Hydroxymethylglutaryl-Coenzyme A Reductase Exhibits Graded Distribution in Normal and Mevinolin-Treated Ileum

Irwin I. Singer, Douglas W. Kawka, Susan E. McNally, Solomon Scott, Alfred W. Alberts, Julie S. Chen, and Jesse W. Huff

Because the small bowel is a site of significant cholesterol synthesis, we determined the ileal distribution of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme of the cholesterol biosynthetic pathway. Immunofluorescence microscopy on unfixed snap-frozen sections of ileum and jejunum from untreated rats or dogs showed HMG-CoA reductase in the absorptive villus epithelial cells and this appeared to be strikingly localized in their apical cytoplasm. This pattern of HMG-CoA reductase staining approximated a gradient along the villus-crypt axis with the distal villi labeling most intensely. Treatment of rats with mevinolin and/or cholestyramine for 12 days induced a 5- to 11-fold increase in ileal HMG-CoA reductase activity, and yielded a higher intensity of immunostaining without altering the pattern of enzyme distribution observed in control intestines. Also, rats with maximal induction of ileal HMG-CoA reductase exhibited a twofold increase in the number of epithelial villus cells containing prominent stacks of smooth-surfaced membranes in their apical cytoplasm as seen with electron microscopy. These observations suggest that the distal villus absorptive epithelial cells of the ileum contain high concentrations of HMG-CoA reductase, and therefore might be capable of contributing significant quantities of cholesterol to the circulation.

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Mevinolin is a very effective serum cholesterol-lowering agent in both humans and dogs, where significant reductions have been observed when the drug is used alone or in combination with bile acid sequestrants. This powerful hypolipidemic effect occurs because mevinolin is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.34), a microsomal enzyme catalyzing the rate-limiting reaction of cholesterol synthesis. However, chronic studies in rats show that compactin, a related but somewhat less active reductase inhibitor, does not reduce blood cholesterol in this species, presumably because treatment with HMG-CoA reductase inhibitors causes a significant increase in enzyme levels in rat livers. In recent immunofluorescence and electron microscopic studies of mevinolin-treated rats, we observed a dramatic induction of HMG-CoA reductase-containing hepatocytes which contained greatly expanded smooth endoplasmic reticulaums, and which were localized peripherally. These data suggest that the periportal regions of the liver might be specialized for heightened cholesterol synthesis. Since the small intestine (particularly the ileum) also plays a key role in mammalian cholesterol synthesis, we have now localized HMG-CoA reductase in the ileum of normal and mevinolin-treated rats and dogs. Contrary to previous biochemical determinations, we find that HMG-CoA reductase is heavily concentrated in the absorptive epithelial cells of the distal villi and greatly reduced in the crypt cells.

Methods

Chemicals

Mevinolin, a fungal metabolite, was isolated from Aspergillus terreus (ATCC #20542) at Merck as described. Purified rat liver HMG-CoA reductase and rabbit antisera to HMG-CoA reductase were provided by Genen C. Ness (University of South Florida, Tampa, Florida). Cholestyramine and fluorescein isothiocyanate-conjugated affinity purified goat antirabbit IgG were obtained from Merck or Boehringer-Mannheim Biochemicals, Indianapolis, Indiana.

Animals

Sprague-Dawley rats (Charles River Company, 200 g, male, 7 weeks old) were housed under reversed lighting (12 hours dark/12 hours light). They consumed Purina rat laboratory chow containing the test compounds (wt/wt percent) ad libitum; controls were fed standard chow. The rats were anesthetized with CO2 and were sacrificed by cervical dislocation at the diurnal high point (10 AM) for HMG-CoA reductase. Beagle dogs (8 to 10 kg, male, 32 to 38 weeks old) were fed Purina dog chow and housed under
normal lighting conditions. They were treated orally once a day with mevinolin capsules (180 mg/kg/day) for 9 weeks; the controls received encapsulated lactose. Sacrifice was performed by ketamine injection and exsanguination. All animals were handled according to the guidelines for the care and use of laboratory animals (Institute of Laboratory Animal Resources, National Research Council).

