

Discordant expression of the sterol pathway in lens underlies simvastatin-induced cataracts in Chbb:Thom rats

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Abstract Simvastatin rapidly induced cataracts in young Chbb:Thom (CT) but not Sprague Dawley (SD) or Hilltop Wistar (HW) rats. Oral treatment for 14 but not 7 days committed CT rat lenses to cataract formation. The cholesterol to phospholipid molar ratio in lenses of treated CT rats was unchanged. Differences between strains in serum and ocular humor levels of simvastatin acid poorly correlated with susceptibility to cataracts. No significant differences were found between rat strains in the capacity of simvastatin acid to inhibit lens-basal sterol synthesis. Prolonged treatment with simvastatin comparably elevated HMG-CoA reductase protein and enzyme activity in lenses of both cataract resistant and sensitive strains. However, in contrast to SD and HW rats, where sterol synthesis was markedly increased, sterol synthesis in CT rat lenses remained at baseline. Discordant expression of sterol synthesis in CT rats may be due to inadequate upregulation of lens HMG-CoA synthase. HMG-CoA synthase protein levels, and to a much lesser extent mRNA levels, increased in lens cortex of SD but not CT rats. Because upregulation of the sterol pathway may result in increased formation of isoprene-derived anti-inflammatory substances, failure to upregulate the pathway in CT rat lenses may reflect an attenuated compensatory response to injury that resulted in cataracts.—Cenedella, R. J., J. R. Kuszak, K. J. Al-Ghoul, S. Qin, and P. S. Sexton. **Discordant expression of the sterol pathway in lens underlies simvastatin-induced cataracts in Chbb:Thom rats.** *J. Lipid Res.* 2003. 44: 198–211.

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The avascular ocular lens grows throughout life by the terminal differentiation of epithelial cells, present as a monolayer on the anterior surface, into vastly elongated fiber cells. Plasma membrane is essentially the only or-

ganelle in these cells since all others disappear after fiber cell elongation. This plasma membrane contains the highest relative content of cholesterol in nature. Human lens fiber membrane accommodates sterol to phospholipid molar ratios of as high as 3:1 [as reviewed in ref. (1)] through formation of cholesterol bilayer domains (2). Possible functions of this high cholesterol include adjusting the order or fluidity of cortical (outer lens) and nuclear (inner lens) membranes to similar states (3) and antagonizing excess membrane binding of the lens soluble proteins, the crystallins (4). The lens is self-dependent on biosynthesis to sustain its life-long need for cholesterol for membrane formation (5, 6) and isoprenes for protein isoprenylation (7), and interference with the lens sterol pathway by genetic defect or drugs can produce cataracts. All known human genetic defects in enzymes of this pathway [mevalonate kinase (8), $\Delta^8\text{-}\Delta^7$ sterol isomerase (X-linked dominant chondrodysplasia punctata) (9), dehydrocholesterol Δ^7 dehydrogenase (Smith-Lemli-Opitz Syndrome) (10), and desmosterol reductase (desmosterolosis) (11)] involve cataracts. Inhibition of cholesterol synthesis with drugs at desmosterol reductase (12), dehydrocholesterol Δ^7 dehydrogenase (13), oxysqualene cyclase (14, 15), and HMG-CoA reductase (HMGR) (16, 17) is associated with cataract formation in humans and/or experimental animals. Triparanol was rapidly withdrawn as a hypocholesterolemic drug after reports in 1962 that it induced cataracts in patients (18, 19).

The ocular safety of the statins, potent competitive inhibitors of HMGR (20), has been a subject of obvious interest since these drugs are intended for lifelong use and are the most prescribed group of drugs in the United States. Although the results of clinical safety trials collec-

Abbreviations: CT, Chbb:Thom; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; HMGs, 3-hydroxy-3-methylglutaryl CoA synthase; HW, Hilltop Wistar; SD, Sprague Dawley; SQS, squalene synthase.

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tively show no higher risk of cataracts in statin-treated patients [as reviewed in ref. (1)], simvastatin use in one study was associated with an increased incidence of cortical cataracts after 2 years of treatment (21), and simvastatin was recently reported to increase the risk of cataracts in patients concomitantly taking erythromycin (22). Animal studies also indicated high ocular safety of statins since cataracts were seen in dogs only after 1 or more years of treatment with high doses (16) and no reports of statin cataracts in rodents had been previously reported.

In the present study, we describe how treatment with simvastatin can induce cataracts strain specifically in rats and probe the biochemical basis for the selective sensitivity and the mechanism of the cataract. Our observations suggest that the sensitivity of Chbb:Thom (CT) rats to cataract formation, compared with Sprague Dawley (SD) and Hilltop Wistar (HW) rats, is related to discordant expression of the sterol synthesis pathway in lens. Lens HMGCR protein and enzyme activity of treated CT rats was markedly increased without an increase in the sterol synthesis pathway. The consequence could be a failed compensatory response to injury. The clue that simvastatin may selectively induce cataracts in CT rats was provided to us by the unpublished observations of Jürgen Güttner, Boehringer Ingelheim Pharma KG. Finding a genetic component of the simvastatin rodent cataract is especially interesting since it suggests the possibility of genetically similar links to toxicity in humans. Human differences in ethnicity and race have apparently not been accounted for in assessing potential risks from statin use.

METHODS

Animals and treatment

SD and HW rats were from Hilltop Lab Animals (Scottsdale, PA) and CT rats were from our breeding colony established with adult female and male rats purchased from Boehringer-Ingelheim, Biberach an der Riss, Germany (contact angelika.bader@boehringer-ingelheim.com). The CT rats are a HW derived strain (Jürgen Güttner, personal communication). Rats were fed Purina rat chow ad libitum and maintained on a 18 PM to 6 AM dark cycle. Animals were killed by carbon dioxide inhalation between hours 1 to 3 of the light cycle.

Simvastatin tablets were dispersed in water, emulsified, and brought to a concentration of 20 mg of simvastatin per ml of water. Rats (male and female) of 19–21 days of age received a single daily oral dose (10 AM) of 100 mg or 200 mg of simvastatin/kg body weight by gastric gavage. With exception of initial studies, simvastatin was given at 200 mg/kg/day. Age-matched controls were untreated. In one study, young adult CT rats (450–490 g males and 255–290 g females) were treated daily with 200 mg simvastatin/kg/day for 8 weeks.

Lens morphology

Eyes were enucleated, an incision was made in the posterior sclera, and the eye fixed for 2–3 days at 4°C in 0.07 M sodium cacodylate buffer containing 2.5% glutaraldehyde. Lenses were then removed, washed in cacodylate buffer, and serially delipidated with ethanol to propylene oxide. Lenses were embedded in epoxy resin, sectioned into 1–2 μm thick slices, mounted on

glass slides, and stained with a 1:1 mixture of methylene blue and azure II as recently described (23). A Vanox AHB53 Olympus microscope equipped with a 35 mm camera was used to photograph the lens sections.

Lens apoptosis

The epithelial cell layer adherent to the lens capsules freshly recovered from control and simvastatin treated CT and SD rats was examined for the presence of apoptotic cells. Lenses were removed and the capsule carefully dissected onto Probe On Plus slides (Fisher Scientific, St. Louis, MO). The tissue was air dried at 37°C for 15 min followed by 15 min fixation in 4% paraformaldehyde. Apoptosis was detected using the DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI), a modified TUNEL assay. Briefly, the tissue was permeabilized by exposure to proteinase K, and then refixed and biotinylated nucleotides were incorporated into 3'OH ends of the DNA using terminal deoxynucleotidyl transferase. Horseradish peroxidase-labeled streptavidin was then bound to the biotinylated nucleotides, and this was detected using the substrate DAB plus hydrogen peroxide. Positive controls consisted of exposing epithelia from control rat lenses to DNase I (1 U/ml) for 10 min prior to incorporation of biotinylated nucleotides.

Lens lipid analysis

Pairs of decapsulated lenses from individual control and treated rats, or pooled from pairs of rats, were weighed, dehydrated (drying oven, overnight), reweighed, and homogenized in 3 ml of 2:1 (v/v) chloroform-methanol as done before (24). Aliquots of the recovered total lipids were analyzed for phospholipid content by colorimetric analysis and cholesterol content by gas liquid chromatography (24).

Measurement of serum levels of simvastatin acid

Forty nine to 58-day-old SD, HW, and CT rats received a single oral dose, by gavage, of simvastatin (200 mg/kg). At various times between 0.5 to 6.0 h after dosage, blood samples (0.5 to 1.0 ml) were collected by cardiac puncture from rats under brief ether anesthesia.

