Tissue-selective acute effects of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase on cholesterol biosynthesis in lens

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Abstract Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the key enzyme that regulates cholesterol synthesis, lower serum cholesterol by increasing the activity of low density lipoprotein (LDL) receptors in the liver. In rat liver slices, the dose-response curves for inhibition of [^{14}C]acetate incorporation into cholesterol were similar for the active acid forms of lovastatin, simvastatin, and pravastatin. The calculated IC_{50} values were approximately 20–50 nM for all three drugs. Interest in possible extrahepatic effects of reductase inhibitors is based on recent findings that some inhibitors of HMG-CoA reductase, lovastatin and simvastatin, can cause cataracts in dogs at high doses. To evaluate the effects of these drugs on cholesterol synthesis in the lens, we developed a facile, reproducible ex vivo assay using lenses from weanling rats explanted to tissue culture medium. [^{14}C]Acetate incorporation into cholesterol was proportional to time and to the number of lenses in the incubation and was completely eliminated by high concentrations of inhibitors of HMG-CoA reductase. At the same time, incorporation into free fatty acids was not inhibited. In marked contrast to the liver, the dose-response curve for pravastatin in lens was shifted two orders of magnitude to the right of the curves for lovastatin acid and simvastatin acid. The calculated IC_{50} values were 4.5 ± 0.7 nM, 5.2 ± 1.5 nM, and 469 ± 42 nM for lovastatin acid, simvastatin acid, and pravastatin, respectively. Thus, while equally active in the liver, pravastatin was 100-fold less inhibitory in the lens compared to lovastatin and simvastatin. Similar selectivity was observed with rabbit lens. Following oral dosing, ex vivo inhibition of [^{14}C]acetate incorporation into cholesterol in rat liver was similar for lovastatin and pravastatin, but cholesterol synthesis in lens was inhibited by lovastatin by as much as 70%. This inhibition was dose-dependent and no inhibition in lens was observed with pravastatin even at very high doses. This tissue-selective inhibition of sterol synthesis by pravastatin was likely due to the inability of pravastatin to enter the intact lens since pravastatin and lovastatin acid were equally effective inhibitors of HMG-CoA reductase enzyme activity in whole lens homogenates. We conclude that pravastatin is tissue-selective with respect to lens and liver in its ability to inhibit cholesterol synthesis. —**Mosley, S. T., S. S. Kalinowski, B. L. Schafer, and R. D. Tanaka.** Tissue-selective acute effects of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase on cholesterol biosynthesis in lens. *J. Lipid Res.* 1989. **30:** 1411–1420.

Supplementary key words liver • lovastatin • pravastatin • simvastatin

Cholesterol and nonsterol products of mevalonate metabolism are essential for cell growth and multivalent feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), a key enzyme in the cholesterol biosynthetic pathway (1). Compactin, an inhibitor of HMG-CoA reductase, was discovered in 1976 (2) and recently analogues of compactin have been approved for use as hypocholesterolemic agents (3). The target organ for these inhibitors of HMG-CoA reductase is the liver since the liver is both the major site of lipoprotein production and low density lipoprotein (LDL) catabolism. Inhibition of hepatic reductase causes an increase in hepatic LDL receptor activity which in turn increases the removal of LDL from plasma (3,4). However, high intracellular concentrations of compactin are toxic to cultured cells even in the presence of excess cholesterol, indicating that nonsterol products of mevalonate metabolism as well as cholesterol are essential for normal cell growth and cell division (1,5,6).

Intense current interest in extrahepatic effects of HMG-CoA reductase inhibitors is based on recent findings that two of these inhibitors, lovastatin and simvastatin, cause cataracts in a dose-dependent fashion in dogs (7,8). Cholesterol synthesis is particularly important in the ocular lens of the eye which consists of two cell types, epithelial cells and fiber cells (9). Lens epithelia divide and differentiate into fiber cells which subsequently cease cell division and lose their nuclei. During this developmental sequence, the lens fiber cells begin a process of elongation which increases their plasma membrane surface area by 1000-fold (10). Moreover, the cholesterol/phospholipid ratio in the lens fiber cell membrane is the highest of any tissue in the body (11). All of these special demands for cholesterol must be met by endogenous syn-

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; LDL, low density lipoproteins; TLC, thin-layer chromatography.

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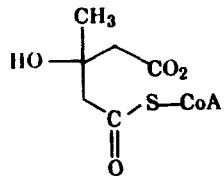
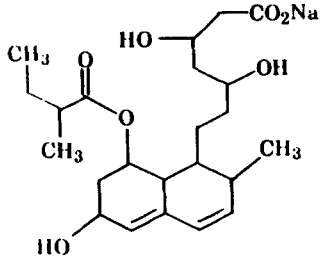
thesis of sterol from acetate since the lens is completely avascular and, unlike most other tissues, cannot obtain cholesterol from the plasma (10,11).

Pravastatin is a newly developed inhibitor of HMG-CoA reductase which, like lovastatin, simvastatin, and mevastatin (compactin), has a dihydroxy heptanoic acid function which resembles the HMG moiety of HMG-CoA (**Fig. 1**). Each of these compounds effectively inhibits cholesterol synthesis in hepatocytes but intact human fibroblasts are resistant to inhibition by pravastatin while they are sensitive to inhibition by lovastatin (12,13). For example, the IC_{50} (the drug concentration at which 50% inhibition is observed) for [^{14}C]acetate incorporation into cholesterol by pravastatin in cultured human fibroblasts is 90- to 180-fold higher than the IC_{50} in primary hepatocyte cultures (12). In contrast, the IC_{50} of lovastatin (in the active open ring form, lovastatin acid) is the same in fibroblasts as in primary hepatocytes. Experiments with radiolabeled analogues of lovastatin acid and pravastatin suggest that this difference is due to lower uptake of pravastatin in nonhepatic tissues and cells (12).

