In vitro effects of simvastatin on tubulointerstitial cells in a human model of cyclosporin nephrotoxicity

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OVER THE LAST DECADE, numerous studies have suggested that 3-hydroxy-3-methylglutaryl CoA reductase inhibitors significantly retard the progression of renal failure in a variety of animal models of chronic renal disease, including subtotal renal ablation (26) and puromycin aminonucleoside nephrosis (15), in obese Zucker rats (35), Dahl salt-sensitive rats (33), and Han:SPRD rats with autosomal dominant polycystic kidney disease (10). Human studies have been much more limited, although beneficial effects have been reported for nondiabetic causes of renal impairment (serum creatinine 0.15–0.4 mmol/l) (36), as well as for nephropathies complicating type 1 diabetes mellitus (41), type 2 diabetes mellitus (25), and a variety of primary glomerulonephritides (5, 11, 39, 42).

Where benefits of HMGCoA reductase inhibitors have been demonstrated for progressive renal disease, they have generally been attributed to decreases in the circulating levels of cholesterol. However, recent evidence suggests that HMGCoA reductase inhibitors may exert renoprotective effects via cholesterol-independent mechanisms. For example, both lovastatin (34) and simvastatin (12) have been shown to inhibit growth factor-stimulated mesangial cell proliferation in rats and humans, respectively. This inhibition was reversed in both cases by mevalonate, but not cholesterol, suggesting that depletion of nonsterol isoprenoid intermediates of the mevalonate pathway by HMGCoA reductase inhibitors may curb glomerulosclerosis as a result of suppression of mesangial proliferation. Although the mechanisms are not fully understood, cell growth may be blocked through reduced synthesis of prenylated proteins (such as ras and G proteins) (4, 23) or glycoproteins (such as Nα-H exchangers (NHE)) (49), which have separately been implicated in the cellular actions of growth factors (4, 47).

Such mechanisms may also contribute to amelioration by HMGCoA reductase inhibitors of tubulointerstitial pathology, which is characterized by initial proximal tubule cell (PTC) hypertrophy and hyperplasia followed by renal cortical fibroblast (CF) proliferation, excessive extracellular matrix deposition, PTC atrophy, and impaired tubular transport function (16, 45). These lesions correlate much better than glomerular pathology with the degree and progression of renal impairment, regardless of the type and anatomic origin of the inciting injury (3). However, apart from one recent study involving rat PTC (48), there have been no studies of the effects of HMGCoA reductase inhibitors on tubulointerstitial biology at the cellular level. Thus, the aims of the present series of experiments were to assess the in vitro effects of clinically relevant concentrations of simvastatin on human PTC and CF viability, growth, NHE activity, collagen synthesis, and growth factor secretion. These parameters were studied both under basal conditions and in the presence of cyclosporin A (CsA), which has previously been validated by us and by other investigators as an in vitro model of tubulointerstitial disease (20, 44, 50).

SUBJECTS AND METHODS
Reagents. Simvastatin (provided by Merck Sharp and Dohme, Sydney, NSW, Australia) was hydrolytically converted from an inactive lactone prodrug form to an active
β-hydroxy open acid form by initially dissolving 15 mg in 1.5 ml 0.25 mol/l NaOH and heating at 37°C for 1 h. The resulting solution was titrated to pH 7.4 with 0.25 mol/l HCl, brought to a final concentration of 5 mg/ml (12 mmol/l), and stored in multiple aliquots at −80°C until use (46). A 1.3 mg/ml (10 mmol/l) stock solution of mevalonate salt was prepared by solubilizing 32.5 mg DL-mevalonic acid lactone [DL- β-hydroxy- β-methylvalerolactone; Sigma, St Louis, MO] in 2 ml absolute ethanol followed by the addition of 23 ml of 1:1 (vol/vol) DMEM and Ham's F-12 (DMEM-F-12; ICN Pharmaceuticals, Costa Mesa, CA) supplemented with 5 µg/ml human transferrin (Sigma) (34). Cholesterol (Sigma) stock solution (100 mmol/l) was freshly prepared by dissolving 38.7 mg in 1 ml preheated (50°C) absolute ethanol (31). A 10 mg/ml stock solution of CsA (provided by Novartis, Basel, Switzerland) was also prepared in absolute ethanol. The final concentration of ethanol was standardized at 0.1% for all wells (including controls). This concentration of alcohol was shown to have no effect on tubule cell and fibroblast viability and function (37, 44). Our results confirmed this for all parameters studied (data not shown).

Patients. Segments of macroscopically and histologically normal renal cortex were obtained aseptically from adult human kidneys removed surgically because of small (<6 cm) renal adenocarcinomas (n = 8), pelviureteric transitional cell carcinomas (n = 2), or benign renal cysts (n = 2). The average patient age was 63.3 ± 4.3 yr, and the male-to-female ratio was 5:7. Patients were otherwise healthy and were on no medications. Informed consent was obtained before each operative procedure, and the use of human renal tissue for primary culture was reviewed and approved by the Royal North Shore Hospital and University of Sydney Human Medical Research Ethics Committee.

