Inhibition of Human Cytochrome P450 3A4 by Cholesterol

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Cholesterol has been shown to be hydroxylated at the 4β-position by cytochrome P450 (P450) 3A4, and the reaction occurs in vivo (Boding, K. et al., J. Biol. Chem. 277, 31534-31540, 2002). If cholesterol is a substrate of P450 3A4 then it follows that it should also be an inhibitor, particularly in light of the high concentrations found in liver. Heme perturbation spectra indicated a Kd of 8 µM for the P450 3A4-cholesterol complex. Cholesterol inhibited the P450 3A4-catalyzed oxidations of nifedipine and quinidine, two prototypic substrates, in liver microsomes and a reconstituted enzyme system with Ki ~ 10 µM, in an apparently non-competitive manner. The concentration of cholesterol could be elevated 4- to 6-fold in cultured human hepatocytes by incubation with cholesterol; the level of P450 3A4 and cell viability were not altered under the conditions used. Nifedipine oxidation was inhibited when the cholesterol level was increased. We conclude that cholesterol is both a substrate and inhibitor of P450 3A4, and a model is presented to explain the kinetic behavior. We propose that the endogenous cholesterol in hepatocytes should be considered in models of prediction of metabolism of drugs and steroids, even in the absence of changes in the concentrations of free cholesterol.

P450 enzymes are nearly ubiquitous in nature. These hemoproteins are involved in the metabolism of numerous steroids, drugs, carcinogens, and other substrates in mammals and, in addition, in many pathways of secondary metabolism and defense in plants and microorganisms (1). Humans have 57 “CYP” genes potentially coding for P450s (2). One of these, P450 3A4, is involved in the metabolism of ~½ of the drugs marketed today (of those known to be oxidized) (2-4). In addition to drugs, this enzyme also has roles in both the bioactivation and detoxication of chemical carcinogens, e.g. aflatoxin B1 (2, 5, 6). P450 3A4 also has relatively high catalytic activities in the oxidation of a number of steroids, e.g. testosterone, progesterone, and 17β-estradiol (7, 8). More recently P450 3A4 has been shown to catalyze the 4β-hydroxylation of cholesterol (9, 10) and the product has been proposed to be an in vivo biomarker of the activity of P450 3A4 (and the related protein P450 3A5, which is generally expressed at lower levels) (11). P450 3A4 has been described as displaying rather schizophrenic behavior towards some ligands, which has been difficult to understand (2). Evidence for cooperativity—both homotropic and heterotropic—was first described in 1993-1994 (12, 13). Subsequently considerable work has provided insight into the phenomenon, although not all aspects have been clarified (reviewed in (2, 14)). A general conclusion is that multiple ligands can be accommodated and that this multiplicity somehow accounts for the cooperative behavior that is seen, e.g. due to forcing substrates into juxtapositions more (or less) conducive for catalysis. This multiple ligand hypothesis is supported by several kinetic studies (13, 15-17) and by fluorescence measurements (18). The binding of testosterone is a relatively slow process and is best described in terms of two (or more?) molecules of testosterone, with a conformational change in the protein (19, 20). X-ray crystallographic studies have provided some evidence for a multi-ligand model of P450 3A4, with some structures indicating progesterone and testosterone bound at a peripheral site (21, 22) and...
two molecules of ketoconazole in a P450 3A4 crystal (23).

Ligand competition is another issue with P450 3A4 and is the basis of many of the drug-drug interaction issues in pharmaceutical development (24). Another consideration, which has received less discussion, is competition between drugs and endogenous compounds, e.g. sterols. Testosterone, progesterone, and estrogens are known substrates (7, 8) but are generally present at only low concentrations in the tissues where P450 3A4 is highly expressed, i.e. liver and small intestine. However, cholesterol is present at high concentrations in liver. Even considering the low solubility of cholesterol and equilibrium with bound forms, the high concentration of cholesterol in the liver is a potential issue.

In consideration of the demonstrated 4β-hydroxylation of cholesterol by P450 3A4 (9, 10), if cholesterol is a substrate of P450 3A4 then it follows that it should also be an inhibitor, which may be an issue in the context of the afore mentioned high concentrations of cholesterol in the liver (and brain). We considered this issue and have described the in vitro kinetics, which appear to follow non-competitive behavior but can be considered in other paradigms presented by Segel (25). The level of cholesterol in cultured human hepatocytes could be elevated by treatment with cholesterol. Elevation of the cholesterol level led to attenuated nifedipine oxidation activity, a marker for P450 3A4 (7). We conclude that consideration should be made for cholesterol inhibition of P450 3A4 in extrapolation to in vivo situations, analogous to binding of drugs and steroids to plasma proteins.

