Effect of lovastatin on small GTP binding proteins and on TGF-β1 and fibronectin expression

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Effect of lovastatin on small GTP binding proteins and on TGF-β1 and fibronectin expression. We have shown that lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, delays development and progression of diabetic nephropathy in streptozotocine-induced diabetic rats through suppression of glomerular transforming growth factor (TGF)-β1 mRNA expression. We have also shown that lovastatin suppresses both control and high glucose (HG)-induced TGF-β1 and fibronectin mRNA expression and protein synthesis by rat mesangial cell (RMC) and that this down-regulation by lovastatin is reversed by mevalonate. It was postulated that this down-regulation may be linked to signaling of small guanine triphosphate (GTP)-binding proteins and mediated by the limitation of isoprenoids such as farnesylpyrophosphate (FPP) and geranylgeranylpyprophosphate (GGPP) in RMC. To determine the isoprenoid and small GTP-binding proteins involved in TGF-β1 and fibronectin expression, FPP or GGPP was added alone or in combination to RMC treated with lovastatin cultured under normal or high glucose condition. Suppression of TGF-β1 and fibronectin expression by lovastatin was reversed effectively when GGPP was added alone. Partial reversal of lovastatin effect on fibronectin and TGF-β1 expression was found when FPP was added alone. Adding both GGPP and FPP resulted in complete reversal of lovastatin effect on fibronectin but not TGF-β1 suggesting that fibronectin and TGF-β1 are regulated differently. Furthermore, luciferase activity of RMC cotransfected with fibronectin promoter reporter system and plasmid-expressing C3 exoenzyme (a specific inhibitor of Rho family GTP binding proteins, pEFC3) was completely suppressed when compared with RMC cotransfected with empty vector, pEF. Because geranylgeranylation is usually involved in post-translational modification and membrane targeting of Rho family small GTP binding proteins, these data indicate that Rho family small GTP binding proteins rather than Ras family small GTP binding proteins may play a key role in the TGF-β1 and fibronectin expression in RMC.

Although hyperlipidemia is considered a potential risk factor for renal fibrosis, the clinical effect of lipid lowering on the prevention of diabetic nephropathy remains unclear. Clinical studies investigating the renal protective effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor in diabetic nephropathy are mostly of short duration and in small populations of patients [1]. The role of HMG CoA reductase inhibitor in experimental diabetes remains incompletely understood. Recently, we demonstrated in streptozotocin (STZ)-induced diabetic rats that lovastatin significantly suppressed glomerular transforming growth factor (TGF)-β1 expression in 4 days and urinary albumin excretion in 4 weeks; it also reduced glomerular volume in 2 weeks after induction of diabetes mellitus [2]. In this study, lovastatin suppressed serum cholesterol level in diabetic rats only after 6 months, suggesting that lovastatin may have renal protective effect independent of lipid lowering action. Park et al [3] found that lovastatin reduced mesangial proliferation and glomerular macrophage infiltration before any increase in extracellular matrix in nephrotic rats. These observations suggest that lovastatin’s effect on albuminuria and glomerular hypertrophy may be related to modulation of early cellular events in a diabetic kidney.

CELLULAR EFFECTS OF HMG CoA REDUCTASE INHIBITOR

HMG CoA reductase is a rate-limiting enzyme in a cholesterol biosynthetic mevalonate pathway (Fig. 1). HMG CoA reductase inhibitor has been shown to produce various effects at the cellular level: suppression of the expression of chemokines [3] and cytokines [2, 4], induction of apoptosis of mesangial cells [5], inhibition of cell proliferation [6], interference with intracellular signaling [7], reduction of extracellular matrix (ECM) production [8] and modulation of ECM protein degradation system [9].

From the mevalonate pathway, several isoprenoids are produced as an intermediate stage, including farnesylpyrophosphate (FPP) and geranylgeranylpyprophosphate (GGPP) as shown in Fig. 1. These isoprenoids are enrolled in post-translational modification [10] and membrane localization of small guanine triphosphate (GTP) binding proteins such as Ras and Rho family proteins.

Key words: HMG CoA reductase inhibitor, mesangial cell, isoprenoids.