Cell culture. The method for primary culture of human PTC and CF is described in detail elsewhere (17, 19). Briefly, renal cortical tissue was dissected from the medulla and minced, digested with collagenase (class 2, 383 U/mg, Worthington, Freehold, NJ), and passed through a 100 µm mesh. Filtered tissue was resuspended in 45% Percoll (Pharmacia, Uppsala, Sweden) and separated into four distinct bands by isopycnic ultracentrifugation. The lowermost band was removed for PTC culture. The uppermost band was removed for CF culture.

PTC were resuspended in serum-free, antibiotic-free, hormonally defined media consisting of 1:1 (vol/vol) DMEM-F-12 (ICN Pharmaceuticals), supplemented with 5 µg/ml human transferrin (Sigma), 5 µg/ml (0.87 µmol/l) bovine insulin (Sigma), 0.05 µmol/l hydrocortisone (Sigma), 10 ng/ml (1.64 nmol/l) epidermal growth factor (Collaborative Research, Bedford, MA), 50 µmol/l PGE2 (Sigma), 50 nmol/l selenium (Sigma), and 5 pmol/l triliodothyronine (Sigma). The tubular fragments were plated at a density of 1.5 mg pellet/cm² (∼5,000 fragments/cm²) in 75-cm² flasks (Corning). Media were changed every 48 h. The cells were incubated in humidified 95% air−5% CO₂ at 37°C and were subcultured at near-confluence using a seeding density of 4,000 cells/cm². Such cells were designated passage 1.

Fibroblasts (CF) were resuspended in antibiotic-free DMEM-F-12 supplemented with 10% fetal calf serum (Trace Biosciences, Sydney, Australia) and were seeded at an initial density of 2.25 mg pellet/cm². Subculture at confluence was subsequently performed using a seeding density of 7,000 cells/cm². Because doubling times were greater for CF than for PTC, the higher seeding densities used in the former group allowed the synchronization of the attainment of confluence in both populations at ∼5 days after plating.

Cytologic examination of PTC and CF preparations from all donors failed to reveal any evidence of cellular atypia. The morphological, biochemical, and functional characteristics of these cells were previously studied in this laboratory and found to reproducibly exhibit the features of PTC and CF in vivo (17, 19).

Experimental protocol. All experiments were performed on confluent, quiescent, passage 2 PTC and CF. Cells were made quiescent by three washes followed by incubation for 24 h in basic media (DMEM-F-12 containing 5 µg/ml human transferrin).

Basic media containing vehicle or various concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, or 10 µmol/l) of simvastatin were added for a further 24 h to simulate clinically relevant or suprapharmacological tissue levelsimvastatin concentrations (1, 9). Cell viability, growth, function, collagen synthesis, and secretion of selected fibrogenic cytokines were then measured. To determine whether the effects of simvastatin on CF growth and on PTC growth and NHE activity were related to deprivation of cholesterol or earlier intermediates in the mevalonate pathway, PTC in some experiments were exposed to 1 µmol/l simvastatin in the presence or absence of mevalonate (200 µmol/l) or cholesterol (100 µmol/l).

In additional experiments, the effects of simvastatin were also evaluated in PTC or CF incubated for 24 h in the presence or absence of 1,000 ng/ml CsA. This concentration approximates renal cortical tissue levels of cyclosporin in clinical settings (27, 40) and was previously demonstrated in this laboratory to induce evidence of toxicity in both PTC and CF (20).

Cell viability. Cell viability was measured by exclusion of trypan blue dye (17). PTC viability was additionally assessed by release of lactate dehydrogenase (LDH), according to the method of Mitchell et al. (30).

Cell growth. Cell growth was measured according to a previously described method (18). Twenty-four hours before study, 4 µCi (0.15 MBq) of [methyl-3H]thymidine (37 MBq/ml, 185 GBq/mmol; Amersham) was added to each milliliter of control or test media that was incubated with cells. At the end of the incubation periods, cells were dislodged with 80 µl/cm² CR-Dispase (Collaborative Research) for 10 min at 37°C and subsequently washed three times in PBS at 1,250 g and 4°C for 5 min. Aliquots of the cell suspensions were retained for cell counting in a standard hemocytometer and for determination of thymidine incorporation, an index of DNA synthesis, by liquid scintillation counting in a β-counter (1215 Rackbeta II, LKB Wallac, Turku, Finland).

PTC functional studies. The ability of human PTC to form a tight, impermeable monolayer was assessed by paracellular diffusion of inulin, as described previously (17). Briefly, confluent cells grown on permeable supports (Millicell-CM inserts, 0.4 µm, 12 mm diameter; Millipore, Bedford, MA) were exposed to media containing appropriate solvent vehicle or various concentrations of simvastatin for 24 h. [3H]Inulin (50 µg/ml, 215 MBq/g, 5.8 mCi/g, NEN Research Products, DuPont, Wilmington, DE) was then added to the basolateral side as a marker for the extracellular space. Aliquots of the basolateral and apical media were collected at times 0, 0.5, 1, 2, 3, 4, 6, 8, and 24 h.

