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Inhibitors of HMG-CoA Reductase Reduce Receptor-mediated Endocytosis in Human Kidney Proximal Tubular Cells

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Abstract. The proximal tubular cells of the kidney are responsible for reabsorption of proteins from the tubular lumen. In a study using Opossum kidney (OK) cells, receptor-mediated protein endocytosis was reduced by statins, inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which are widely used for therapeutic reduction of plasma cholesterol levels. To explore the possible clinical relevance of the observations in OK cells, protein endocytosis in human kidney tubular cells was investigated in the presence and absence of statins.

The uptake of FITC-labeled albumin in these cultures of human kidney tubular cells was investigated by microscopy, flow cytometry and spectrofluorometry. Protein uptake occurred selectively into proximal tubular cells while it was absent in distal tubular/collecting duct cells. Three statins (simvastatin, pravastatin, and rosuvastatin) significantly inhibited the uptake of protein in a concentration-dependent way. This inhibitory effect of statins could be prevented by the co-addition of mevalonate, the product of HMG-CoA reductase. This effect was not the result of a statin-induced cytotoxicity since cell-viability was unaffected. Finally, it was demonstrated that statins strongly inhibited cholesterol synthesis in the human kidney tubular cells.

These data suggest that statins have the potential to inhibit albumin uptake by the human proximal nephron as a result of inhibition of HMG-CoA reductase in the proximal tubule cells. Taken into account the data of the accompanying manuscript this inhibitory effect most probably results from a reduced prenylation of some proteins critically involved in endocytosis. It is suggested that these data help to explain the occurrence of proteinuria in some patients treated with high statin doses.

Statins, by their ability to inhibit 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzyme of the sterol pathway, are potent inhibitors of sterol biosynthesis (1). As a result of the reduction of cellular sterol pools, there is compensatory upregulation of cell-surface receptors for cholesterol-containing low density lipoproteins (LDL), an effect which takes place mainly in the liver (2–4). This mechanism underlies the therapeutic use of the statins to lower plasma cholesterol and particularly the levels of LDL. However, many additional effects of statins on cell function have been described in the literature (5). These appear to be independent of cellular cholesterol homeostasis and are collectively termed "pleiotropic effects". Many of these have been shown to result from the depletion of mevalonate (the HMG-CoA conversion product)-derived intermediates of the sterol pathway, particularly the isoprenoid pyrophosphates such as geranylgeranyl pyrophosphate (GGPP). Isoprenoid pyrophosphates are required by the cells for the post-translational modification of a range of proteins, especially GTP-binding proteins. In the accompanying publication (6), it has been shown that receptor-mediated endocytosis by opossum kidney (OK) cells was inhibited by statins, an effect that could be prevented by the addition of mevalonate and GGPP, but not cholesterol.

In the phase III studies of a new statin, rosuvastatin, which included comparisons with other statins and placebo, there was an observation of proteinuria in some subjects, most frequently in those taking rosuvastatin 80 mg (above the currently recommended dose range). The proteinuria observed with rosuvastatin was generally transient, not associated with worsening renal function and mainly of tubular type, suggesting reduced reabsorption of normally filtered proteins (7–9). The observations made in OK cells suggest that the mechanism for a reduced rate of protein reabsorption is linked to inhibition of HMG-CoA reductase in the proximal tubule cells which in turn leads to a depletion of the cellular GGPP pool and thereby to reduced function of one or more GTP-binding proteins, known to be involved in the process of endocytosis (6,10–12).

The OK cell line has been widely used as a cell model of protein reabsorption by proximal tubular cells, and the mechanisms of effect of drugs and other substances that cause tubular proteinuria have been studied in these cells. However, the OK cells are a non-human, stable cell line and it is possible that the rate-limiting factors for protein endocytosis could be quite different from those in human proximal cells. Therefore, it was important to establish whether the effects of statins on

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receptor-mediated endocytosis could also be observed in other cells, ideally primary human kidney tubular cells.

During the past years, methods for culturing primary human kidney tubular cells have been optimized (13–15). As well as proximal tubular cells, these cultures also contain distal tubular and collecting duct cells that can be distinguished from each other by means of the specific markers, *i.e.* leucine aminopeptidase (LAP) for proximal tubular cells and epithelial membrane antigen (EMA) for distal tubular and collecting duct cells (13–15).

In this study we investigated (i) whether primary cultures of human kidney (proximal tubular, distal tubular and collecting duct) cells endocytose a measurable amount of protein, (ii) which cell type is responsible for protein uptake, (iii) if statins (simvastatin, pravastatin and rosuvastatin) have an effect on cellular protein uptake and (iv) to what extent mevalonate prevents statin induced effects. It has been shown that the proximal tubular cells are mainly responsible for protein endocytosis in the mixed cell culture system. In agreement with the results in OK cells, the three statins inhibited protein endocytosis in a concentration-dependent fashion, and the inhibition could be prevented by mevalonate.

