differed from that obtained with progesterone in the accumulation of the two substrates for Δ^{8} isomerase [2 and 4]. However, compounds indicative of a block in Δ^{24} reductase were observed for treatment with both tamoxifen [3, 4, and 8] and progesterone [3, 6, and 8]. To evaluate the time dependency of these observations, the accumulation profile of steroid intermediates with time in the presence of tamoxifen was investigated (Fig 10). The three largest peaks, Δ^{8} cholestenol, zymosterol, and desmosterol, were evaluated for 72 hours, and the results showed that Δ^{8} cholestenol and desmosterol accumulate as if they are end products of a biosynthetic pathway, whereas the pattern of zymosterol more nearly fit a profile characteristic of an intermediate in a pathway that increases to a new higher steady-state level to offset partial inhibition downstream. The total accumulation of sterol intermediates over 72 hours in the presence of tamoxifen is considerable, about 12% as large as the total free cholesterol observed at this time in the presence of tamoxifen. In addition, Δ^{8} cholestenol was heavily labeled when ^{13}C acetate was included in the medium (data not shown), indicating that this intermediate was derived from de novo synthesis occurring while the cells were incubated with tamoxifen and labeled acetate.

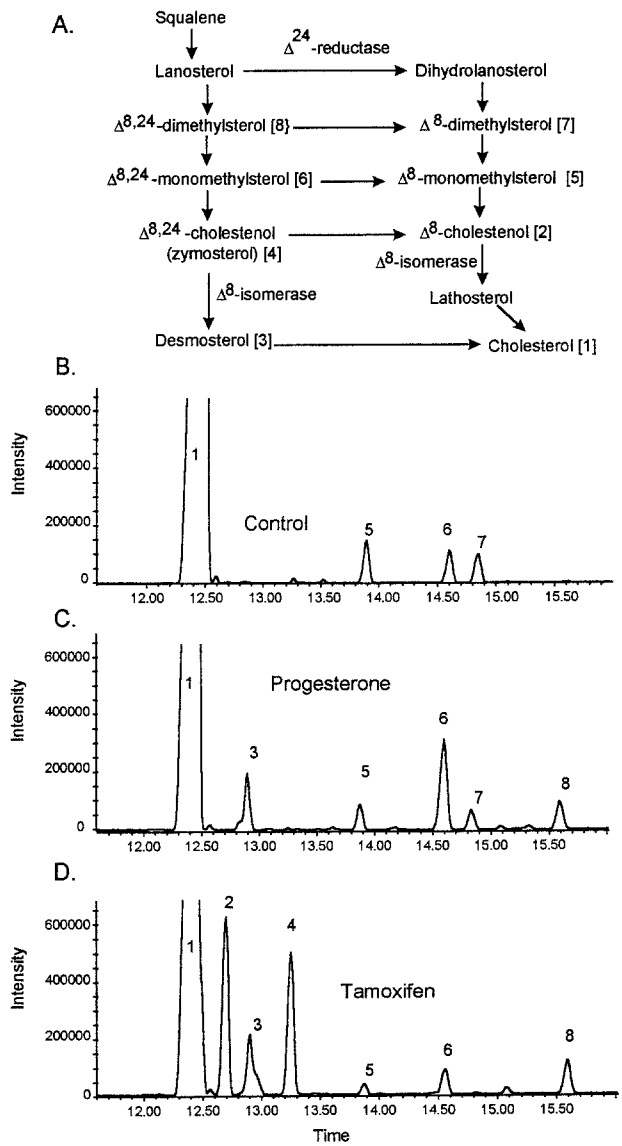

Fig 9. GC-MS chromatogram of cholesterol and sterol intermediates present after saponification of hexane/ethyl ether extract of total lipids from HepG2 cells. (A) Cells incubated 72 hours in the presence of 25 mmol/L glucose, 4 mmol/L glutamine, and 10% CPSR. (B) Cells incubated as in the control and in the presence of 10 μmol/L progesterone. (C) Cells incubated as in the control and in the presence of 10 μmol/L tamoxifen. Identification of peaks: 1 = cholesterol, molecular ion (MI) 458; 2 = Δ^{8} cholestenol, MI 458; 3 = desmosterol and an unidentified sterol, desmosterol MI 456; 4 = zymosterol, MI 456; 5 = monomethyl sterol, no $\Delta^{24,25}$ double bond, MI 472; 6 = monomethyl sterol with $\Delta^{24,25}$ double bond, MI 470; 7 = dimethyl sterol, no $\Delta^{24,25}$ double bond, MI 486; 8 = dimethyl sterol with $\Delta^{24,25}$ double bond, MI 484.