PTC monolayer integrity was further evaluated by measuring the formation of transepithelial Na⁺ and K⁺ gradients, which depends on intact monolayers with functional active transport mechanisms (17). Media were collected from the apical and basolateral compartments of confluent monolayers grown on permeable supports after 24 h incubation in control or test media. Na⁺ and K⁺ concentrations were measured by
L-ascorbic acid (Sigma), and 60 µg/ml predominate route of apical sodium uptake by PTC (32). Cells assessed, inasmuch as this antiporter has been linked to PTC flame photometry (Instrumentation Laboratory IL943, Lexington, MA).

The effect of simvastatin on apical NHE activity was also assessed, inasmuch as this antiporter has been linked to PTC growth (18), is inhibited by CsA (20), and represents the predominant route of apical sodium uptake by PTC (32). Cells were acetylated by a 20 mmol/l NH₄Cl pulse. Apical NHE activity was then measured as the ethylisopropylamiloride (EIPA)-sensitive component of ²²Na⁺ uptake during the first minute after the apical addition of uptake buffer containing (in mmol/l): 135 NaCl, 4 KCl, 1.2 CaCl₂, 0.8 MgCl₂, 28.3 HEPES, and 17.7 Tris base and 100 µmol/l ouabain (Sigma) and 0.2 µCi carrier-free ²²Na (1 mCi/ml, sp act ~50 Ci/µmol, NEN Research Products). Basolateral Na⁺ transport was inhibited by the simultaneous addition of Na⁺-free buffer (in mmol/l: 135 choline chloride, 4 KCl, 1.2 CaCl₂, 0.8 MgCl₂, 28.3 HEPES, and 17.7 Tris base and 100 µmol/l ouabain, pH 7.4).

Results were corrected for cellular protein content, which was measured by a commercially available protein assay (Protein Assay Kit II, Bio-Rad, Hercules, CA) using bovine serum albumin (fraction V, RIA grade; Sigma) as the standard. The validity of this experimental system for measuring apical NHE activity has been previously confirmed in our laboratory (17, 18).

CF collagen synthesis assay. CF collagen synthesis was measured by tritiated proline incorporation (14). After 24 h incubation in control or test media, cells were washed and incubated for a further 3 h under the same conditions in the presence of 20 µCi/ml (2,3-H)proline (Amersham), 50 µg/ml L-ascorbic acid (Sigma), and 60 µg/ml β-aminopropionitrilone (Sigma). Media containing secreted, noncrosslinked collagen was incubated for 1 h at 37°C with 400 µl Tris-CaCl₂-N-ethylmaleimide buffer (50 mmol/l Tris·HCl, 5 mmol/l CaCl₂, 2.5 mmol/l N-ethylmaleimide, 0.02% NaN₃) in both the presence and absence of 2 mg/ml collagenase (Worthington Biochemicals). The reaction was terminated by precipitation with ice-cold 250 µl 40% TCA and 250 µl 2% tannic acid for 1 h at 4°C. Samples were centrifuged at 1,250 g for 5 min. Supernatants were counted in a beta counter, and counts obtained from samples that were not exposed to collagenase served as background values. Pellets derived from the collagenase group were solubilized in 200 µl 0.2 mol/l NaOH and counted to measure proline incorporation into collagenase-insensitive proteins. The percent of total protein synthesized as collagen (%collagen) was calculated as the ratio of collagenase-releasable dpm divided by total dpm (supernatant plus pellet) as follows: %collagen = (C/P)/[(5.4 × (1 - C/P)] + (C/P)) × 100, where C is collagenase-releasable dpm in supernatants and P is collagenase-insensitive dpm in pellets.

A correction factor of 5.4 for noncollagen protein was used to adjust for the relative abundance of proline and hydroxyproline in proteins containing collagen (14).

Measurement of growth factors in conditioned media in the presence and absence of simvastatin. It has previously been demonstrated in this laboratory that human CF secrete insulin-like growth factor-I (IGF-I), IGF-I binding protein-2 (IGFBP-2), and IGFBP-3, whereas PSC release transforming growth factor-β1 (TGF-β1) and the AB heterodimer of platelet-derived growth factor (PDGF-AB) (19, 21). Moreover, the secretory profiles of these fibrogenic agents are significantly altered by exposure of CF and PTC to CsA (20). Therefore, the effects of various concentrations of simvastatin on secretion of these fibrogenic growth factors into CM were measured under basal conditions and in the presence of CsA.

