Effect of lipid-lowering strategies on tubular cell biology

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Background. Interstitial fibrosis and the development of renal cysts are crucial phenomena in renal disease progression. While 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have been shown to reduce the progression of several experimental nephropathies, the mechanism of their potential protective effect remains unclear.

Methods. The antiproliferative, apoptotic, and fibrinolytic effects of HMG-CoA reductase inhibitors were assessed in primary cultured rat (rPTCs) and mouse proximal tubule cells (mPTCs), in isolated rat proximal tubules, and in vivo in 5/6 nephrectomized rats (Nx).

Results. In vitro, lovastatin inhibited rPTC proliferation in a manner selectively prevented by mevalonate, farnesyl-, or geranylgeranyl-pyrophosphate (FPP or GGPP). Lovastatin reduced membrane-bound p21ras and fetal calf serum-induced c-fos and c-jun protein expression. Gel shift assay showed that lovastatin reduced activated protein-1 (AP-1) binding activity. In vivo, lovastatin inhibited tubular cell proliferation after Nx, as measured by proliferative cell nuclear antigen staining. Lovastatin-treated mPTCs displayed nucleus cleavage and DNA ladder formation, which were prevented by GGPP. Like C3 exoenzyme, lovastatin induced actin filament disruption, which preceded evidence of apoptosis. Lovastatin increased tissue-type plasminogen activator (PA) and decreased PA inhibitor activities and antigens; these effects were prevented by mevalonate and GGPP but not FPP, and were reproduced by C3 exoenzyme in a manner insensitive to GGPP.

Conclusions. HMG-CoA reductase inhibitors decreased proliferation, increased apoptosis, and enhanced fibrinolytic activity of renal tubular cells via modulation of different isoprenylated proteins. These effects could participate to reduce the progression of renal diseases.

Tubular cyst formation and interstitial fibrosis are crucial phenomena in the progressive deterioration of renal function in the course of renal diseases. Moreover, interstitial fibrosis has long been recognized as a main prognostic factor regarding renal function outcome in both human glomerular and tubulointerstitial nephropathies [1].

Sustained tubular cell proliferation and dysregulation of programmed cell death are pivotal in the development of renal cysts in both genetic and acquired nephropathies; tubular cell proliferation is also an early component of renal lesions in human glomerular and tubular diseases [2]. Growth factors are key mediators in these settings, as they may induce cell proliferation and promote cell survival. Epidermal growth factor (EGF) and other tyrosine kinase-dependent signaling pathways activate immediate early genes through the p21ras molecular switch. Increased expression of c-myc, c-jun, and c-fos were observed in response to ischemic acute tubular necrosis or subtotal nephrectomy and in the polycystic cpk/cpk mouse [3–5]. On the other hand, interstitial fibrosis results from unbalanced extracellular matrix (ECM) production and proteolysis. Interestingly, ECM products, as well as cytoskeleton components, may participate in the control of cell proliferation and apoptosis.

The newly developed cholesterol synthesis antagonists 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors competitively block production of mevalonate, the rate-limiting step in isoprenoid and cholesterol synthesis [6]. The modulatory effects of these agents on the cell response to various hormones, growth factors, and mediators have been demonstrated in different cell systems. Interestingly, in cultured human mesangial cells, simvastatin inhibited platelet-derived growth factor (PDGF)-induced DNA synthesis and lipopolysaccharide-induced nuclear factor κB (NF-κB) activation and reduced monocyte chemoattractant protein-1 (MCP-1) production. In cultured rat mesangial cells, simvastatin inhibited arginine vasopressin-evoked calcium peak and subsequent activation of mitogen-activated protein kinase [7–10]. In the proximal tubule cell line LLCPK2, simvastatin blocked the stimulatory properties of high-density lipoprotein on the protein content and the monolayer resistance [11]. On the other hand, isoprenylated proteins are required for cell cycle progression and the control of cell growth [12]. Lovastatin and farnesyl-transferase antagonists inhibited p21ras isoprenylation and p21ras-dependent cell growth in vitro and in vivo in p21ras transformed and untransformed cells [13]. Interestingly, growth arrest in lovastatin-treated mesangial cells was associated with an increased cyclin-dependent kinase (CDK) regulator p27kip1 and inhibition of PDGF-stimu-
lated CDK2 and CDK4 activities [14]. Finally, in lovastatin-treated mesangial cells, the inhibition of Rho protein geranylgeranylation prevented intracellular actin polymerization and induced apoptosis [15].