Materials and Methods

Isolation, Purification and Culture of Primary Human Kidney Tubular Cells

Human kidney tubular epithelial cells were isolated as described previously (14,15). Briefly, normal human kidney tissue, that became available through nephrectomies performed for oncological indications, was collected and processed in a sterile manner. The use of this tissue for the purpose of cell culture was approved by the local ethical committee. The average age of the donors was 65 and none of them had received statin treatment. Normal tissue was decapsulated macroscopically. The cortex and outer stripe of outer medulla were dissected, cut into pieces of about 1 mm^3 and digested in collagenase D solution (Roche, Ottweiler, Germany), supplemented with DNAse (Sigma, St Louis, Missouri, USA). The suspension was shaken vigorously for 2 h at 37°C and sieved through a 120 μm sieve. The resulting cell suspension was loaded on top of a discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient with densities of 1.04 and 1.07 g/ml. After centrifugation, cells from the intersection were carefully aspirated, washed and brought into culture as a mixed population of proximal tubular (PTC-LAP positive), distal tubular and collecting duct cells (DTC-EMA positive). Cells were seeded in 24 well plates (25000 cells /well) in α-MEM medium (Life Technologies, Rockville, Maryland, USA) modified according to Gibson d'Ambrosio (16), supplemented with 10% fetal calf serum and grown until confluence (± 6 d). Sixteen hours before study of the protein uptake, the serum-containing medium was replaced by serum-free α-MEM Gibson d'Ambrosio.

Statin and Mevalonate Incubation

Rosuvastatin was synthesized by AstraZeneca and pravastatin and simvastatin were purified from proprietary tablet formulations with simvastatin converted to the sodium salt. Confluent cultures were incubated with simvastatin, pravastatin or rosuvastatin for 16 h in serum-free medium. Simvastatin is a relatively lipophilic molecule, easily taken up by cells and thus a relatively potent inhibitor of cellular HMG-CoA reductase in non-hepatic cells (17). It was used in the following concentrations: 50, 10, 1 and 0.1 μM. In contrast, pravastatin and rosuvastatin are relatively hydrophilic molecules, and as a result of lower cellular uptake relatively less potent in cell culture and therefore used at higher concentrations, 500, 100, 10 and 1 μM. Statins were dissolved as stock solutions in dimethylsulfoxide (II) DMSO and then diluted into culture medium so that the final concentration of DMSO was 0.1%. The cell cultures used as control contained 0.1% DMSO. To investigate the effect of mevalonate, the compound was supplied to the cultures at a concentration of 100 μM, dissolved in water.

Detection of Albumin Uptake

Spectrofluorometry. Protein uptake was determined by incubating confluent cultures (either pre-incubated with statins or not) with fluorescein-isothiocyanate (FITC) labeled human albumin (Sigma) at 37°C, and at 4°C (control) for 1 h. After incubation with FITC-albumin, cells were extensively rinsed with ice-cold PBS (3x) and lysed during 20 min with 0.1% triton in PBS to release FITC-label. Fluorescence intensity was quantified using spectrofluorometry (485 nm excitation and 538 nm emission wavelengths). Lysates of cells not incubated with FITC-albumin were used as blanks.

Microscopy. Confluent cell cultures were incubated with FITC-labeled human albumin (75 μg/ml) at 37°C for 1 h, extensively rinsed (3x) with PBS, fixed for 10 min in 4% formaldehyde and subsequently stained for EMA by immuno-fluorescence. After overnight incubation with rat-anti-human EMA antibody (Seralab, Leicestershire, UK) and rinsing, the cells were incubated for 2 h with Cy3 labeled goat-anti-rat antibody (Jackson, West Grove, Pennsylvania, USA).

Flowcytometry. Confluent cell cultures, pre-incubated either with or without statins were incubated with FITC-labeled human albumin (500, 50, 20, 10 and 5 μg/ml) at 37°C for 1 h, extensively rinsed (3x) with PBS and trypsinized. The resulting cell suspension was subsequently incubated with mouse-anti-human LAP primary antibody (18) and rabbit-anti-mouse, phycoerythrin labeled secondary antibody, for 30 min each. Before and after incubation with secondary antibody, cells were rinsed 3x with PBS. Finally, cells were fixated with 2.5% paraformaldehyde.

Flowcytometric analysis was performed using a FACSVantage flow cytometer (Becton Dickinson, San Diego, California, USA). Single labeling of endocytosis and LAP and a negative control of unstained cells were performed to regulate fluorescence compensation settings.

Determination of Total Protein Concentration

To correct for variations in cell number, albumin uptake was calculated per μg cellular protein. Protein concentration of the cell lysates was determined using a BCA kit (Perbio, Belgium) according to manufacturers instructions.

Statin Toxicity

Cytotoxicity of statins on the tubular cells was measured with a cytotoxicity assay based on MTT activity (Easy for you, Biomedica Group). Confluent cell cultures were incubated with different concentrations of simvastatin, pravastatin, and rosuvastatin or with vehicle alone during 16 h. The cytotoxicity test was performed according to the manufacturer's instructions.