The effect of simvastatin on basal and CsA-stimulated CF secretion of IGF-I into culture media was determined by specific RIA after Bio-Spin P-10 chromatographic separation of IGFBPs, as previously described (19). IGFBP-3 and IGF-FBP-2 levels in CM samples were measured using specific RIA kits (19).

TGF-β1 levels in CM- and PTC-CM were measured using a commercially available ELISA kit (Promega TGF-β1 ELISA, Promega, Madison, WI). Total (active plus latent) TGF-β1 was determined after transient acidification of CM. Briefly, 1 ml of CM was incubated with 30 µl 1 mol/l HCl for 10 min at room temperature before neutralization with an equivalent volume of 1.2 mol/l NaOH-0.5 mol/l HEPES. The minimum detectable concentration of TGF-β1 by the assay was 15.6 pg/ml.

PDGF-AB was also measured in CM-CM samples by commercial ELISA kit (human PDGF-AB Biotrak assay, Amersham). Cross-reactivities with the AA and BB dimers of PDGF were 10 and 2%, respectively. The minimum detectable concentration of PDGF-AB by the assay was 8.4 pg/ml. Intra- and interassay coefficients of variation for both ELISA kits were <5% and 10%, respectively.

Statistical analysis. All studies were performed in triplicate from PTC and CF cultures obtained from at least three separate human donors. Each experiment contained internal controls originating from the same culture preparation. For the purposes of analysis, each experimental result for the growth, apical NHE, and LDH release studies was expressed as a change from the control value, which was regarded as 100%, and analyzed independently. Results are expressed as means ± SE. Statistical comparisons between groups were made by ANOVA. Pairwise multiple comparisons were made by Fisher’s protected least-significant differences test. Analyses were performed using the software package Statview version 4.5 (Abacus Concepts, Berkeley, CA). P values <0.05 were considered significant.

RESULTS

Simvastatin exerts cholesterol-independent inhibition of both CF and PTC growth without affecting cellular viability. CF and PTC viability, as determined by trypan blue exclusion, were unaltered by simvastatin concentrations up to 1 µmol/l (Table 1). Similarly, LDH release by PTC was not significantly altered after exposure to 0.1 µmol/l (98.1 ± 4.5% of controls, P = NS) or 1 µmol/l simvastatin (98.6 ± 4.0%, P = NS).

However, simvastatin significantly inhibited CF and PTC thymidine incorporation at the highest concentration tested of 1 µmol/l (Table 1). This inhibition of DNA synthesis was completely prevented in CF by exogenous mevalonate, but not cholesterol (Fig. 1). Exogenous mevalonate also abrogated the suppressive effects of simvastatin on PTC DNA synthesis and resulted in significantly higher thymidine incorporation rates than those found in PTC exposed to the combination of simvastatin and cholesterol (Fig. 1). Neither mevalonate nor cholesterol alone exerted any significant effects on cellular growth (data not shown).
Simvastatin exerts cholesterol-dependent inhibition of PTC apical NHE activity. Total apical \( {^{22}}\text{Na}^+ \) uptake by PTC was inhibited by simvastatin in a concentration-dependent fashion. Significant suppression was observed at simvastatin concentrations of 0.5 \( \mu \text{mol/l} \) (67.2 \( \pm \) 10.0\% of controls, \( P < 0.05 \)) and 1 \( \mu \text{mol/l} \) (62.0 \( \pm \) 9.3\% of control, \( P < 0.05 \)). No significant change was observed at 0.1 \( \mu \text{mol/l} \) (83.4 \( \pm \) 17.2\% of controls).

Apical NHE activity, which accounted for \( \sim 35\% \) of total \( {^{22}}\text{Na}^+ \) uptake under control conditions (0.81 \( \pm \) 0.01 \text{nmol·mg protein}^{-1} \cdot \text{min}^{-1}, \text{respectively}), was significantly inhibited by simvastatin at a concentration of 1 \( \mu \text{mol/l} \) (60.9 \( \pm \) 15.2\% of control values, \( P < 0.05 \)), but not 0.5 \( \mu \text{mol/l} \) (86.0 \( \pm \) 17.5\%, \( P = \text{NS} \)) or 0.1 \( \mu \text{mol/l} \) (92.6 \( \pm \) 19.6\%, \( P = \text{NS} \)). In separate experiments, the effects of 1 \( \mu \text{mol/l} \) simvastatin were completely reversed by either 200 \( \mu \text{mol/l} \) mevalonate or 100 \( \mu \text{mol/l} \) cholesterol (Fig. 2). These concentrations of mevalonate and cholesterol did not affect apical NHE activity under basal conditions (data not shown).