Experimental Procedures

Chemicals—Cholesterol, 17α-ethynylestradiol, dansyl chloride, 4,4-dimethylaminopyridine, 2-hydroxypropyl-β-cyclodextrin (HPβCD), miconazole, nifedipine, quinidine, L-α-1,2-diloleoyl-sn-glycero-3-phosphocholine, L-α-1,2-dilauroyl-sn-glycero-3-phosphoserine, and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO), and L-α-1,2-dilauroyl-sn-glycero-3-phosphocholine was purchased from ENZO Life Sciences International (Plymouth Meeting, PA). Nifedipine was recrystallized from hot C₂H₅OH in amber glass. 4β- and 7α-OH cholesterol were purchased from Steraloids (Newport, RI). 3S-Hydroxyquinidine and quinidine N-oxide were obtained from Toronto Research Chemicals (North York, ON, Canada). Quinine was purchased from the New York Quinine and Chemical Works (New York City, NY). HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA).

Oxidized nifedipine (pyridine derivative product) was synthesized by HNO₃ oxidation as described elsewhere (26).

Enzyme Preparations—Recombinant P450 3A4 with a C-terminal (His)₅ tag (27) was expressed in Escherichia coli and purified as described elsewhere (16). Rat NADPH-P450 reductase (28) and human cytochrome b₅ (29) were expressed in E. coli and purified as described elsewhere. Human liver samples (for preparation of microsomes) were from organ donors and obtained through Tennessee Donor Services (Nashville, TN) in accord with Vanderbilt Institution Review Board policies.

MS and Spectroscopy—A ThermoFinnigan TSQ Quantum mass spectrometer (ThermoFinnigan, Sunnyvale, CA) connected to a Waters Acquity UPLC system (Waters, Milford, MA) was used for quantitation of dansylated compounds.

UV-visible spectra were recorded with an Aminco DW2/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA).

Measurement of Cholesterol 4β-Hydroxylation Activity with P450 3A4 Reconstituted System—A “5× P450 premix” was prepared with slight modification (30). Briefly, a 5× protein premix including 2.5 µM P450 3A4, 5 µM NADPH-P450 reductase, 2.5 µM cytochrome b₅, 150 µg/ml phospholipid mixture (L-α-1,2-diloleoyl-sn-glycero-3-phosphocholine:L-α-1,2-dilauroyl-sn-glycero-3-phosphoserine:L-α-1,2-dilauroyl-sn-glycero-3-phosphocholine, 1:1:1, w/w/w), 0.81 mM sodium CHAPS (0.50 mg/ml), and 3.0 mM GSH in 50 mM potassium HEPES buffer (pH 7.4), and a 5× buffer mix including 12 mM GSH and 150 mM MgCl₂ in 200 mM potassium HEPES buffer (pH
7.4) were prepared. A typical 500-µl enzyme reaction mixture was prepared by mixing 100 µl of 5× P450 3A4 protein premix, an equal volume of 5× buffer mix, 5 µl of cholesterol solution in 310 mM HPβCD (45% HPβCD (w/v)), and 220 µl of H2O. Incubations were initiated by the addition of 75 µl of an NADPH-generating system (31). Reactions were quenched with 2.0 ml of CH2Cl2 including 150 pmol of 17α-ethynylestradiol (as the internal standard) and mixed with a vortex device. Following centrifugation (2 × 10³ g, 5 min), 1.4 ml of the organic layer was transferred and taken to dryness under an N2 stream. A general method for derivatization with dansyl chloride was used with slight modification (32). Briefly, the samples were dissolved in 200 µl of CH2Cl2 containing 2 mg of dansyl chloride, 0.5 mg of 4,4-dimethylaminopyridine, and 2 µl of triethylamine and incubated at 65°C for 1 h. The samples were dried under an N2 stream and then dissolved in 100 µl of CH3CN for analysis.

Dansylated products were analyzed with LC-MS on a UPLC system connected to a TSQ Quantum mass spectrometer using a Hypersil GOLD octadecylsilane column (2.1 mm × 150 mm, 3 µm, Thermo Scientific). LC conditions were as follows: Solvent A contained 95% H2O, 5% CH3CN, and 0.1% HCO2H (v/v) and solvent B contained 5% H2O, 85% CH3CN, 10% tert-butyl methyl ether, and 0.1% HCO2H (v/v). The column was maintained at the initial condition of 50% B (v/v) for 0.5 min with a flow rate of 350 µl/min, followed by a linear gradient increasing to 100% B (v/v) over 2.0 min. This condition was maintained for 7.5 min and then returned to the initial condition over 0.05 min and maintained until the end of a 13-min run. The column temperature was maintained at 40°C. The injection volume onto the column was 15 µl. MS analyses were performed in the positive ion electrospray mode. Quantitation was based on MRM (dansylated 4β-OH cholesterol: m/z 636 → 252, collision energy, 22 V; dansylated 17α-ethynylestradiol: m/z 530 → 171, collision energy, 40 V). The following (optimized) parameters were used for the detection of the analyte and the internal standard: N2 sheath gas, 27 psi; N2 auxiliary gas, 21 psi; spray voltage, 5.0 kV; capillary temperature, 270°C; capillary offset, 35 V; tube lens voltage, 220 V; Ar collision gas, 1.5 mTorr; scan time, 50 ms; Q3 scan width, 1 m/z; Q1/Q3 peak widths at half-maximum, 0.7 m/z. The data were collected and quantified using Thermo-Finnigan Xcalibur version 1.0 software.