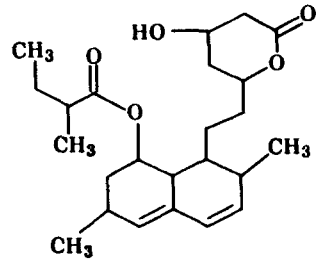
Because of the potential for adverse effects due to inhibition of the sterol pathway in the lens, we have developed methods to examine directly the ability of HMG-CoA reductase inhibitors to affect cholesterol synthesis in rat liver and lens organ culture, both ex vivo and after an oral dose and we compare this to the ability to inhibit HMG-CoA reductase enzyme activity in broken cell preparations made from liver and lens. These studies show that pravastatin, while equally effective in liver, is 100-fold less potent in inhibition of cholesterol synthesis in the intact lens compared to two other HMG-CoA reductase inhibitors, lovastatin and simvastatin.



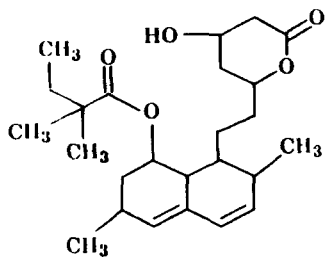
HMG-CoA



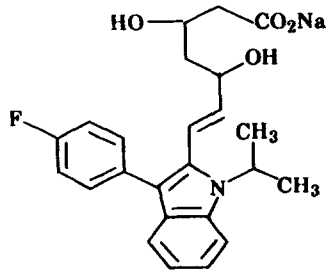
Pravastatin (Squibb)



Lovastatin (Merck)



Simvastatin (Merck)



Fluindostatin (Sandoz)

Fig. 1. Chemical structures of HMG-CoA, pravastatin, lovastatin, simvastatin, and fluindostatin.

MATERIALS AND METHODS

Materials

Pravastatin was used as the active, open-ring form in all experiments. Lovastatin or simvastatin were used as the lactone in experiments where drug was administered orally to rats or as the active open-ring forms, lovastatin acid or simvastatin acid, in experiments where drug was added to ex vivo incubations. Both lovastatin acid and simvastatin acid were derived from their corresponding lactones by treatment with 0.1 N NaOH and were used as sodium salts. Pravastatin and lovastatin were provided by Sankyo, Inc., Japan. Simvastatin was a gift from Merck Sharp and Dohme, Inc. Fluindostatin (SRI62320), fluorophenyl indole dihydroxy acid, was a gift from Sandoz, Inc., and was used as the sodium salt. These compounds were used as supplied by the manufacturer and were determined to be ≥ 98% pure. [1-^{14}C]Acetic acid was from DuPont New England Nuclear or from ICN, Inc. DL-3-Glutaryl-[3-^{14}C]hydroxy-3-methylglutaryl coenzyme A ([^{14}C]HMG-CoA), [1,2-^{3}H]cholesterol, and [1-^{14}C]octanoic acid were from DuPont New England Nuclear.

Cholesterol synthesis in rat lenses ex vivo

Sprague-Dawley rats (21 days old, 50–60 g) were received from either Taconic or Camm Labs, were housed on a 12-h light-dark cycle, were given Purina Rat Chow 5001 and water ad libitum, and were used within 5 days of receipt. Under light ether anesthesia, animals were killed at the third hour of the light cycle by cervical dislocation and the lenses were removed, cleaned of connective tissue, and placed in 10 ml of Minimal Essential Medium (MEM, GIBCO, Inc.) supplemented with 200 U/ml penicillin and 200 μg/ ml streptomycin, MEM vitamin solution, and 2 mM glutamine (GIBCO, Inc.). Lenses that were damaged during removal from the eye rapidly became opaque in MEM, were easily distinguished from undamaged lenses, and were discarded. Intact lenses were transferred to 20 × 150 mm incubation tubes, four lenses per tube, in 1 ml of fresh MEM to which various amounts of the test compounds were added. The final concentrations of lovastatin acid or simvastatin acid were 0,0.3,1,3,10,30,100, and 300 nM; the concentrations of pravastatin were 0,10,30,100,300,1,000,3,000, and 10,000 nM. The incubation tubes were maintained at 37°C under 5% CO_2 atmosphere with agitation at 90 oscillations per min for 30 min after which [^{14}C]acetic acid (131 dpm/pmol) was added to each tube (final concentration: 9 μCi/ml, 150 μM) and the incubation was continued for 4 h. [^{14}C]Acetate incorporation was stopped by the addition of 60 μl 3N NaOH to each incubation tube which then remained overnight at room temperature. The lenses were disrupted by vortexing the incubation tubes for 30 sec and aliquots were taken from each tube for protein determination. Each aliquot was centrifuged for 1 min in an Allied microcentrifuge at 10,000 *g*, and the amount of protein in the supernate was measured using the Bradford assay (14). [^{3}H]Cholesterol was added to the remainder of each incubation tube (50,000 dpm/tube) as an internal recovery standard along with 150 μg unlabeled cholesterol and 20 μg squalene as markers for visualization by thin-layer chromatography (TLC). Each incubation tube received 3.5 ml of ethanolic KOH (ethanol with 5.7% (w/v) KOH) and was saponified for 1 h at 90°C. The tubes were cooled to room temperature and the contents of each tube were extracted twice with 7 ml petroleum ether. The pooled extracts were evaporated to dryness under nitrogen, brought up in 60 μl chloroform–hexane 1:1, spotted on Whatman plastic-backed PE Sil G TLC plates, and developed in $CHCl_3$. The region corresponding to cholesterol (R_f = 0.12) was cut out, placed in 7 ml Optifluor (Packard, Inc.) and counted in a Beckman LS 5801 liquid scintillation counter. The data are expressed as pmol [^{14}C]acetate incorporated into cholesterol per mg protein, corrected for recovery. All values are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated. Several types of control incubations were performed including pre-killed blanks (addition of NaOH prior to the addition of [^{14}C]acetate), no-cell blanks, or incubations containing whole lenses in the presence of a high concentration of lovastatin acid to inhibit cholesterol biosynthesis (see below). Each of these negative controls had values that were usually 1–5% of the no-drug positive control samples. Other experiments (not shown) evaluated the effects of different media, [^{14}C]acetate concentrations, and other incubation conditions. For example, 140 μM [^{14}C]acetate was sufficient to saturate the cholesterol biosynthetic pathway in these experiments since higher concentrations of [^{14}C]acetate (up to 1.4 mM) had no effect on the rate of incorporation. Measurements of the incorporation of octanoate into cholesterol were performed in the same manner as described for the incorporation of [^{14}C]acetate into cholesterol, but [^{14}C]octanoate (53 mCi/mmol, 138 μM) was used as the radiolabeled precursor.