Aliquots (0.2 ml) of the recovered serum were brought to 1.0 ml with 0.1 M potassium phosphate buffer (pH 7.2), 50 ng of lovastatin acid was added in 50 μl as internal standard, and the statins extracted using 1 ml C2 Bond-Elute columns (Varian, Harbor City, CA) as described by Stubbs et al. (25). Statins were ultimately eluted with 0.4 ml of acetonitrile-water (75:25, v/v), lyophilized, the residue dissolved in 100 μl of 75:25 acetonitrile-water and briefly microfuged. Samples (50 μl to 100 μl) were injected into a C₁₈ Vydac reverse phase column (25 cm × 4.6 mm, 300 Å, 5 μm). The statins were eluted at room temperature with 1.5 ml/min of an isocratic mixture of 50% acetonitrile and 50% aqueous buffer (0.05 M Na₂HPO₄, 0.042 M H₃PO₄, pH 4.0). Absorbance was monitored at 238 nm. Lovastatin acid eluted at about 3.5 min and simvastatin acid at 5.2 min. Standard mixtures containing equal masses of lovastatin acid and simvastatin acid were run with each group of unknowns. The UV absorbance (areas under the absorbance curve) by equal masses of lovastatin and simvastatin acids were essentially 1.0. The lactones of pure lovastatin (Merck, Co., Rahway, NJ) and simvastatin (Merck) were converted to the acid forms by mild alkaline hydrolysis (26).

Measurement of ocular humor levels of simvastatin acid

SD, HW, and CT rats (40–42 days old) were given a single oral 200 mg/kg dose of simvastatin. Rats were killed in groups of 3–5 at 2, 4, 6, and 10 h after injection. Eyes were enucleated, washed in PBS, and gently dried. A puncture was made at the center of the cornea with a 27-gauge needle and ocular humors were col-

lected into a capillary tube at the cornea-tube interface. Ocular humor was pooled from 6–10 eyes at each time point and the volume accurately determined by weighing (70–170 μ l). After adding 10 ng of lovastatin acid as an internal standard, the ocular humor samples were brought to 1.0 ml with the phosphate buffer and analyzed for simvastatin acid content as done with the serum samples.

HMGR kinetics

Possible strain differences in the kinetic properties of HMGR were examined using solubilized liver enzyme from the three rat strains rather than lens enzyme, since recovery of adequate microsomes for these assays from rat lens was considered impractical. Twenty-day-old rats were treated for 7 days with 200 mg/kg of simvastatin to upregulate the enzyme and killed 22 h after the last dose. Although we assume that the kinetic properties of the liver enzyme are characteristic of those in other tissues of a given rat, we recognize that marked differences in at least the specific activity of HMGR can exist between tissues (27). The cytoplasmic domain of HMGR was partially purified from liver microsomes using freeze/thaw treatment and ammonium sulfate precipitation as described by Heller and Gould (28). The HMGR assay was performed essentially according to Endo et al. (29). To 40 μ l of 1.25 \times assay buffer (29) was added 3 μ l of water or 3 μ l of 0.51 μ M simvastatin acid and 4.0 μ l of DL-3-[glutaryl- 14 C]HMG-CoA (57.8 mCi/mmol, New England Nuclear). The reaction was started by addition of 2 μ g of microsomal enzyme protein in 3 μ l. HMG-CoA concentrations ranged from 0.025 mM to 0.100 mM. The simvastatin acid, when added, was 31 nM. After 20 min incubation at 37°C, the reaction was stopped by adding 20 μ l of 6N HCl (containing 200 μ g of unlabeled mevalonic acid). Twenty-five microliters was subjected to TLC on silica gel G plates in a sandwich TLC chamber according to Shapiro (30). The TLC zone containing the 14 C-mevalonate was recovered, counted, and enzyme activity expressed as nmol of 14 C-mevalonate formed/min/mg protein. Lineweaver-Burk plots of the data provided estimates of K_m and K_i values.

Western blots: HMGR and squalene synthase

Changes in the relative lens concentrations of HMGR and squalene synthase (SQS) protein in response to treatment of rats with simvastatin were examined in protein recovered from the lens epithelium and outer lens cortex, the region accounting for most of the lens' cholesterol synthesis (31). Lens capsules containing attached epithelium were pooled from 6–8 lenses and homogenized in 1.0 ml of lysis buffer (5 mM Tris, 1 mM EDTA, 5 mM EGTA, 5 mM β -mercaptoethanol, and 0.2% SDS, v/v). Very gently stirring of the remaining decapsulated lenses in 3 ml of the lysis buffer for 5 min solubilized about 10% of the lens radius (31). Because we previously observed that the relative concentrations of HMGR in the lens epithelium and outer cortex following treatment of rats with lovastatin changed in parallel (32), we initially examined enzyme protein changes only in the epithelium. Samples of lysed tissue containing 50–75 μ g of protein were lyophilized, dissolved in SDS/sample buffer, and separated by SDS-PAGE in 13% gels. After transfer to ProBlott membrane (Applied Biosystems, Foster City, CA), they were probed with the A9 monoclonal antibody to HMGR diluted 1:100 as described before (33). Membranes were at times stripped and reprobed with rabbit anti rat SQS diluted 1:1,000 (gift from Ishaiaha Shechter, Uniform Services University of the Health Sciences, Bethesda, MD). Specific binding was visualized by chemiluminescent detection (33).

Western blots: 3-hydroxy-3-methylglutaryl CoA synthase

An affinity purified rabbit-anti-rat-polyclonal antibody to a peptide corresponding to the carboxy terminal 14 amino acids

[(C)SGEPESAVISNGEH] of cytosolic 3-hydroxy-3-methylglutaryl CoA synthase (HMGS) was prepared for us by Zymed Labs (San Francisco, CA). Royo et al. (34) had prepared an antibody to the carboxy terminus of hamster-cytosolic HMGS whose terminal 10 amino acids are identical to that of the rat protein. Rats were killed between hours 2–3 of the light cycle that corresponded to hours 21–22 after the last dose of simvastatin. The lens epithelium and cortex (separately recovered by dissection) from SD and CT rats treated from 1 to 14 days with 200 mg simvastatin/kg/day, or untreated controls were Dounce homogenized in the sucrose containing Buffer A described by Royo et al. (34) and centrifuged at 100,000 $g \times 1$ h. The SDS-containing lysis buffer used to recover lens proteins for the HMGR and SQS Western blot assays dissolves organelles and, therefore, could not be used in these studies. Immunoblots were performed against the PAGE separated cytosolic proteins using the purified antiserum at a 1:5,000 dilution and peroxidase conjugated goat-anti-rabbit secondary antibody at 1:80,000. The antibody recognized a single polypeptide of about 54 kDa.

RT-PCR estimation of changes in cytosolic HMGS mRNA levels in lens cortex

Total RNA was recovered from the outer cortex of lenses from the untreated control and treated SD and CT rats as described before (33). Rats were treated from 8 to 14 days with 200 mg/kg/day of simvastatin. Single stranded cDNA was synthesized from 0.5 μ g of total RNA by the Gene Amp RNA PCR kit (Roche Applied Biosystems, Branchburg, NJ) using 2.5 μ M random hexamers and standard conditions (10 mM Tris, 50 mM KCl, 5 mM MgCl₂, 1 mM dNTPs, 1 U/ μ l RNase inhibitor, and 2.5 U/ μ l of MULV reverse transcriptase; 15 min at 42°C and then 5 min at 95°C). PCR reactions were performed using 20 μ l of RT reaction mixture to which was added 80 μ l of PCR master mix to give 0.025 U/ μ l of HotStar Taq DNA polymerase (QIAGEN), 0.24 μ M sense and antisense primers and 10.0 μ M digoxigenin-11dUTP (Boehringer Mannheim, Indianapolis, IN). HMGS sense (AAGT CGGGACAGAGACAATCAT-3') and antisense (5'-CCTCGGTCA AAAATTACAGGAG-3') primers were constructed to amplify a 304 bp segment of HMGS, nucleotides 272–576 of cytosolic HMGS (35). The sequence of the amplified segment matched that for cytosolic HMGS (recovered product sequenced by Commonwealth Biotechnologies, Richmond, VA). Changes in the relative concentration of HMGS were normalized by comparison to lens levels of constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A 576 bp segment of GAPDH was simultaneously amplified using sense (5'-CCATCACCATCTTCCA GGAG-3') and antisense (5'-CCTGCTTCACCACCTTCTTG-3') primers described by Vos et al. (36). Hot Start-Touchdown PCR conditions were 94°C for 15 min; 3 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; 3 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min; 3 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; 3 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; 23 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and finally 72°C for 5 min. PCR products were detected by chemiluminescence as performed previously (33). Changes in the relative concentrations of HMGS and GAPDH were estimated by densitometric scanning of bands in the X-ray film.

Lens cortex HMGR activity

SD and CT rats (20 day old) were orally dosed for 14 days with 200 mg simvastatin/kg/day. At 22 h after the last dose, lenses were collected from individual treated rats and age-matched (untreated) control rats and decapsulated. The outer cortex from individual pairs of lenses was solubilized by stirring for 4 min in 0.25 ml of lysis buffer (see above) prepared with 0.25% Tergitol

(Sigma) rather than SDS. The enzyme assay is possible in the presence of Tergitol. Lenses were placed in 35 mm culture dishes inclined at about 30 degrees and oscillated on a rotatory shaker at about 100 oscillations per min for 4 min. Between 700 μ g and 900 μ g of protein was solubilized per pair of lenses. HMGR activity was measured essentially as described above. Four microliters of water containing [14 C]HMG-CoA (5 nmol at 58.9 mCi/mmol) was added to 10 μ l of 5 \times assay buffer. Reactions were started by addition of 36 μ l of lens lysate and incubated for 2 h at 37°C. Reactions were stopped by adding 20 μ l of 6 N HCl containing 200 μ g of mevalonic acid and [14 C]mevalonate isolated by TLC, recovered, and radioactivity measured as described above. HMGR activity was expressed as pmol of mevalonate formed/min/cortex (two lenses). Preliminary assays showed that enzyme activity was linear for at least 2 h.