**Microscopy**

Segments of ileum or jejunum (5 cm in length) were rapidly dissected and irrigated with 0.1 M phosphate buffer (pH 7.2); liver samples were also taken from each animal. For immunofluorescence (IF) microscopy, tissues were mounted on cork slices and quenched in liquid nitrogen-cooled Freon 22. Cryostat sections (5 μm) were stained for HMG-CoA reductase using monospecific rabbit IgG as previously described. To avoid nonspecific labeling of intestinal tissue, we used a modified “blotto” method. Before staining, the sections were incubated for 20 minutes with blotto supernatant (5% wt/vol) nonfat dry milk in 0.1 M phosphate buffer (pH 7.8) containing 0.1% NaN₃ and 0.1% bovine serum albumin (BSA) centrifuged at 15,000 g for 10 minutes. After washing in 0.1 M Tris buffer (pH 7.8), the sections were stained with purified (protein A-Sepharose) monospecific rabbit anti-HMG-CoA reductase IgG (100 μg/ml) followed by the fluorochrome conjugate (1:25 dilution) in 0.1% dry milk, 0.1% BSA, 0.1% NaN₃ and 0.1 M phosphate buffer (pH 7.8) clarified at 15,000 g. Coverslips were mounted on the stained sections with glycerol containing 4% n-propyl gallate which greatly reduced fading of the fluorescent label during microscopy.

Immunofluorescent micrographs were taken with a Zeiss Photomicroscope III equipped with an epifluorescence condenser III RS, and X10, X16, or X63 Neofluar objective lenses using Ilford HP5 film, and were processed with Microphen fine grain developer at 1600 ASA. Exposures were made with the use of two different methods: either the automatic mode or the standard mode. With the automatic method, the microscope selected the proper exposure time in relation to the fluorescence intensity of the slide, which varied from one sample to another. Therefore, we used this method to document our initial description of the distribution of HMG-CoA reductase within the ileum of control and compound-treated animals. The standard exposure mode was used in semiquantitative experiments performed to compare the relative amounts of reductase staining among various specimens. With this method, the microscope photometer was used to determine the proper exposure time on the most intensely labeled sample, and all subsequent specimens were photographed with that exposure setting. For electron microscopy (EM), the ileal mucosa was rapidly dissected out, macerated finely in fixative (2% glutaraldehyde, 3.5% paraformaldehyde, 0.1 M sucrose, 0.1 M phosphate buffer, pH 7.2), and processed as previously described.

**Results**

**Localization of Intestinal HMG-CoA Reductase**

When sections of ileum obtained from rats fed a standard diet were labeled with monospecific HMG-CoA reductase IgG, IF microscopy showed that this enzyme was strikingly localized in the intestinal epithelium (Figure 1A and 1C). HMG-CoA reductase was most concentrated in the apical portions of the epithelial cells above their nuclei (Figure 1A). The labeling intensity appeared reduced at the basal portions of the villi, and was markedly diminished in the crypts; the lamina propria and epithelial goblet cells were unlabeled (Figure 1C). No background staining was seen in control sections stained with nonimmune rabbit IgG followed by fluorescein-conjugated goat antirabbit IgG using the modified blotto protocol (Figure 1B). Very similar staining patterns were observed in the ilea of rats given mevinolin (Figure 1D and 1E), 3% cholestanol (not shown), or cholestanol plus mevinolin (Figure 1F to 1H). All drug-treated rats exhibited a staining gradient for HMG-CoA reductase with the high point in the distal villi, and the nadir localized in the crypts (Figure 1D and 1F). Corresponding liver sections from these rats exhibited the expected increases in HMG-CoA reductase-containing periporal hepatocytes (not shown) that have been reported previously. Further substantiate these results, the intestines of dogs were also stained for HMG-CoA reductase. Control dogs showed similar distal-to-basal gradients of HMG-CoA reductase distribution and zones of enzyme concentration in the apical absorptive cell cytoplasm (Figure 1I); these patterns were also observed in the ileum and jejunum of mevinolin-treated animals (Figure 1J and 1K).

**Induction of HMG-CoA Reductase Staining**

Several techniques were used to determine whether mevinolin and cholestanol treatment induced increases in the content of ileal HMG-CoA reductase. Using an in vitro enzyme assay system, 5.6- to 11.8-fold elevations in reductase activity were observed in pooled ileal epithelial microsomes after compound administration (Table 1). Evidence that these increases in activity reflect an actual induction in the ileal content of HMG-CoA reductase was obtained from electrophoretic transfer-blot experiments (Figure 2). Western blots of crude microsome pellets isolated from control ilea exhibited a reductase band at the 52 kD position (Lane 2), and showed a conspicuous induction of HMG-CoA reductase-stained bands after treatment with mevinolin and cholestanol (Lane 1). A similar, but more striking, stimulation of reductase staining was observed in liver microsomes isolated from the same animals (Lanes 3 and 4). (These data lend further credence to the specificity of our HMG-CoA reductase antibodies since all the stained proteins seen in our Western blots migrated at the molecular masses of native reductase, or its proteolytic cleavage products.) As a final check of mevinolin-induced reductase staining in the ileum, semiquantitative immunofluorescence staining experiments were performed using a standardized exposure mode (Figure 3). A very obvious increase in reductase staining intensity was observed in the ileal villi of rats treated with cholestanol and mevinolin relative to the control specimens (Figure 3A and 3C). Both levels of staining were completely eliminated by preincubating the reductase antibodies with excess purified HMG-CoA reductase (Figure 3B and 3D).
Electron Microscopy of Ileum from Drug-Treated Rats