**Measurement of Cholesterol 4β-Hydroxylation Activity with Human Liver Microsomes**—The amount of cholesterol in a set of 10 pooled human liver microsomes (31) (7 males and 3 females) was first quantified as described elsewhere (33). Aliquots of 10 pooled human liver microsomal samples, including exactly 25 nmol of cholesterol, were added to 1.0 ml of reaction mixture containing different amounts of cholesterol (0-225 nmol) and 3.1 mM HPβCD in 50 mM potassium phosphate buffer (pH 7.4). The assays were conducted at 37°C for 30 min. Incubations were initiated by the addition of an NADPH-generating system (31). Following extraction, the derivatization and quantitation steps were conducted as described above.

**Inhibition Studies with Human Liver Microsomes and a P450 3A4 Reconstituted System**—Reaction mixtures with human liver microsomes included 0.10 mg/ml microsomal protein, 5.8 µM endogenous cholesterol, 0-60 µM exogenous cholesterol, and 3.1 mM HPβCD in 100 mM potassium phosphate buffer (pH 7.4). The assays were conducted at 37°C for 20 min and 20 min for quinidine, respectively. Reaction mixtures with recombinant P450 3A4 included 0.10 µM P450 3A4, 0.20 µM NADPH-P450 reductase, 0.10 µM cytochrome bs, 40 µg/ml phospholipid mixture, 0.1 mg/ml of CHAPS, 3.0 mM GSH, and 30 mM MgCl2, 0-60 µM of cholesterol, and 3.1 mM HPβCD in 50 mM potassium HEPES buffer (pH 7.4). Incubation times were 10 min (nifedipine) and 5 min (quinidine). A typical volume of enzyme reaction mixture was 500 µl, and the substrate concentrations ranged from 5 to 200 µM (nifedipine) and 10 to 300 µM (quinidine). After a 3 min preincubation, enzyme reactions were initiated by addition of 75 µl of an NADPH-generating system (31). The nifedipine reactions were terminated by addition of 200 µl of CH3CN including 20 µM miconazole (as the internal standard), and the quinidine reactions were terminated by addition of 20 µl of 5% CF3CO2H (v/v) and 2 µl of a 100 µM...
quinine solution in C$_2$H$_5$OH (as the internal standard). Samples were centrifuged at $1.2 \times 10^4 \times g$ for 10 min, and the supernatants were collected for LC analyses.

Samples for nifedipine oxidation studies were analyzed with a UPLC system connected to an Acquity UV detector using an Acquity UPLC BEH C18 octadecylsilane column (2.1 mm $\times$ 50 mm, 1.7 µm, Waters). LC conditions were as follows: Solvent A contained 45% H$_2$O and 55% CH$_3$OH (v/v) and solvent B was 100% CH$_3$OH (v/v). The column was maintained at an initial condition of 0% B (v/v) for 2 min with a flow rate of 500 µl/min, followed by a linear gradient increasing to 100% B (v/v) over 1.5 min. This condition was maintained for 1.5 min and then returned to the initial condition over 0.1 min and maintained until the end of a 7-min run. The column temperature was maintained at 40 °C. The injection volume was 15 µl. Absorbance was monitored at 269 nm (oxidized nifedipine) and 230 nm (miconazole).

Quinidine oxidation samples were analyzed with a UPLC system connected to an Acquity fluorescence detector using an Acquity UPLC BEH C18 octadecylsilane column (2.1 mm $\times$ 50 mm, 1.7 µm, Waters). LC conditions were as follows: Solvent A contained 90% H$_2$O, 10% CH$_3$CN, and 0.1% CF$_3$CO$_2$H (v/v) and solvent B contained 10% H$_2$O, 90% CH$_3$CN, and 0.1% CF$_3$CO$_2$H (v/v). The column was maintained at the initial condition of 0% B (v/v) for 0.5 min with a flow rate of 350 µl/min, followed by a linear gradient increasing to 5% B (v/v) over 3 min. After that, the percentage of B was increased with a linear gradient to 100% over 1 min. This condition was maintained for 1.5 min and then returned to the initial condition over 0.1 min and maintained until the end of a 7.7-min run. The column temperature was maintained at 40 °C. The injection volume was 7.5 µl. Fluorescence measurements were made using an excitation wavelength of 360 nm and an emission wavelength of 415 nm.

