Inhibitors of 3-Hydroxy-3-Methylglutaryl–CoA Reductase Reduce Receptor-Mediated Endocytosis in Opossum Kidney Cells

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Abstract. Renal proximal tubule cells are responsible for the reabsorption of proteins that are present in the tubular lumen. This occurs by receptor-mediated endocytosis, a process that has a requirement for some GTP-binding proteins. Statins are inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase used for the therapeutic reduction of cholesterol-containing plasma lipoproteins. However, they can also reduce intracellular levels of isoprenoid pyrophosphates that are derived from the product of the enzyme, mevalonate, and are required for the prenylation and normal function of GTP-binding proteins. The hypothesis that inhibition of HMG-CoA reductase in renal proximal tubule cells could reduce receptor-mediated endocytosis was therefore tested. Five different statins inhibited the uptake of FITC-labeled albumin by the proximal tubule–derived opossum kidney cell line in a dose-dependent manner and in the absence of cytotoxicity. The reduction in albumin uptake was related to the degree of inhibition of HMG-CoA reductase. Simvastatin (e.g., statin) inhibited receptor-mediated endocytosis of both FITC-albumin and FITC-β2-microglobulin to similar extents but without altering the binding of albumin to the cell surface. The effect on albumin endocytosis was prevented by mevalonate and by the isoprenoid geranylgeranyl pyrophosphate but not by cholesterol. Finally, evidence that the inhibitory effect of statins on endocytosis of proteins may be caused by reduced prenylation and thereby decreased function of one or more GTP-binding proteins is provided. These data establish the possibility in principle that inhibition of HMG-CoA reductase by statins in proximal tubule cells may reduce tubular protein reabsorption.

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate, the major rate-limiting step of the sterol pathway (1). The statins are widely used for the therapeutic reduction of cholesterol-containing atherogenic lipoproteins (2–4). This is brought about as a result of depletion of intracellular mevalonate leading to reduction of the regulatory sterol pool, which in turn causes upregulation of HMG-CoA reductase and other enzymes of the sterol pathway, most importantly the LDL receptors principally in the liver (5–7). However, a range of additional effects of statins on cells that are independent of cholesterol homeostasis has been described; these include, proliferation (8), signal transduction (9), and apoptosis (10). Many of these cholesterol-independent effects of statins have been shown to result from depletion of mevalonate and its nonsterol metabolites, particularly the isoprenoid pyrophosphates, such as farnesol pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), that are required for the posttranslation modification of a variety of cell proteins, including the superfamily of small GTP-binding proteins (11–13). Endocytosis is one cell function that requires multiple GTP-binding proteins (14,15) and therefore might be affected by statins. However, this possibility has not been investigated.

Rosuvastatin is a new member of the statin class recently approved in the United States and other countries at doses from 5 to 40 mg. It has lipid-lowering effects that are superior to other statins with an overall incidence of side effects similar to other statins (3,16–18). During routine clinical monitoring in the Phase III studies that included atorvastatin, simvastatin, pravastatin, and placebo as comparators, proteinuria, based on dipstick testing, was observed in some subjects, most frequently with rosuvastatin 80 mg. Further studies with rosuvastatin demonstrated that the proteinuria was generally transient and not associated with worsening renal function (16,17). The finding of elevated levels of low molecular weight proteins such as β2-microglobulin in the urine of subjects who developed dipstick-positive proteinuria suggested a tubular cause of the proteinuria, with a specific effect on protein reabsorption (17).

Protein reabsorption by the proximal tubular cells proceeds by receptor-mediated endocytosis using the multiligand recep-
tors megalin and cubilin with subsequent trafficking of endosomes to lysosomes for protein degradation or transcytosis (19). Several stable cell lines derived from proximal tubules are available to study the process of protein reabsorption in tissue culture. These include the opossum kidney cell line (OK), which retains characteristics of proximal tubular epithelial cells, particularly a high rate of uptake of albumin by endocytosis (20), and has been used to study the mechanisms and regulation of protein endocytosis (21–23).

In the experiments reported here, we used the OK cell line to test the hypothesis that inhibition of HMG-CoA reductase can result in reduced efficiency of protein reabsorption by renal proximal tubule cells. We show that statins inhibit the uptake of albumin and β2-microglobulin by OK cells, that the degree of reduction of uptake is related to the degree of inhibition of HMG-CoA reductase, and that this occurs in the absence of cell toxicity. Furthermore, this effect of statins results from depletion of metabolites of mevalonate other than cholesterol and is likely to be due to reduced prenylation of one or more GTP-binding proteins. In the accompanying article (24), similar experiments are described using primary cultures of human tubular cells.

