Pitavastatin Enhanced Lipoprotein Lipase Expression in 3T3-L1 Preadipocytes

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It is known that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) enhance the expression of the low-density lipoprotein (LDL) receptor and lower the level of LDL cholesterol in the blood. But, a triglyceride (TG)-lowering effect is also observed during their administration. To clarify the possibility that statins enhance LPL activity and its mechanism, the effects of statins on the expression of LPL in adipocytes were studied. When statins (pravastatin, simvastatin, atorvastatin and pitavastatin) were added to the culture medium of mouse 3T3-L1 preadipocytes at final concentrations of 1 µM for 3 days, LPL activity increased. Pitavastatin increased the activity the most. Western and Northern blotting showed that LPL protein and mRNA were strongly expressed on the addition of pitavastatin. With the addition of mevalonate (10 µM, 3 days), LPL activity weakened significantly. Statins, especially pitavastatin, increased the expression of LPL in 3T3-L1 preadipocytes. The TG-lowering effect of pitavastatin might be mediated by enhancement of LPL production in adipocytes. J Atheroscler Thromb, 2005; 12: 163–168.

Key words: Statin, Adipocyte, Triglyceride, Pleiotropic effect

Introduction

In the cholesterol bio-synthetic pathway, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a key enzyme which synthesizes mevalonate from HMG-CoA. Inhibitors of this enzyme (statins) reduce the concentration of low-density lipoprotein (LDL) cholesterol in the blood (1–4), by enhancing the activity of LDL receptors. Furthermore, a triglyceride (TG)-lowering effect and a high-density lipoprotein (HDL) cholesterol-raising effect were also observed (5–10). Saito Y et al. (5) reported in a dose-finding study using a double-blind comparison in hyperlipidemia patients that pitavastatin reduced TG levels by 22–30% in patients with a baseline TG level of ≥ 150 mg/dl. Various mega trials using statins, showed that administration of statins decreases the incidence of cardiovascular events (6–8, 10–13). Precise analysis showed that the inhibition of early cardiac events is not only due to the LDL-lowering effect (14), but also due to anti-atherogenic effects, such as the improvement of endothelial cells (15), anti-inflammatory effects (16), suppression of the migration and proliferation of smooth muscle cells (SMCs) (17), induction of the apoptosis of SMCs (18), and suppression of the cellular uptake of oxidized-LDL by monocytes. (19)

The mechanism by which statins reduce serum triglyceride (TG) level is not controversial. It is considered that the TG-lowering effect was due to a reduction in the synthesis of very low-density lipoprotein (VLDL) in the liver (20–22). But, little is known about the molecular mechanism underlying the catabolism.

Lipoprotein lipase (LPL), a key enzyme present on the surface of endothelial cells (23), hydrolyzes TG in lipo-
proteins. This enzyme is believed to be produced by adipose tissue, skeletal muscle and heart. Therefore, we hypothesize that statins could enhance LPL expression in adipocytes.

Endo K, et al reported that atorvastatin and pravastatin increased LPL mass in cases of type2 diabetes with hypercholesterolemia (24). Verd JC, et al reported that simvastatin treatment caused an increase (72%) in LPL activity in rabbits (25). Whether statins enhance the LPL expression in vitro is not yet clear. In the present study, we investigated the effect of various statins on LPL activity, protein, and m-RNA levels in preadipocytes (3T3-L1) and its mechanism in relation to mevalonate and peroxisome proliferator-activated receptor (PPAR) γ.

Materials and Methods

3T3-L1 cell culturing

Mouse 3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s minimal essential medium, supplemented with a 10% mixture of delipidated fetal calf serum/delipidated calf serum (v/v), and 2 mmol/l L-glutamine and 40 μg/ml gentamycin, at 37°C under 5% CO₂. The cells (2.5 × 10⁴) were dispersed in 12-well plates and incubated for 2 days. Then, the cells were incubated in the above-mentioned medium supplemented with dexamethasone (0.25 μM) and insulin (10 μg/ml) for another 2 days.

When the culture was 50% confluent, various statins were added. The statins used were as follows; pravastatin (endowed by Sankyo Co., LTD), simvastatin (endowed by Merk Co., LTD), atorvastatin (endowed by Pfizer Inc) and pitavastatin (Kowa Co., LTD). These agents were dissolved in ethanol. Statins were added to the culture medium at final concentrations of 1 μM during 3 days.

Measurements of LPL activity

For the assay of LPL activity, 3T3-L1 cells were harvested, dissolved in 0.1M Tris buffer (pH 7.4), and sonicated on ice for 30 sec using an Ultrasonic Generator model US-50 (NISSEI, Japan), and the homogenates were centrifuged at 2,000 × g for 10 min (himac CF 15D2, HITACH, Japan). The supernatants were used for the enzyme assay.