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LOVASTATIN DOWN-REGULATES EXPRESSION OF TGF-β1 IN RAT GLOMERULI AND MESANGIAL CELLS

We demonstrated that the glomerular TGF-β1 expression in STZ-induced diabetic rats was suppressed by lovastatin at day 2 after treatment [4] and this effect by lovastatin was sustained for up to 3 months [2]. Increased TGF-β1 expression by HG (30 mmol/L) in rat mesangial cells (RMC) was also suppressed by lovastatin [2]. Therefore, it appears that the suppressive effect of lovastatin on the HG-induced glomerular TGF-β1 expression is through a direct effect at cellular level rather than any cholesterol lowering effect. Although the intracellular signaling pathway responsible for TGF-β1 expression induced by HG is not yet clearly defined, it appears that Rho family small GTP binding protein(s) is involved in this pathway. As shown in Fig. 3, the addition of mevalonate or GGPP but not FPP to the lovastatin-treated RMC cultured under control or HG reversed the suppressive effect of lovastatin on TGF-β1 expression.

TGF-β1 expression is up-regulated by HG in both in vivo and in vitro experiments but it is still unclear as to what transcription factor(s) is responsible for the up-regulation. Three different AP-1 binding sites are located on the TGF-β1 promoter region; they are the major elements responsible for the tetradecanoylphorbol acetate (TPA) activation and TGF-β1 autoinduction [17]. Other transcription factor binding sites including Sp1, CRE, NF-κB, and Egr-1 are also found in the TGF-β1 promoter region. Other evidence suggests that Sp1 is responsible for the expression of TGF-β1 and TGF-β3 [18]. A putative glucose responsible element (GLURE), CAC GTG was also found in the promoter region (−1040 to −1009) of human TGF-β1 gene as was shown in mouse TGF-β1 gene [19].

SAPK/JNK or p38 MAPK signaling pathways are presumably activated by Rho family small GTP-binding proteins rather than Raf-MEK-ERK pathway activated by Ras protein may regulate TGF-β1 expression because the SAPK/JNK and p38 MAPK cascades also activate AP-1. In addition, ERK MAPK can be activated by Rho A through unspecified signaling pathway. Therefore, it is suggested that lovastatin suppresses expression of TGF-β1 induced by control or HG through decreased activation of the transcription factors as a result of the reduction of functional Rho family small GTP-binding proteins on plasma membrane.

Another important intracellular signaling molecule activated by HG is protein kinase C (PKC) [12]. It has been established that certain isoforms of PKC stimulate Ras and/or Raf resulting in triggering of Raf-MEK1-ERK cascade [13, 14]. Some evidence suggests that Rho family small GTP binding proteins are involved in PKC activation [15] and translocation to plasma membrane [16]. However, the precise signaling network between Rho family GTP binding proteins and an individual isoform of PKC still remains to be elucidated.
**Fig. 2. Major intracellular signaling pathways mediated by small GTP-binding proteins.**

Three different MAPK signaling pathways named ERK pathway (Ras → Raf → MEK1/2 → ERK1/2), SAPK/JNK pathway (MEKK1 → SEK/MKK4 → SAPK/JNK), and p38 pathway (TAK1 → MKK3/6 → p38) are well established and involved in the intracellular signaling of the small GTP-binding proteins. From these MAPK pathways, various transcription factors such as Elk1, ATF-2 (CREB), AP-1, NF-κB, and Sp1 are activated and start the transcription of target genes.

**Fig. 4. Effect of FPP and GGPP on the expression of fibronectin mRNA.** After 4 h of treatment with liposome containing FPP and/or GGPP, cells were washed twice with media without serum, and incubated another 24 h under control or HG conditions with 10 μmol/L of lovastatin. Total RNA was isolated and Northern blot analysis for fibronectin mRNA was performed.