The data collection and quantitative analysis were conducted with Waters MassLynx version 4.1 and QuanLinx version 4.1 software, respectively.

**Hepatocyte Incubations**—Cryo-preserved human hepatocytes (lots Hu1126 and Hu4242) were purchased from Invitrogen Corp. (Carlsbad, CA), stored frozen in liquid N$_2$ until use, and thawed according to the supplier’s recommended procedure. The cell number and viability were assessed using a Trypan Blue exclusion test. Cells were seeded at $0.9 \times 10^5$ cells/well in collagen Type #1 precoated 96-well plates in culture medium composed of William’s E medium including 10% FBS (v/v). The cells were maintained in a humidified incubator at 37 °C (5% CO$_2$, v/v) for 24 h. The medium was changed to FBS-free Hepato STIM medium (BD Biosciences, Bedford, MA) including 200 µM cholesterol (0.31 mM HPβCD) or 30 µM pravastatin to modify the cholesterol levels in the cells. After 24 h, the leakage of lactate dehydrogenase was measured with a Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Indianapolis, IN). The cells were washed twice with 100 µl of pre-warmed PBS buffer and P450 3A4 activities were measured using 200 µM nifedipine (in FBS-free Hepato STIM medium) with 30 min incubation. The media were collected and stored at -20 °C until analysis.

**Quantitation of Nifedipine Product in Media**—The media (100 µl, following incubation with nifedipine) was mixed with 100 µl of CH$_3$CN containing 1 µM miconazole (as the internal standard). Samples were centrifuged at $1.2 \times 10^4 \times g$ for 10 min, and the supernatants were collected and analyzed with LC-MS on a UPLC system connected to a TSQ Quantum mass spectrometer using an L-column2 ODS (2.1 mm $\times$ 150 mm, 5 µm, Chemicals Evaluation and Research Institute, Saitama, Japan). LC conditions were as follows: Solvent A contained 95% H$_2$O, 5% CH$_3$CN, and 0.1% HCO$_2$H (v/v) and solvent B contained 5% H$_2$O, 95% CH$_3$CN, and 0.1% HCO$_2$H (v/v). The column was maintained at the initial condition of 20% B (v/v) for 1.0 min with a flow rate of 350 µl/min, followed by a linear gradient increasing to 100% B (v/v) over 3.5 min. This condition was maintained for 2.0 min and then returned to the initial condition over 0.1 min and maintained until the end of a 9-min run. The column temperature was maintained at 40 °C. The injection volume onto the column was 10 µl. MS analyses were
Quantitation was based on MRM (oxidized nifedipine: m/z 345 → 284, collision energy, 26 V; miconazole: m/z 415 → 159, collision energy, 32 V). The following (optimized) parameters were used for the detection of the analyte and the internal standard: N₂ sheath gas, 40 psi; N₂ auxiliary gas, 15 psi; spray voltage, 5.0 kV; capillary temperature, 270 °C; capillary offset, 12 V; tube lens voltage, 146 V; Ar collision gas, 1.9 mTorr; scan time, 50 ms; Q3 scan width, 1 m/z; Q1/Q3 peak widths at half-maximum, 0.7 m/z. Data were collected and quantified using Thermo-Finnigan Xcalibur version 1.0 software.

Quantitation of Cholesterol in Hepatocytes—After treatment with 200 µM cholesterol or 30 µM pravastatin, cells were washed twice with 100 µl of pre-warmed PBS buffer (pH 7.4) and collected with 200 µl of 0.25% (w/v) trypsin solution to separate the cell solutions with 2.0 ml of CH₂Cl₂ including 500 pmol of 7α-OH cholesterol (as the internal standard) and mixed with a vortex device. Following derivatization and quantitation, steps were conducted as reported previously (33).

Immunooquantitation of P450 3A4 in Hepatocytes—After the treatment with 200 µM cholesterol or 30 µM pravastatin, the cells were washed twice with 100 µl of pre-warmed PBS buffer and the proteins in the cells were collected with TRIZOL® Reagent (Invitrogen). The protein concentration was determined by a bicinchoninic acid assay (BCA, Pierce, Rockford, IL). The proteins extracted from hepatocytes (2-2.5 µg of protein per well, in triplicate) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis ((31, 34), 7.5%, w/v), along with standard samples varying amounts of purified P450 3A4 (0-100 fmol) per well. The proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (BioRad, Hercules, CA). A primary rabbit serum including polyclonal antibodies raised against human P450 3A4 (35) was used (125-fold dilution in PBS). The secondary antibody was goat anti-rabbit IRD800CW, which emits infrared light at 800 nm (detected using an Odyssey Li-Cor instrument (Li-Cor, Lincoln, NE)). The slope of the standard curve (based on the spiked samples) was used to quantify the amount of P450 3A4 in proteins extracted from hepatocytes.