LPL activity was measured using triolein (Sigma, St. Louis) as a substrate. The substrate solution was prepared as follows; 100 mg of Triolein, and 7.5 ml of 0.2% Triton X-100, in a final volume of 7.5 ml of 1 M Tris-HCl (pH 8.0), was sonicated on ice for 10 min. For the measurement of LPL activity, the reaction mixture contained 50 μl of substrate solution,25 μl of 20% fatty acid-free bovine serum albumin (Sigma, St. Louis) (pH 8.0), 5 μl of HDL (3 mg protein/ml) as apo CII, and an appropriate amount of sample (170 μl). After incubation for 60 min at 37°C, the enzyme reaction was terminated by addition of 10 μl of di-isopropylfluorophosphate. At 4°C, the amount of free fatty acids released in the mixture was measured using an enzymatic method (Nescauto NEFA V2; Azwell, Inc. Osaka, Japan).

Western blot analysis of LPL protein from 3T3-L1 cells

LPL protein was detected by Western blot analysis. Cells were suspended in a lysis buffer containing 10mM Tris-HDL (pH 7.5), 150 mM NaCl, 0.5% TritonX-100, 0.5 mM PMSF, and 1 mM EDTA for 4 hours at 4°C. After centrifugation at 12,000 × g, the concentration of proteins in the supernatant was measured using the Bio-Rad protein assay. Samples were diluted 1: 1 with electrophoresis sample buffer containing 100 mM Tris (pH 6.8), 10% SDS, a 10% glycerol, 0.1% bromophenol blue and 5% b-mercaptoethanol, then boiled for 5 min and electrophoresed on 10% SDS-polyacrylamid gel. The proteins were transferred into Hybond-ECL nitrocellulose (Amersham). After blotting, the membrane was washed with Tris Buffer Saline (TBS, 100mM Tris, pH 7.5, 0.9% NaCl) and blocked with 5% BSA in TBS, then briefly washed in TTBS (TBS plus 0.1%Tween-20) and incubated with a chicken antiserum against mouse LPL (kindly provided by Masuno H, Department of Medical Laboratory Technology, Ehime Collage of Health Science, Japan and Olivecrona T, Division of Nephrology, Department of Internal Medicine, Umea University, Sweden) at a dilution of 1: 500 for 2 hours at room temperature. After a wash with TTBS, the blot was incubated with peroxidase-labeled affinity purified antibody to chicken IgG (H + L) (KPL, UK) at a dilution of 1: 1500 for 1 hour at room temperature, washed with TTBS, and incubated with diluted (1: 3000) biotinylated HRP atreptavidin complex for 1 hour at room temperature. Visualization of the antigen-antibody complex was performed by photo detection. The products were quantified with digital scanning and Scion Image software.

Northern blot analysis of LPL m-RNA from 3T3-L1 cells

LPL m-RNA was detected by Northern blot analysis. Total RNA was isolated from 3T3-L1 preadipocytes grown in 6-well plates using a RNeasy Mini Kit (QIAGEN). The amount of RNA was calculated with the absorbance at 260nm. RNA samples (5 μg) were electrophoresed on a 1.5% agarose gel containing 6.5% formaldehyde, 20 mM MOPS, 1mM EDTA, and 8 mM sodium acetate, and stained with 1mg/ml ethidium bromide. After the electrophoresis, the RNA was visualized with a UV cross-linker (Spectro Linker XL-1000; SPECTRONICS CORPORATION), and transferred to a Hybridization Transfer Membrane (PerkinElmer Life Sciences, Inc. USA). Human LPL cDNA clones were used as probes. cDNA clones for β-actin were used as control probes. The cDNA probes
for the Northern blotting were labeled using Random Primer DNA Labeling Kit Ver 2.0 (Takara, Japan). The RNA blots were prehybridized and hybridized with a $^{32}$P-labeled dCTP cDNA probe. Membranes were hybridized and washed as previously described (26). The membranes were analyzed by visual imaging using a Storm Phosphorimager System™ (Molecular Dynamics, Inc. Sunnyvale, CA). The products were quantified using digital scanning and Scion Image software.

**Addition of mevalonate and pioglitazone**

The effect of mevalonate on LPL activity in the presence of pitavastatin (1 $\mu$M, 3 days) was studied. Mevalonate (10 $\mu$M) dissolved in dimethyl sulfoxide (DMSO) was added to the culture medium containing pitavastatin.

The effect of PPAR$\gamma$ was investigated using pioglitazone as follows; at 4 days after passage, pioglitazone, which is a stimulator for PPAR$\gamma$, was added to the culture medium at a final concentration of 10 $\mu$M dissolved in DMSO for 4 days. Pitavastatin was added to the medium on the fourth day after the preincubation with pioglitazone.

**Statistical analysis**

Stat View-J 5.0 software was used for all statistical analyses. Paired t-test was carried out to determine if the differences between groups were statistically significant. A volume of $p < 0.05$ was considered to be significant.

**Results**

**LPL expression in 3T3-L1 preadipocytes by statins**

LPL activity was measured in 3T3-L1 preadipocytes in the presence of various statins. Addition of pravastatin, simvastatin, atorvastatin and pitavastatin, at final concentrations of 1 $\mu$M to the 3T3-L1 cells and incubation for 3 days increased the LPL activity in the cells to various degrees. Simvastatin and pitavastatin both increased LPL activity significantly. Pitavastatin, however, increased LPL activity the most (Fig. 1).