Fatty acid synthesis

The aqueous phases from the [^{14}C]acetate petroleum ether extractions were acidified with HCl and extracted twice with petroleum ether. The organic phases were then evaporated to dryness, dissolved in 60 μl of chloroform–hexane 1:1 and subjected to TLC on silica gel G in heptane–diethyl ether–acetic acid 90:30:1. The spot corresponding to fatty acid (R_f = 0.3) was visualized by iodine vapor, cut out, and counted as described above. The data are expressed as pmol [^{14}C]acetate incorporated into fatty acids per mg of lens protein.

Rat liver

Livers from the rats used in the lens studies were excised, rinsed in phosphate-buffered saline, and sliced with an automated tissue slicer (Mickle Laboratory Engineering Co. Ltd.) to produce 1-mm cubes. These cubes were rinsed in MEM and incubated with various concentrations (0–1,000 nM) of the test drugs as indicated. Each tube contained four 1-mm liver cubes (~1 mg protein). The samples were processed for [^{14}C]acetate incorporation into cholesterol by TLC as described above except that the liver samples were homogenized by eight strokes in a Dounce homogenizer prior to saponification.

Rabbit lenses

Female weanling rabbits were euthanized with CO_2, after which the eyes were removed and the lenses were obtained by careful dissection of each eye. Rabbit lenses were pooled and treated as described above for rat lenses except that each lens was incubated in 6 ml of MEM in a 35-mm well of a 6-well culture plate, one lens per well, with various concentrations of the test drugs. Lenses were incubated for 15–30 min with pravastatin or lovastatin acid prior to the addition of [^{14}C]acetate. After 4 h at 37°C, the reactions were stopped with 3N NaOH and processed as described above.

HMG-CoA reductase assay

HMG-CoA reductase enzyme activity was measured by a radiometric assay. Rat lenses were homogenized in buffer containing 200 mM KCl, 50 mM KPO_4, 5 mM EDTA, 5 mM DTT, 0.1 mM leupeptin, and 0.25% (v/v) Kyro EOB, pH 7.4, at room temperature (15). Lenses were either used immediately after removal from the eye, or were quick-frozen in liquid nitrogen and stored at −76°C until use. For homogenization, the ratio of lens to homogenization buffer was one lens per 0.1 ml of buffer. Frozen lenses were thawed in homogenization buffer maintained at 37°C and homogenized by 10–15 strokes in a 2 ml Dounce homogenizer. The homogenate was spun at 10,000 *g* for 5 min, after which aliquots were taken for protein measurement and measurement of enzyme activity. Aliquots of the supernatant cell homogenate were preincubated for 10 min at 37°C in reaction buffer which contained 100 mM KPO_4, 6 mM DTT, 20 mM D-glucose-6-phosphate and 2 mM NADPH, pH 7.4. The reaction was initiated by the addition of [^{14}C]HMG-CoA (final concentration, 30 μM; specific activity, 120 dpm/pmol) and proceeded at 37°C for the indicated time intervals. The reaction was stopped with 50 μl 5 N HCl and [^{14}C]mevalonolactone was isolated from the 10,000 *g* supernatant of the reaction mixture as described using [^{3}H]-mevalonolactone as a recovery standard (15). Values are expressed as pmol of [^{14}C]HMG-CoA reduced to [^{14}C]-mevalonolactone per mg of cell protein, corrected for recovery and counting efficiency.

Oral dose experiments

Female weanling rats were given pravastatin, lovastatin (lactone), or vehicle alone (1% carboxymethyl cellulose) via gavage tubes. At the specified times after dosing the animals were killed and tissue samples were immediately removed and pulse-labeled with [^{14}C]acetate as described above except that test compounds (pravastatin or lovastatin) were not added to the medium.

RESULTS

Inhibition of cholesterol synthesis in liver

We measured the ability of lovastatin acid, simvastatin acid, and pravastatin to inhibit cholesterol synthesis in fresh liver samples taken from weanling rats. In all of these ex vivo experiments, the active acid form of each of the drugs was used. As shown in **Fig. 2**, the amount of inhibition of [^{14}C]acetate incorporation into cholesterol in the liver samples increased with increasing concentration of drug. This dose-response was similar for all three drugs at concentrations ranging from 1 to 1,000 nM, with simvastatin acid being slightly more potent than lovastatin acid and pravastatin.

Cholesterol synthesis in the whole lens

To study cholesterol synthesis in lens a similar ex vivo assay was developed using lenses freshly explanted to tissue culture medium. When lenses from weanling rats were quickly removed and placed in MEM medium in the

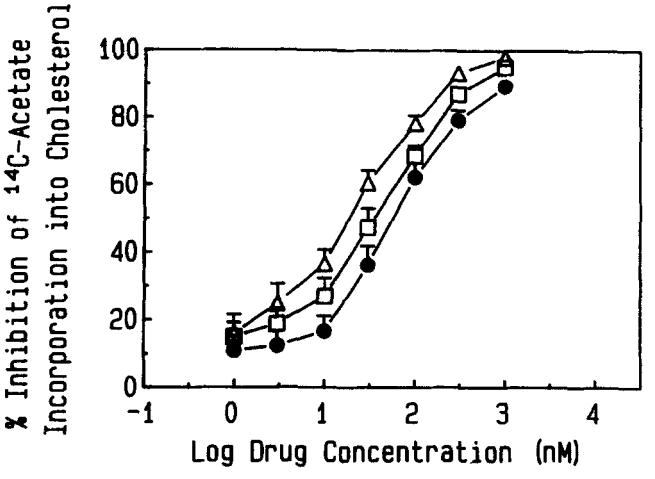


Fig. 2. Dose-response curves for inhibition of [^{14}C]acetate incorporation into cholesterol in rat liver ex vivo. Liver samples from female weanling rats were incubated with increasing amounts of simvastatin acid (△), lovastatin acid, (□), or pravastatin (●) and were pulsed with [^{14}C]-acetate as described in Materials and Methods. Each data point represents the mean ± SEM of 12–18 individual incubations from six separate experiments.