HMG-CoA reductase inhibitors were shown to reduce progressive glomerulosclerosis and tubulointerstitial fibrosis in different models of renal injury. In Dahl salt-sensitive rats on a high-salt diet, 22 weeks treatment with lovastatin reduced serum cholesterol, albuminuria, blood pressure, and focal glomerulosclerosis, but did not alter glomerular hemodynamics [16]. Similarly, in 5/6 nephrectomized rats and in obese Zucker rats, HMG-CoA reductase inhibition lessened albuminuria and mesangial matrix expansion and cellularity [17, 18]. Uninephrectomized nephrotic rats treated for 60 days with lovastatin had similar proteinuria but better inulin clearance and less pronounced glomerulosclerosis as compared with placebo-treated rats [19]. Importantly, in the Han:SPRD rat model of hereditary polycystic kidney disease, lovastatin-treated heterozygous males had milder disease with smaller cysts and lower plasma urea as compared with untreated animals [20].

However, the precise mechanisms of these protective effects remain unclear. In the rat remnant kidney model, the protective effect of lovastatin on renal function two weeks after nephrectomy was associated with elective vasoconstriction of glomerular efferent vessels and enhanced reactivity to acetylcholine of afferent glomerular vessels [21]. Lovastatin reduced MCP-1 expression and macrophage infiltration in nephrotic rats [22]. In the anti-Thy-1.1 rat model of glomerulonephritis, simvastatin suppressed mesangial cell proliferation and macrophage infiltration and reduced matrix expansion, although it did not affect circulating lipids [23]. Clearly, a large amount of data has now established that HMG-CoA reductase inhibitors could regulate cell functions in vitro and in vivo independently from a cholesterol-lowering effect.

We evaluated the antiproliferative and apoptotic effects and the fibrinolytic activity of different HMG-CoA reductase inhibitors in primary cultured proximal tubular cells and in isolated proximal tubules.

**HMG-CoA reductase inhibitors inhibit renal cell growth**

Treatment of RPT cells during 48 hours with lovastatin, simvastatin, and compactin reduced fetal calf serum (FCS)-induced [3H]-thymidine incorporation in a concentration-dependent manner, with IC_{50} values in the micromolar range [24]. Likewise, lovastatin blocked BrdUrd incorporation, as assessed by immunocytochemical studies. The proliferative effect of EGF was similarly abolished by lovastatin. Tyrosine kinase and mitogen-activated protein kinase inhibitors, AP-1 antisense oligonucleotides as well as the farnesyl-transferase inhibitor hydroxy-farnesyl-phosphonic acid (HFPA) decreased FCS-induced rat proximal tubule cells (rPTCs) proliferation.

The effect of lovastatin was prevented by mevalonate, farnesyl-phoshate (FPP,) and geranylgeranyl-phosphophate (GGPP), whereas cholesterol and other products of the mevalonate pathway were inactive. Immunoblot analysis showed that lovastatin decreased membrane-bound p21_{ras} and inhibited FCS-induced c-fos and c-jun expression in rPTCs. Furthermore, gel shift assay demonstrated the functional impairment of AP-1 DNA binding activity in nuclear extracts from lovastatin-treated cells. Much like the lovastatin effects on upstream components of p21_{ras} activated AP-1 pathway, the decrease of AP-1 binding activity was totally prevented by mevalonate.

*In vivo*, tubular cell proliferation and c-fos protein expression were increased two weeks after nephrectomy, as measured by Western blot and immunohistochemical analysis of proliferative cell nuclear antigen staining (6.3 vs. 1.6 labeled cell per field, \( P < 0.02 \)). Lovastatin administration (4 mg/kg/d) reduced c-fos protein expression and tubular cell proliferation (6.3 vs. 3.5 labeled cell per field, \( P < 0.02 \)) two weeks after nephrectomy.

In summary, these results demonstrated that HMG-CoA reductase inhibitors were antiproliferative both *in vitro* and *in vivo* in epithelial tubule cells and that this effect was exerted via inhibition of the p21_{ras}-activated and AP-1-dependent mitogenic cascade (Fig. 1A).

**HMG-CoA reductase inhibitors induce apoptosis in proximal tubular cell**

Twenty-four hour HMG-CoA reductase inhibitor-treated mouse proximal tubule cells (mPTCs) displayed typical features of apoptosis, as evidenced by nucleus cleavage after staining with Hoescht 33258, DNA ladder pattern on gel electrophoresis, and DNA fragmentation as measured by the ratio of fragmented DNA over intact DNA following [3H]-thymidine labeling [25]. HFPA reproduced the apoptotic effect of lovastatin; conversely, lovastatin-induced apoptosis was totally prevented by GGPP but only partially by FPP.