Kinetic Analyses—The kinetic parameters were calculated from untransformed data using GraphPad Prism (GraphPad, San Diego, CA). The models used in this study were as follows:

\[
v = \left\{ \frac{V_{max}}{1 + [I]} \right\} \times [S] \times \left( \frac{K_m + [S]}{K_m} \right) \quad (Eq. 1)
\]

\[
v = \left\{ \frac{V_{max}}{1 + [I]^h / K_i^h} \right\} \times [S] \times \left( \frac{K_m + [S]}{K_m} \right) \quad (Eq. 2)
\]

where \(V_{max}\) is the maximal formation rate (\(k_{cat}\) was used with purified P450 3A4), \([I]\) is the inhibitor concentration, \(K_i\) is the inhibitor constant, \([S]\) is the substrate concentration, \(K_m\) is the Michaelis constant, and \(h\) is a Hill coefficient. The Hill constant-incorporated non-competitive inhibition model (Eq. 2) was applied for the analysis of nifedipine metabolism by human liver microsomes, and the usual non-competitive inhibition model (Eq. 1) was applied for the rest of the analyses.

Results

Cholesterol 4β-Hydroxylation Activity with Human Liver Microsomes and a P450 3A4 Reconstituted System—The cholesterol 4β-hydroxylation activity of P450 3A4 was measured in the presence of 3.1 mM HPβCD. Cholesterol binding to HPβCD has been reported with a \(K_d\) value of 1.1 mM (36). Free cholesterol concentrations were calculated using the \(K_d\) value of the cholesterol-HPβCD complex and a simple 2-state binding model (i.e. cholesterol + HPβCD ⇌ cholesterol•HPβCD). These free cholesterol concentrations were used for further kinetic analyses.

The calculated \(V_{max}\) and \(K_m\) values in human liver microsomes were 1.0 ± 0.2 pmol/min/mg protein and 27 ± 9 µM, respectively (Fig. 1A). The calculated \(k_{cat}\) and \(K_m\) values for the reconstituted system were 2.2 ± 0.2 pmol/min/nmol P450 and 6.4 ± 2.1 µM, respectively (Fig. 1B), yielding an estimated catalytic efficiency of \(3.4 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}\) (6 M⁻¹ s⁻¹).
Cholesterol Binding to P450 3A4—
Binding of cholesterol to P450 3A4 induced a substrate-type (“Type I”) difference spectrum (37) (Fig. 2, inset). The calculated $\Delta A\text{max}/\text{nmol} \text{ P450}$ and $K_d$ values (hyperbolic binding model) were $0.005 \pm 0.001$ and $8.3 \pm 1.0 \mu M$, respectively (Fig. 2).

Inhibitory Effect of Cholesterol towards P450 3A4 Activity in Human Liver Microsomes and Reconstituted System—Lineweaver-Burk plots for nifidipine metabolism in human liver microsomes (with varying cholesterol concentrations) indicated that the inhibition type of cholesterol was apparently non-competitive (supplemental Fig. S1). However, the plots of nifidipine concentrations vs. rates of nifidipine oxidation in human liver microsomes could not be fit well to the basic non-competitive inhibition model (Eq. 1). Instead of this model, the non-competitive model including an exponent (Hill coefficient) was used (Eq. 2). This model provided a good fit to the curve, and the calculated $K_i$ value and the Hill coefficient were $11.4 \pm 0.3 \mu M$ and $2.8 \pm 0.2$, respectively (Fig. 3A, Table 1). On the other hand, plots of nifidipine concentrations vs. rates of nifidipine oxidation in the P450 3A4 reconstituted system could be fit well to the basic non-competitive inhibition model (Eq. 1), with a $K_i$ value of $9.2 \pm 0.3 \mu M$ (Fig. 4A, Table 1, supplemental Fig. S2).

Cholesterol showed apparent non-competitive inhibition with quinidine 3S-hydroxylation activity in both human liver microsomes and reconstituted system, and the $K_i$ values were calculated as $11.1 \pm 1.0 \mu M$ and $9.1 \pm 0.6 \mu M$, respectively, with good fitting to the basic non-competitive inhibition model (Eq. 1) (Figs. 3B and 4B, Table 1, supplemental Figs. S1 and S2).