Since HMG-CoA reductase is a microsomal membrane-intercalated protein localized predominantly in the smooth endoplasmic reticulum (SER), our IF and enzyme assay data prompted us to determine whether increased membrane accumulation occurs in the ileum of mevinolin/cholestyramine-treated rats. Electron micrographs from specimens with the highest levels of induced intestinal HMG-CoA reductase activity (11.8-fold increase following 3% cholestyramine and 0.25% mevinolin) showed conspicuous accumulations of dense stacks of smooth membranes in the apical regions of ileal villus ab-

Figure 1. Immunofluorescence microscopy of intestinal HMG-CoA reductase distribution in normal and mevinolin-treated rats and dogs. Specimens were stained and photographed in the automated mode described in Methods. A. HMG-CoA reductase is localized in the apical absorptive cell cytoplasm (arrowhead) distal to the nucleus (arrow) in the control rat ileal villus epithelium. (Bar = 20 μm.) B. Specific staining is eliminated from the rat epithelium (arrowhead) when HMG-CoA reductase antibodies are replaced by nonimmune IgG. (Bar = 20 μm.) C. HMG-CoA reductase distribution in the control-fed rat ileum approximates a gradient with maximal staining in the distal villus epithelium (ev), less staining at the villus base (b), and only slight staining in the crypts (c). Goblet cells (g) and the lamina propria (Ip) are unstained. (Bar = 30 μm.) D. Ileum of mevinolin-treated rat (0.075%, 12 days) shows a similar gradient in HMG-CoA reductase labeling with the zenith at the distal villi (d), the nadir in the crypts (c), and the basal portions of the villi (b) exhibiting intermediate staining levels. (Bar = 100 μm.) E. Higher magnification of specimen shown in D exhibits a uniform concentration of HMG-CoA reductase in the apical cytoplasm of the ileal absorptive epithelial cells; the perinuclear regions (arrowhead) and goblet cells (g) are only sparsely stained. (Bar = 10 μm.) F, G, H. Ileal epithelium of rats given 3% cholestyramine (9 days) and 0.25% mevinolin plus 3% cholestyramine (3 days). F. The gradient of HMG-CoA reductase staining along the villus-crypt axis persists when maximal levels of enzyme activity are induced by this treatment (d = distal villus, b = basal villus, and c = crypts). (Bar = 100 μm.) G. Apical concentration of HMG-CoA reductase (arrowhead) remains unchanged in vertical sections of the villus absorptive cell cytoplasm. (Bar = 20 μm.) H. Oblique section of the villus epithelium shows apparent enzyme labeling in the basal epithelial cell cytoplasm (arrowhead). This artifact is seen because the section passes into the apical portions of adjacent absorptive cells. (Bar = 10 μm.) I. HMG-CoA reductase is localized in the distal ileal absorptive cell cytoplasm of control dogs. (Bar = 50 μm.) J, K. Apical concentrations of HMG-CoA reductase persist in the small intestines of mevinolin-treated dogs (180 mg/kg/day for 9 weeks); J shows the ileum, K shows the jejunum. (Bars = 50 μm.)
**Table 1. Effects of Hypolipidemics on Ileal Epithelial Cell Levels of HMG-CoA Reductase in Rats**

<table>
<thead>
<tr>
<th>Diet</th>
<th>HMG-CoA reductase activity (µM mevalonate/mg/min)</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard chow</td>
<td>13.3</td>
<td>—</td>
</tr>
<tr>
<td>3% Cholestyramine*</td>
<td>75.5</td>
<td>5.6</td>
</tr>
<tr>
<td>0.075% Mevinolin*</td>
<td>99.3</td>
<td>7.4</td>
</tr>
<tr>
<td>3% Cholestyramine plus 0.25% mevinolin</td>
<td>157.3</td>
<td>11.8</td>
</tr>
</tbody>
</table>

*Ilea were cut open, washed thrice with phosphate buffer; the epithelia were isolated using a Stomacher extractor. Enzyme activities were determined on pooled ileal epithelial microsomes (4 rats per group) as previously described. The compounds were given for 12 days.

