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Simvastatin enhances induction of inducible nitric oxide synthase in 3T3-L1 adipocytes

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Abstract

The present study was designed to determine whether hydroxymethylglutaryl-CoA reductase inhibitors (statins) modulate the NO production via iNOS in adipocytes stimulated by lipopolysaccharide (L) and tumour necrosis factor-α (T). Well-differentiated 3T3-L1 adipocytes significantly produced NO by LT-treatment. Pre-incubation with simvastatin, a lipophilic statin, pravastatin, a hydrophilic one, or Y27632, an inhibitor of Rho kinase, further enhanced the production of NO. The effect of simvastatin was offset by mevalonate and geranylgeranyl pyrophosphate (GGPP) but not by squalene. The mRNA level for iNOS parallelled the NO production. The NF-κB was activated by the LT-treatment and was further enhanced by simvastatin, pravastatin or Y27632 addition. Mevalonate and GGPP completely offset the effect of simvastatin. Statins and Y27632 also further increased the interleukin-6 secretion in the LT-treated 3T3-L1 adipocytes. These results suggest that statins, especially lipophilic type, enhance induction of iNOS by inhibiting the small GTP-binding protein signal in adipocytes.

Keywords: *HMG-CoA reductase inhibitor, pravastatin, nuclear factor-κB, small G protein, cholesterol*

Introduction

Nitric oxide (NO), a bioactive free radical messenger molecule, is involved in vascular homeostasis, immune system and neurotransmission. Nitric oxide synthase (NOS) synthesizes NO using L-arginine as the substrate [1,2]. Basically NOSs are divided into two categories. One is a constitutive NOS, endothelial NOS (eNOS) and neuronal NOS (nNOS), whose activity is dependent on intracellular calcium ion. The other is an inducible NOS (iNOS), which is synthesized *de novo* in response to a variety of stimuli such as cytokines, stress and radiation in various cell types.

At low concentrations, NO has been shown to play a role in various physiological processes, whereas it can be toxic at high concentrations. Once iNOS is induced, it remains active for a certain period of time and continuously produces NO excessively. Peroxy nitrite ($ONOO^-$), generated by reaction of NO with superoxide (O_2^-), is a reactive nitrogen species (RNS) and plays a major role in the cytotoxic process via nitration [3]. Thus, iNOS-mediated NO production can be both beneficial and detrimental to tissues.

Nuclear factor-κB (NF-κB) is characterized as an activator of the expression of genes including cytokines and iNOS, etc., in various cell types [4,5]. In the resting state, I-κB forms a complex with NF-κB in the cytosol, thereby making the nuclear localization signal inaccessible to nuclear membrane. On exposure to lipopolysaccharide (LPS) and/or cytokines, I-κB is

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phosphorylated through activation of I-κB kinase and then NF-κB is released from the degraded I-κB. Free NF-κB, an active form, migrates to the nucleus and trigger transcription of genes [6]. In adipocytes, activation of NF-κB is considered to play the central role for regulating adipocytokine productions such as adiponectin, plasminogen activator inhibitor-1 (PAI-1) and interleukin-6 (IL-6) [7].

Hydroxymethylglutaryl (HMG)-CoA reductase inhibitors, which are collectively called statin, inhibit mevalonate synthesis mainly in liver, thereby blocking the synthesis of cholesterol via squalene. Therefore, statin depletes isoprenoids such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). These isoprenoids enhance the binding of small GTP-binding proteins (Rho, Ras, Rac, etc.) to plasma membrane by prenylation, thereby playing an important role in their kinase activities [8]. Recently, inhibition of isoprenoid synthesis is considered to relate to pleiotropic effects of statin such as improvement of vascular endothelial function [9], inhibition of macrophage proliferation [10] and antioxidant activity [11]. There are several reports regarding the effect of statins on regulation of iNOS in many types of cells [12–18].

LPS and cytokines are known to induce iNOS in adipocytes, which thereby produce significant NO [5,19,20]. The NO production via iNOS in adipocytes is postulated to relate to insulin resistance [20]. To our knowledge, there are no reports concerning the effect of statins on induction of iNOS in adipocytes.

Materials and methods

Reagents and cell culture

Simvastatin and pravastatin were purchased from LKT Laboratories, Inc. (St. Paul, MN). Mevalonate, geranylgeranylpyrophoshate (GGPP), squalene, Y276 32, LPS from Escherichia coli and Nω-nitro-L-argi nine methyl ester (L-NAME) were from Sigma Chemical Co. (St. Louis, MO).

3T3-L1 pre-adipocytes (American Type Culture Collection, Rockville, MD, No. CCL 92.1) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum (Gibco BRL, Rockville, MD) and were differentiated as described previously [5,7,19]. Well-differentiated ($>90\%$) 3T3-L1 mature adipocytes treated for 3 h with statins (simvastatin or pravastatin) were then exposed to the combination of 5 μg/ml of LPS and 100 ng/ml of recombinant TNF-α (kindly provided from Dainihon Pharmaceutical Co., Osaka, Japan) for the next 24 h in the presence or absence of mevalonate, GGPP, squalene, Y27632, L-NAME and aminoguanidine hydrochloride (AG, Wako Pure Chemicals, Osaka, Japan). As controls, 3T3-L1 adipocytes were cultured without these reagents for the same length of time.