The inhibition of quinidine N-oxidation by cholesterol in both enzyme sources was also apparently non-competitive, and the $K_i$ values calculated with the basic non-competitive inhibition model (Eq. 1) were $9.2 \pm 0.7 \mu M$ and $15.0 \pm 0.8 \mu M$, respectively (Figs. 3C and 4C, Table 1, supplemental Figs. S1 and S2).

Effect of Cholesterol on P450 3A4 Activity in Hepatocytes—Cholesterol concentrations were quantified in a preliminary study in hepatocytes (lot Hu1126) following treatment with 200 $\mu M$ cholesterol or 30 $\mu M$ pravastatin (supplemental Fig. S3). The cholesterol amount in the cells treated with 200 $\mu M$ cholesterol was increased up to 48 h, and that in the cells treated with 30 $\mu M$ pravastatin was decreased by up to 30% after 24 and 48 h. However, lactate dehydrogenase leakage from the cells after 48 h treatment with either cholesterol or pravastatin was increased, although those after 24 h treatment were not changed (data not shown). These results indicated that 24 h treatment with cholesterol or pravastatin could modify the cholesterol amount in the cells without causing cytotoxicity. The P450 3A4 contents in human hepatocytes (lots Hu1126 and Hu4242) after 24 h treatment with 200 $\mu M$ cholesterol (or 30 $\mu M$ pravastatin) were not significantly changed compared with those in the cells without any treatment (Table 2).

The nifedipine oxidation activities of human hepatocytes were measured after 24 h treatment with 200 $\mu M$ cholesterol. Nifedipine oxidation activities in the cells (lot Hu1126) treated with 200 $\mu M$ cholesterol were significantly decreased (59%), compared with the activities of no treatment cells (Fig. 5A). Nifedipine oxidation activity in the lot Hu4242 cells treated with 200 $\mu M$ cholesterol was significantly decreased (by 63%) (Fig. 5B).

Discussion

The purpose of this study was to determine the inhibitory effects of cholesterol on P450 3A4, the major human P450 in liver and small intestine, in light of reports that cholesterol is a substrate for 4β-hydroxylation by P450 3A4 (9-11) (Fig. 1). Cholesterol was shown to bind to P450 3A4 ($K_a$ 8 $\mu M$) (Fig. 2) and to inhibit prototypic P450 3A4 reactions with a $K_i$ of $\sim \ 10$ $\mu M$ (Figs. 3 and 4, Table 1). These results, considered in the context of the known concentration of cholesterol in liver, suggest that cholesterol inhibition occurs in cells. Raising the level of cholesterol in cultured human hepatocytes resulted in the inhibition of nifedipine oxidation by P450 3A4 (Fig. 5).

Cholesterol inhibition studies were done with human liver microsomes and a purified enzyme system (reconstituted with NADPH-P450...
reductase and cytochrome $b_5$). Steroids (e.g., testosterone) and other hydrophobic substrates could not be utilized as substrates in these assays because they are bound to HPβCD, resulting in the inhibition of hydroxylation activity in the absence of cholesterol. Therefore we used the drug nifedipine, the first P450 3A4 substrate identified (7). HPβCD did not inhibit nifedipine or quinidine oxidation activity. The inhibition of these activities by cholesterol appeared to be non-competitive, and the data were fit to a classic model (Eq. 1) to obtain $K_i$ values (Table 1). In the case of the results obtained with liver microsomes (Fig. 3, supplemental Fig. S1) a poor fit was obtained but could be improved by the inclusion of an exponent (Hill factor) (Eq. 2). However, we do not have a specific physical interpretation of this factor for inhibition, as in the case of Hill coefficients applied to cooperative phenomena observed with P450 3A4 substrates (2, 15, 38).

Although the simplistic view of non-competitive inhibition is that the inhibitor is interacting at a site distinct from that of the substrate (39), a number of possibilities exist (25) (Fig. 6), particularly in a case in which there is extensive evidence for the presence of multiple ligands in a large active site (15, 16, 23, 40). In the model shown in Fig. 6, the substrate is nifedipine or quinidine (S). Cholesterol is the inhibitor (I) but it is also a substrate (the EI’ complex). However, in the configurations of EI and ESI, cholesterol (I) can block the oxidation of other substrates without being in the “S” site. This model comes from the classic text of Segel (41) and can explain the results observed here. Further, the proposed proclivity for multiple modes of binding can explain the relatively weak absorbance changes observed when cholesterol binds to P450 (Fig. 2), in that only a small population of the modes of binding could result in $\Delta H_2O$ displacement (as an iron ligand) and spin-state shift. The multiplicity of configurations would also be an issue contributing to the low rate of catalysis, i.e. 4$\beta$-hydroxylation (Fig. 1).