Discussion

Using IF microscopy on cryostat sections of freshly frozen unfixed rat ileum, we observed that HMG-CoA reductase was localized (Figure 1E and 1G). These membranous stacks were composed of flattened, smooth-surfaced saccules associated with rough endoplasmic reticulum (Figure 4B and 4C). Similar membranous structures were found in control ileal villus cells (Figure 4D), but they were much less numerous and did not extend into the apical cytoplasm. Double-blind morphometric analysis of our micrographs showed that 75% of the ileal villus absorptive cells from mevinolin/cholestyramine-treated rats exhibited prominent stacks of smooth membranes, while only 35% of the villus cells from control animals contained these membranes (Figure 5). No other morphological differences were observed between the intestines of compound-treated and control rats.

**Figure 2. Electrophoretic transfer-blot analysis of crude microsome pellets isolated as previously described from control and mevinolin/cholestyramine-treated rat ileal and liver fractions.** This blot shows specific labeling of HMG-CoA reductase and induction of increased reductase content in the compound-treated tissues. Staining was performed with 86 µg/ml rabbit anti-HMG-CoA reductase IgG, followed by peroxidase-conjugated goat antirabbit IgG using the "blotto" method.

We believe that the staining in our immunofluorescent micrographs is specific for ileal HMG-CoA reductase because this staining is eliminated by preincubating the reductase antibodies with excess purified HMG-CoA reductase before use in our labeling protocol. Also, no staining is seen if nonimmune rabbit IgG is substituted for reductase antibodies in our procedure. In addition, the fact that we observed an induction of HMG-CoA reductase immunostaining in both our ileal micrographs and our electrophorograms, which correlates with increased reductase activity after mevinolin/cholestyramine treatment, further strengthens this belief.

Morphometric analysis of our electron micrographs of the ileum showed that the number of absorptive enterocytes containing prominent stacks of smooth-surfaced membranes increased by more than twofold in mevinolin/cholestyramine-treated rats exhibiting maximal induction of HMG-CoA reductase. These membranous stacks were often found to be closely associated with the endoplasmic reticulum. Because HMG-CoA reductase is an integral membrane protein shown to be mainly situated in the endoplasmic reticulum, one would expect to see increases in the content of endoplasmic reticulum membranes to accommodate newly synthesized reductase in...
response to mevinolin treatment. Although we have observed membrane accumulation associated with mevinol
in/cholestyramine administration, not all of the absorptive epithelial cells had stacks of smooth-surfaced mem-
branes, despite the uniform induction of reductase immu
nofluorescence staining in the distal villus epithelium. Simi
larly, the less intensely labeled, but uniformly stained, control ileal villus epithelium also exhibited smooth mem-
branous stacks in 38% of the enterocytes, while the re
mainder of the cells sampled lacked such membranes. These results suggest that a loose association exists be
 tween the quantities of reductase and microsomal mem-
brane synthesized on a per-cell basis.

The dense stacks of smooth-surfaced membranes ob-
served in compound-treated enterocytes have the mor-
phological characteristics of the Golgi apparatus,20'21 but we do not have any other cytochemical evidence showing that these membranes are Golgi-related. Such morpho
logical observations suggest that increased cholesterol synthesis stimulates Golgi activity. This hypothesis is sup
ported by the appearance of a conspicuous Golgi appara
tus in corpus luteal cells during maximal progesterone se
cretion,22 and the formation of striking stacks of flattened membranous sacculles in the juxtanuclear cytoplasm of hepatocytes synthesizing very high levels of HMG-CoA reductase.12 However, HMG-CoA reductase has un
processed high manose sugars suggesting that this en
zyme does not pass through the Golgi apparatus. The homogeneous distribution of HMG-CoA reductase fluores
ce labeling in the apical ileal cytoplasm without ap
parent concentration in the Golgi region favors this sug
 gestion. Therefore, it is likely that the induced smooth-
surfaced membranes that we observed are components of the smooth endoplasmic reticulum.