Materials and Methods

Reagents and Materials

Cell culture medium was from Invitrogen (Paisley, UK). Rosuvastatin was synthesized by AstraZeneca, and the other statins were purified from proprietary tablet formulations (simvastatin was converted to the sodium salt). Mevalonolactone, cholesterol, GGPP, FITC-BSA, FITC-inulin, ATP Bioluminescence Assay kit, and somatic cell ATP releasing reagent were from Sigma (Poole, UK). FTY-727 and GGTI-2147 were from Calbiochem (La Jolla, CA). Human β2-microglobulin was obtained from ICN (Basingstoke, UK) and labeled with the Fluoresporter FITC labeling kit from Molecular Probes, (Eugene, OR). [2-14C] acetate and ECL plus kit were from Amersham (Little Chalfont, UK). Goat anti-human Rap1A, anti-human actin and horseradish peroxidase–conjugated anti-goat secondary antibody were from Santa Cruz (Santa Cruz, CA). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Billerica, MA). All other reagents and chemicals were of the highest grade.

Cell Culture and Treatment

OK cells were maintained in DMEM-Ham’s F-12 mix supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air-5% CO2 at 37°C. The cells were used between passage numbers 68 and 80 and cultured for 6 d after seeding at 1 × 105 cells/cm2 in 24-well plates for uptake/binding assays and six-well plates for cholesterol synthesis experiments. Test agents were prepared as concentrated stocks in ethanol (cholesterol), media (GGPP, mevalonic acid lactone), or DMSO (all other test agents). An appropriate vehicle control was included in all experiments. The cells were incubated in serum-free media for 24 h before addition of test agents.

Uptake, Binding, and ATP Cytotoxicity Assays

To measure rates of uptake, we incubated the cells for 15 or 30 min at 37°C with FITC-BSA, FITC-β2-microglobulin, or FITC-inulin at various concentrations. To measure cell surface binding, we chilled the cells for 1 h before the addition of FITC-BSA at various concentrations at 4°C for an additional 24 h. The medium was removed, and cells were washed three times with cold PBS and then lysed in somatic cell ATP-releasing reagent. The fluorescence of the released label was measured at 485 nm excitation and 538 nm emission wavelengths. Uptake and binding results were expressed per milligram of cell protein. Specific binding was calculated by subtracting nonspecific binding (measured with excess unlabeled albumin) from total binding. In preliminary experiments, statins did not modulate albumin binding to the cells, and as this was <10% of the cell associated fluorescence, it was considered unnecessary to correct the observed uptake values. ATP levels were determined from protein uptake samples by bioluminescence and expressed per milligram of cell protein.

Cholesterol Synthesis Assay

After addition of 10 μCi [2-14C] acetate for 3 h, the cells were washed in cold PBS, solubilized with 0.1 M sodium hydroxide, and saponified. Nonsaponifiable lipid was extracted with isohexane. Labelled cholesterol was measured by flow scintillography after HPLC as described previously (25). Cholesterol synthesis was expressed per milligram of cell protein.

Western Blot Analysis

OK cells in 80-cm2 flasks were rinsed twice in cold PBS and lysed in 50 mM Tris-HCl (pH 7.4), 1% Nonidet-P40, 150 mM sodium chloride, and 0.1% SDS. The protein extracts were separated by SDS-PAGE and electroblotted to PVDF membrane. The membrane was probed with antibodies for Rap1A or actin and then the secondary antibody, and bound antibodies were detected using ECL Plus.

Statistical Analyses

Inhibition constants (IC50) and kinetic and binding parameters were calculated by nonlinear regression using GraphPad Prism software (San Diego, CA). When appropriate, statistical significance was assessed by unpaired t test.

Results

Statins Inhibit Albumin Uptake and HMG-CoA Reductase Activity in OK Cells

As shown in Figure 1A, statins exemplified by rosuvastatin, pravastatin, and simvastatin inhibited the uptake of FITC-BSA into OK cells in a concentration-dependent manner. In these experiments, the cells were incubated with statins for 22 to 24 h before the measurement of protein uptake. Atorvastatin and fluvastatin also inhibited uptake in a similar manner, and Table 1 documents IC50 values for all of the statins examined for inhibition of protein uptake. The concentration-dependent inhibitory effect of the statins on cholesterol synthesis from acetate, which was used as an index of inhibition of HMG-CoA reductase within the cells, was measured 3.5 h after addition of the compounds with the results illustrated in Figure 1B and IC50 values shown in Table 1. This was a suitable time at which to compare the potencies of the statins for inhibition of cellular HMG-CoA reductase, because at later times and low statin concentrations, interpretation of the data is complicated by the compensatory homeostatic response of the cells, presumably as a result of upregulation of the enzyme (see Figure 2B).