Quantification of Cholesterol Synthesis

Primary human kidney tubular cells were seeded in 6 well plates. Confluent cultures were pre-incubated with different statins or with vehicle for 0.5 h. Then, 10 μCi [2-^{14}C] acetate was added to the

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culture medium and the cells incubated for a further 3 h. To measure the incorporation into cholesterol, the cells were washed in cold PBS, solubilized with 0.1M sodium hydroxide and saponified. Non-saponifiable lipid was extracted with isohexane. Labeled cholesterol was measured by flow scintillography after isolation with HPLC as described previously (19). Cholesterol synthesis was expressed per mg cell protein.

Statistics

The spectrofluorometry data on uptake of FITC-albumin are the combined results of experiments on 5 kidney specimens using 4 wells per condition per kidney. To compare results from different kidney specimens, albumin uptake was expressed as percentage of the mean of their respective controls. The data are expressed as the mean ± SD

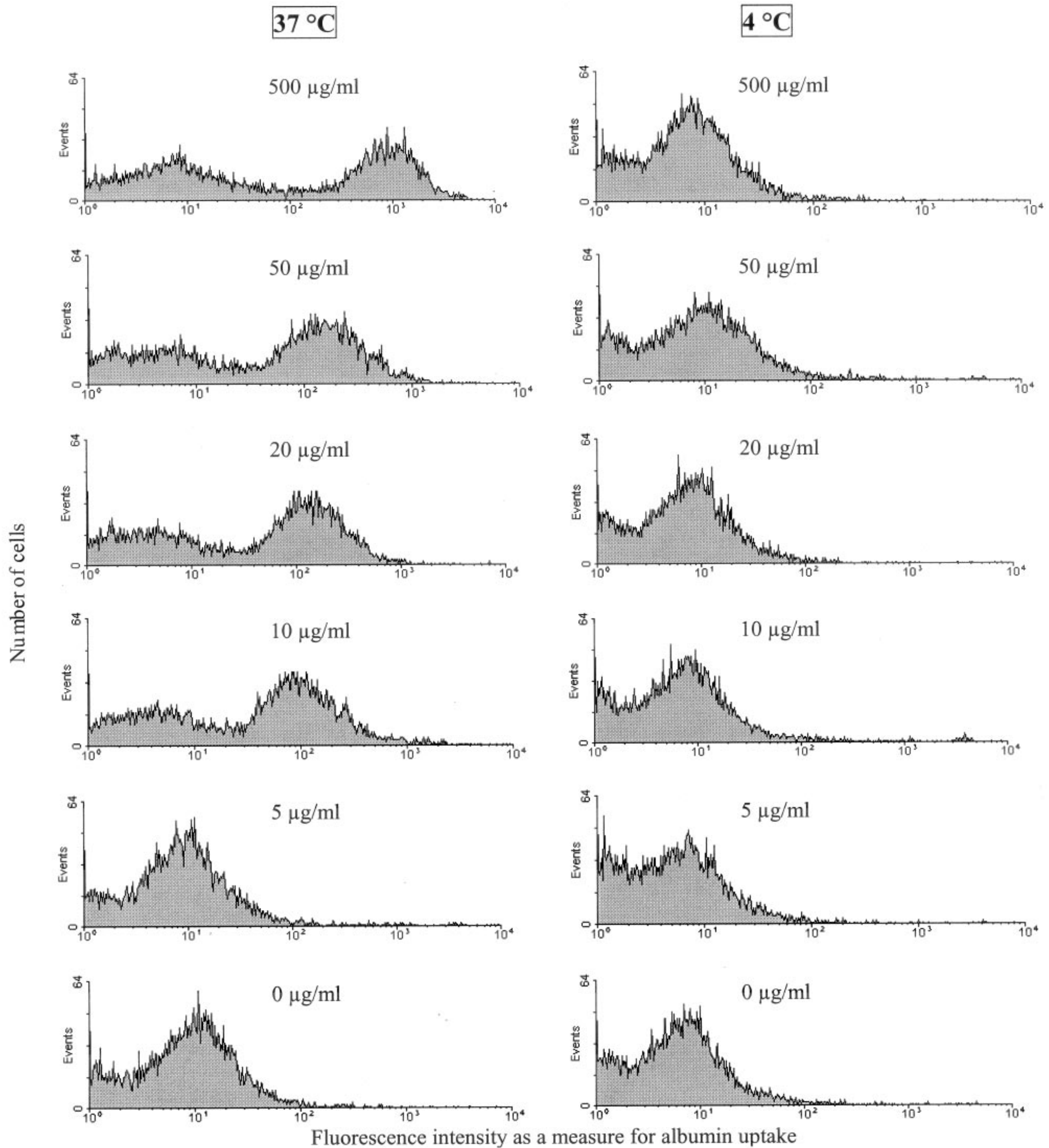

Figure 1. Flowcytometric analysis of human kidney tubular cell cultures that were incubated with different concentrations of FITC-albumin (500, 50, 20, 10, 5 μg/ml) during 1 hour at 37°C or 4°C. Albumin endocytosis occurred at 37°C as indicated by the shift in the distribution of the cells to higher intensity fluorescence, and not at 4°C as no fluorescence shift is observed. Histograms show the results from a representative experiment on one kidney specimen out of 3 experiments on different kidneys.