NO production

NO synthesis was estimated by the assay for nitrite, a stable reaction product of NO and molecular oxygen [5,7,19]. Briefly, 600 μl of culture supernatant was incubated with 100 μl of Griess-Romijn reagent (Wako Pure Chemicals). After 15 min of incubation at 25°C, the optical density of the assay samples was measured spectrophotometrically at 520 nm. Nitrite concentrations were calculated from a standard curve derived from nitrite ion standard solution (Kanto Chemical Co., Tokyo, Japan). Fresh culture medium (DMEM) was used as the blank. The protein concentration of the cells was determined by the method of Bradford [21].

Reverse-transcriptase coupled polymerase chain reaction (RT-PCR) analysis

Six hours after the each treatment, cells were rinsed twice with PBS and total RNA was extracted using a SV Total RNA Isolation system (Promega Co, Madison, WI). RNA concentrations were determined by absorbance at 260 nm.

For complementary DNA (cDNA) synthesis, 2 μg of total RNA with oligo (dT) 15 primer, RNasin (Promega), dNTPmix (Takara Bio Inc. Shiga, Japan), SuperScript III RT (Invitrogen Co, Rockville, MD, USA) was incubated at 42°C for 50 min and the reaction was terminated at 95°C for 15 min. Real-time PCR was performed by the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Each PCR was carried in a total 50 μl of the reaction mixture containing 25 μl of 2 × SYBR Green Master Mix (Applied Biosystems), 5 μl of sense and anti-sense primer (0.1 μM), 13 μl diethylpyrocarbonate-treated water and 2 μl of cDNA. The PCR conditions were 1 cycle of 50°C for 2 min, then 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. The primers for iNOS and β-actin, as control, have been described previously [5]. The PCR products were visualized under a UV transilluminator by electrophoresis in a 2% agarose gel with 0.5 × TBE Buffer (45 mM Tris-borate, 1 mM EDTA), followed by staining with ethidium bromide (0.1 μg/ml).

Preparation of nuclear extracts

Sixty minutes after the treatment of combination with LPS and TNF-α, the cells were rinsed twice with ice-cold PBS with phosphatase inhibitors (pH 7.4) and were scraped and corrected by centrifugation at 3000 × g for 5 min at 4°C. The pellet was then resuspended and incubated for 30 min in ice-cold hypotonic buffer (20 mM HEPES, 5 mM NaF, 10 μM

Na_2MoO_4, 0.1 mM EDTA, 0.5% Nonidet P-40, pH 7.5) and centrifuged at 14 000 × g for 1 min at 4°C. The nuclear pellet was resuspended in ice-cold lysis buffer (20 mM HEPES pH 7.5, 0.35 M NaCl, 20% glycerol, 1% Igepal-CA630, 1 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM DTT) containing a protease inhibitor cocktail. After 10 min of incubation at 4°C, the lysate was centrifuged for 10 min at 15000 × g and the supernatant was stored at −80°C, as described previously [5,7].

Quantification of NF-κB activity

The nuclear extracts were applied to the sensitive ELISA-based assay kit (Active Motif North America, CA) and quantified for NF-κB activity according to the manufacturer's protocol as follows. The 5 μg protein of nuclear extracts were incubated for 1 h at 25°C with oligonucleotides containing an NF-κB binding consensus which was coated to the microwells, in the presence of competitive binding with the wild-type or mutated consensus oligonucleotides (the latter has no effect on NF-κB binding). Then 1:1000 rabbit anti-NF-κB p65 antibodies were added to each well and incubated for 1 h at 25°C, followed by the incubation with 1:1000 peroxidase-conjugated goat anti-rabbit IgG for 1 h at 25°C. The peroxidase activity was visualized by tetramethylbenzidine reaction and the optical density was read at 450 nm [5,7].

IL-6 production

Twenty-four hours after the LPS and TNFα treatment, culture medium was collected from each sample. IL-6 concentrations in each medium were measured by using a mouse IL-6 ELISA kit (PIERCE Endogen, Rockford, IL). The concentration was calculated from the standard curve and expressed as per mg of the total cell protein content.

Statistics

Data are presented as the means and standard deviations (SD). The difference between the means was estimated by one way ANOVA and Tukey's test. The values were considered to be statistically significant at $p < 0.05$.