Lens sterol synthesis

In vitro: dose response curves to simvastatin. Using 24-well culture plates, individual lenses from untreated 20-day-old CT, SD, and HW rats were incubated in 1 ml of TC199 media (37) containing 0 mM to 100 mM simvastatin acid. Each concentration was tested with 3–6 (but usually 5–6) lenses. Sterol synthesis was measured from incorporation of Na[14 C]acetate (54.7 mCi/mmol, New England Nuclear), 9.2 μ Ci added in 3 μ l of ethanol to give 0.17 mM sodium acetate. Lenses were incubated 4 h at 37°C with gentle rocking. After removing the media, individual lenses were washed twice with ice-cold PBS containing 15 mM unlabeled sodium acetate and twice with plain PBS. Individual intact lenses were saponified for 4 h at 100°C in 1.0 ml of ethanolic KOH (1 N in 67% ethanol) containing 100 μ g of unlabeled cholesterol. After adding 1 ml of water, nonsaponifiable sterols were extracted with 2–4 ml aliquots of hexane. The pooled hexane extracts were washed with an equal volume of water, recovered, evaporated, and the sterols separated by TLC (silica gel G using a solvent of n-hexane-diethylether-glacial acetic acid, 73:25:2, v/v/v). The cholesterol band, visualized by brief exposure to iodine vapor, was recovered and the 14 C content measured by scintillation counting. Sterol synthesis was expressed as pmol [14 C]acetate incorporated into sterols per lens.

In vivo: acute treatment with simvastatin. Twenty-day-old CT, SD and HW rats were given a single oral dose of simvastatin (200 mg/kg) or were untreated (controls). Animals were killed at 2, 6, 10, and 22 h later, individual lenses (5–6) incubated with 1 ml of TC199 containing [14 C]acetate, and sterol synthesis measured as described above. In parallel experiments, lens capsules (epithelial cell layer) were recovered at each time and drug induced changes in HMGR protein levels assessed by Western blotting.

In vivo: prolonged treatment with simvastatin. Twenty-day-old CT, SD, and HW rats were given a daily (10 AM) oral dose of simvastatin (200 mg/kg/day) for 7 or 14 days. On the 7th and 14th day, treated animals were given one final dose of simvastatin and killed from 2 h

to 22 h later. Individual lenses collected at selected times (7–12 lenses/time) were incubated with 14 C-acetate to assess sterol synthesis. Sterol synthesis of lenses from untreated, age-matched control rats was also measured. Lens HMGR protein levels were examined at the same selected times by Western blotting in parallel experiments. In some cases, changes in SQS and HMGS concentrations were also estimated by Western blots in lens fractions from control and treated rats.

In order to assess the effects of simvastatin treatment on “late-stage control” of sterol synthesis, individual lenses from control and treated CT, SD, and HW rats (treated for 7 or 14 days) were incubated in 1 ml of TC199 media containing 58 μ Ci of 5- 3 H]mevalonolactone (15 Ci/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO) for 4 h at 37°C. The lenses were incubated for 30 min in the media containing 10 μ M simvastatin prior to addition of the labeled substrate to minimize dilution of the [3 H]mevalonolactone with freshly generated mevalonate. Sterols were recovered from individual control and treated rats (4–8 lenses/group) and radiolabel measured as described above.

Synthesis of lens sterol intermediates: prolonged treatment with simvastatin. Twenty-day-old SD and CT rats were treated for 14 days with 200 mg/kg simvastatin. Twenty-two hours after the last dose, individual lenses from treated and untreated age-matched control rats were incubated with [14 C]acetate as described above. Six to eight individually incubated lenses were pooled from each group and saponified in the presence of 100 μ g of unlabeled squalene, lanosterol, lathosterol, 7-dehydrocholesterol, desmosterol, and cholesterol. Duplicate pools of incubated lenses were assayed. Nonsaponifiable lipids were extracted into hexane and fractionated by TLC using silica gel G plates and the solvent described above. After location of lipid fractions by brief exposure to iodine vapor, the squalene and lanosterol bands were directly recovered and counted. The silica gel band bearing the sterols was recovered, extracted with hexane, and the sterols rechromatographed on silica gel G TLC plates impregnated with 7% silver nitrate as described before in detail (38). The second TLC separates sterols into lathosterol, desmosterol, 7-dehydrocholesterol, and cholesterol. Zymosterol, the immediate precursor of lathosterol, comigrates with cholesterol. The gel zones bearing the individual sterols or sterol fractions were extracted with diethylether (the silver nitrate interferes with the scintillation counting) and the radiolabel content measured.

RESULTS

The studies described below were intended to address two questions: why are CT rats selectively sensitive to the development of cataracts following treatment with simvastatin and what is the mechanism of the simvastatin cataract?

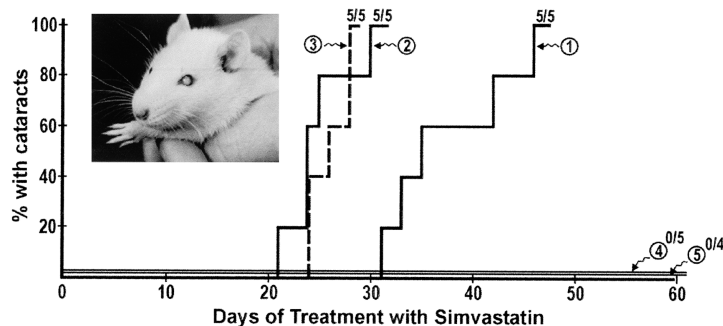


Fig. 1. Rat strain specific induction of cataracts by simvastatin. Beginning at 20 days of age, rats received a single daily dose of simvastatin by gastric gavage. 1) Chbb:Thom (CT) rats given 100 mg/kg b.w./day. 2) CT rats given 200 mg/kg/day for 10 days and 100 mg/kg/day thereafter. 3) CT rats given 200 mg/kg/day. 4) Hilltop Wistar (HW) rats given 100 mg/kg/day. 5) Sprague Dawley (SD) rats given 100 mg/kg/day.

Cataract formation

All CT rats developed cataracts after 31 to 46 days of receiving 100 mg simvastatin/kg/day (Fig. 1, line 1) when treatment was started at 20 days of age. None of the SD or HW rats developed cataracts after 60 days of treatment; however, one lens of one HW rat showed an opacity on day 61. The simvastatin cataracts rapidly progressed from trace clouding in the center of the lens to complete opacification in 2–3 days. Opaque lenses were grossly hydrated and lost about one-third of their soluble protein (data not shown). Giving 200 mg simvastatin/kg/day induced cataracts in all CT rats after only 24 to 27 days of treatment (Fig. 1, line 3).

This dose was selected for future studies because of the short treatment time needed for cataract formation.

Histological changes in lens structure were evident after 10 but not 5 days of treatment (Fig. 2). Initially, both the anterior and posterior ends of fiber cells were irregularly swollen. By the 15th day these changes were more pronounced and included comparable changes in the equatorial portions of fibers as well as committing the lens to opacification, since all CT rats developed cataracts by day 30 when treatment was stopped at day 14 (Fig. 3). No identically treated SD rats developed cataracts. Damage which may have occurred to the CT lens by the 7th day of