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of the percentage inhibitions. A Mann-Whitney U test with Bonferroni correction was performed to determine the significance of effect on endocytosis in statin treated cells compared to controls. Furthermore, albumin uptake in statin treated cultures with and without the co-addition of mevalonate was also compared using a Mann-Whitney U test.

Results

Endocytosis of FITC-albumin by Human Kidney Tubular Cells

Flowcytometric measurement of albumin uptake by human kidney tubular cell cultures, incubated with 5 to 500 μg/ml FITC-albumin for 1 h at 37°C (Figure 1, left panel), indicated these cultures were able to endocytose a measurable amount of the protein, as indicated by the shift in the distribution of the cells to higher fluorescence intensity. As expected, incubation at 4°C resulted in significantly less endocytosis (Figure 1, right panel). Based on the data found for various kidney specimens ($n = 3$), a concentration of 75 μg/ml of FITC-albumin (the concentration at which a measurable uptake was seen in the different cell preparations) was chosen to investigate the effect of statins on cellular protein uptake in further experiments. Control cultures (vehicle) endocytosed 4.4 ± 2.1 μg albumin per mg total cellular protein over the different experiments performed to investigate the effect of statins on proximal tubular cell endocytosis.

Albumin Uptake in Proximal Tubular Cells

Since in previous studies, LAP and EMA were found to be specific markers of proximal and distal tubular cells respectively (13–15), these markers were used to determine whether albumin endocytosis took place into one or both of the tubular cell populations. Microscopic analysis of albumin uptake was combined with immunofluorescent EMA staining (Figure 2). EMA positive or distal tubular/collecting duct cells showed no albumin uptake while the EMA negative cells (proximal tubular cells) showed a clear intracellular fluorescence signal, indicating uptake of FITC-albumin. This was confirmed by flow cytometric detection of the albumin uptake (Figure 3), after LAP immunostaining. The average number of LAP positive cells or proximal tubular cells in the different cultures under study was 42 ± 6.5% (mean ± SD). LAP positive cells demonstrated a clear FITC-albumin signal whereas LAP negative cells (distal tubular/collecting duct cells) were negative for the FITC signal. This confirms the findings by microscopy.

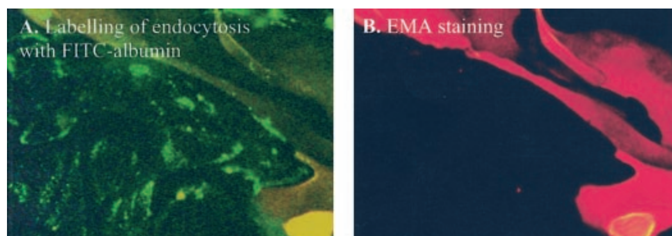

Figure 2. Microscopic images (630x) of a cell culture incubated with 75 μg/ml FITC-albumin for 1 h and stained for EMA. Albumin uptake (green color) is shown in panel A. EMA staining (red color) at exact the same localization in the culture is shown in panel B. Endocytosis did not occur in EMA positive, distal tubular/collecting duct cells but is confined to EMA negative, proximal tubular cells. This figure shows a representative picture of one experiment out of 3 experiments on 3 different kidney specimens.

Effect of Simvastatin Pravastatin and Rosuvastatin on Proximal Tubular Cell Endocytosis

Figure 4 shows that 16 h exposure of the cultures to the 3 statins of interest (simvastatin, pravastatin and rosuvastatin), all resulted in a significant and concentration-dependent inhibition of albumin uptake in proximal tubular cells ($P < 0.02$). However, even at the highest concentrations of the statins, endocytosis was not completely inhibited. Spectrofluorometric results were confirmed by flow cytometric analysis of one representative example of the cell preparations (Figure 5).

Effect of Mevalonic Acid

As shown in Figure 6, the co-addition of mevalonate to the cultures incubated for 16 h with the statins prevented the inhibitory effects of the statins on albumin uptake. Cell cultures that were exposed to statins in combination with mevalonate showed an endocytosis significantly higher than that of cell cultures that received statins in the absence of mevalonate ($P < 0.05$).

Cytotoxicity of Statins

To exclude the possibility that the observed effect of statins on endocytosis was a result of a cytotoxic action of the compounds, cell viability was assessed using an MTT based assay after 16 h incubation of the cell cultures in either the presence or absence of the statins. Figure 7 clearly shows that cell viability is not decreased in statin treated cultures as compared to those treated with vehicle.