**Fig. 3. Effect of FPP and GGPP on the expression of TGF-β1 mRNA.** FPP (15 μmol/L) and GGPP (15 μmol/L) were mixed alone or together with cationic liposome and added in rat mesangial cell culture treated with lovastatin under control or HG conditions. After 4 h of treatment with liposome containing FPP and/or GGPP, cells were washed twice with media without serum and incubated another 24 h under control or HG with 10 μmol/L of lovastatin. Total RNA was isolated and Northern blot analysis for TGF-β1 and GAPDH was carried out.

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**EFFECTS OF LOVASTATIN ON ECM METABOLISM**

Recent studies suggested that lovastatin and other inhibitors of HMG CoA reductase reduce ECM accumulation through two possible ways: the suppression of ECM synthesis and the enhancement of ECM degradation. Nishimura et al [8] reported that pravastatin inhibited fetal calf serum–stimulated type IV collagen secretion and mRNA expression by mesangial cells and that GGPP and mevalonate reversed the effects of pravastatin nearly completely. The pravastatin effects on type IV collagen secretion and expression may depend on Rho family small GTP-binding protein(s) rather than Ras. Essig et al [9] reported that lovastatin modulated the plasminogen activator/plasmin system of rat proximal tubular cells both in vivo and in vitro. In the in vivo study, lovastatin induced an increase in activity and amount of tPA and uPA, and a decrease in plasminogen activator inhibitor-1 (PAI-1). A similar result was obtained from the cultured primary renal proximal tubular cells. In the cell culture study, however, no uPA activity was detected. Changes in plasminogen activator/plasmin system by lovastatin were reversed by mevalonate and GGPP and by treatment with C3 exoenzyme, an inactivator of Rho family GTP-binding proteins.

We have also demonstrated that lovastatin suppressed fibronectin mRNA and protein synthesis induced by HG in RMC. This lovastatin effect was reversed by the addition of GGPP (Fig. 4) as well as mevalonate [2]. Addition of FPP allowed only partial reversion of suppressive effect of lovastatin on fibronectin expression. By adding
Lovastatin exerts its renoprotective effects in experimentally induced diabetes through inactivation of Rho family small GTP-binding proteins.

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both GGPP and FPP, however, a synergistic effect on fibronectin expression was found. This suggests that the intracellular signaling pathway for fibronectin expression enhanced by HG is somewhat different from that for TGF-β1 expression. To confirm the involvement of Rho family small GTP-binding proteins rather than Ras, RMC were cotransfected with a fibronectin promoter reporter system containing approximately 1.9 kb fibronectin promoter followed by the luciferase gene [20] and the plasmid-expressing C3 exoenzyme pEFC3 [21]. Luciferase activity of RMC cotransfected with the reporter system and empty vector pEF increased by treatment with HG (Fig. 5). In contrast, the luciferase activity of RMC cotransfected with the reporter system and pEFC3 was suppressed completely (Fig. 5). It is therefore clear that overexpression of fibronectin induced by HG is regulated by the function of Rho family GTP-binding proteins.

CRE binding protein (CREB) has been recognized as the major transcription factor responsible for fibronectin expression enhanced by HG [22]. The SAPK/JNK and p38 MAPK cascades induced by Rho family GTP-binding proteins activate CREB and ATF-2 binding to CRE (Fig. 2).

Based on these observations, we propose that the Rho family small GTP binding proteins are deeply involved in the synthesis of ECM proteins such as type IV collagen and fibronectin, as well as in the degradation of ECM proteins through activation of plasmin system and that

Fig. 5. Effect of C3 exoenzyme on fibronectin expression. Rat mesangial cells were cotransfected with fibronectin reporter system (pRFNluc) containing about 1.9 kb of rat fibronectin promoter region followed by luciferase gene and pEFC3 harboring C3 exoenzyme gene or empty vector, pEF and β-galactosidase expressing plasmid, pCMVβ. All plasmids, 3 μg (pRFNluc); 2 μg (pEFC3); 1 μg (pCMVβ) were mixed with liposome and introduced into rat mesangial cells under serum-free conditions. At 4 h later, the cells were washed twice with serum-free media and incubated in control or HG media for 24 h. These cells were disrupted, and luciferase and β-galactosidase activities were measured by the manufacturer’s description. Luciferase activity was normalized by β-galactosidase activity and represented by relative activity.