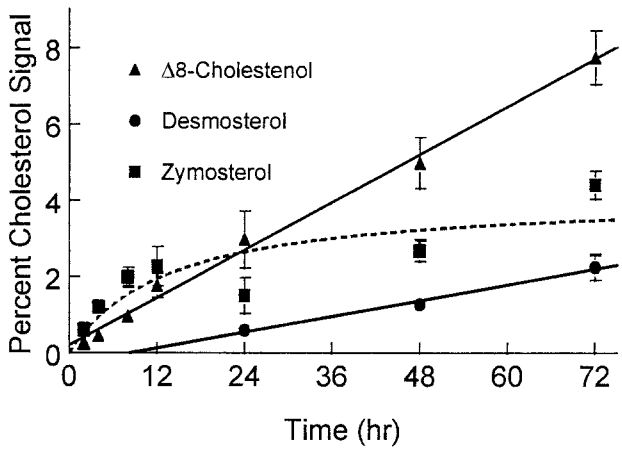

Fig 10. Accumulation of major sterols in HepG2 cells measured as a percent of GC-MS cholesterol signal. Cells were incubated in the presence of 10 μmol/L tamoxifen with incubation conditions as noted in Fig 2. Error bars represent the range of duplicates. Data for Δ^8-cholestenol and desmosterol fitted by linear regression, $R^2 = .99$. Data for zymosterol fitted by saturable Michaelis-Menten plot.

DISCUSSION

The use of ^{13}C-enriched precursors and ISA demonstrated that tamoxifen and progesterone are inhibitors of de novo cholesterol synthesis in cellular models of liver. Although these studies may have been performed using radioisotopes, an advantage of ISA over radioisotope studies is that precursor and product dilution are determined separately. Radioisotope studies do not distinguish between treatment effects on enrichment of the precursor compartment and treatment effects on rates of synthesis. ISA demonstrated that the effects of tamoxifen and progesterone were to decrease the rate of cholesterol synthesis and not to affect enrichment of the precursor compartment, lipogenic acetyl CoA. The decrease in cholesterol synthesis was indicated by a decrease in g(48 h) of free cholesterol in cells incubated in the presence of tamoxifen and progesterone (Fig 3). The parameter g(48 h) is the fraction of sampled free cholesterol derived from newly synthesized free cholesterol (Fig 1). ^{13}C enrichment of free cholesterol will decrease if the synthesis from a ^{13}C-enriched precursor is decreased or if the contribution of unlabeled free cholesterol is increased due to either a decrease in the turnover of preexisting free cholesterol or hydrolysis of cholesterol esters. We found that in the case of tamoxifen and progesterone, the total compartment of free cholesterol was unchanged (Fig 6), demonstrating that a decrease in synthesis was responsible for the decrease in fractional enrichment of free cholesterol. In the case of 25OHC, a known inhibitor of cholesterol synthesis, the total compartment of free cholesterol was decreased (Fig 6) and the g(48 h) value was greatly decreased (Fig 3), demonstrating that free cholesterol synthesis was decreased. In fact, because of the decrease in the compartment of free cholesterol, the decrease in synthesis was actually greater than indicated by simply comparing g(48 h) values between control and 25OHC incubations.

The ISA experiments in Figs 3 to 6 were performed using [1-^{13}C]acetate as the labeled cholesterol precursor. The lack of any effect of tamoxifen on the precursor enrichment of various additional ^{13}C substrates, mevalonate, palmitate, and glucose (Fig 7A), indicated that the decreased cholesterol synthesis observed in the presence of tamoxifen was not due to an effect on lipogenic precursor enrichment. Thus, although the amount of new cholesterol was decreased by tamoxifen, the relative contribution of each of these precursors to lipogenic pathways was not altered and tamoxifen did not alter the relative flux of carbon into the cholesterol synthesis pathway from these precursors. Because mevalonate enters the cholesterol synthesis pathway after the highly regulated HMG CoA step, experiments with [4,5-^{13}C]mevalonate provided insight into the role of external sources of mevalonate in the flux of acetyl units to cholesterol. In keeping with the well-established effect of mevalonate to reduce HMG CoA reductase activity, [4,5-^{13}C]mevalonate present in the medium comprised more than 90% of the precursor carbon for cholesterol synthesis. ISA indicated that $F_{(mevalonate)}$ was close to 1 (Fig 7A). This analysis indicating the fraction of the precursor pool contributed by mevalonate is a unique feature of ISA allowing a quantitative understanding of its role in cholesterol synthesis. In addition, ISA revealed a mechanism by which 25OHC differs from tamoxifen and progesterone in inhibiting cholesterol synthesis (Fig 7B). Although all three compounds decrease cholesterol synthesis, only 25OHC produced a differential effect for the two labeled substrates in terms of the fraction of new synthesis. This finding indicating that 25OHC acts at least in part to block the flux of acetate to mevalonate, characteristic of a compound reducing the activity of HMG CoA reductase, was revealed only through ISA. Since the total amount of cholesterol was increased by the addition of mevalonate to the medium, ^{14}C incorporation studies perhaps would have revealed a difference in incorporation, but it would not be possible to attribute this difference quantitatively to an inhibition of de novo synthesis without ISA. In summary, the data in Fig 7 demonstrate that tamoxifen and progesterone inhibit cholesterol synthesis by acting at steps beyond HMG CoA reductase.