The dose-dependency of the effect of pitavastatin on the enhancement of LPL activity was examined. At up to 1 $\mu$M of pitavastatin, LPL activity was increased. But at over 10 $\mu$M, not only activity but also the amount of cell protein dropped markedly, possibly due to damage to the cell (Fig. 2).

Next, LPL protein expression in the presence of pitavastatin (final concentration of 1 $\mu$M during 3 days) was studied by Western blotting. LPL protein was strongly expressed (+ 42%) on the addition of pitavastatin, compared to that without pitavastatin, whereas to expression of $\beta$-actin was un-changed (Fig. 3).

Then, Northern blotting was also performed. The analysis showed that expression of LPL m-RNA was enhanced (+ 26%) by the addition of pitavastatin, whereas that of $\beta$-actin was un-changed (Fig. 4).

**Mechanism by which pitavastatin induced LPL activity in 3T3-L1 preadipocytes**

To understand the mechanism by which pitavastatin induced LPL activity, the effects of mevalonate and/or pioglitazone were investigated.

We measured LPL activity, when pitavastatin (1 $\mu$M) and mevalonate (10 $\mu$M) were added at the same time. With the addition of mevalonate plus pitavastatin, the LPL activity enhanced by pitavastatin, was reduced signifi-
In the absence of pitavastatin, there was no significant difference between the absence or presence of mevalonate (Fig. 5).

The effect of pioglitazone was studied. After preincubation with pioglitazone (10 µM, 72 hours), which is a ligand for PPARγ, pitavastatin was added and incubated for 72 hours, and the LPL activity was measured. In the presence of pioglitazone only, LPL activity increased to 13.2 µEq/hr/µg released FFA. Furthermore, with the addition of pitavastatin, LPL activity increased further (p < 0.05) (Fig. 6).

**Discussion**

In 3T3-L1 preadipocytes, various statins enhanced LPL activity. Notably pitavastatin increased the level of LPL activity by 30%. Pitavastatin also increased LPL protein and m-RNA expression in 3T3-L1 preadipocytes significantly. These results suggested that pitavastatin enhanced LPL activity by increasing LPL expression. However, these findings might be different from the TG-lowering effect of the various statins used in clinical studies, because simvastatin and atorvastatin are considered...
pdrugs and pravastatin is regarded as a hydrophilic inhibitor (27). Although the main mechanism of the TG-lowering effect of statins is considered to be a reduction in the synthesis of VLDL in the liver, pitavastatin also might reduce TG levels by increasing LPL expression.

Next, the mechanism by which pitavastatin increased LPL expression was studied. LPL expression induced by pitavastatin was suppressed by mevalonate. Statins essentially reduce levels of metabolites such as farnesylpyrophosphate and geranylgeranylpyrophosphate. These metabolites are known to have effects on cell proliferation, differentiation and apoptosis due to the isoprenylation of proteins (28–29). Then, to clarify the pathway by which mevalonate products affect the production of LPL, mevalonate was added to the preadipocytes in the presence of pitavastatin. As shown in Fig. 5, mevalonate inhibited LPL activity, which was enhanced by pitavastatin. We speculate that the metabolites from mevalonate act on the promoter region of LPL and suppress it. In other words, pitavastatin may release the suppression of mevalonate, and increase LPL expression in adipocytes. Therefore, we consider that pitavastatin lowers serum TG levels by increasing in LPL activity and also raising levels of HDL cholesterol. These improvements might be part of the pleiotropic effect on the progression of atherosclerosis.

Furthermore, the relation of PPAR-γ to enhancement of LPL expression was studied. Pioglitazone is known as a stimulator for PPAR γ. Shirai K reported (30) that troglitazone which is a ligand for PPAR γ reduced the serum TG level significantly and gradually increased preheparin LPL mass (31) in non-insulin-dependent diabetes mellitus patients. Therefore we hypothesized that enhancement of LPL expression might be due to the activation of PPAR γ by pitavastatin. When pioglitazone was added, the LPL activity increased. Further addition of pitavastatin also increased LPL activity. In this study, pitavastatin in addition to pioglitazone had an effect. The result indicated a mechanism by which induction of LPL expression by pitavastatin might not be due to PPAR γ activation. Inoue et al. (32) reported that cerivastatin, fluvastatin, pitavastatin were not ligands for PPAR α, δ, and γ in an in vitro assay. PPAR α, which regulates LPL expression, is considered to exist in the liver, kidney, vascular endothelial cells and vascular and smooth muscle cells. LPL expression in 3T3-L1 preadipocytes is not considered to relate with PPAR α. Examination of this point is required.

In Summary

Statins especially pitavastatin enhanced LPL activity in 3T3-L1 preadipocytes. This enhancement was due to an increase of LPL protein, and the process might be due to another pathway not involving PPAR-γ.

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