As in the general case of prediction of drug-drug interactions involving P450s and other enzymes, the $K_i$ can be compared with the estimated free concentration of the inhibitor. A typical concentration of total cholesterol in human serum is $\sim$ 5 mM (i.e., 20 mg/ml or 200 mg/dl). One estimate of the amount of unesterified cholesterol in human liver is 840 $\mu$mol, based only on the concentration of unesterified cholesterol in human liver microsomes (~56 nmol/mg protein (33)) multiplied by a typical recovery factor of 10 mg microsomal protein/g liver (42). For a liver (of 1.5 kg), this would give an “average” concentration of 560 $\mu$M in the liver. Our own work with human liver microsomes (mean value of 56 pmol unesterified cholesterol/mg microsomal protein, or $\sim$100 nmol free (unesterified) cholesterol/nmol (total) P450. Some of the cholesterol is bound to lipoprotein complexes, so the exact concentration of free cholesterol is not precisely known. Another approach to the estimation is to consider the solubility of cholesterol in buffer (5.2 $\mu$M (43)), although this value may not be appropriate in considering the overall equilibrium among various proteins. However, using a conservative value of 5 $\mu$M for an “effective” cholesterol concentration and a $K_i$ of 10 $\mu$M, Eq. 1 would predict an increase in the “area under the curve” (integral) for an in vivo pharmacokinetic profile of 50% (i.e. 50% more exposure of the tissues to drug). If cholesterol were treated as an experimental drug candidate in development, this degree of inhibition would suggest caution. It is possible that the effective concentration of free cholesterol P450 3A4 encounters is higher, given the ratio of 100 nmol unesterified cholesterol/nmol P450 in liver microsomal membranes. To put this in perspective, the decrease in nifedipine oxidation activity observed in the hepatocytes was $\sim$ 60% (Fig. 5) after raising the cholesterol concentration. However, this experiment does not directly address the question of how much inhibition was produced by the basal level of cholesterol in the hepatocytes.

Use of an animal model to test the hypothesis that cholesterol inhibits P450 3A4 function in cells would not be trivial in that P450 3A4 is not present in mice and mice contain seven Subfamily 3A P450s, thus requiring a complex transgenic model. Accordingly we designed experiments with cultured human hepatocytes and modulated the level of cholesterol in the cells. We considered reducing the level of cholesterol in the hepatocytes with the use of statin inhibitors of 3-hydroxymethylglutaryl CoA reductase, but many of the statins are P450 3A4 substrates themselves.
and could not be used (44). Pravastatin is not a P450 3A4 substrate, but we were only able to achieve an attenuation of ~ 30% of the cholesterol level with this statin in the cells (supplemental Fig. S3), which is very significant clinically but in subsequent experiments was not sufficient to modify rates of nifedipine oxidation (at least at the level of statistical significance, results not presented). Cholesterol levels in the hepatocytes could be elevated 4- to 6-fold by incubation with 200 µM cholesterol, concordant with the knowledge that hepatocytes are known to have active cholesterol uptake transporters (45).

Three individual lots of cultured human hepatocytes were used in this work. In one set, the viability decreased during the course of the experiment and was reversed by the presence of the added cholesterol, thus confounding any interpretation of results (not presented). In the other two sets of experiments, nifedipine oxidation (a P450 3A4 marker (7)) was significantly decreased when the level of cholesterol increased (Fig. 5). Nifedipine oxidation is considered a classic and reliable marker of P450 3A4 (7); we have not extended these studies to other substrates.

In summary, we evaluated the inhibitory effect of cholesterol towards P450 3A4 activities in human liver microsomes, a reconstituted system, and human hepatocytes. Cholesterol showed apparent non-competitive inhibition towards three oxidation reaction catalyzed by P450 3A4, with \( K_i \) values comparable to that of the clinically established typical P450 3A4 inhibitor verapamil (46). The increased cholesterol could cause attenuation of P450 3A4 activity in hepatocytes (Fig. 5), indicating that cholesterol in human liver could affect the metabolism of drugs and other compounds by P450 3A4. This conclusion is relevant to the basal level of cholesterol as well as elevated levels.

**REFERENCES**

FOOTNOTES

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1-S3.

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2 The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FBS, fetal bovine serum; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPβCD, 2-hydroxypropyl-β-cyclodextrin; LC, liquid chromatography; MS, mass spectrometry; MRM, multiple reaction monitoring; OH, hydroxyl; P450, cytochrome P450; PBS, phosphate-buffered saline (10 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl).

FIGURE LEGENDS

FIGURE 1. Rates of cholesterol 4β-hydroxylation by human liver microsomes and recombinant P450 3A4. The resulting data points were fit to the Michaelis-Menten equation (GraphPad Prism software). A, microsomes, $K_m = 27 \pm 9 \mu M$, $V_{max} = 1.0 \pm 0.2$ pmol/min/mg protein; B, recombinant P450 3A4, $K_m = 6.4 \pm 2.1 \mu M$, $k_{cat} = 2.2 \pm 0.2$ pmol/min/nmol P450.