presence of [^{14}C]acetate, the synthesis of [^{14}C]cholesterol increased as the number of lenses increased (data not shown). [^{14}C]Acetate incorporation was linear for at least 6 h, and incorporation was completely inhibited by a high concentration of lovastatin acid (data not shown).

To evaluate the effects of reductase inhibitors on cholesterol synthesis in lens, rat lenses were incubated with increasing amounts of lovastatin acid, simvastatin acid, or pravastatin and the incorporation of [^{14}C]acetate into cholesterol was measured as described above for liver samples. As with liver, inhibition of cholesterol synthesis increased in the presence of increasing concentrations of drug compared to control incubations (**Fig. 3**). In marked contrast to the liver, however, the dose-response curve for lens for pravastatin was shifted by two orders of magnitude to the right of lovastatin acid and simvastatin acid (Fig. 3). From these dose-response experiments, the average IC_{50} values for liver and lens were calculated by linear regression analysis and are shown in **Table 1**. In liver, the IC_{50} values for all three drugs were very similar: for pravastatin and lovastatin acid, the IC_{50} values were 48 ± 11 nM and 33 ± 13 nM, respectively, and were not significantly different. For simvastatin acid in liver, the IC_{50} value was 19 nM, slightly more potent than pravastatin but not significantly more potent than lovastatin acid. However, the IC_{50} value for pravastatin in the rat lens was 469 ± 42 nM compared to 4.5 ± 0.7 and 5.2 ± 1.5 nM for lovastatin acid and simvastatin acid, respectively (Table 1). By comparison, 5 nM pravastatin had no detectable effect on cholesterol synthesis in lens. These dose-response experiments demonstrated that, although only a small difference was observed among these inhibitors in liver, in lens pravastatin was 100-fold less potent in the inhibition of cholesterol biosynthesis compared to either lovastatin acid or simvastatin acid.

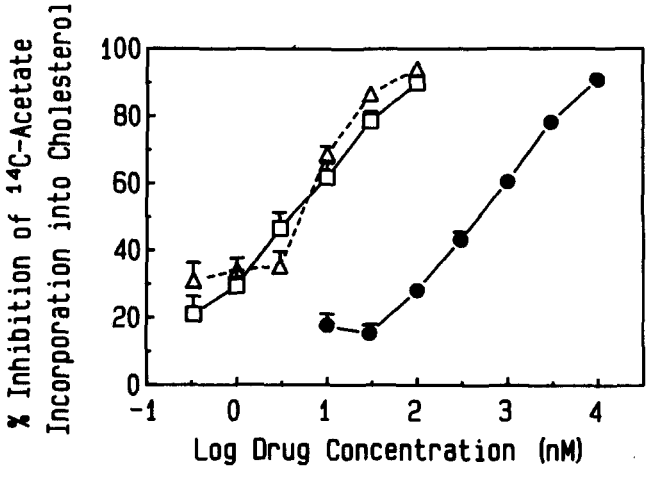


Fig. 3. Dose-response curves for inhibition of [^{14}C]acetate incorporation into cholesterol in rat lenses ex vivo. Lenses were incubated with increasing amounts of simvastatin acid (△), lovastatin acid (□), or pravastatin (●), and were pulsed with [^{14}C]acetate as described in Materials and Methods. Each data point represents the mean ± SEM of 6–38 samples from 6–12 separate experiments.

TABLE 1. Average IC_{50} values for inhibition of cholesterol synthesis by pravastatin, lovastatin acid, or simvastatin acid in freshly explanted rat tissues

Drug	IC_{50} Values for [^{14}C]Acetate Incorporation into Cholesterol			
	Liver	n	Lens	n
	nM		*nM*	
Pravastatin	48.1 ± 10.9	6	468.7 ± 42.1	12
Lovastatin Acid	33.4 ± 12.6	6	4.5 ± 0.7	7
Simvastatin Acid	18.6 ± 6.0	6	5.2 ± 1.5	6

Each sample contained four lenses or four 1-mm liver samples and there were two to four incubations at each drug concentration. Each experiment used six to eight different drug concentrations chosen to give 20–80% inhibition. [^{14}C]Acetate incorporation into cholesterol in control (no drug) lenses was 6.05 ± 0.22 pmol/4 h per mg in these experiments. Control liver samples incorporated 50 ± 5.2 pmol/4 h per mg. Values are given as mean ± SEM; n, number of experiments.

Another way to measure cholesterol synthesis is to use [1-^{14}C]octanoic acid, a medium chain length fatty acid which is converted to cytosolic acetyl-CoA by mitochondrial oxidation (16). The IC_{50} values for inhibition of [^{14}C]octanoate incorporation into cholesterol in rat lens by pravastatin and lovastatin acid were 389 nM and 4.2 nM, respectively, the same 100-fold difference observed using [^{14}C]acetate as the cholesterol precursor.

Sex, age, and species specificity

To see whether a sex difference existed in the effects of these drugs on the lens, a dose-response experiment was done in male rats and the results were essentially the same. The IC_{50} values for pravastatin and lovastatin were 472 nM and 3.14 nM, respectively, a difference of 150-fold (**Table 2**). Weanling rats were used in these experiments because younger rats have a higher rate of cholesterol biosynthesis compared to mature rats (7,8). However, the IC_{50} value for pravastatin in lenses from older rats (8–10 weeks old) was 966 nM, still 200-fold higher than for lovastatin acid, which was 4.13 nM (Table 2).