Tamoxifen also differed from 25OHC in other aspects of cholesterol metabolism. In the presence of 25OHC, free cholesterol was decreased for both rat hepatocytes and HegG2 cells (Fig 6). Tamoxifen, which produced a similar effect on fractional synthesis, g(48 h) (Fig 3), did not cause a decrease in free cholesterol levels. This comparison indicated that cells incubated in the presence of 25OHC do not have the same ability to maintain free cholesterol levels as cells incubated with tamoxifen. One explanation for this difference is that 25OHC is known to increase the rate of cholesterol esterification,[26] leading to a decrease in the level of free cholesterol. No such effect has been reported for tamoxifen. Another difference between the two compounds is that tamoxifen does not affect LDL association in HepG2 cells, whereas 25OHC decreased LDL association (Fig 8). 25OHC is also a well-known inhibitor of LDL uptake in CHO cells, and has been used to elucidate the mechanism by which LDL receptor expression and cholesterol biosynthesis enzymes are simultaneously regulated to control the level of cellular free cholesterol.[27] Since tamoxifen has been shown to be an effective LDL-cholesterol–lowering agent in postmenopausal women,[5] it may be predicted that it should increase, or at least not decrease, the expression of LDL receptors.

While our evidence indicates that tamoxifen does not act by the same mechanism as 25OHC, the actions of tamoxifen are similar to those of progesterone. Both compounds have been shown to inhibit P-glycoprotein transport activity[28] and both cause accumulation of sterol precursors, consistent with inhibition of later steps in the cholesterol synthesis pathway. However, an important finding of our study is that differences in the type and amount of sterol precursor were observed between the two compounds (Fig 9), but these differences may be due to the lesser potency of progesterone as a cholesterol synthesis inhibitor and may not have been observed at a higher concentration of progesterone. Both compounds produce more Δ^{24} sterol intermediates than intermediates derived from dihydrolanosterol. This pattern of sterol accumulation is consistent with inhibition of Δ^{24} reductases. Δ^{24} Reductase inhibition is also attributed to triparanol, an anticholesterolemic drug that also causes cataracts.[29] Cataract formation is a possible side effect of tamoxifen treatment.[11] Inhibition of side-chain double-bond reduction may be part of a general mechanism of many compounds that inhibit cholesterol synthesis. The ability to observe sterol precursors and to identify those derived from lanosterol and dihydrolanosterol is one of the advantages of GC-MS over other forms of detection.

The effect of tamoxifen on cholesterol synthesis does not appear to be mediated via the classic estradiol receptor mechanism. Incubation of tamoxifen and estradiol together did not diminish the effect of tamoxifen on cholesterol synthesis (data not shown). Furthermore, the antiestrogen ICI 182,780, which binds the receptor but has no estrogen agonist effect,[30] did not affect cholesterol synthesis (Fig 3). The finding that tamoxifen does not act by a receptor-mediated mechanism to reduce cholesterol synthesis indicates that this effect of tamoxifen is not related to the mechanism by which estradiol promotes low LDL-cholesterol levels. In summary, our data indicate that tamoxifen inhibits de novo cholesterol synthesis in HepG2 and rat liver cells by acting at a step beyond HMG CoA reductase. The GC-MS analysis of intermediates indicates an accumulation of zymosterol and supports the hypothesis that tamoxifen inhibits Δ^{24} reductase. We suggest that this inhibition of Δ^{24} reductase is related to the observation that tamoxifen administration decreases LDL-cholesterol in postmenopausal breast cancer patients.

REFERENCES

1. Kellen JA: Introduction: The enigma of tamoxifen, in Kellen JA (ed): Tamoxifen: Beyond the Antiestrogen. Boston, MA, Birkhauser, 1996, pp 1-23

2. Bagdade JD, Wolter J, Subbaiah PV, et al: Effects of tamoxifen treatment on plasma lipids and lipoprotein lipid composition. J Clin Endocrinol Metab 70:1132-1135, 1990

3. Love RR, Newcomb PA, Wiebe DA, et al: Effects of tamoxifen therapy on lipid and lipoprotein levels in postmenopausal patients with node-negative breast cancer. J Natl Cancer Inst 82:1327-1332, 1990

4. Love RR, Wiebe DA, Newcomb PA, et al: Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann Intern Med 115:860-864, 1991