For all five statins tested, the relationship between inhibition of albumin uptake and sterol synthesis was such that inhibition
of albumin uptake of 20% or more (measured at 22 to 24 h) was observed only in association with inhibition of cholesterol synthesis of 80% or more (measured at 3.5 h). In Figure 1C, this is illustrated for pravastatin, simvastatin, and rosuvastatin. In these experiments, ATP levels were also measured in cell extracts (Figure 1D) and normalized to cell protein level. ATP levels were not reduced, even at the highest concentrations. Thus, the effect of statins on albumin uptake was not the result of cytotoxicity.

Table 1. IC$_{50}$ values for inhibition of albumin uptake and cholesterol biosynthesis by various statins

<table>
<thead>
<tr>
<th>Statin</th>
<th>Protein Uptake IC$_{50}$ (μM)</th>
<th>Cholesterol Synthesis IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluvastatin</td>
<td>0.11 (0.10 to 0.12)</td>
<td>0.04 (0.03 to 0.04)</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>0.29 (0.20 to 0.43)</td>
<td>0.01 (0.01 to 0.02)</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0.94 (0.49 to 1.79)</td>
<td>0.07 (0.04 to 0.15)</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>1.69 (1.08 to 2.63)</td>
<td>0.13 (0.05 to 0.35)</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>26.46 (4.46 to 156.86)</td>
<td>0.55 (0.12 to 2.62)</td>
</tr>
</tbody>
</table>

* Values were determined from dose-response curves for each statin and are the combined results of two to eight independent experiments, expressed as mean IC$_{50}$ value with 95% confidence intervals shown in brackets.

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**Time Course of Inhibition of Albumin Uptake in Relation to HMG-CoA Reductase Activity**

Because simvastatin was a potent inhibitor of both albumin uptake and HMG-CoA reductase within the cells, it was used as a representative statin to understand further the mechanism of effect on protein uptake. In a time-course experiment (Figure 2), 10 μM simvastatin resulted in progressive inhibition of albumin uptake over 48 h (Figure 2A), and this was associated with a high degree of inhibition of HMG-CoA reductase throughout the experiment (Figure 2B). In contrast, the lower concentration of simvastatin (0.1 μM) resulted in a strong inhibition of HMG-CoA reductase at the start of the experiment (3.5 h), but this lessened with time and showed a rebound at 24 and 48 h. In this case, there was moderate inhibition of albumin uptake at the earlier time points, but this did not progress with time. These results suggest that both prolonged and high-degree inhibition of HMG-CoA reductase is required to manifest the effect on protein uptake.
Inhibition of Receptor-Mediated Endocytosis of Low Molecular Weight Proteins in OK Cells

Albumin and β₂-microglobulin are absorbed by receptor-mediated endocytosis in OK cells, whereas the polysaccharide inulin is taken up by fluid-phase endocytosis (26,27). As shown in Figure 3, the uptake of FITC-BSA (5 or 50 mg/L) and FITC-β₂-microglobulin (2.5 or 25 mg/L) was inhibited by ~75% (P < 0.001) compared with control after incubation with simvastatin for 24 h. However, the internalization of FITC-inulin (500 mg/L) was unaffected, indicating that receptor-mediated endocytosis was specifically inhibited by statins.

Effect on FITC-BSA Uptake Kinetics and Cell Surface Binding

Figure 4A shows the effect of incubation with 10 μM simvastatin for 24 h on uptake measured over a wide range of FITC-BSA concentrations. The uptake was saturable and inhibited by simvastatin at all FITC-BSA concentrations, resulting in a decrease in the apparent maximal rate of uptake (Vₘₐₓ of 1.9 ± 0.1 versus 3.5 ± 0.2 μg/mg in control). In the experiment shown in Figure 4B, the binding of FITC-BSA to the cell surface was measured at 4°C, after 24 h of incubation with 10 μM simvastatin. Because it is known that the binding of albumin to OK cells requires several hours to equilibrate (28), this was determined 24 h after the addition of FITC-BSA. Simvastatin had no effect on the number of albumin binding sites (Bₘₐₓ of 7.6 ± 1.2 versus 8.8 ± 0.5 μg/mg in control) or binding affinity (Kd of 197 ± 60.4 versus 190 ± 24.2 μg/ml in control).