Is this tissue-selective property of pravastatin a species-specific phenomenon restricted to the rat? To answer this question, we determined the IC_{50} values for pravastatin or lovastatin acid in the ex vivo rabbit lens. Lenses from young female New Zealand white rabbits were isolated and incubated with various concentrations of pravastatin or lovastatin acid and pulse labeled with [^{14}C]acetate for 4 h as described in Methods. Control lenses incorporated 2–4 pmol of [^{14}C]acetate into cholesterol per mg of lens protein, a value similar to that in the young rat lens. As in the rat, the IC_{50} values in the rabbit lens were markedly different, 226 nM and 5.91 nM for pravastatin and for lovastatin acid, respectively (Table 2), a 40-fold difference.

TABLE 2. IC_{50} values for [^{14}C]acetate incorporation into cholesterol in lenses from male rats, adult female rats, and from rabbits

Drug	IC_{50} [^{14}C]Acetate Incorporation into Cholesterol		
	Young Male Rats	Adult Female Rats	Female Rabbits
	nM		
Pravastatin	472	966	226 ± 65.5
Lovastatin Acid	3.14	4.13	5.91 ± 0.21

Rat lenses were incubated in various concentrations of pravastatin or lovastatin acid from 0 to 3 μM for 30 min prior to the addition of [^{14}C]acetate (150 μM, 130 dpm/pmol) as described in Materials and Methods. The incorporation was carried out for 4 h. Experiments with rabbit lenses were similar to those with rat lenses except that each sample contained one lens and there were two to six samples at each drug concentration. In the absence of drug, [^{14}C]acetate incorporation into cholesterol (pmol/4 h per mg protein) was 4.61 ± 0.53, 1.00 ± 0.10, and 2.33 ± 0.53 for weanling male rat lens, adult female rat lens, and female rabbit lens, respectively.

Preliminary experiments using lenses isolated from mongrel dogs gave a selectivity similar to that observed with rabbit lenses (data not shown).

Effects of inhibitors on FFA synthesis in lens

Inhibition by pravastatin or lovastatin is specific for HMG-CoA reductase since acetate incorporation into fatty acids in cultured cells such as human fibroblasts is unaffected at concentrations where incorporation into cholesterol is maximally inhibited (17). Similarly, pravastatin and lovastatin acid had no inhibitory effect on incorporation of [^{14}C]acetate into free fatty acids in lens at concentrations which inhibited [^{14}C]acetate incorporation into cholesterol by 80–90% (**Table 3**).

An indole-based HMG-CoA reductase inhibitor

We tested another potent HMG-CoA reductase inhibitor, fluindostatin, which is similar to pravastatin in that it is administered in the active acid form but which has an indole ring system in place of the decalin ring (Fig. 1). The IC_{50} for fluindostatin in rat lens was 6.20 ± 2.11 nM (mean ± SEM, n = 2), a value similar to lovastatin acid and simvastatin acid, but 76-fold more potent than pravastatin in the lens. In liver, the IC_{50} values of fluindostatin and pravastatin were similar based on studies in primary cultures of rat hepatocytes (data not shown). Thus, of the four reductase inhibitors examined, all showed similar inhibition in liver and three were similar in lens with the exception of pravastatin, which was markedly less potent in lens.

Inhibition after oral dose

If this same tissue selectivity occurs in vivo, it should be possible to detect changes in cholesterol synthesis in the lens after administering these drugs to rats per os. Two hours after an oral dose of pravastatin, lovastatin (as the lactone), or vehicle alone, rats were killed and lenses were removed immediately and pulsed with [^{14}C]acetate ex vivo as described above. **Fig. 4** shows that at a dose of 25 mg/kg, there was no inhibition of cholesterol biosynthesis in lenses from the pravastatin-treated rats but there was an 18% inhibition in the lenses from the rats that had received lovastatin. At higher doses, the amount of inhibition increased to 65% in the lovastatin group, but there was still no inhibition in the lenses of rats receiving pravastatin even at doses as high as 1,000 mg/kg (Fig. 4). At the same time, inhibition of [^{14}C]acetate incorporation into cholesterol was measured in liver samples from these same rats. Both pravastatin and lovastatin inhibited liver to the same extent, from 36–97% for both drugs (not shown). When the time interval between the oral dose and killing was extended to 3 or 4 h, pravastatin and lovastatin inhibited equally in the liver, but only lovastatin inhibited in the lens (data not shown).

In these in vivo experiments, we were concerned with the possibility that pravastatin or lovastatin acid might wash out of the tissues during the [^{14}C]acetate pulse since both pravastatin and lovastatin acid are easily washed out of cells in culture (17,18). However, these drugs were not washed out of liver samples after an oral dose and accumulation in the liver in vivo. Triplicate samples of liver were taken from rats that had received pravastatin, lovastatin, or vehicle alone. Each sample consisted of four 1-mm cubes of liver in 1 ml of MEM. The samples were washed 0, 1, or 2 times with 1 ml of MEM after which [^{14}C]acetate incorporation into cholesterol was measured. Control liver samples incorporated 249 pmol of [^{14}C]acetate/mg cell protein, while samples from pravastatin- and lovastatin-treated animals incorporated 57 and 43 picomol, respectively (**Table 4**). After two washes, the values were essentially unchanged (Table 4). Similar results were obtained when lenses were washed except that no inhibition by pravastatin was observed. Thus, after oral dosing these drugs were not washed out of tissues to any significant extent and, therefore, differential loss of drug to the

TABLE 3. [^{14}C]Acetate incorporation into cholesterol and fatty acids (FA) in the presence of pravastatin or lovastatin acid

Treatment	[^{14}C]Acetate Incorporation		% Inhibition	
	Cholesterol	FA	Cholesterol	FA
	pmol/mg per 4 h		%	
No addition	5.67 ± 0.35	8.41 ± 0.66	0	0
Pravastatin (3 μM)	1.55 ± 0.05	8.72 ± 0.19	73	0
Lovastatin acid (0.1 μM)	0.32 ± 0.00	14.11 ± 1.70	94	0

Lenses were incubated in pravastatin, lovastatin acid, or no drug for 30 min prior to the addition of [^{14}C]acetate (140 μM, 130 dpm/pmol) as described in Methods. Values expressed are pmol [^{14}C]acetate incorporated per mg protein for a 4-h incubation (mean ± SEM; n = 3).