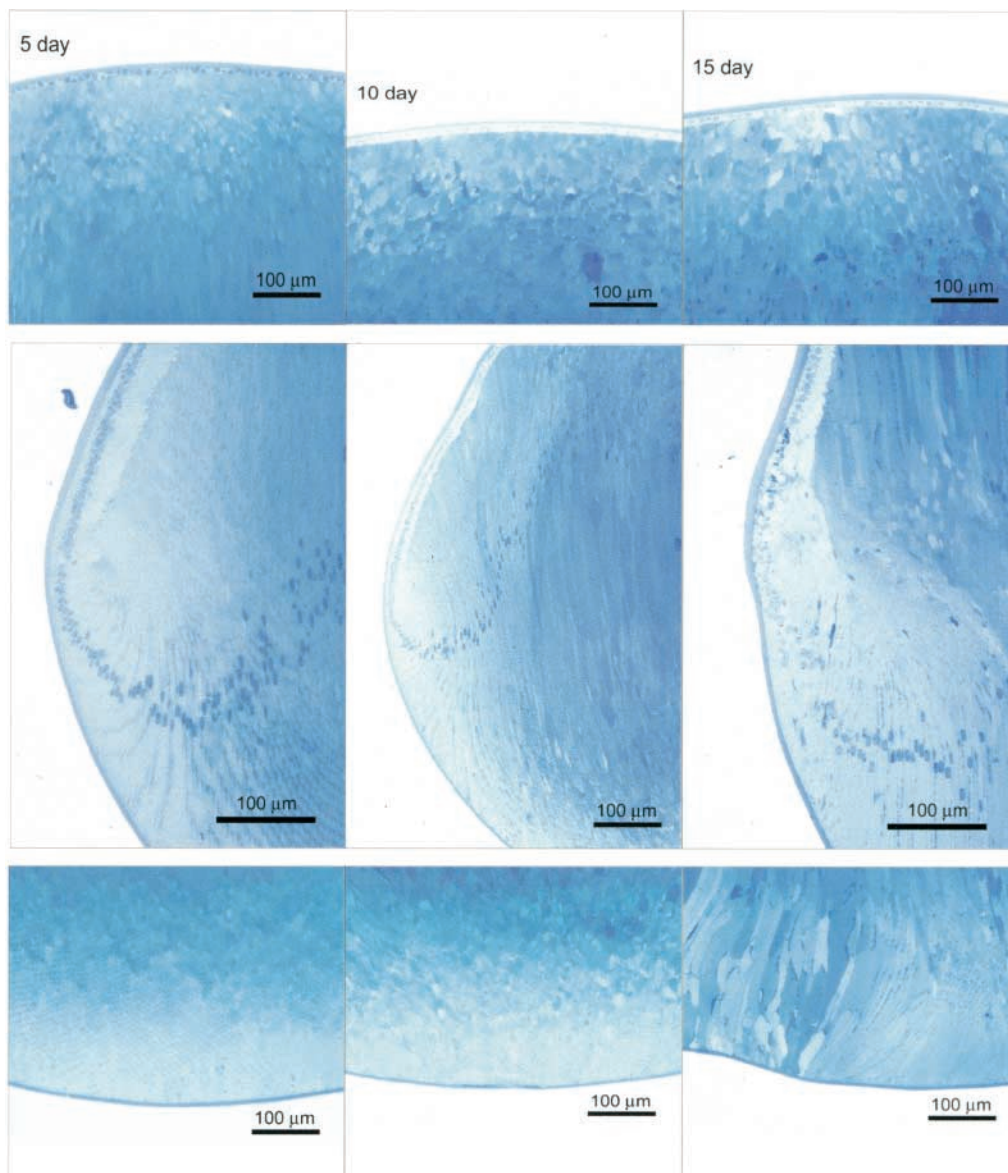


Fig. 2. Lens histology with duration of treatment (left column, 5 days; middle column, 10 days; and right column, 15 days) of CT rats with 200 mg/kg/day of simvastatin beginning at 20 days of age. After 5 days of treatment, the ordered structure of the lens necessary for transparency is still apparent in all regions (top row, anterior; middle row, equatorial; and bottom row, posterior) of the lens. However, after 10 days, irregular swelling of anterior and posterior fiber ends had occurred though lenses were still grossly transparent. After 15 days, the above described swelling had increased and the equatorial portions of fibers were also abnormally swollen.

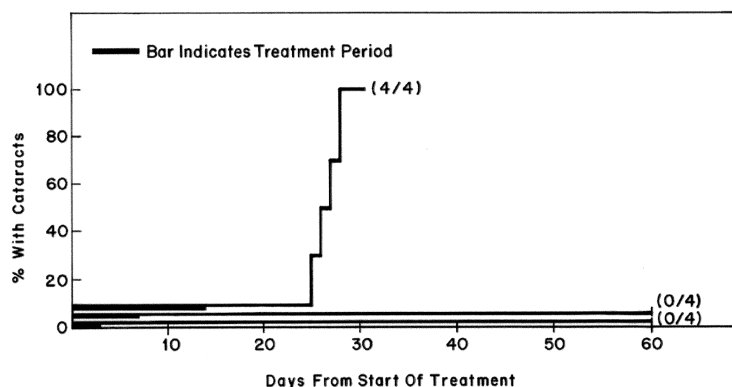


Fig. 3. Effects of duration of simvastatin treatment on induction of cataracts. CT rats (20 days old) were treated for 3, 7, or 14 days with 200 mg/kg/day. Treatment was then stopped.

treatment with simvastatin was apparently reversible or below some critical threshold, since no rats developed cataracts if treatment was stopped after 7 days. In view of the importance of this time interval to cataract formation, we focused on changes in lens chemistry and metabolism following 7 and 14 days of treatment.

Simvastatin can also induce cataracts in older CT rats. Four young adult CT rats (8 weeks of age at start) were treated with simvastatin (200 mg/kg/day) for 8 weeks. Two of four rats developed cataracts. They were seen in one rat by slit lamp examination after 6 weeks of treatment and in the second at 8 weeks by histological examination. Jürgen Güttner (Boehringer-Ingelheim, Inc.) described in a personal communication that 11 of 20 young adult CT rats (11 weeks old at start) developed cataracts following 8 weeks oral treatment with simvastatin.

Apoptosis

The epithelial cell layer adherent to the lens capsule from CT rats was examined for the presence of apoptotic cells at 2 h and 22 h after a single 200 mg/kg dose of simvastatin and after 7 or 14 consecutive days of treatment (22 h after the last dose). Cells were examined at 2 h and 22 h after the single dose to test the possibility that, if nuclear fragments were seen at 2 h (time when ocular humor levels of simvastatin are highest, **Table 1**), they might have been cleared by 22 h. None of the simvastatin treatments produced an obvious increase in then incidence of apoptotic cells (**Fig. 4**). Apoptosis is also not a significant feature of human senile cataracts (39) or in some other experimental models of rodent cataracts (M.E. Verdugo-Gazdik, Pfizer, Inc., Groton, CT; personal communication).

Lens lipids and weights

The simvastatin cataract does not appear to be due to a selective reduction in the cholesterol content of lens membranes, since the cholesterol to phospholipid molar ratio was unchanged between lenses of 14-day-treated and age-matched control CT rats (Table 1). This possibility is supported by finding that simvastatin induced cataracts in adult sized CT rats where lens growth, and thus the need for cholesterol, is slight. Lenses from 14-day treated rats were about 10% smaller, slightly dehydrated, and contained 10–15% lower concentrations of both cholesterol and phospholipid. These lens changes were not seen in SD rats treated for 14 days (data not shown).

Serum and ocular humor concentrations of simvastatin acid

Peak serum levels of simvastatin acid reached about 1,000–1,100 ng/ml or about 2.5 μ M in CT rats versus about 600 ng/ml in SD rats at 1 h after giving a single oral dose of 200 mg/kg (**Fig. 5**). However, since there was little difference in serum levels between CT and HW rats, it seems unlikely that serum levels of simvastatin are important to explaining the special sensitivity of CT rats to cataract formation.

Ocular humor levels of simvastatin acid were expectedly lower than serum levels in all rat strains, and there was some indication of higher ocular humor levels in the CT rats at 2 h to 4 h after dosage (**Table 2**). However, by 6 h and 10 h after treatment, differences between strains were not clear. For example, at 6 h the concentration of simvastatin acid in one of the two ocular humor pools from SD, HW, and CT rats were 204 nM, 231 nM, and 284 nM, respectively (Table 2). We assume that these concentrations

TABLE 1. Effect of treatment with simvastatin for 14 days on lens lipids and weights

	Lipids			Weights		
	Cholesterol	Phospholipid	C/P	Wet Weight	Dry Weight	%H ₂ O
	<i>μmol/g dry lens</i>			<i>mg/lens</i>		
Lens						
Control	3.96 \pm 0.09	5.99 \pm 0.14	0.661 \pm 0.011	21.60 \pm 0.24	8.38 \pm 0.09	61.21 \pm 0.14
Treated	3.44 \pm 0.11 ^a	5.16 \pm 0.08 ^a	0.667 \pm 0.018	19.48 \pm 0.26 ^a	7.83 \pm 0.06 ^a	59.97 \pm 0.32 ^a

Chbb:Thom (CT) rats (20 days old) were orally treated or not treated (controls) for 14 days with 200 mg/kg/day of simvastatin. Six lens pairs were used for each experimental group.

^a P(t) of differences from control < 0.005.