Modulation of Isoprenoid Products of HMG-CoA Reductase Is Responsible for Statin-Induced Inhibition of Protein Uptake

The mechanism of the statin-mediated inhibition of albumin uptake was investigated further in the experiments shown in Figure 5. The effect of 10 μM simvastatin could almost completely be prevented by the co-addition of 10 μM mevalonate or 10 μM GGPP, whereas addition of 100 μM cholesterol had no effect. Mevalonate also prevented the inhibition of albumin uptake by atorvastatin and rosuvastatin (data not shown). This suggests that as a result of inhibition of HMG-CoA reductase, the depletion of GGPP or one of its products is critical to the effect of statins on protein reabsorption.

To determine whether the effect of simvastatin on protein uptake was consistent with the time course of depletion of cellular GGPP, we assessed the geranylgeranylation of Rap1A (as an example of a GTP-binding protein) by Western blotting using an antibody that recognizes only the unprenylated form of the protein (29). As shown in Figure 6, unprenylated Rap1A was not observed in control cells. However, it was detected in the cells 4 h after incubation with simvastatin and increased over 24 h, whereas actin levels were unchanged.

Statin-induced inhibition of protein uptake was prevented by...
co-addition of GGPP. This could be due to reduced prenylation of one or more GTP-binding proteins involved in the process of endocytosis. Two related enzymes, farnesyl transferase and class I geranylgeranyl transferase, are responsible for prenylation of the majority of GTP-binding proteins (13). As shown in Figure 7, incubation of cells with up to 10 μM mevalonate, 10 μM GGPP, or 100 μM cholesterol in the absence and presence of 10 μM simvastatin for 24 h and 10 mg/L FITC-BSA for the final 30 min. Results are expressed as mean ± SEM of FITC-BSA uptake from six or more replicate samples per condition. **p < 0.001 versus control.

Discussion
The experiments described in this report were designed to test the hypothesis that statins, through inhibition of HMG-CoA reductase, could reduce the rate of receptor-mediated endocytosis of proteins by renal proximal tubule cells. The experiments used the OK cell line, which has been used widely to study this process. The results show that statins inhibit protein endocytosis into OK cells by a mechanism linked to inhibition of HMG-CoA reductase. The statins did not cause overt cell damage as shown by the lack of effect on ATP levels.

The OK cell line, derived from kidney tubules, retains the capacity to take up albumin across the apical membrane at a rate comparable to that observed in proximal tubules in vivo (26). The particular cells used in these experiments took up FITC-BSA at rates similar to those reported in previously published work (28,32,33). Simvastatin, used as an example of the statin class, inhibited the uptake of FITC-BSA and FITC–β2-microglobulin but not of FITC-inulin—a marker of fluid-phase fluid endocytosis. Albumin and β2-microglobulin bind to the cubilin/megalin receptor system that is present in OK cells and in clathrin-coated pits in the brush border of proximal tubular epithelial cells (27,34). These receptors are responsible for the binding and internalization of multiple proteins from the glomerular filtrate, including albumin, peptide hormones, and vitamin binding proteins (35,36). In OK cells, simvastatin did not reduce the number of cell surface receptors for albumin but...
Reduced the rate of uptake, suggesting that statins modulate one of the processes of early endocytosis, subsequent to the interaction of proteins with the megalin/cubulin complex.

The transport of the protein ligands into cells is mediated by the fusion of the endocytic clathrin-coated vesicles with early endosomes followed by acidification and further trafficking of the vesicles within the cells. Agents such as chlorpromazine and cytochalasin D (21), which directly disrupt these processes (by effects on clathrin and the actin-cytoskeleton, respectively), exert rapid effects (within 30 min) on endocytosis as do agents that inhibit endosomal acidification (37,38). This is in contrast to the slow rate of onset of inhibition observed with simvastatin, only half complete by 24 h at 10 μM, suggesting instead that statins cause a gradual depletion or inactivation of co-factors required in the early uptake process.