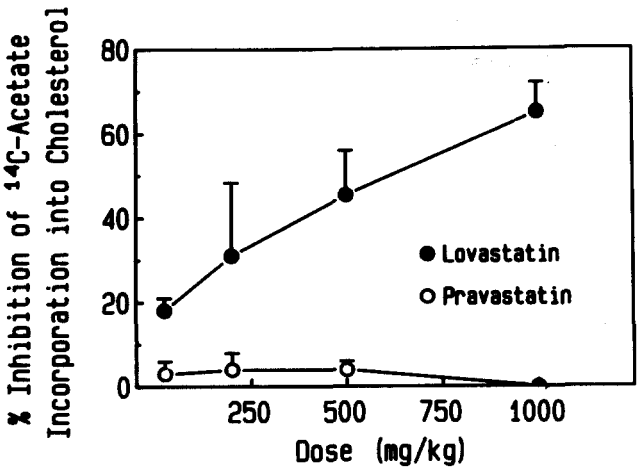


Fig. 4. Inhibition of cholesterol biosynthesis in isolated rat lens after an oral dose of pravastatin (○) or lovastatin (●). Rats were given the indicated drug or vehicle alone via gavage tubes. Two hours after dosing, lenses were obtained and pulse-labeled with [^{14}C]acetate for 4 h as described in Methods. The [^{14}C]acetate incorporated into cholesterol was determined for each pair of lenses. Each data point represents the mean ± SEM from two to four experiments and within each experiment individual treatment groups had seven rats.

medium cannot explain the difference in tissue selectivity between pravastatin and lovastatin.

HMG-CoA reductase inhibition

It was possible that the 100-fold difference between pravastatin and lovastatin acid in rat lens was not due to some tissue-selective property of pravastatin, but rather was due to a different sensitivity towards the HMG-CoA reductase enzyme found in the lens. Therefore, we measured the inhibition of HMG-CoA reductase by pravastatin or lovastatin acid in broken lens cell preparations. Whole lens homogenates were prepared and incubated with [^{14}C]HMG-CoA and the conversion to [^{14}C]mevalonate was linear for 30–120 min (**Fig. 5**, panel A). When pravastatin and lovastatin acid were added to the homogenates, the amount of inhibition of the reductase was the same for both drugs (Fig. 5, panel B). The calculated IC_{50} values for the inhibition of HMG-CoA reductase in these experiments were 37.4 ± 7.21 nM and 30.0 ± 3.29 nM for pravastatin and lovastatin acid, respectively, and were not significantly different.

DISCUSSION

Tsujita et al. (12) originally reported that while pravastatin and lovastatin were equally potent in liver, lovastatin was significantly more potent in inhibition of [^{14}C]acetate incorporation into cholesterol in several nonhepatic rat tissues including kidney, lung, spleen, adrenal, and muscle. In our experiments, all of the reductase inhibitors tested, lovastatin, simvastatin, pravastatin, and fluindostatin, were potent inhibitors of cholesterol synthesis in rat liver preparations and had IC_{50} values around 20–50 nM. Indeed, the liver is the target organ for these drugs and clinical studies show that their cholesterol-lowering effects are similar (3,19,20).

To evaluate the effects of HMG-CoA reductase inhibitors on cholesterol synthesis in the lens, we developed a reproducible, facile, and sensitive assay using freshly explanted lenses from weanling rats. In this system, all of the lenses needed for a single experiment could be excised, cleaned of connective tissue, and placed in the incubator within about 45 min. The rate of cholesterol biosynthesis was linear with both time and protein and no cholesterol synthesis or reductase activity was detectable in the presence of an inhibitor of HMG-CoA reductase. In developing this lens culture system, over 50 IC_{50} experiments were performed to verify these results and pravastatin was always less inhibitory in the lens compared to lovastatin acid and simvastatin acid. In lens, as in other cells (18), the effects of reductase inhibitors were limited to inhibition of cholesterol biosynthesis inasmuch as fatty acid biosynthesis, DNA synthesis (not shown), or protein glycosylation (not shown) were unaffected.

The current experiments demonstrate that the IC_{50} value for inhibition of sterol synthesis by pravastatin in the rat lens ex vivo was 100-fold higher than for the other re-

TABLE 4. Effect of multiple washes on inhibition of [^{14}C]acetate incorporation into cholesterol in liver slices removed from rats 2 h after an oral dose of pravastatin (500 mg/kg), lovastatin (500 mg/kg), or vehicle alone

Drug	[^{14}C]Acetate Incorporation into Cholesterol		
	No Wash (%)	One Wash (%)	Two Washes (%)
	pmol/mg protein		
None	249.0 ± 25.7 (100)	324.0 ± 64.3 (100)	322.0 ± 50.5 (100)
Pravastatin	57.1 ± 6.06 (23)	80.2 ± 2.91 (24)	76.1 ± 7.85 (23)
Lovastatin	43.7 ± 20.5 (17)	44.7 ± 18.4 (14)	47.0 ± 17.2 (15)

Samples of rat liver were washed with 1 ml of MEM as indicated in the table and incubated with [^{14}C]acetate for 4 h at 37°C as described in Materials and Methods. Each value represents the mean ± SEM (n = 4).