Cholesterol Synthesis

Figure 8 shows that cholesterol synthesis in confluent human epithelial kidney cell cultures was strongly inhibited by treating the cultures with the various statins for 3.5 h.

Discussion

In the accompanying manuscript (6) the statin induced inhibition of protein uptake by proximal tubular cells was assessed in opossum kidney (OK) cells. This effect was seen with five members of the statin class. It was related to the degree of inhibition of HMG-CoA reductase and prevented by co-addition of mevalonate. The data indicated that the statin-inhibition is most probably the result of a diminished isoprenylation of one or more proteins that play a key role in the early stages of endocytosis. The OK cells serve as an excellent model to study the possible mechanisms of drug effects on protein uptake. However, to determine whether this mechanism was likely to operate in the clinical situation, it was important to determine whether the same effect could be observed in a primary preparation of human kidney tubular cells.

The human cell culture system, described in this manuscript, consists of primary cells isolated from the cortex and outer

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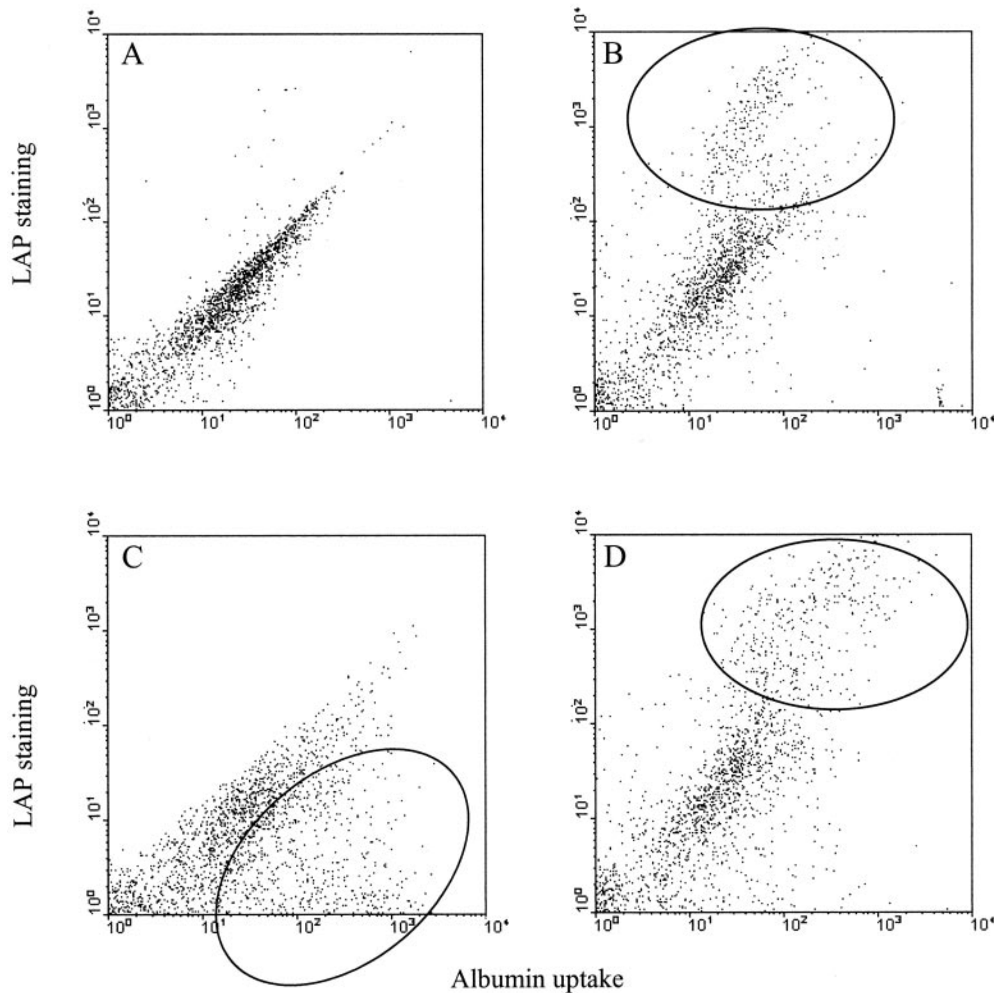

Figure 3. Flowcytometric analysis of albumin uptake. (A) Negative control of cells not incubated with albumin, not stained for LAP. (B) Cells stained for LAP, not incubated with albumin. The LAP positive cells are clearly shifted along the y-axis and are present in the oval (C) Cells incubated with albumin (75μg/ml, 1 h), not stained for LAP. Cells that have taken up FITC-albumin are shifted along the x-axis and are present in the oval (D) Cells incubated with albumin (75μg/ml, 1 h) and stained for LAP. The vast majority of cells that have taken up FITC-albumin are also LAP positive (shift of these cells along the y-axis compared to C). Or else, LAP positive cells have taken up FITC-albumin (shift of these cells along the x-axis compared to B).