The observation that reductions in the rate of uptake of FITC-BSA into OK cells caused by the statins could be overcome by the addition of the product of the enzyme, mevalonate, is strong evidence for the linkage to inhibition of HMG-CoA reductase. Mevalonate is a precursor of a variety of nonsterol metabolites that include isoprenoid pyrophosphates such as FPP and GGPP, which have key roles in the posttranslational processing by prenylation of numerous cell proteins, notably the superfamily of GTP-binding proteins. Prenylation at the C-terminus is a requirement for membrane association and thereby the normal function of these proteins (39). Addition of GGPP in the OK cell experiments could prevent the statin-induced inhibition of protein reabsorption, but the addition of cholesterol did not. Moreover, the time course of appearance of unprenylated Rap1A was similar to the time course of inhibition of protein endocytosis, supporting the idea that statins, as a result of inhibition of HMG-CoA reductase, cause a gradual depletion of the intracellular pool of GGPP and, thereby, the progressive inactivation of one or more of the GTP-binding proteins involved in endocytosis. A number of cellular GTP-binding proteins are known to be involved in the process of endocytosis, including Rho, Rac, and several members of the Rab family (13,14,40). Further evidence was obtained using inhibitors of protein prenylation (30,31). It was shown that 10 μM GTI-2147 (a selective inhibitor of geranylgeranyl transferase I), added for 6 h, prevented endocytosis of albumin, whereas the farnesyl transferase inhibitor FTI-277 did not. The current experiments leave open the question of which protein or set of proteins are most critically involved in the statin-induced inhibition of endocytosis.

According to the hypothesis, the effects of statins on tubular protein reabsorption in vivo would be in proportion to the degree of inhibition of HMG-CoA reductase exerted in the proximal cells. This in turn would be determined by the rates of influx and efflux of the statins to and from the proximal cells and by the intrinsic potencies of the inhibitors. Of the five statins tested, rosuvastatin is the most potent inhibitor of cell-free HMG-CoA reductase, and pravastatin is the least (41). In the experiments in OK cells, the rank order of IC50 values for inhibition of cholesterol synthesis did not follow the order of intrinsic potencies of the statins on HMG-CoA reductase but did follow the order of the relative lipophilicities (log D at pH 7.4) of the compounds (42). This would be expected if, under the conditions of the experiments in OK cells, the intracellular concentrations of the statins were mainly determined by the rate of passive diffusion into the cells.

However, in vivo, the proximal tubular cells are responsible for the active transport of various organic anions, including statins from the basolateral to the apical surfaces. Pravastatin, for example, is known to undergo active tubular secretion as intact compound to the extent of 47% of whole-body clearance (43). Rosuvastatin is transported in a similar way, because it is known that 28% of an intravenous dose is cleared by the kidney and this rate can be accounted for only by active tubular secretion (44). Active transporters located on both basolateral and apical surfaces of the cells drive such tubular secretion. OK cells are known to transport organic anions such as para-aminohippuric acid (45). The para-aminohippuric acid transporter has been cloned from human kidney and is known as hOAT1, but additional homologues, including hOAT3, have also been identified (46). Rosuvastatin is a good substrate for hOAT3 but has markedly lower affinity for hOAT1 (CDA Brown, University of Newcastle, England, personal communication, 2003). OK cells seem to express mainly OAT1 (45); therefore, the full set of transporters relevant to the tubular transport of statins in vivo may not be present in these cells. Thus, although the OK cells in culture may be a good model of tubular protein reabsorption, they may not reproduce all of the features of active secretion of drugs in vivo.

Observations of the effects of pharmacodynamically active compounds on cells in culture have to be interpreted with an understanding of the factors that control the disposition and the intracellular levels of the drugs in vivo. The results of the experiments with statins in the OK cell model establish the possibility, in principle, that inhibition of HMG-CoA reductase in proximal tubular cells could be linked to lower rates of tubular protein reabsorption in vivo and suggest a plausible
mechanism for the tubular proteinuria that has been observed in some patients who are treated with the 80 mg dose of rosuvastatin. Inhibition of receptor-mediated endocytosis by proximal tubular cells of 20% or less could easily explain in quantitative terms the degree of proteinuria observed at this dose in vivo. It is possible that OK cells could be a misleading model if, for example, there were important differences in the rate-limiting factors involved in protein endocytosis in human proximal tubule cells. However, this is unlikely, because statins have also been shown to inhibit protein endocytosis in a mevalonate-dependent manner in primary cultures of human tubular epithelial cells (24).

A variety of experiments in animal models of kidney disease have provided evidence of renal protective effects of statins (47). These have been found to be independent of cholesterol lowering and therefore are in the category of the so-called pleiotropic effects, which include anti-inflammatory actions and other cellular effects such as on proliferation and differentiation (47). It has been postulated that in patients with progressive renal disease, excess uptake of albumin damages the proximal cells and exerts proinflammatory actions that contribute to interstitial fibrosis (48). The evidence in this report is that statins can inhibit the uptake of proteins into proximal cells as a result of inhibition of HMG-CoA reductase. Whether this and other pleiotropic effects that stem from the pharmacologic action of statins in the proximal cell could contribute to their overall beneficial effects in kidney disease warrants further investigation.

Acknowledgments

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References