Simvastatin increases PTC monolayer permeability. PTC monolayer integrity was significantly impaired even at the lowest tested simvastatin concentration of 0.1 \( \mu \text{mol/l} \), as evidenced by increased paracellular

### Table 1. Concentration-dependent effects of simvastatin on CF and PTC

<table>
<thead>
<tr>
<th></th>
<th>Simvastatin Concentration (( \mu \text{mol/l} ))</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>CF</td>
<td></td>
</tr>
<tr>
<td>Viable cells, %total cells counted</td>
<td>97.8 ( \pm ) 0.3</td>
</tr>
<tr>
<td>Thymidine uptake, %control value</td>
<td>100</td>
</tr>
<tr>
<td>Collagen synthesis, %total protein synthesis</td>
<td>11.1 ( \pm ) 1.7</td>
</tr>
<tr>
<td>Protein secretion, ( \mu \text{g/well} )</td>
<td>8.0 ( \pm ) 1.2</td>
</tr>
<tr>
<td>IGF-I, ng·mg cellular protein(^{-1})·day(^{-1})</td>
<td>4.18 ( \pm ) 0.9</td>
</tr>
<tr>
<td>IGFBP-2, ng·mg cellular protein(^{-1})·day(^{-1})</td>
<td>501 ( \pm ) 129</td>
</tr>
<tr>
<td>IGFBP-3, ng·mg cellular protein(^{-1})·day(^{-1})</td>
<td>1790 ( \pm ) 513</td>
</tr>
<tr>
<td>PTC</td>
<td></td>
</tr>
<tr>
<td>Viable cells, %total cells counted</td>
<td>96.3 ( \pm ) 0.5</td>
</tr>
<tr>
<td>Thymidine uptake, %control value</td>
<td>100</td>
</tr>
<tr>
<td>Apical NHE, %control value</td>
<td>100</td>
</tr>
<tr>
<td>Na(^+) gradient, mmol/l</td>
<td>2.03 ( \pm ) 0.46</td>
</tr>
<tr>
<td>K(^+) gradient, mmol/l</td>
<td>0.75 ( \pm ) 0.21</td>
</tr>
<tr>
<td>TGF-β1, ng·mg cellular protein(^{-1})·day(^{-1})</td>
<td>0.51 ( \pm ) 0.14</td>
</tr>
<tr>
<td>PDGF, ng·mg cellular protein(^{-1})·day(^{-1})</td>
<td>0.93 ( \pm ) 0.23</td>
</tr>
</tbody>
</table>

Values are expressed as means \( \pm \) SE of 6–18 experiments. Confluent, quiescent, passage 2 human proximal tubule cells (PTC) and cortical fibroblasts (CF) were incubated for 24 h with various concentrations of simvastatin (open acid form), as indicated. IGF, insulin-like growth factor; IGFBP, IGF binding protein; TGF, transforming growth factor; PDGF, platelet-derived growth factor; NHE, sodium-hydrogen exchange. *\( P < 0.05 \) vs. control.

Fig. 1. Effect of exogenous cholesterol and mevalonate on inhibition of cortical fibroblast (CF) and proximal tubule cell (PTC) DNA synthesis by simvastatin. Confluent, quiescent CF (n = 6) and PTC (n = 9) were incubated in media containing vehicle (control) or 1 \( \mu \text{mol/l} \) simvastatin in the presence or absence of either cholesterol (100 \( \mu \text{mol/l} \)) or mevalonate (200 \( \mu \text{mol/l} \)). DNA synthesis was measured as thymidine incorporation into cells. *\( P < 0.05 \) vs. control; \#\( P < 0.05 \) vs. simvastatin + simvastatin + cholesterol.

Fig. 2. Effect of exogenous cholesterol and mevalonate on inhibition of PTC apical Na\(^+\)/H\(^+\) exchange (NHE) activity caused by simvastatin. Confluent, quiescent PTC were incubated in media containing vehicle (control) or 1 \( \mu \text{mol/l} \) simvastatin in the presence or absence of either cholesterol (100 \( \mu \text{mol/l} \)) or mevalonate (200 \( \mu \text{mol/l} \)). Apical NHE activity was measured as ethylisopropylamiloride (EIPA)-sensitive apical \( {^{22}}\text{Na}^+ \) uptake after intracellular acidification by 20 \( \mu \text{mol/l} \) NH\(_4\)Cl. Results represent means \( \pm \) SE of 12 experiments. *\( P < 0.05 \) vs. all other groups.
Simvastatin and the Kidney

Simvastatin modulates the effects of CsA on human tubulointerstitial cells. The effects of 0.1 µmol/l simvastatin on CsA-induced changes in the above parameters were studied. This concentration of simvastatin was chosen on the basis of the results of the above studies, which showed that the drug at this level minimally affects basal cellular characteristics. Moreover, pharmacokinetic studies performed by other investigators (1, 9) suggested that this concentration more closely approximates clinically relevant tissue levels than the micromolar concentrations used in previous in vitro studies (12, 48).