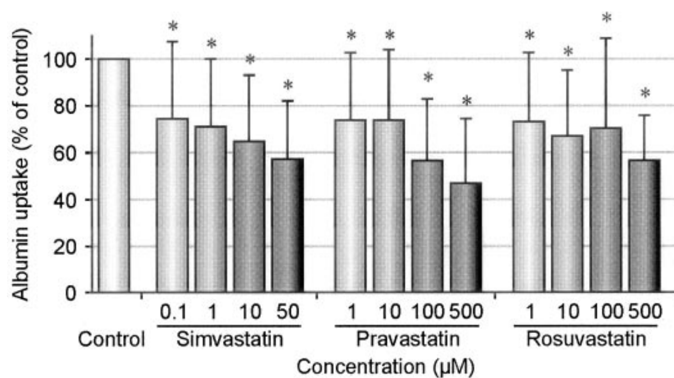

Figure 4. Spectrophotometric analysis of albumin uptake in human tubular kidney cells incubated with different concentrations of three statins or with vehicle. Uptake of FITC-albumin was normalised to total protein level and expressed as percentage of FITC-albumin uptake in controls. Data represent the mean +SD of experiments on 5 different kidneys (20 culture dishes). All three statins inhibited endocytosis significantly (* $p < 0.02$) and dose-dependently.

stripe of the outer medulla of normal human kidney tissue. Although the resulting cultures contain proximal tubular, distal tubular as well as collecting duct cells, the different cell types can be identified and distinguished from each other by using specific markers for proximal tubular (LAP, leucine aminopeptidase) and distal tubular/collecting duct cells (EMA, epithelial membrane antigen) (13–15). A similar percentage of proximal and thus endocytosing cells (42 $\pm$ 6.5% over 9 kidneys) was present in the cell cultures that were derived from the kidney specimen obtained from different donors. Furthermore these cells retain their specific functional characteristics with regard to glucose uptake and hormonal (PTH and vasopressin) activation (14). In addition, these cell cultures were the appropriate model to study synthesis and localization of osteopontin in the tubular epithelium (20).

To the best of our knowledge this is the first study assessing protein uptake in primary human cell cultures. First, it was determined whether these cultures were able to endocytose

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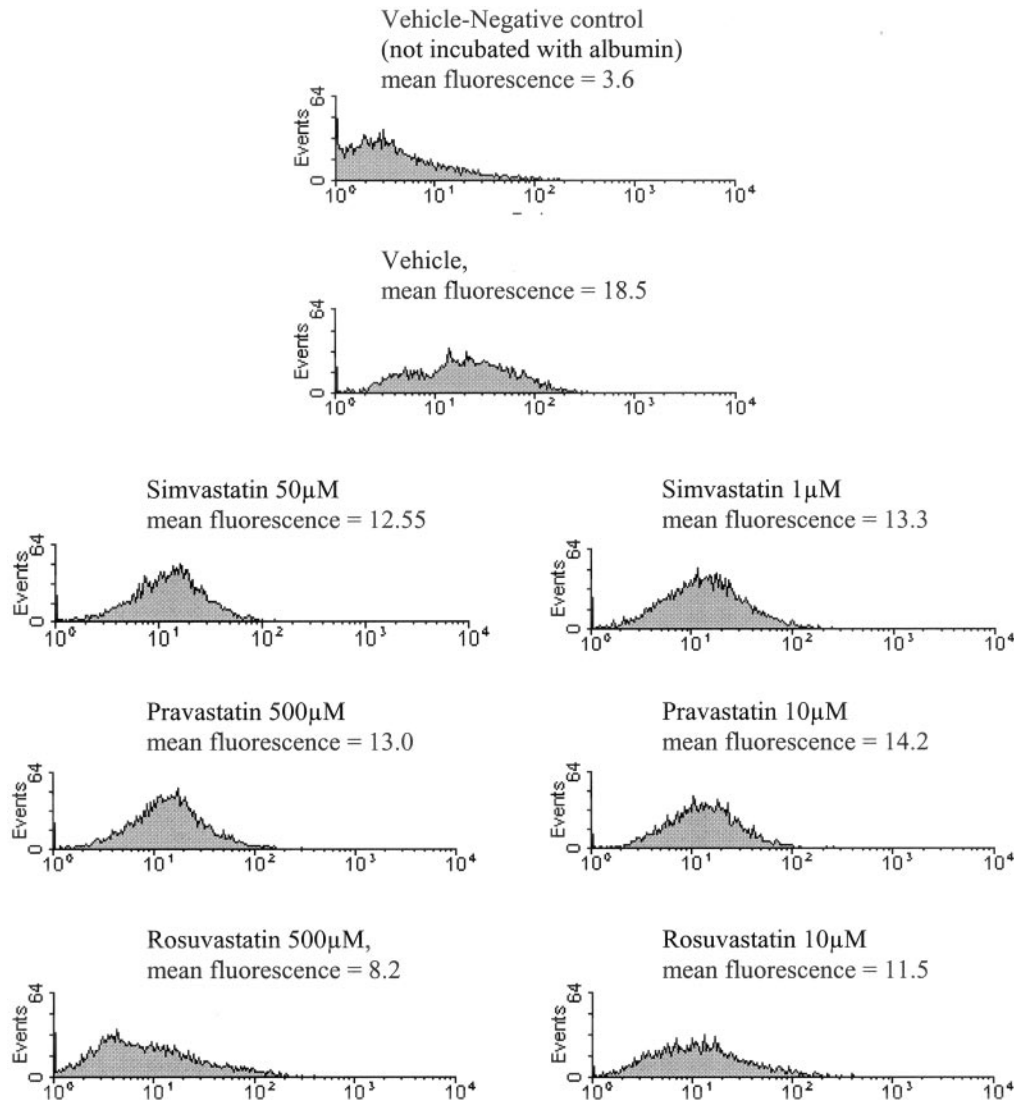