Several workers have attempted to measure regional differences in intestinal cholesterol synthesis and HMG-CoA reductase activity using biochemical or physical methods to separate villus epithelial cells from crypt cells, and their results have been contradictory. While two groups23'24 showed that the ileal villi exceed the crypts in

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Immunofluorescence micrographs made under standardized conditions (see Methods) illustrating a dramatic increase in HMG-CoA reductase staining in the rat ileal epithelium following mevinolin and cholestyramine administration. (Bar = 50 μm). A. Ileal epithelium of control rat shows a moderate level of HMG-CoA reductase immunofluorescence localized in the apices of the absorptive cells (arrowhead). B. Preparation similar to that in A stained with anti-HMG-CoA reductase IgG that was pre-incubated with excess purified rat liver HMG-CoA reductase. This treatment eliminated the immunolabeling of the ileal epithelium (arrowhead). C. Substantial HMG-CoA reductase staining is found in the rat ileum (arrowhead) following treatment with 3% cholestyramine (3 days) and 0.25% mevinolin plus 3% cholestyramine (3 days). D. Intense HMG-CoA reductase staining similar to that shown in C, in the mevinolin/cholestyramine-treated ileum (arrowhead), is blocked by pretreating the HMG-CoA reductase antibody with purified HMG-CoA reductase.
Figure 4. Electron microscopy of ileal villus absorptive cells from rats given 3% cholestyramine (9 days) and 0.25% mevinolin plus 3% cholestyramine (3 days) to induce maximal HMG-CoA reductase activity. A. Many dense stacks of membranes (arrowheads) are seen at low magnification in the apical cytoplasm between the nucleus (n) and the brush border of microvilli (m). (Bar = 1 μm.) B. Higher resolution micrograph of a cross-section cut midway through the apical absorptive cell cytoplasm shows that the dense stacks of smooth membranes (arrowheads) are present in each cell, and they are often (e.g., at arrow) closely apposed to the endoplasmic reticulum (er). (Bar = 1 μm.) C. Higher magnification of the smooth membranous array depicted by the arrow in B. It is composed of many parallel membranous sacculles (arrowheads), bulbous elements (crossed arrow), and tubular or rounded vesicles (arrow) apparently contiguous with membranes of the smooth (ser) and rough endoplasmic reticulum (er). (Bar = 0.5 μm.) D. Smooth membranous stacks seen in longitudinal section (arrowhead) and cross section (arrow) just apical to the nucleus of a control rat ileal villus absorptive enterocyte. (Bar = 0.5 μm.)
both cholesteryl synthesis and HMG-CoA reductase activity, another laboratory\textsuperscript{13,14} found that the lower villi had a higher rate of cholesteryl synthesis than the crypts and upper villi; still others\textsuperscript{15} showed that HMG-CoA reductase activity was highest in the crypt cells, or that the rate of villus sterol synthesis was equal to that of the crypts. These discrepancies are probably caused by technical problems such as poor separation of cell types, cell lysis, incomplete enzyme recovery, enzyme inactivation, or pool studies of Mak and Trier\textsuperscript{28} who observed that the upper ileal villi had the greatest quantities of mevalonate incorporation into cholesterol, while reduced labeling was found in the lower villi and crypts. Although it is possible that the apical villus concentrations of HMG-CoA reductase labeling could signify inactive enzyme (i.e., due to phosphorylation\textsuperscript{27}), we believe, based on the above autoradiographic observations,\textsuperscript{26} and the careful cell fractionation work of Merchant and Heller,\textsuperscript{23} that this staining pattern represents high concentrations of functional reductase. Taken together, these observations suggest that the absorptive cells of the distal villi in the ileum are probably capable of active cholesterol synthesis, and thus may contribute significantly to the plasma cholesterol pool.

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References

10. Edwards PA, Lan SF, Fogelman AM. Alterations in the rates of synthesis and degradation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase, and therefore probably active in cholesterol synthesis. Furthermore, since we studied freshly frozen unfixed tissue, enzyme inactivation and loss should be minimal. Our results are in complete agreement with the autoradiographic studies of Mak and Trier\textsuperscript{26} who observed that the upper ileal villi had the greatest quantities of mevalonate incorporation into cholesterol, while reduced labeling was found in the lower villi and crypts. Although it is possible that the apical villus concentrations of HMG-CoA reductase labeling could signify inactive enzyme (i.e., due to phosphorylation\textsuperscript{27}), we believe, based on the above autoradiographic observations,\textsuperscript{26} and the careful cell fractionation work of Merchant and Heller,\textsuperscript{23} that this staining pattern represents high concentrations of functional reductase. Taken together, these observations suggest that the absorptive cells of the distal villi in the ileum are probably capable of active cholesterol synthesis, and thus may contribute significantly to the plasma cholesterol pool.

Index Terms: cholesterol synthesis inhibitors • small intestine • gradients in ileal hydroxymethylglutaryl-coenzyme A reductase
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