As demonstrated previously (20), CsA exerted significant effects on CF, characterized by augmented synthesis and secretion of both collagen and IGFBP-3 and by reduced viability and DNA synthesis (Table 2, Figs. 4 and 5). The main parameter significantly modified by coadministration of simvastatin was collagen synthesis, in which the 30% increase above control values provoked by 1,000 ng/ml CsA alone was completely abrogated by concomitant incubation with 0.1 µmol/l simvastatin (CsA-simvastatin). This concentration of simvastatin did not affect collagen synthesis under basal conditions. Results represent means ± SE of 6–18 experiments. Confluent, quiescent, passage 2 human CF and PTC were incubated for 24 h in vehicle (control), or 1,000 ng/ml cyclosporin (CsA) in the presence or absence of 0.1 µmol/l simvastatin. This concentration of simvastatin did not effect any of the measured parameters under basal conditions (see Table 1). *P < 0.05 vs. control; †P < 0.05 vs. CsA alone.

**Table 2. Effects of simvastatin on CsA-induced CF and PTC toxicity**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CsA</th>
<th>CsA + Simvastatin</th>
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<tbody>
<tr>
<td>CF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymidine uptake, %control value</td>
<td>100</td>
<td>62.0 ± 9.8*</td>
<td>49.2 ± 9.9*</td>
</tr>
<tr>
<td>Trypan blue, %total cells counted</td>
<td>99.2 ± 0.2</td>
<td>97.2 ± 0.7*</td>
<td>98.8 ± 0.3*</td>
</tr>
<tr>
<td>LDH release, %control value</td>
<td>100</td>
<td>110.9 ± 2.8*</td>
<td>126.5 ± 4.1†</td>
</tr>
<tr>
<td>Apical NHE, %control value</td>
<td>100</td>
<td>65.9 ± 12.0*</td>
<td>61.8 ± 14.7*</td>
</tr>
<tr>
<td>TGF-β1, ng·mg cellular protein 1·day⁻¹</td>
<td>0.89 ± 0.07</td>
<td>1.13 ± 0.09*</td>
<td>1.09 ± 0.10</td>
</tr>
<tr>
<td>PDGF, ng·mg cellular protein 1·day⁻¹</td>
<td>1.31 ± 0.29</td>
<td>1.85 ± 0.43</td>
<td>1.73 ± 0.26</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6–18 experiments. Confluent, quiescent, passage 2 human CF and PTC were incubated for 24 h in vehicle (control), or 1,000 ng/ml cyclosporin (CsA) in the presence or absence of 0.1 µmol/l simvastatin. This concentration of simvastatin did not effect any of the measured parameters under basal conditions (see Table 1). *P < 0.05 vs. control; †P < 0.05 vs. CsA alone.

**Fig. 3. Effect of simvastatin on transepithelial permeability in human PTC.** Confluent, quiescent PTC grown on permeable supports were exposed to various concentrations of simvastatin, as indicated. [14C]Inulin was instilled into the basolateral compartment, and its appearance in the contralateral apical compartment was subsequently measured. Results represent means ± SE of 6 separate experiments. At each time point, apical-to-basolateral ratios of [14C]inulin concentrations were significantly higher in the presence of each concentration of simvastatin tested compared with controls.

**Fig. 4. Effect of simvastatin on cyclosporin A (CsA)-induced CF collagen synthesis.** Collagen synthesis was measured as [3H]proline incorporation into collagenase-sensitive proteins contained in media conditioned by confluent, quiescent CF incubated for 24 h in vehicle (control), 1,000 ng/ml CsA, or 1,000 ng/ml CsA and 0.1 µmol/l simvastatin (CsA-simvastatin). This concentration of simvastatin did not affect collagen synthesis under basal conditions. Results represent means ± SE of 8 experiments, each performed in triplicate. *P < 0.05 vs. all other groups.
both human PTC and CF. This finding is potentially associated with appreciable suppression of growth in rat PTC (48), inhibition of HMGCoA reductase was rat mesangial cells (34), human mesangial cells (12), kidney cells (38), Madin-Darby canine kidney cells (46), as human preputial fibroblasts (47), baby hamster unchanged fibroblast cytotoxicity and possibly induced collagen synthesis and IGF-I secretion by CF.

lial permeability, but completely prevented CsA-induced alterations in CF secretion of insulin-like growth factor (IGF)-I, IFG binding protein (IGFBP)-2, and IGFBP-3. IGF-I, IGFBP-2, and IGFBP-3 levels were measured by specific RIAs in media conditioned by confluent, quiescent CF (n = 9) incubated for 24 h in vehicle (control), 1,000 ng/ml CsA, or 1,000 ng/ml CsA and 0.1 µmol/l simvastatin (CsA + simvastatin). This concentration of simvastatin did not effect any of the measured parameters under basal conditions. Results are normalized for cellular protein and are expressed as a percentage of control values. *P < 0.05 vs. control and CsA + simvastatin. exposed to CsA, although the differences did not achieve statistical significance. None of these effects could be reversed by concomitant incubation with simvastatin. PTC viability, estimated by trypan blue exclusion, was not significantly different between any of the groups (Table 2). However, using LDH release as an independent measure of cytotoxicity, PTC viability was significantly reduced after incubation with CsA and impaired further by incubation with both CsA and simvastatin (P < 0.001 vs. CsA alone) (Table 2).