Figure 5. Histograms representing albumin uptake in proximal tubular cells. Statin or vehicle treated cultures of human tubular kidney cells were incubated with FITC-albumin (except the negative control) and flowcytometrically analysed: a histogram plot of albumin uptake in LAP positive, endocytosing cells (not LAP negative cells) was designed. The mean of albumin fluorescence was calculated is a measure for albumin uptake. The three statins clearly inhibited FITC-albumin uptake as indicated by the shift in the distribution of the cells to lower intensity fluorescence and the lower mean fluorescence, compared to the vehicle. Histograms show the results from a representative experiment on one kidney specimen out of 5 experiments on different kidneys.

FITC labeled albumin since albumin represents a substantial proportion of the proteins present in the glomerular ultrafiltrate (21). Albumin uptake could be demonstrated by microscopy as well as flow cytometry and spectrofluorometry. Second, using flow cytometry and microscopy, it was shown that protein uptake occurred in proximal tubular cells and not in distal tubular and collecting duct cells, thereby reflecting the *in vivo* situation in which proteins are well known to be taken up by the proximal tubular epithelium. The human tubular cell cultures endocytosed 4.4 μg/mg total protein, which is in the same order as observed in the OK cell cultures (3.5 μg/mg). These primary tubular cell cultures of human origin thus offer a suitable, clinically relevant, *in vitro* model to study protein uptake in the human kidney.

In agreement with the results obtained in OK cells, a range of statins significantly inhibited protein uptake in human proximal tubular cells in a concentration-dependent way without exerting a toxic effect. In the human cell cultures, the effect of statins on proximal tubular cell endocytosis shows a higher variance compared to OK cells. For the experiments on human kidney tubular cells, cultures from different kidney donors are used; this self-evidently results in the high variance representing the biologic variation between individuals. Analogous to OK cells, co-addition of mevalonate to statin treated cultures reversed the statin induced reduction in protein uptake. Moreover, it is known from OK cell experiments that co-addition of GGPP, a mevalonate-metabolite and substrate molecule for prenylation of many proteins, also prevents the statin induced

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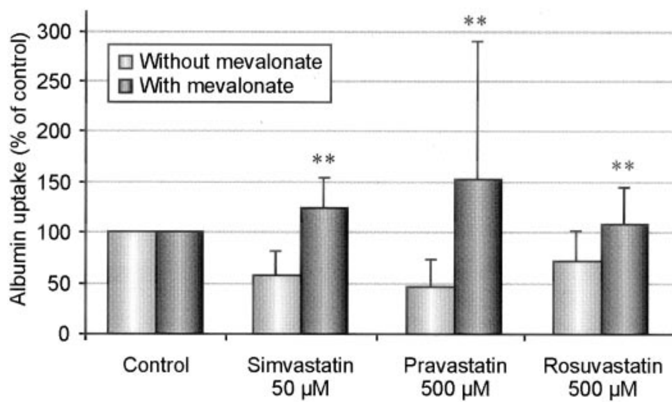

Figure 6. Effect of mevalonate on the statin induced inhibition of albumin endocytosis by human kidney tubular cells. Albumin uptake was normalised to total protein concentration and expressed as percentage of the FITC-albumin uptake in vehicle-treated control. Albumin uptake in cultures incubated with statin and mevalonate was significantly higher than (* $p < 0.005$) in cultures treated with statin alone. Data represent the mean +SD of experiments on 5 different kidneys (20 culture dishes).

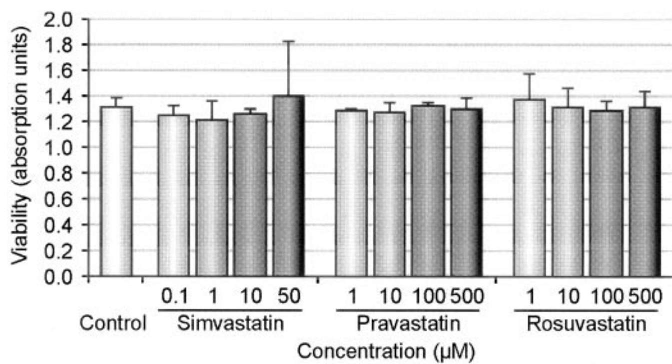

Figure 7. Effect of statins on cell viability. Confluent cultures were incubated for 16h with increasing concentrations of simvastatin, pravastatin and rosuvastatin. Subsequently cell viability was measured by an MTT based test and expressed in absorption units. No statin induced effect on cell viability could be observed.