5. Love RR, Wiebe DA, Feyzi JM, et al: Effects of tamoxifen on cardiovascular risk factors in postmenopausal women after 5 years of treatment. J Natl Cancer Inst 86:1534-1539, 1994

6. Ellsworth JI, Chandrasekaran C, Cooper AD: Evidence for sterol-independent regulation of low-density lipoprotein receptor activity in Hep-G2 cells. Biochem J 279:175-187, 1991

7. Thangaraju M, Kumar K, Gandhirajan R, et al: Effect of tamoxifen on plasma lipids and lipoproteins in postmenopausal women with breast cancer. Cancer 73:659-663, 1994

8. Dnistrian AM, Schwatz MK, Greenberg EJ, et al: Effect of tamoxifen on serum cholesterol and lipoproteins during chemohormonal therapy. Clin Chim Acta 223:43-52, 1993

9. Wiseman H, Cannon M, Arnstein HRV, et al: Enhancement by tamoxifen of the membrane antioxidant action of the yeast membrane sterol ergosterol: Relevance to the antiyeast and anticancer action of tamoxifen. Biochim Biophys Acta 1181:201-206, 1993

10. Anker G, Lonning PE, Ueland PM, et al: Plasma levels of the atherogenic amino acid homocysteine in post-menopausal women with breast cancer treated with tamoxifen. Int J Cancer 60:365-368, 1995

11. Gylling H, Pyrhonen S, Mantyla E, et al: Tamoxifen and toremifene lower serum cholesterol by inhibition of Δ^8-cholestenol conversion to lathosterol in women with breast cancer. J Clin Oncol 13:2900-2905, 1995

12. Cypriani B, Tabacik C, Descomps B: Effect of estradiol and antiestrogens on cholesterol biosynthesis in hormone-dependent and -independent breast cancer cell lines. Biochim Biophys Acta 972:167-178, 1988

13. Brown MS, Goldstein JL: The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89:331-340, 1997

14. Wakeling AE, Dukes M, Bowler J: A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867-3873, 1991

15. Panini SR, Gupta A, Sexton RC, et al: Regulation of sterol biosynthesis and 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in cultured cells by progesterone. J Biol Chem 262:14435-14440, 1987

16. Metherall JE, Waugh K, Li H: Progesterone inhibits cholesterol biosynthesis in cultured cells. J Biol Chem 271:2627-2633, 1996

17. Butler JD, Blanchette-Mackie J, Goldin E, et al: Progesterone blocks cholesterol translocation from lysosomes. J Biol Chem 267: 23797-23805, 1992

18. Metherall JE, Li H, Waugh K: Role of multidrug resistance P-glycoproteins in cholesterol biosynthesis. J Biol Chem 271:2634-2640, 1996

19. Lange Y: Cholesterol movement from plasma membrane to rough endoplasmic reticulum. J Biol Chem 269:3411-3414, 1994

20. Kelleher JK, Kharroubi AT, Aldaghlas TA, et al: Isotopomer spectral analysis of cholesterol synthesis: Applications in human hepatoma cells. Am J Physiol 266:E384-E395, 1994 (suppl)

21. Berry MN, Friend DS: High yield preparation of isolated rat liver parenchymal cells. J Biol Chem 43:506-520, 1969

22. Kharroubi AT, Masterson TM, Aldaghlas TA, et al: Isotopomer spectral analysis of triglyceride fatty acid synthesis in 3T3-L1 cells. Am J Physiol 263:E667-E675, 1992 (suppl)

23. Brooks CJW, Horning EC, Young JS: Characterization of sterols by gas chromatography–mass spectrometry of the trimethylsilyl ethers. Lipids 3:391-402, 1968

24. Gerst N, Ruan B, Pang J, et al: An updated look at the analysis of unsaturated C_{27} sterols by gas chromatography and mass spectrometry. J Lipid Res 38:1685-1701, 1997

25. Stephan ZF, Yurachek EC: Rapid fluorometric assay of LDL receptor activity by Dil-labeled LDL. J Lipid Res 34:325-330, 1993

26. Lange Y, Steck TL: Cholesterol homeostasis. Modulation by amphiphiles. J Biol Chem 269:29371-29374, 1994

27. Wang X, Sato R, Brown MS, et al: SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell 77:53-62, 1994

28. Field FJ, Born E, Chen H, et al: Esterification of plasma membrane cholesterol and triacylglycerol-rich lipoprotein secretion in CaCo-2 cells: Possible role of p-glycoprotein. J Lipid Res 36:1533-1543, 1995

29. Laughlin RC, Carey TF: Cataracts in patients treated with triparanol. JAMA 181:339-340, 1962

30. Osborne CK, Coronado-Heinsohn EB, Hisenbeck SG, et al: Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. J Natl Cancer Inst 87:746-750, 1995