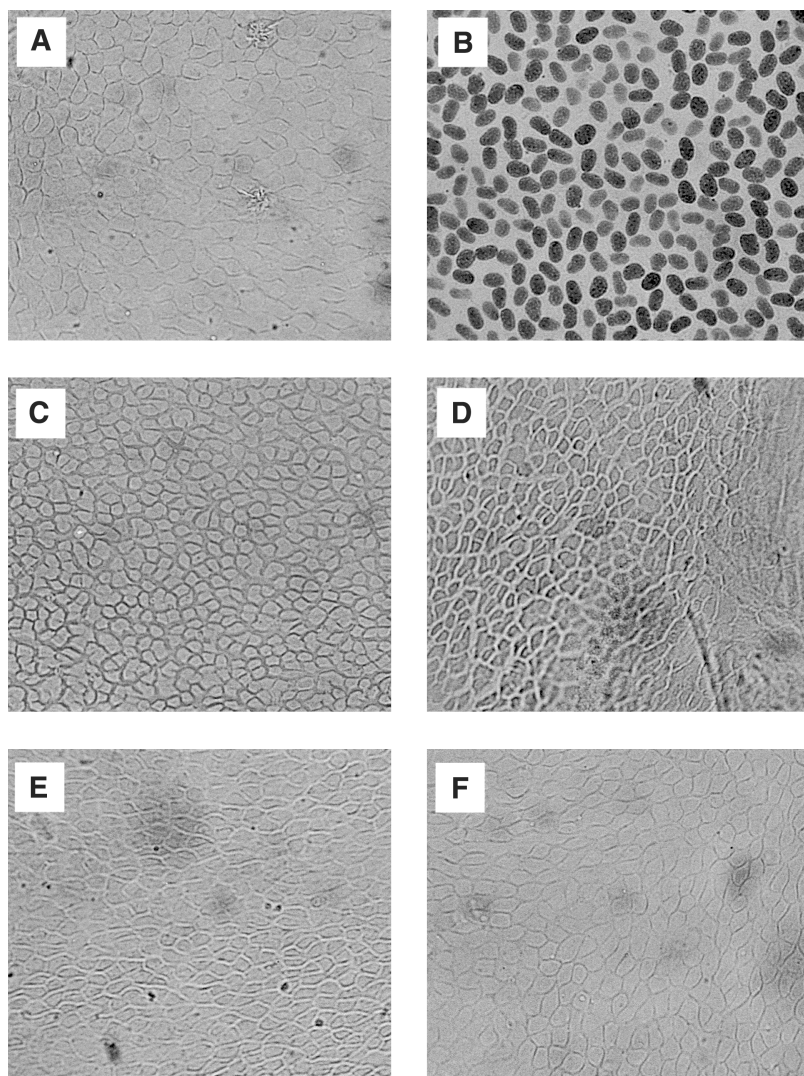


Fig. 4. Effect of acute and prolonged simvastatin treatment on apoptosis of lens epithelia. CT rats were treated with 200 mg/kg/day simvastatin and killed 2 h (C) or 22 h (D) after a single dose, or after daily doses for 7 days (E), or 14 days (F) beginning at 20 days of age. Epithelium from an untreated rat (27 days old) is shown in A. DNase I treated epithelium from an untreated 27-day old rat is shown in B and serves as a positive control. Epithelia were stained using a modified TUNEL assay as described in Methods. Epithelia from 3 rats were stained per time point. Representative fields are shown. Magnification $\times 200$.

approximate those in the lens. Simvastatin is apparently cleared from the lens by 22 h after dosage in all rat strains, since lens HMGR protein levels and sterol synthesis were both increased 3–5-fold over basal levels in all rats at this time (Fig. 6).

Response of lenses to acute inhibition of sterol synthesis

We considered the possibility that the selective sensitivity of CT rats to cataract formation was due to increased sensitivity to inhibition of the lens sterol synthesis pathway by simvastatin because of lower lens concentrations of HMGR protein or to kinetically altered protein. No significant differences were seen in the capacity of simvastatin acid to inhibit basal levels of lens sterol synthesis (Fig. 7). The estimated ID_{50} concentrations of simvastatin acid for inhibition of sterol synthesis from [^{14}C]acetate were about 6 nM, 9 nM, and 10 nM for SD, HW, and CT lenses, respectively. This observation appears consistent with detecting similar basal levels of HMGR protein in lenses of untreated CT, SD, and HW rats (Fig. 6, Figs. 8–10) and measuring similar K_m and K_i values for HMGR from liver of the various strains (Fig. 11). We assume that the similar kinetic properties of liver HMGR (K_m 29 μM to 47 μM and

simvastatin K_i of 1.8 nM and 2.0 nM) between strains also applies to the lens.

Lens sterol synthesis decreased to almost zero at 2 h after giving a single 200 mg/kg oral dose of simvastatin to 20-day-old CT, SD, and HW rats (Fig. 6). Although sterol synthesis was still suppressed at 6 h posttreatment, by 10 h synthesis was equal to or greater than control levels (untreated rats, zero time) in all three strains and 3- to 5-fold greater than control for all at 22 h after dosage. The increased synthesis measured at 22 h correlated well with the marked upregulation of HMGR protein levels seen at this time in all lenses. Lens epithelial levels of HMGR, which mimicked changes in the lens fiber cells (Figs. 9, 10), were increased 5-fold or more at both 6 h and 22 h after dosage in all three rat strains. The inhibition of lens sterol synthesis evident at 6 h (Fig. 6) obviously reflected the continued presence of adequate simvastatin in the lens to inhibit expression of the extra HMGR protein. Simvastatin was apparently cleared from the lenses of all rats by 22 h after dosage, since lens sterol synthesis was increased in proportion to the increases in HMGR protein mass at this time (Fig. 6). Although the rapid upregulation of HMGR protein expression in the lens may seem un-

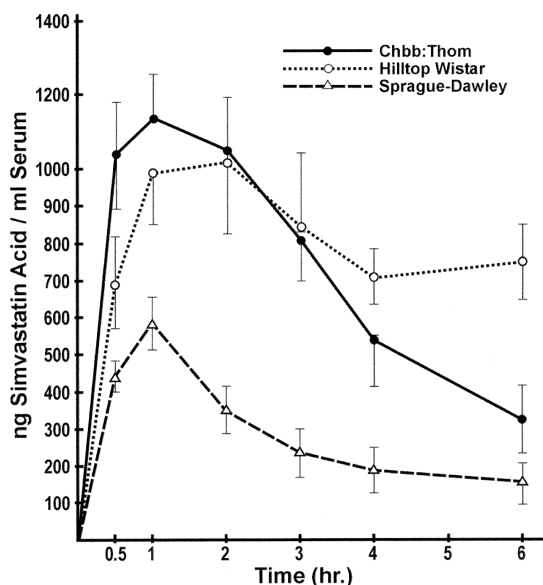


Fig. 5. Serum levels of simvastatin acid with time after acute dosage. CT, HW, and SD rats (49–58 days of age, 5–7 rats/strain) received a single oral dose of simvastatin (200 mg/kg) by gavage. Blood samples were collected by cardiac puncture under brief ether anesthesia, and serum drug levels were measured by HPLC analysis. Points are the mean \pm SEM (bars).

usual, we had previously shown that lens HMGR protein can turnover rapidly, <1 h for cultured lens cells (40). We had also previously seen that treatment of SD rats with lovastatin induced similar upregulation of HMGR protein levels in the lens epithelium and cortical fiber cells (32).

Responses of lenses to prolonged inhibition of sterol synthesis

Results to this point showed no significant differences in lens HMGR protein or in the acute response of lenses from the three strains to inhibition of sterol synthesis by simvastatin. We considered the possibility that differences in sterol synthesis might be induced after prolonged treatment.

Following 1 or 2 weeks of treatment with simvastatin, HMGR protein levels in lens epithelium were increased at

TABLE 2. Ocular humor levels of simvastatin acid

Time (h)	Simvastatin Acid		
	SD	HW	CT
	<i>nM</i>		
2	186 (243, 128)	356 (410, 302)	631 (665, 607)
4	118 (109, 127)	208 (210, 206)	593 (847, 339)
6	129 (204, 53)	153 (74, 231)	390 (284, 495)
10	26 (35, 16)	30 (35, 24)	93 (43, 142)

Forty 42-day-old rats were given a single oral dose of simvastatin (200 mg/kg). Rats were killed in groups of 3–5 at 2, 4, 6, and 10 h after dosage. Aqueous humor was pooled from each set of eyes and assayed by HPLC for simvastatin acid. Values are the average of two experiments (individual values in parentheses).

least 5-fold in all three rat strains (Fig. 8). The marked increase in HMGR protein was accompanied by comparable increases in incorporation of [14 C]acetate into sterols by the intact lens of SD and HW rats but not CT rats (Fig. 8). The increased incorporation in SD and HW rat lenses was most evident at 10 h and 22 h after the last dose, likely reflecting the clearance of simvastatin by these times. There was obvious dissociation of upregulated lens HMGR protein levels from sterol synthesis in CT rats treated for 7 days, which progressed to complete dissociation by 14 days of treatment. Thus, in spite of greatly increased concentrations of HMGR protein, CT lens sterol synthesis remained at baseline levels; i.e., indistinguishable from that of untreated-age matched controls. Higher simvastatin acid concentrations in CT rat lenses seemed unlikely to account for this dissociation of lens HMGR protein levels from sterol synthesis, since there were no consistent differences between strains in serum or ocular humor levels of the drug (Fig. 5 and Table 2), and the complete dissociation in CT rat lens was still evident at 22 h after the last dose of simvastatin (Fig. 8), a time when the drug should have been cleared from the lens (Fig. 6).

We considered two possible explanations for the discordant regulation of sterol synthesis in CT rat lenses: there was blockage or failure to upregulate an enzyme at a metabolic site downstream of HMGR in the lens sterol synthesis pathway of CT rats or there was failure to upregulate enzymes upstream of HMGR, specifically at HMGS.

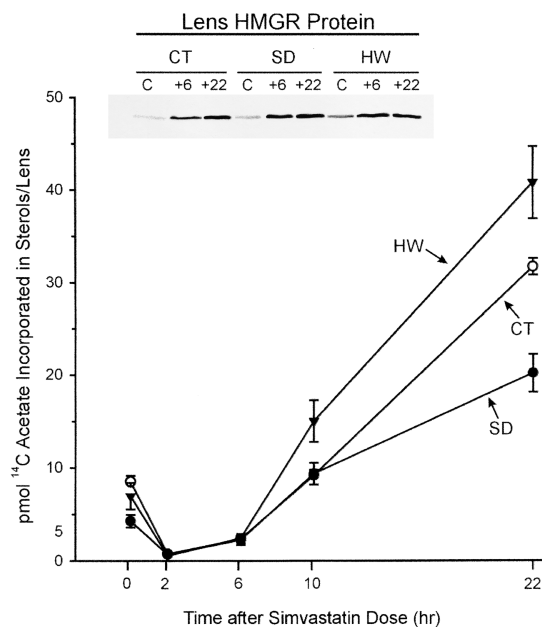


Fig. 6. Effects of acute treatment with simvastatin on lens sterol synthesis and HMGR protein levels. CT, SD, and HW rats (20 days old) were given a single oral 200 mg/kg dose of simvastatin or were untreated (0 time). Individual lenses from rats killed at various times after dosages were incubated with [14 C]acetate as described in Methods. Sterols were recovered from saponified lenses and 14 C content measured. Each value is the mean \pm SEM of 4–6 lenses. HMGR protein levels in the lens epithelium of identically treated rats were estimated by Western blotting (75 μ g of protein/lane).