FIGURE 2. Binding of cholesterol to P450 3A4. Titration of 2.0 µM P450 3A4 was done with increasing concentrations of cholesterol (in the presence of 3.1 mM HPβCD). The plot (—) is a fit of the changes in absorbance (●) vs. the calculated free cholesterol concentration using a hyperbolic binding equation (GraphPad Prism software). An inset shows the spectral changes. $K_d = 8.3 \pm 1.0 \mu M$. Averaged absorbance values were used to analyze the data, i.e. $A_{390}$ means the averaged value of absorbance data between 389 nm and 391 nm and $A_{413}$ means the averaged value of data between 412 nm and 414 nm.

FIGURE 3. Inhibitory effects of cholesterol in human liver microsomes. A, Nifedipine oxidation (5-200 µM); B, quinidine 3S-hydroxylation (10-300 µM); C, quinidine N-oxidation (10-300 µM). The data points represent means of duplicate determinations. The lines represent a simultaneous fit to the corresponding models defined in Experimental Procedures at free cholesterol concentrations of 1.5, 6.6, 11.7, and 16.8 µM. See Table 1 for $K_i$ values.

FIGURE 4. Inhibitory effects of cholesterol on recombinant P450 3A4. A, Nifedipine oxidation (5-200 µM); B, quinidine 3S-hydroxylation (10-300 µM); C, quinidine N-oxidation (10-300 µM). The data points represent the means of duplicate determinations. The lines represent a simultaneous fit to the corresponding models defined in Experimental Procedures at free cholesterol concentrations of 0, 2.5, 5.1, 10.2, and 15.3 µM. See Table 1 for $K_i$ values.

FIGURE 5. Effects of cholesterol levels on P450 3A4 catalytic activity in human hepatocytes. Human hepatocytes (lot No. Hu1126 and Hu4242) were treated with FBS-free Hepato STIM medium (□, no
treatment) or 200 µM cholesterol (■) in FBS-free Hepato STIM medium for 24 h. The catalytic activities of human hepatocytes towards nifedipine (in FBS-free Hepato STIM medium) were measured with 30 min incubation. A, nifedipine oxidation in lot Hu1126 cells; B, nifedipine oxidation in lot Hu4242 cells. The bars represent means of quadruplicate determinations ± SD. *: p < 0.05, **: p < 0.005.

### Table 1

**Calculated $K_i$ values of cholesterol towards activities of human P450 3A4**

<table>
<thead>
<tr>
<th>Oxidation reaction</th>
<th>Enzyme source</th>
<th>$K_i$ (µM)</th>
<th>Hill constant</th>
<th>Apparent inhibition type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifedipine oxidation</td>
<td>Human liver microsomes</td>
<td>$11.4 \pm 0.3^a$</td>
<td>$2.8 \pm 0.2^a$</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td></td>
<td>Reconstituted system</td>
<td>$9.2 \pm 0.3^b$</td>
<td>-</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>Quinidine 3S-hydroxylation</td>
<td>Human liver microsomes</td>
<td>$11.1 \pm 1.0^b$</td>
<td>-</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td></td>
<td>Reconstituted system</td>
<td>$9.1 \pm 0.6^b$</td>
<td>-</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>Quinidine N-oxidation</td>
<td>Human liver microsomes</td>
<td>$9.2 \pm 0.7^b$</td>
<td>-</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td></td>
<td>Reconstituted system</td>
<td>$15.0 \pm 0.8^b$</td>
<td>-</td>
<td>Noncompetitive</td>
</tr>
</tbody>
</table>

$^a$The $K_i$ value was calculated using Eq. 2 in Experimental Procedures.

$^b$The $K_i$ value was calculated using Eq. 1 in Experimental Procedures.
Table 2

Cholesterol and P450 3A4 levels in human hepatocytes

<table>
<thead>
<tr>
<th>Hepatocyte lot</th>
<th>Treatment</th>
<th>Cholesterol (nmol/10^6 cells)</th>
<th>P450 3A4 (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu1126</td>
<td>No treatment</td>
<td>8.7 ± 0.5</td>
<td>15.8 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>200 µM Cholesterol</td>
<td>36.1 ± 1.7</td>
<td>12.8 ± 0.6</td>
</tr>
<tr>
<td>Hu4242</td>
<td>No treatment</td>
<td>3.7 ± 0.8</td>
<td>46.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>200 µM Cholesterol</td>
<td>23.8 ± 2.2</td>
<td>42.7 ± 5.2</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 3
Inhibition of human cytochrome P450 3A4 by cholesterol
Raku Shinkyo and F. Peter Guengerich

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