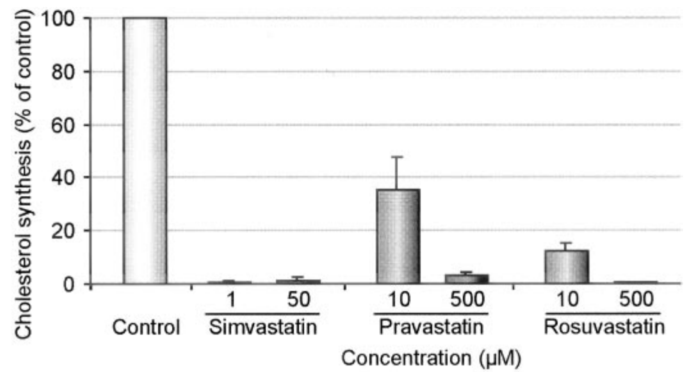

Figure 8. Effect of statins on cholesterol synthesis as measured by the flux of [^{14}C] labelled acetate to cholesterol. Cholesterol synthesis was normalised to total cell protein levels and expressed as % of control. Data represent the mean +SD of experiments on 3 different kidneys (6 cultures dishes).

inhibition of endocytosis. Furthermore, in those cells it was shown that prenylation of Rap1, is reduced by statins with a time course similar to the inhibition of albumin uptake, consistent with the idea that statins are causing a gradual depletion of the critical isoprenoid pyrophosphates. It is already known that statins, via this pathway, exert a range of additional effects on cells including proliferation, signal transduction and apoptosis, independent of their effect on cholesterol homeostasis (5,22–25).

Statin treatment during 3.5 h resulted in a dramatic inhibition of HMG-CoA reductase, as indicated by the amount of labeled acetate incorporation into cholesterol, at concentrations that only partially inhibited albumin uptake. This is consistent with the OK cell results as well as with the fact that production of essential molecules for isoprenylation of proteins, including GGPP, can be maintained, at least to a certain extent, despite strong inhibition of HMG-CoA reductase (3).

The observed effect of statins on the *in vitro* albumin uptake in OK cells and human cells suggests a plausible explanation for the transient proteinuria seen in patients treated with the higher doses of rosuvastatin. This particular proteinuria was mild ($<$1g/ 24h) and based on electrophoretic analysis of the urine was of tubular type. Statins have been shown to reduce proteinuria of glomerular origin in patients with type 2 diabetes and IgA nephropathy (26,27). Although a low incidence of transient, tubular proteinuria was observed in patients treated with the higher doses of rosuvastatin, the use of this statin in patients over short (approximately 8 wk) or longer ($\geq$ 96 wk) periods was shown to result in either no change or an increase in GFR compared to baseline (9), suggesting, in common with other statins, a protective effect in the glomerulus. It is therefore reasonable statins could have the potential to elicit transient tubular proteinuria but also have a reno-protective effect.

The statin concentrations used for these experiments were rather high, compared to the circulating drug levels in humans especially those for pravastatin and rosuvastatin. However, pravastatin and rosuvastatin are relatively hydrophilic organic anions with relatively low rates of uptake into cells by passive diffusion (17) compared to lipophilic statins such as simvastatin. Cellular entry of such molecules is therefore dependent on active uptake by organic anion transporters (28–30). *In vivo* these are mainly present on the basolateral side of the renal epithelium (28,29). Since cultured cells are attached with their basolateral membrane to the culture dish, access of pravastatin and rosuvastatin to the cells may be relatively restricted. Moreover, at present we do not know to what extent the transporters which are responsible for the known flux of these compounds through the proximal tubule cells in humans *in vivo* (31,32) remain expressed in the cell culture system. Further studies investigating both of these issues are ongoing.

The current studies in cells suggest that statins have the potential to reduce protein reabsorption *in vivo* providing there was sufficient inhibition of HMG-CoA reductase in the proximal tubule cells. However, the degree to which this is manifest with the different statins in clinical use is likely to depend on

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a number of factors such as the intrinsic inhibitory potency of the compound, the particular rates of influx and efflux of the compound to and from the proximal cells and on the extent to which the compound is excreted in a non-metabolized form by the proximal tubules. All of these factors are, or are likely to be, different between the particular statins.

In conclusion, this study indicates that statins, in the absence of cellular toxicity, inhibit protein uptake by the human proximal nephron via inhibition of HMG-CoA reductase and reduced prenylation of proteins involved in endocytosis. This in turn may result in tubular proteinuria in some patients treated with high doses of rosuvastatin.

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