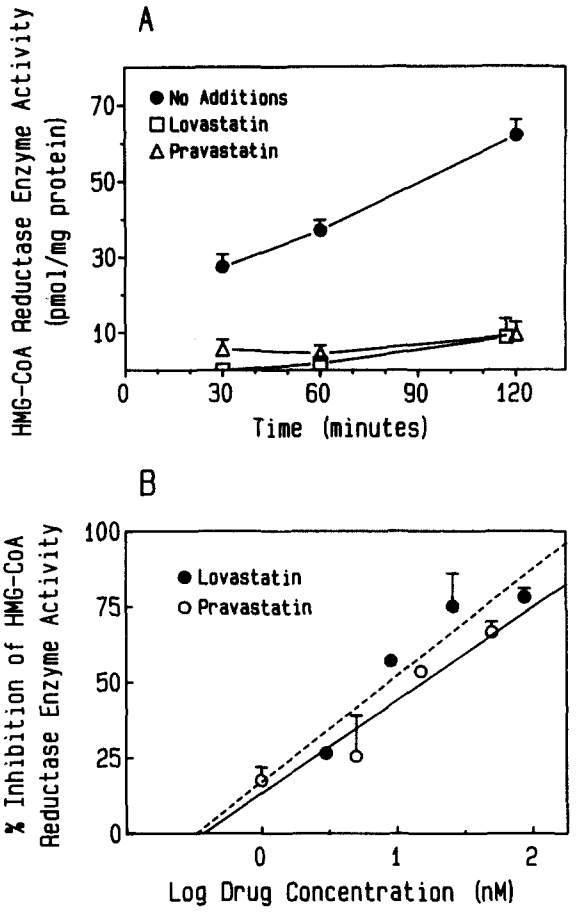


Fig. 5. Panel A: HMG-CoA reductase enzyme activity in homogenates made from rat lens was linear from 30–120 min. Each incubation contained 300 μg of cell homogenate protein with either no additions (●), 1 μM lovastatin acid (□), or 1 μM pravastatin (△). Each data point represents the mean ± SEM of three duplicate incubations in three separate experiments. Panel B: Dose-response curves for inhibition of HMG-CoA reductase enzyme activity by lovastatin acid or pravastatin in homogenates made from isolated rat lens. Aliquots of cell homogenate were incubated with 0–90 nM pravastatin or lovastatin acid as indicated. Data are the mean ± SEM of duplicate incubations. Lines were determined by linear regression analysis.

ductase inhibitors tested. This was true for both young and old lenses, lenses from rats of either sex, and for rabbit lenses as well. Moreover, when rats were given single oral doses of lovastatin or pravastatin, both drugs inhibited cholesterol biosynthesis in liver to the same extent whereas lovastatin, but not pravastatin, inhibited in lens as well in a dose-dependent manner, indicating that this tissue selectivity also occurs in vivo. We examined cholesterol biosynthesis in lens at different times between 1 and 4 h after an oral dose and at every time interval examined, lovastatin was inhibitory while pravastatin showed no inhibition in lens compared to control lenses. This inhibition of [^{14}C]acetate incorporation by lovastatin or lovastatin acid in the lens was not due to nonspecific toxicity since fatty acid biosynthesis in the lens was unaffected. Also, by 24 h after an oral dose with lovastatin, cholesterol biosynthesis in lens was no longer inhibited (not shown). These results imply that, given a dose sufficient to inhibit cholesterol synthesis in the liver, pravastatin would be much less likely to interfere with cholesterol biosynthesis in the lens compared to lovastatin.

Very large oral doses of lovastatin or pravastatin were used in these comparative studies in order to demonstrate that even with large doses of pravastatin, no inhibition occurred in the lens. Under normal circumstances, plasma levels of lovastatin in man are usually quite low (21). However under certain circumstances, such as in transplant patients on cyclosporine therapy, lovastatin levels in plasma can become markedly elevated (22). Thus, under certain circumstances, plasma levels of a reductase inhibitor can rise to levels much higher than in normal patients. Whether this results in accumulation in the human lens or whether reductase inhibitors have any long-term effects in the human lens is not known.

These experiments represent the first direct measurement of HMG-CoA reductase enzyme activity in freshly isolated whole lens. Because the lens must synthesize virtually all of its cholesterol from acetate, the activity of reductase might be expected to be relatively high compared to other cells or tissues. Our value of approximately 1 pmol/min per mg for HMG-CoA reductase enzyme activity in whole lens homogenate appears low compared to cultured cells or liver microsomes but is consistent with the relatively low metabolic activity of the lens as a whole. Only a small portion of lens cells are epithelial cells and only a small portion of these lens epithelial cells are active at any one time (9). Hitchener and Cenedella (23) measured reductase activity in log phase primary cultures of bovine lens epithelial cells and found that these cells, grown in the presence of cholesterol, had reductase activities of 165–240 pmol/min per 10^6 cells which is relatively high compared to other cultured cells (24,25).

It was interesting to note that sterol synthesis in liver was inhibited in our ex vivo assay by over 80% following oral dosing in rats. We expected that, as observed in cultured cells, at least some of the inhibitor would wash out of the liver samples. Repeated washings did not change the amount of inhibition in either lens or liver when the drugs had been administered orally. In vivo, a large fraction of the plasma burden of pravastatin or lovastatin is bound to circulating protein, and some sort of intracellular protein binding may account for the inability of these inhibitors to be washed out of tissue samples ex vivo following an oral dose. These washing experiments also suggest that the decreased [^{14}C]acetate incorporation into cholesterol in the liver samples was not due to the presence of inhibitor in the residual blood or extracellular space. Contaminating blood was not a factor in the lens, which is avascular.

The mechanism responsible for pravastatin's tissue selectivity is unclear, but it seems unlikely that a specific hepatic transport mechanism for pravastatin is involved since a specific transport system for reductase inhibitors has not been demonstrated. Rather, it may be more reasonable to speculate that some property of pravastatin, such as its increased polarity, might contribute to its ability to be selectively excluded from different tissues. The intact lens might be especially resistant to pravastatin because of protection by the lens capsule and/or the blood-ocular barrier (9,10). Greater inhibition by lovastatin acid and simvastatin acid in the lens could have been due to some unique property of the lens HMG-CoA reductase enzyme, but the fact that pravastatin and lovastatin acid were equally potent inhibitors of HMG-CoA reductase activity in lens cell homogenates demonstrates that the difference lies in the abilities of the drugs to gain access to the reductase enzyme. This tissue-selective property of pravastatin is supported by other experiments in which pravastatin and lovastatin acid display similar effectiveness against primary liver cell cultures and a large difference in other cultured cell lines such as fibroblasts (12).