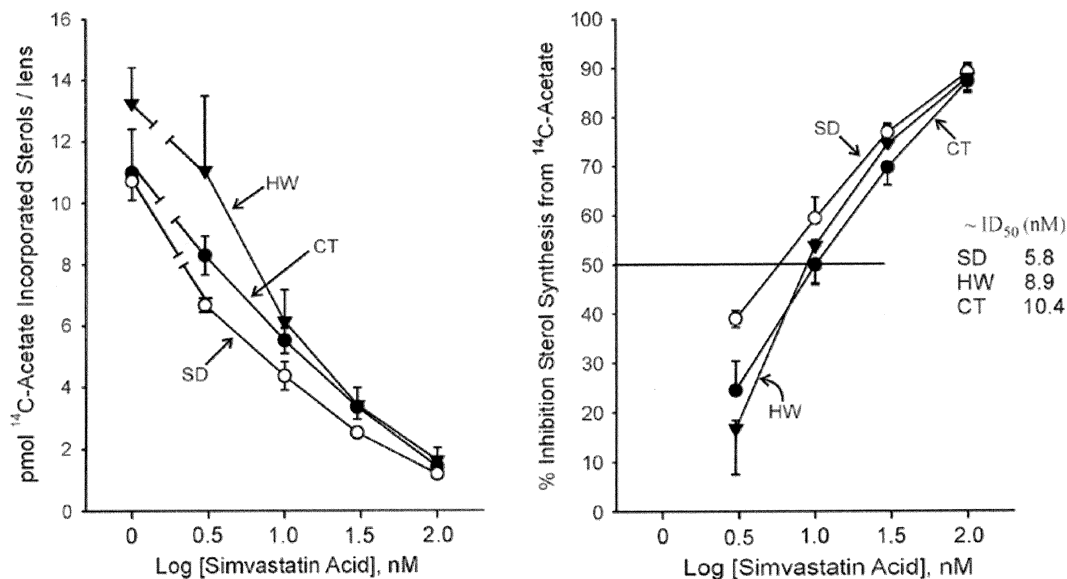


Fig. 7. Simvastatin acid inhibition of basal sterol synthesis by rat lenses in vitro. Individual lenses from 20-day-old untreated SD, HW, and CT rats were incubated for 4 h at 37°C in 1 ml of TC199 media containing Na[1-¹⁴C]acetate (9.2 μ Ci/ml, 54.7 μ Ci/ μ mol) and 3 nM to 100 nM of simvastatin acid. Controls were incubated without simvastatin. Values are mean \pm SEM of 4–6 lenses/point.

Late stage regulation of sterol synthesis

Because a block at a late metabolic site in sterol synthesis might explain the upregulation of HMGR in CT rat lenses without a concomitant increase in cholesterol formation, synthesis of late stage intermediates was assessed. Incorporation of [¹⁴C]acetate into squalene, lanosterol, lathosterol, 7-dehy-

drocholesterol, desmosterol, and cholesterol was markedly increased in lenses from SD rats treated with simvastatin for 14 days as compared with their controls (**Fig. 12**). There was essentially no increased incorporation of radiolabel into any of these intermediates or cholesterol in lenses from CT rats. Thus, there appears to be no block in sterol synthesis between

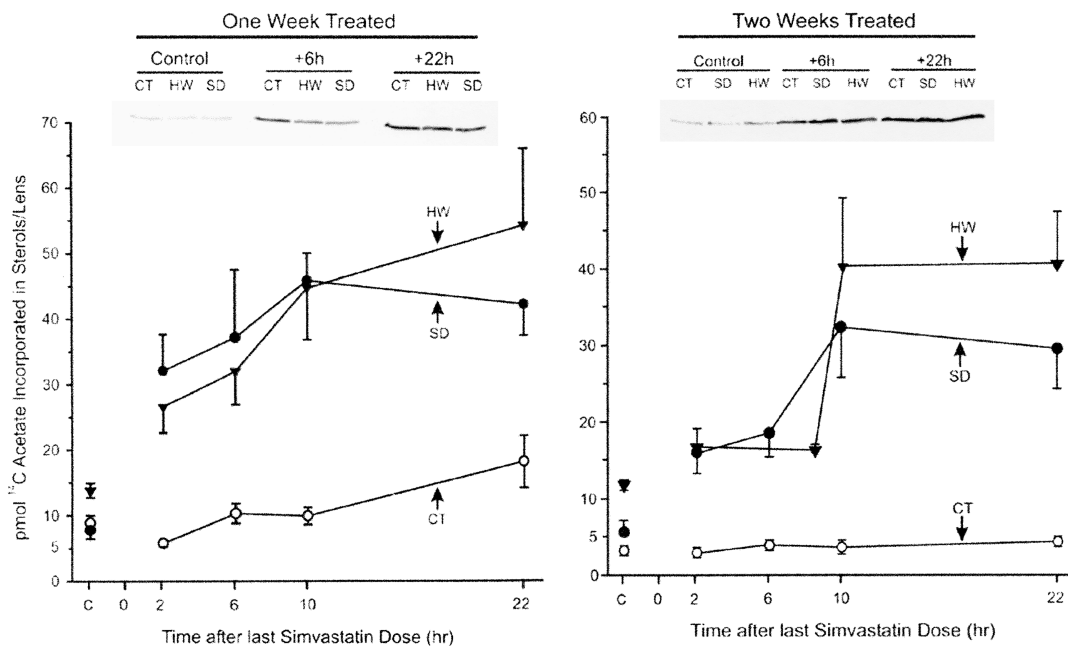


Fig. 8. Effects of prolonged treatment with simvastatin on lens sterol synthesis and HMGR protein levels. CT, SD, and HW rats (20 days old) were orally treated with 200 mg simvastatin/kg/day for 7 or 14 days. Age-matched controls (C) were untreated. At specific times after the last dose, usually 2, 6, 10, and 22 h, lenses were removed and individually incubated with [1-¹⁴C]acetate. Labeled sterols were recovered as described in Methods and the ¹⁴C content measured. Each point is the mean \pm SEM of 8–12 individual lenses. HMGR protein levels in lens epithelium of identically treated rats were estimated by Western blotting (65–70 μ g protein/lane).

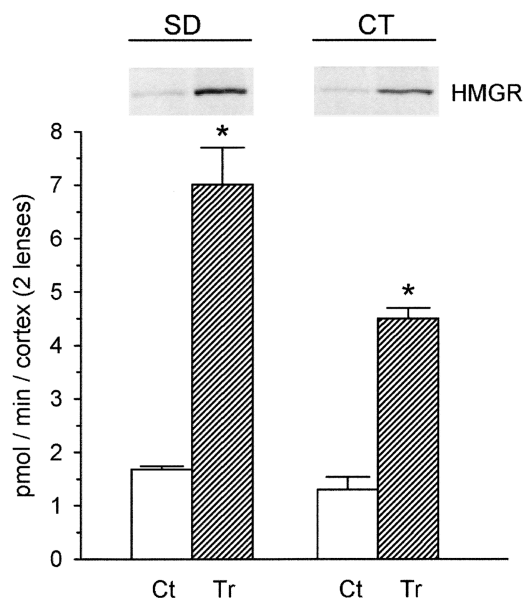


Fig. 9. Changes in lens cortex HMGR protein and enzyme activity following prolonged treatment with simvastatin. SD and CT rats (20 days old) were treated (Tr) for 14 days with simvastatin. Age-matched controls (Ct) were untreated. Cortical lysates were examined for HMGR protein and assayed for HMGR activity at 22 h after the last dose of drug. Enzyme activity was expressed as pmol of mevalonate formed per min per cortex (two lenses). Each value is the mean SEM (bars) of four separate pairs of lenses from individual rats. Equal aliquots of protein was pooled from each lens pair in the control and treated groups and 80 μ g applied to each gel lane. **P*(*t*) of differences from controls <0.001.

squalene and cholesterol in lenses of CT rats; i.e., we did not see increased incorporation of [14 C]acetate into any of the intermediates examined.