DISCUSSION

The present study is the first reported study of the effects of HMGCoA reductase inhibitors on the human cortical tubulointerstitium. Simvastatin, in micromolar concentrations, resulted in cholesterol-independent anti-proliferative effects in both human PTC and renal CF, as well as cholesterol-dependent inhibition of apical NHE activity in PTC. CF synthesis and secretion of IGF-I and IGFBP-3 were also inhibited by simvastatin in a concentration-dependent fashion, but CF collagen synthesis was unaffected under basal conditions. Submicromolar concentrations of simvastatin did not affect any baseline cellular parameters, except for transepithelial permeability, but completely prevented CsA-induced collagen synthesis and IGF-I secretion by CF. However, this beneficial effect was associated with unchanged fibroblast cytotoxicity and possibly increased proximal tubule cytotoxicity.

As has been found in numerous other cell types, such as human preputial fibroblasts (47), baby hamster kidney cells (38), Madin-Darby canine kidney cells (46), rat mesangial cells (34), human mesangial cells (12), and rat PTC (48), inhibition of HMGCoA reductase was associated with appreciable suppression of growth in both human PTC and CF. This finding is potentially relevant to human renal pathophysiology, because augmented growth of PTC (and subsequently of CF) is one of the earliest findings in several experimental models of chronic renal disease, such as diabetic nephropathy (43) and subtotal renal ablation (16). Such enhanced growth has been linked to the development of nephrosclerosis and ensuing renal impairment (16, 45) in much the same way that glomerular growth has been linked to glomerulosclerosis (24).

Although it is therefore tempting to attribute at least some of the documented renoprotective effects of HMG-CoA reductase inhibition in both clinical (5, 11, 25, 36, 39, 41, 42) and experimental renal disease (10, 15, 26, 33, 35) to suppression of tubulointerstitial growth, such inhibition was only apparent in vitro at a concentration of 1 µmol/l. This concentration is lower than has generally been employed for simvastatin (5–25 µmol/l) (12, 48) and lovastatin (5–30 µmol/l) (46–48) in previous renal and extrarenal cellular growth-suppression studies, but is still substantially higher than anticipated clinically relevant concentrations. For example, the reported peak plasma concentration in humans receiving 20 mg simvastatin per os was 0.05 ± 0.01 µmol/l (as free acid equivalents) (1). Renal cortical tissue levels may be up to six times higher than plasma levels (9), but are still likely to be one order of magnitude less than those required for significant renal antiproliferative effects. Nevertheless, these effects may become more relevant as clinicians prescribe progressively higher doses of HMGCoA reductase inhibitors (e.g., up to 160 mg/day of simvastatin) (6).

Inhibition of tubulointerstitial growth by simvastatin was completely reversed by mevalonate, implying that the effect was specific for HMGCoA reductase inhibition, but was not affected by the addition of exogenous cholesterol (100 µmol/l). This concentration of cholesterol has previously been shown to be sufficient for the proliferation of cultured cells (38). Thus DNA synthesis in these cells appeared to require mevalonate or one of its nonsterol derivatives. Similar results were obtained by Vrtovsnik et al. (48), who observed that the antiproliferative effects of 5 µmol/l lovastatin on rat PTC proliferation were completely reversed by mevalonate, farnesyl-pyrophosphate, and geranylgeranyl-pyrophosphate, but not by farnesol, isopentyladenine, dolichol, ubiquinone, or cholesterol. This raises the possibility that tubulointerstitial cell growth may be blocked by HMGCoA reductase inhibition as a result of depletion of isoprenylated proteins, such as ras and heterotrimeric G proteins, that function in growth factor signal transduction (4, 23). Interestingly, in the present study, inhibition of CF growth by simvastatin was associated with a concomitant reduction in the secretion of the autocrine growth factor IGF-I, which is known to signal through the ras pathway (28).

PTC growth has additionally been linked to NHE activation (18), and interestingly, simvastatin exerted concentration-dependent inhibition of PTC apical NHE activity in a manner that closely paralleled the dose-response curve for PTC growth. However, whereas the antiproliferative effects of simvastatin were cholesterol
independent, suppression of PTC apical NHE activity was completely reversed by supplementing the culture media with cholesterol. The ratio of cholesterol to phospholipids in the cell membrane is known to influence both its physical properties and active ion transport processes (2), and strong correlations have been observed between various membrane transporters and plasma lipid levels (2, 49). Although there have been no other studies of the effects of HMGCoA reductase inhibitors on the activities of apical NHE isoforms (NHE3 and/or NHE2) per se, both human and experimental studies have suggested a reduction in NHE1 activity by simvastatin and lovastatin (31, 49). In contrast to our study, this inhibition was abrogated by mevalonate, but not by squalene, and appeared to occur despite a paradoxical increase in cellular cholesterol content. The apparent disparity between these results and those of the present study may relate to altered NHE regulation in the lymphoblasts studied and to structural differences between NHE1 (which is both N- and O-linked glycosylated) and the predominant NHE isofrom on the apical membranes of PTC (NHE3) (which is nonglycosylated) (32). Nonsterol intermediates in the mevalonate pathway, such as dolichol phosphate, have been shown to be important in the synthesis of glycoproteins (31) and may explain why NHE1 activity appears to be dependent on mevalonate derivatives other than cholesterol, whereas PTC apical NHE activity is not.