Actin filament disruption by cytochalasin D or C3 exoenzyme, a selective inhibitor of the geranyleranlated activated Rho protein, induced apoptosis in mPTCs. Similarly, lovastatin caused early actin filament network disruption, as evidenced by immunofluorescence study of rhodamine phalloidin staining. This effect was reversed by GGPP and preceded evidence of apoptosis. Interleukin-1β-converting enzyme inhibitor II, a protease inhibitor, had no effect on the apoptosis induced by either cytochalasin D or lovastatin.

This suggested that in mPTCs, the inhibition of isoprenoid production was instrumental in HMG-CoA reductase inhibitor-induced apoptosis, and that inactiva-
tion of Rho protein and disruption of actin filaments might play a role in the induction of apoptosis (Fig. 1B).

**HMG-CoA reductase inhibitors and the tubular fibrinolytic system**

Proximal tubules freshly isolated from rats treated for two days with lovastatin (4 mg/kg/day) showed increased tissue-type plasminogen activator (tPA) and urokinase (uPA) activities and antigens [26]. Incubation with lovastatin (5 μM) of proximal tubules isolated from untreated rats induced a time-dependent (6 to 48 hr) increase in tPA and uPA and a decrease in PA inhibitor-1 (PAI-1) activities. *In vitro*, supernatants, cytosols, and membranes of renal proximal tubular cells in primary culture had no detectable uPA activity, and lovastatin (0.1 to 10.0 μM) induced an increase in tPA and a decrease in PAI-1 activities and antigens.

These effects were selectively prevented by mevalonate and GGPP. Accordingly, HFPA did not reproduce the effect of lovastatin. C3 exoenzyme reproduced the effect of lovastatin on tPA and PAI-1 activity, and furthermore, it blocked the preventive effect of GGPP. The
effect of lovastatin on actin stress fibers network was subsequently analyzed. The results of immunofluorescence studies demonstrated disruption of cellular actin stress fibers in lovastatin-treated cells, which was reversed by GGPP and reproduced by the C3 exoenzyme. These results suggested that the cytoarchitecture of the cells regulated tPA and PAI-1 activities and that lovastatin induction of tPA and inhibition of PAI-1 synthesis might result from modifications of the cytoskeleton proteins, most likely via inhibition of geranylgeranylated Rho proteins.

**DISCUSSION**

The results presented earlier in this article showed that HMG-CoA reductase inhibitors exert a full range of biological effects in tubular cells. Supplementation with mevalonate—the product of the reaction that is blocked by HMG-CoA reductase inhibitors—totally abrogated these effects, thus demonstrating that they did not result from nonspecific cytotoxicity. Importantly, these in vitro effects were independent from a cholesterol-lowering effect, and rather were related to the inhibition of nonsterol mevalonate-derived compound, most probably inhibition of isoprenoid synthesis. As many as 2 to 3% of all cellular proteins are reported to be posttranslationally modified through isoprenylation [27]. However, the tubular effects of HMG-CoA reductase inhibitors appeared to be closely related to the inhibition of specific isoprenylated proteins. Indeed, the time course, the concentration threshold, and the sensitivity to isoprenoid supplementation of the apoptotic, anti proliferative, and fibrinolytic effects were different.

Dysregulation of renal cell growth is clearly a major point in the development of renal cysts. The control of tubular cell proliferation and programmed cell death could represent a promising strategy to reduce the progression of renal diseases. Experimental data suggest that HMG-CoA reductase inhibitors, at least in vitro, might display these effects. Moreover, these antagonists modulate the tubular plasminogen-plasmin pathway, one of the most efficient systems involved in ECM remodeling. The resulting increase of proteolytic activity of tubular cells might contribute to prevent ECM deposition and renal interstitial fibrosis. Further research is clearly needed to elucidate whether these drugs may offer, besides their plasma cholesterol-lowering effect, therapeutic advantages in the treatment of renal diseases.

**CONCLUSION**

In renal tubular cells, the inhibition of isoprenoid synthesis by lovastatin resulted in decreased proliferation, increased apoptosis, and enhanced fibrinolytic activity. Inhibition of the p21<sup>ras</sup>/API cascade is critical in the anti proliferative effect of lovastatin; on the other hand, disruption of actin filaments and inhibition of geranylgeranylation of Rho protein appear pivotal in the apoptotic and fibrinolytic activity of lovastatin.

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