Regulation of lens sterol synthesis downstream of mevalonic acid

Regulation of sterol synthesis independent of HMGR was assessed by examining incorporation of [3 H]mevalonate into lens total sterols of control rats and rats treated



Fig. 10. Changes in lens epithelium and cortical HMGR and squalene synthase (SQS) protein following treatment with simvastatin. SD and CT rats (20 days old) were treated (T) for 14 days with simvastatin. Age-matched controls (C) received no treatment. Protein of the lens epithelium (Epi) and cortex (Cort) (about outer 10% of lens radius) was examined for HMGR and SQS levels by Western blotting (75 μ g of protein/lane) at 12 h after the last dose of drug. Membranes were probed for HMGR, stripped and re-probed for SQS.

for 7 or 14 days with simvastatin. Sterol synthesis was increased about 1.4-fold in CT rat lenses and 2.1-fold in lenses of SD and HW rats treated for 7 days, indicating some upregulation of one or more enzymes downstream of mevalonate in all rat strains (Table 3), and perhaps less upregulation at this time in the CT rat lens. After 14 days of treatment, incorporation of [3 H]mevalonate into lens sterols was increased 2.1- to 2.6-fold in SD and HW rats, but not significantly changed for the CT rat lenses. This observation, together with finding no increased incorporation of radiolabel into sterol intermediates between squalene and cholesterol, suggests a block in sterol synthesis between mevalonate and squalene.

HMGR protein and enzyme activity in lens cortex

HMGR protein levels were markedly increased in the lens cortex of both SD and CT rats following 14 days treatment with simvastatin (Figs. 9, 10). The cortex accounts for most of the lens' total cholesterol synthesis (31). Lens epithelial HMGR protein was also increased (Fig. 10) in response to treatment as seen before (Figs. 6, 8). The increase in lens cortex HMGR protein levels following prolonged dosage of SD and CT rats was accompanied by comparable increases in cortical HMGR enzyme activity (Fig. 9).

SQS protein in the lens epithelium and cortex

The cellular concentrations of SQS, the first committed enzyme in cholesterol synthesis, usually change in parallel with those of HMGR (41). As compared with control lenses, following 14 days treatment with simvastatin the epithelium of treated CT and SD rats contained clearly increased concentrations of SQS, about a 3- to 4-fold increase (Fig. 10). The concentration of SQS in the cortex of control lenses (C) was greater than in control epithelium in both strains. However, the relative increase in cortical concentration of SQS following treatment with simvastatin was only slight (1.4- to 1.8-fold) compared with the increase in HMGR. As discussed below, the much greater increase in lens levels of HMGR than SQS in the lens cortex (Fig. 10), coupled with increased activity of the lens sterol synthesis pathway in treated SD and HW rats (Fig. 8), might protect lenses of SD and HW rats from cataracts due to shunting of increased levels of nonsterol intermediates to formation of protective substances.

Cytosolic HMGS protein and mRNA in lens

Cytosolic HMGS provides the substrate, HMG-CoA, for HMGR and is a regulated enzyme in the sterol synthesis pathway (34, 42). Goldstein and Brown identified HMGR and HMGS as the two major regulated enzymes of the sterol synthesis pathway (41). Expression of upregulated HMGR could be dependent on increased availability of its substrate. Treatment of SD rats for 8 or 14 days resulted in about an average 12-fold increase in the relative concentration of HMGS protein and about a 2-fold increase in HMGS mRNA in the lens cortex (Fig. 13). This lens region accounts for about 85% of the lens' total cholesterol synthesis (31, 43) and 85–90% of the total HMGS protein (estimated from the intensities of the bands scanned in

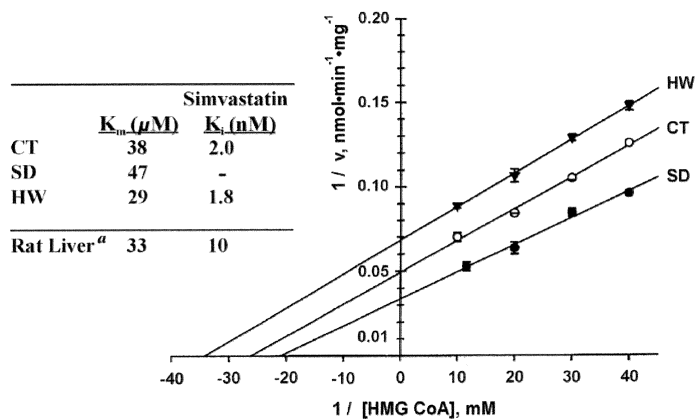


Fig. 11. Michaelis-Menten kinetics of liver HMGR. HMGR protein was solubilized and partially purified from the livers of 20-day-old CT, SD, and HW rats. Protein ($2 \mu\text{g}/50 \mu\text{l}$ reaction mixture) was incubated for 20 min with [^{14}C]HMG-CoA ($0.025\text{--}0.10 \text{ mM}$, 57.8 mCi/mmol) in the absence or presence of simvastatin (31 nM). [^{14}C]mevalonolactone was recovered by TLC and radiolabel content measured. ^aThe published K_m for rat liver HMGR and the K_i for a mevinolinic acid derivative is provided by Endo et al. (29).

Fig. 13 and the total protein content of the epithelial and cortical fractions). In contrast to the cataract resistant SD rats, the concentration of HMGS protein and mRNA in the cortex of chronically treated CT rats was little affected (Fig. 13). The strain differences in upregulation of the cortical protein were less apparent after a single dose of simvastatin (Fig. 13, day 1), and HMGS protein appeared comparably increased in the epithelium of both strains at all times.

DISCUSSION

Risk of simvastatin-induced cataracts may be age related. Although simvastatin treatment damaged lenses of both weanling and young adult CT rats, the speed of onset, incidence, and severity of cataracts was less in the older animals. This difference might reflect superior drug metabolism in the older rats or reduced susceptibility of the older lenses to stress.

Although statins have not previously been reported to induce cataracts in living rats, lovastatin at high concentration (0.1 mM) was shown to cause opacification of intact rat lenses in long-term (7 days) organ culture (44) and to disrupt the cytoskeleton of cultured lens epithelial cells (45). The current study shows that, compared with SD and HW rat strains, young CT rats were especially sensitive to formation of permanent cataracts following relatively short-term dosage with simvastatin (Fig. 1). How-

ever, the sensitivity may not be exclusive, since the reference strains were only treated for 60 days and one HW rat did develop a cataract at day 61. A high dose of simvastatin, 200 mg/kg/day , was chosen to examine the basis of this selective sensitivity and the mechanism of cataract formation because lens opacification developed in all treated CT rats within only 3 to 4 weeks. A lower dose of 100 mg/kg/day led to cataracts in 5 to 7 weeks. About 50% of young adult rats develop cataracts after 8 weeks of treatment. Cataract development at lower doses for longer times was not tested.

Assuming that the simvastatin-rat cataract is related to effects on lens HMGR, our studies focused on changes in this lens enzyme and lens sterol synthesis. The basal lens concentrations of HMGR protein (Figs. 6, 8–10), basal levels of sterol synthesis (Figs. 6–8), and sensitivity to inhibition of basal synthesis (Fig. 7) by simvastatin were not different between strains. Kinetic properties of liver HMGR were similar between strains (Fig. 11). When rats of each strain were treated with a single oral dose of simvastatin, lens concentrations of HMGR protein and rates of sterol synthesis were comparably and rapidly upregulated (Fig. 6). However, after 1 week of daily treatment, lenses from CT rats displayed discordant expression of the sterol synthesis pathway (Fig. 8). A 3- to 5-fold increase in lens HMGR protein was unmatched by comparable increases in sterol synthesis in CT rats. This was in contrast to comparable 3- to 5-fold increases in both HMGR protein and sterol synthesis in the other two strains. By the second

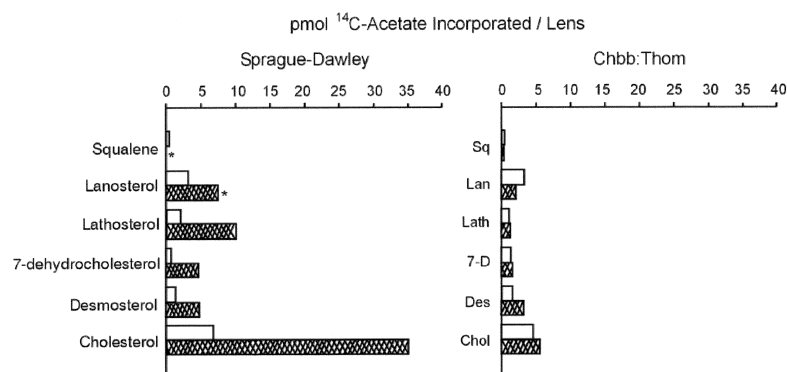


Fig. 12. Effect of prolonged simvastatin treatment upon synthesis of sterol synthesis intermediates. Twenty-two hours after the last dose of simvastatin, individual lenses from SD and CT rats treated for 14 days were incubated 4 h in 1 ml of TC199 media containing [^{14}C]sodium acetate as described. Individual lenses from untreated-age matched controls were identically incubated. Six to eight lenses from each group were pooled in duplicate, saponified, and lipids extracted and fractionated by various TLC systems. Values for the duplicate pools differed by an average 15%. Treated, hatched bars; Control, open bars. *Squalene samples inadvertently pooled with the lanosterol samples.