We conclude from these experiments that pravastatin is tissue-selective with respect to lens and liver in its ability to inhibit cholesterol biosynthesis. The experimental systems described here will be useful for studying the regulation of sterol synthesis and HMG-CoA reductase in the lens both in vivo and in vitro and may be useful for evaluating the potential for adverse effects on lens by other agents that interfere with normal cellular metabolism in the lens.

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REFERENCES

1. Brown, M. S., and J. L. Goldstein. 1980. Multivalent feedback regulation of HMG-CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J. Lipid Res.* **21:** 505–517.
2. Endo, A., M. Kuroda,, and K. Tanzawa. 1976. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B, fungal metabolites having hypocholesterolemic activity. *FEBS Lett.* **72:** 323–326.
3. Grundy, S. M. 1988. HMG-CoA reductase inhibitors for treatment of hypercholesterolemia. *New Engl. J. Med.* **319:** 24–32.
4. Brown, M. S. and J. L. Goldstein. 1981. Lowering plasma cholesterol by raising LDL receptors. *New. Engl. J. Med.* **305:** 515–517.
5. Siperstein, M. D. 1984. Role of cholesterogenesis and isoprenoid synthesis in DNA replication and cell growth. *J. Lipid Res.* **25:** 1462–1468.
6. Ryan, J., E. C. Hardeman, A. Endo, and R. D. Simoni. 1981. Isolation and characterization of cells resistant to ML236B (compactin) with increased levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **256:** 6762–6768.
7. Tobert, J. A. 1987. New developments in lipid-lowering therapy: the role of inhibitors of hydroxymethylglutaryl-coenzyme A reductase. *Circulation.* **76:** 534–538.
8. MacDonald, J. S., R. J. Gerson, D. J. Kornbrust, M. W. Kloss, S. Prahalada, P. H. Berry, A. W. Alberts, and D. L. Bokelman. 1988. Preclinical evaluation of lovastatin. *Am. J. Cardiol.* **62:** 16J–27J.
9. Cotlier, E. 1987. The lens. *In* Adler's Physiology of the Eye. C. V. Mosby Company, St. Louis, MO. 268–290.
10. Cenedella, R. 1982. Sterol synthesis by the ocular lens of the rat during postnatal development. *J. Lipid Res.* **23:** 619–626.
11. Zelenka, P. 1984. Lens lipids. *Curr. Eye Res.* **3:** 1337–1359.
12. Tsujita, Y., M. Kuroda, Y. Shimada, K. Tanzawa, M. Arai, I. Kaneko, M. Tanaka, H. Masuda, C. Tarumi, Y. Watanabe, and S. Fujii. 1986. CS-514, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase: tissue-selective inhibition of sterol synthesis and hypolipidemic effect on various animal species. *Biochim. Biophys. Acta.* **877:** 50–60.
13. Alberts, A. 1988. HMG-CoA reductase inhibitors—The development. *Atheroscler. Rev.* **18:** 123–131.
14. Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–252.
15. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–260.
16. Andersen, J. M., and J. M. Dietschy. 1979. Absolute rates of cholesterol synthesis in extrahepatic tissues measured with ^{3}H-labeled water and ^{14}C-labeled substrates. *J. Lipid Res.* **20:** 740–752.
17. Mabuchi, H., T. Haba, R. Tatami, S. Miyamoto, Y. Sakai, T. Wakasugi, A. Watanabe, J. Koizumi, and R. Takeda. 1981. Effects of an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase on serum lipoproteins and ubiquinone-10 levels in patients with familial hypercholesterolemia. *New Engl. J. Med.* **305:** 478–482.
18. Brown, M., J. Faust, and J. Goldstein. 1978. Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. *J. Biol. Chem.* **253:** 1121–1128.
19. Pan, H. Y., D. A. Willard, P. T. Funke, and D. N. McKinstry. 1987. The clinical pharmacology of SQ31,000 (CS514) in healthy subjects. *In Drugs Affecting Lipid Metabolism.* R. Paoletti, et al., editors. Springer-Verlag, Berlin Heidelberg. 255–259.
20. Nakaya, N., Y. Homma, H. Tamachi, and Y. Goto. 1987. The effect of CS-514 on serum lipids and apolipoproteins in hypercholesterolemic subjects. *J. Am. Med. Assoc.* **257:** 3088–3092.
21. East, C., P. A. Alivizatos, S. M. Grundy, P. H. Jones, and J. A. Farmer. 1988. Rhabdomyolysis in patients receiving lovastatin after cardiac transplantation. *New Engl. J. Med.* **318:** 47–48.
22. Norman, D. J., D. R. Illingworth, J. Munson, and J. Hosenpud. 1988. Myolysis and acute renal failure in a heart-transplant recipient receiving lovastatin. *New Engl. J. Med.* **318:** 46–47.
23. Hitchener, W. R., and R. J. Cenedella. 1987. HMG-CoA

reductase activity of lens epithelial cells: compared with true rates of sterol synthesis. *Curr. Eye Res.* **6:** 1045–1049.
24. Mosley, S. T., J. L. Goldstein, M. S. Brown, J. R. Falck, and R. G. W. Anderson. 1981. Targeted killing of cultured cells by receptor-dependent photosensitization. *Proc. Natl. Acad. Sci. USA* **78:** 5717–5712.
25. Mosley, S. T., M. S. Brown, R. G. W. Anderson, and J. L. Goldstein. 1983. Mutant clone of Chinese hamster ovary cells lacking 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **258:** 13875–13881.