IGFBP-3 is another N-glycosylated protein (8) that was found in the present investigation to be inhibited by simvastatin in a concentration-dependent fashion, possibly as a result of depletion of mevalonate derivatives such as dolichol phosphate. On the other hand, CF secretion of IGFBP-2, which is not glycosylated (8), was not suppressed. The significance of these changes is uncertain, because both IGFBP-2 and IGFBP-3 have been shown to either inhibit or potentiate the actions of IGF-I, depending on the species, cell type, and experimental conditions (8). However, a change in the relative abundance of these two IGFBPs may be important in targeting the cell and tissue specificity of locally produced IGF-I (13, 19, 22). Thus IGFBPs may determine whether local IGF-I predominantly stimulates interstitial fibroblast proliferation and matrix production (8, 29) or proximal tubule cell regeneration (8, 19).

Transepithelial permeability was markedly increased by simvastatin, even at the lowest concentration tested (0.1 µmol/l). The dependence of this process on mevalonate and cholesterol deprivation was not formally assessed, but was not likely to represent a nonspecific toxic effect because no change was observed in cell viability, as determined by two independent measures. Indeed, PTC toxicity was only apparent at 10 µmol/l (data not shown), which is in agreement with Vrtovsnik et al. (48), who observed that simvastatin only induced detectable cytotoxicity in rat PTC at a concentration between 5 and 10 µmol/l. Similarly, the concentration at which simvastatin significantly inhibited apical NHE activity (1 µmol/l) was 10-fold higher than the concentration at which paracellular inulin permeability increased, thereby implying that the two events were not directly related. The possible confounding effect of increased access of sodium to basolateral NHE was also excluded by exposing basolateral membranes to sodium-free buffer.

In view of the fact that several studies in patients with various types of renal disease have observed a beneficial effect from standard hypcholesterolemic doses of HMGCoA reductase inhibitors (5, 11, 25, 36, 39, 41, 42), the present investigation also examined the effects of a submicromolar (i.e., 0.1 µmol/l) concentration of simvastatin on the in vitro tubulointerstitial toxicity of CsA. For CF, a significant finding was prevention by simvastatin of CsA-induced collagen synthesis. This profibrotic effect of CsA has previously been demonstrated to be mediated by induction of autocrine IGF-I action (20), which was also antagonized by simvastatin in the present investigation. Although such actions may translate into a reduction of interstitial fibrosis complicating CSA therapy in vivo, this has not been subjected to clinical study.

An important caveat is that the potentially beneficial effects of simvastatin on CsA-induced interstitial matrix deposition may be partially offset by an additive cytotoxic effect of simvastatin and CSA on PTC, as indicated by enhanced LDH release. Although this was not detected by trypan blue exclusion, the former test is likely to be more sensitive (30). Theoretically, concerns have been raised that HMGCoA reductase inhibitors may potentiate CsA toxicity by upregulating the expression of low-density lipoprotein receptors on cell surfaces, thereby facilitating intracellular uptake of CsA (7). Clearly, further clinical studies are required in this area.

In conclusion, simvastatin inhibits proximal tubule growth, monolayer barrier function, and apical NHE activity and suppresses CF growth and IGFBP-3 secretion. As has been found to be the case in previous studies of rat glomerular growth (12, 34) and proximal tubular growth (48), these effects were only observed at micromolar concentrations and their relevance to the in vivo situation is uncertain. In submicromolar concentrations, simvastatin exerted minimal effect on the human tubulointerstitium under basal conditions, but abrogated CsA-augmented interstitial collagen synthesis and IGF-I secretion. This was offset by evidence of enhanced proximal tubule cytotoxicity. The results of the present study therefore suggest that HMGCoA reductase inhibitors may ameliorate interstitial fibrosis complicating CSA therapy via direct actions on renal cortical fibroblasts, but that there is also the potential for enhanced proximal tubule injury when the two agents are administered concurrently.

The invaluable assistance by the urologists at the Royal North Shore Hospital in the procurement of human renal tissue is gratefully acknowledged.

D. W. Johnson is supported by a National Health and Medical Research Council of Australia Postgraduate Medical Research Scholarship. The study was supported, in part, by funds from the Australian Kidney Foundation, Clive and Vera Ramaciotti Foundation, Concord Repatriation General Hospital, Royal North Shore Hospital, and the National Health and Medical Research Council of Australia.
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