TABLE 3. Late stage upregulation of lens sterol synthesis following treatment with simvastatin

	7 Days Treated			14 Days Treated		
	Control	Treated	T/C	Control	Treated	T/C
	<i>pmol [³H]mevalonate incorporated into sterols/lens</i>					
Rat Strain						
CT	0.384 ± 0.033	0.523 ± 0.022 ^a	1.36	0.135 ± 0.008	0.118 ± 0.0008	0.87
SD	0.260 ± 0.028	0.538 ± 0.051 ^b	2.07	0.113 ± 0.007	0.238 ± 0.016 ^b	2.11
HW	0.393 ± 0.028	0.809 ± 0.050 ^b	2.06	0.214 ± 0.018	0.552 ± 0.034 ^b	2.58

Twenty-day-old rats were treated for 7 or 14 days with simvastatin (200 mg/kg/day). Age-matched controls were untreated. Individual lenses were incubated with 10 μM simvastatin in 1 ml TC199 media. After 30 min, 5-[³H]mevalonate (58 μCi, 15 Ci/mmol) was added and lenses incubated 4 h. Sterols were isolated by TLC after saponification and radiolabel measured. Each value is the mean ± SEM of synthesis by 4–8 individually incubated lenses.

^a *P*(t) < 0.006.

^b *P*(t) < 0.0005.

week of treatment, the markedly upregulated HMGR protein levels and enzyme activity in CT rat lenses sharply contrasted with no increase in lens sterol synthesis.

The most obvious possible explanation for the failure to upregulate sterol synthesis in CT rat lenses is that these lenses accumulated simvastatin. This possibility seems unlikely because blood and ocular humor levels of simvasta-

tin (Fig. 5, Table 2) were little different between rat strains, and simvastatin was obviously cleared from CT rat lenses by 22 h after acute dosage since lens sterol synthesis and HMGR protein levels were comparably increased at this time (Fig. 6).

A block at a metabolic site in the sterol synthesis pathway downstream of mevalonic acid might account for the disconnection between lens HMGR activity and sterol synthesis following prolonged treatment of CT rats with simvastatin. The absence of increased incorporation of ¹⁴C-acetate into intermediates between squalene and cholesterol of CT rat lenses (Fig. 12) argues against a block after squalene. However, a block may exist in CT lenses between mevalonate and squalene since incorporation of [³H]mevalonate into lens sterols was increased 2–2.5-fold in SD and HW rats following 14 days treatment but was unchanged in CT rat lenses (Table 3). These combined observations suggest that there is failure to upregulate one or more enzymes of the sterol pathway between mevalonate and squalene, such as for example, mevalonate kinase or isopentenyl pyrophosphate isomerase.

We also considered the possible involvement of HMGS in the failed upregulation of sterol synthesis in CT rat lenses, since it was difficult to reconcile how loss of a 2-fold increased sterol synthesis from mevalonate following prolonged treatment with simvastatin (Table 3) could account for loss of a 5-fold increased synthesis from acetate (Figs. 8, 12). Prolonged treatment of SD rats with simvastatin resulted in more than a 10-fold elevation of HMGS protein in the lens cortex and about a 2-fold increase of cortical HMGS mRNA (Fig. 13). There was little or no upregulation of HMGS protein and mRNA in the lens cortex of identically treated CT rats. Thus, if markedly increased concentrations of HMG-CoA are necessary for expression of the extra HMGR protein, failure to achieve this increase in CT rat lenses could leave sterol synthesis idling at the observed basal rate (Fig. 8). Though the combined results indicate that there may be multiple defects in regulation of sterol synthesis in the lens of CT rats, failed upregulation of cortical HMGS could obscure the relevance of any post-mevalonate defects.

Lens levels of HMGS protein could be controlled by both transcriptional and posttranscriptional mechanisms. Post-

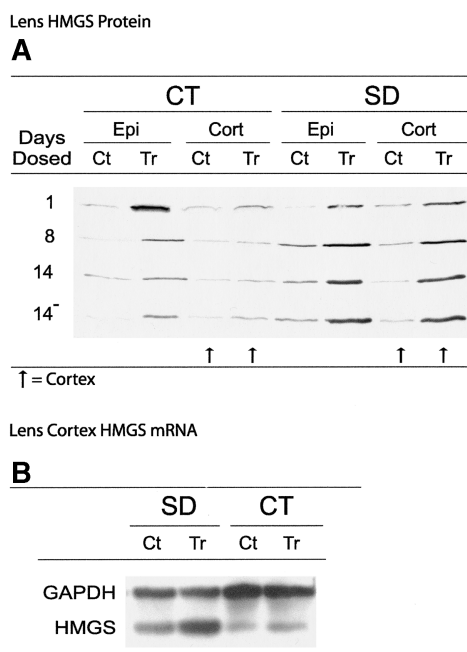


Fig. 13. Changes in lens epithelial and cortical HMG-CoA synthase (HMGS) protein and mRNA following acute and chronic dosage with simvastatin. SD and CT rats (20 days old) were treated once or for 8 or 14 consecutive days with 200 mg simvastatin/kg/day. A: Cytosolic protein from the lens epithelium (Epi) (17–20 μg per lane) and cytosolic protein from the cortex (Cort) (70 μg per lane) were separated by SDS-PAGE and immunoblotted with a rabbit-antirat polyclonal antiserum to cytosolic HMGS. The 14' samples are from a second 14-day experiment. Controls for the 14 and 14' were the same. Arrows identify the lens cortical samples of control and treated CT and SD rats. B: RT-PCR amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and HMGS cDNA segments from 0.5 μg of outer lens cortex total RNA of control (Ct) and treated (Tr) rats. GAPDH cDNA product, 576 bp; HMGS cDNA product, 304 bp. Rats were treated for 8–14 days.

transcriptional regulation may be quantitatively more important, since the lens cortex HMGS protein level increased much more than the mRNA level in simvastatin treated SD rats (Fig. 13). This response is similar to changes in lens HMGR seen in SD rats following chronic treatment with lovastatin. Here, lens cortex HMGR protein increased by more than 6-fold but mRNA copy number increased by only about 40% (32). Thus, the failure of prolonged treatment with simvastatin to increase lens cortex HMGS protein in CT rats could reflect defects at both transcriptional and posttranscriptional levels, with perhaps the posttranscriptional level being more important. The upregulation of HMGS in the lens epithelium but not cortex of CT rats following prolonged treatment with simvastatin implies that control of expression of this enzyme may be different in the two lens compartments. The possibility that different forms of HMGS could be expressed in epithelial and fiber cells might be considered, since two forms of the cytosolic HMGS gene have been identified in *Blattella germanica* (46).

The cause of simvastatin cataract remains unknown. However, it does not appear to be due to selectively lowering the cholesterol content of the lens. The lens cholesterol to phospholipid molar ratio of CT control and treated rats was unchanged after 2 weeks of treatment, a time at which the lens is damaged and committed to cataract formation (Table 1 and Figs. 2, 3) and simvastatin induced cataracts in grown rats where lens net sterol synthesis is negligible. Since basal levels of lens sterol synthesis were not decreased in CT rat after either one or 2 weeks of treatment (Fig. 8), the cataract appears to more likely reflect failure to upregulate the sterol synthesis pathway rather than to inhibition of the pathway. The coordinate increase seen in SD and HW rat lenses of HMGR and sterol synthesis is far beyond that needed to maintain cellular cholesterol levels. What is the purpose of this increase beyond need? The increase might reflect a successful response of SD and HW rat lenses to stress. Treatment of hamsters with proinflammatory cytokines or endotoxin resulted in marked increases of liver HMGR but not of other key regulatory enzymes of sterol synthesis (47) and in decreases of SQS mRNA and protein levels (48). Lens cortex HMGR protein also increased much more than SQS protein following treatment with simvastatin (Fig. 10). The purpose of this unbalanced upregulation could be shunting of sterol synthesis intermediates to formation of nonsterol factors important to counteracting inflammation, such as geranylgeranyl and isoprenylated proteins. Geranylgeranylated proteins are potent inhibitors of cyclooxygenase 2 (49). Increased formation of these factors may not occur in lenses of CT rats due to the lack of increased substrate flow into the sterol pathway and, thus, the simvastatin cataract could represent a failed compensatory response to cellular stress. The stress might be related to actions of statin acids or their lactones, which are independent of inhibiting sterol synthesis (50, 51). An alternative hypothesis is that simvastatin treatment lowered the concentration of sterol pathway derived factors essential for maintaining lens cells, such as the Rho GTPases needed for maintaining the cytoskeleton (45). An argu-

ment against this possibility is that lens sterol synthesis in chronically treated CT rats was not lower than that in control CT rats (Figs. 8, 12). Rather, it seems more likely that the CT lenses were deprived of increased amounts of factors necessary to prevent lens opacification.

The present study supports the idea that genetic defects in one or more enzymes of the sterol synthesis pathway can increase the risk of cataracts from simvastatin in rats. Polymorphism in one of more of the enzymes in the sterol synthesis pathway, in the numerous proteins that mediate the pleiotropic actions of the statins (52), or in the enzymes that metabolize statins could influence the ocular safety of these drugs. Schlienger et al. (22) recently reported that "concomitant use of erythromycin and simvastatin may increase the risk for cataracts" in humans. Erythromycin, along with other drugs, can inhibit the cytochrome P-450 enzymes that metabolize simvastatin and increase its serum levels (53) and, thus, risk for toxicities. Broad human polymorphism in cytochrome CYP2D6 may influence the toxicity of simvastatin (54). ■

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