Results

Effect of simvastatin and pravastatin on nitrite production in LPS/ TNF-α-stimulated 3T3-L1 adipocytes

In previous studies [5,19], the stimulus by LPS or TNF-α alone did not enhance the production of NO in well-differentiated 3T3-L1 adipocytes. In the present study, combined stimulus of 5 μg/ml of LPS and 100 ng/ml of TNF-α (LT) to adipocytes enhanced the NO production significantly (Figure 1). The production of NO by LT-stimulus in adipocytes

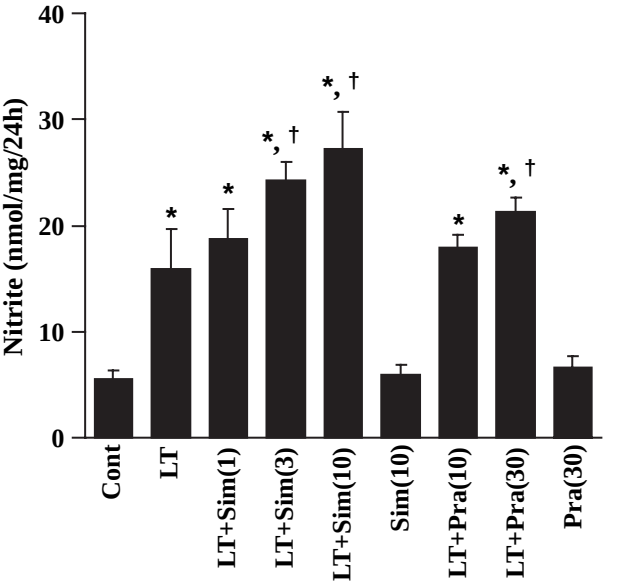

Figure 1. Effect of simvastatin and pravastatin on nitrite production in LPS/ TNF-α-stimulated 3T3-L1 adipocytes. Adipocytes were pre-incubated with various concentrations of simvastatin (0–10 μM) or pravastatin (10 or 30 μM) for 3 h and then stimulated with LPS (5 ng/ml) and TNF-α (100 ng/ml) (LT-stimulus). After 24 h, nitrite concentration in the culture medium was assayed and standardized with total protein contents of the cells. Data are $M \pm$ SD of 5–7 observations. Figures in the parentheses indicate the concentrations of statins. * $p < 0.05$ vs Cont, † $p < 0.05$ vs LT. Cont: Control, LT: lipopolysaccharide + tumour necrosis factor-α, Sim: simvastatin, Pra: pravastatin.

appeared to be further increased dose-dependently by the pre-incubation with 1–10 μM of simvastatin. The NO production was increased by 71% ($p < 0.05$) at simvastatin concentration of 10 μM and by 34% ($p < 0.05$) at pravastatin concentration of 30 μM (Figure 1). The effect of 30 μM of pravastatin on NO production appeared to be less than that of 3 μM of simvastatin.

Effect of mevalonate, geranylgeranylpyrophoshate, squalene and Y27632 on the action of simvastatin in 3T3-L1 adipocytes

We investigated the effect of the products of mevalonate cascade and Y 27632, a specific Rho kinase inhibitor, on statin-induced NO production in 3T3-L1 adipocytes (Figure 2). Mevalonate and GGPP inhibited the simvastatin-induced NO production by 68% and 79% ($p < 0.05$), respectively, but squalene did not. Y 27632 enhanced LT-induced NO production 2-fold. AG and L-NAME, the inhibitors of iNOS, appeared to abolish the LT and simvastatin-induced NO production.

Effect of simvastatin on iNOS expression in 3T3-L1 adipocytes

Figure 3 summarizes the effect of simvastatin and Y 27632 on iNOS expression determined by real time

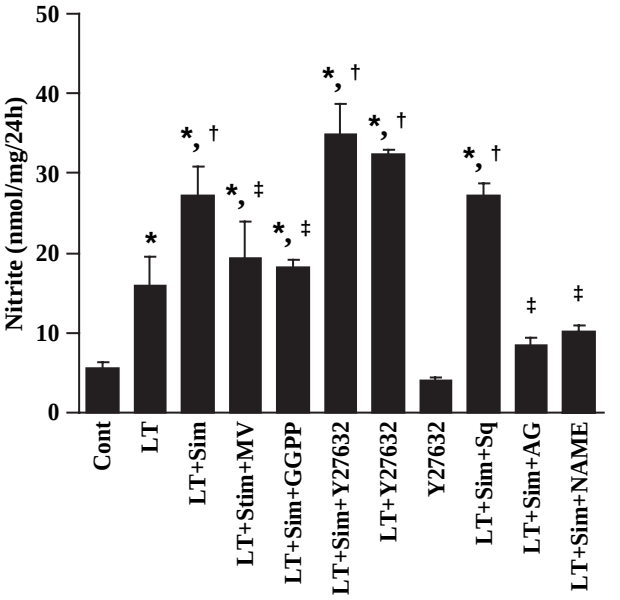

Figure 2. Effect of mevalonate, geranylgeranylpyrophoshate, squalene and RhoA inhibitor (Y 27632) on simvastatin-induced nitrite production in 3T3-L1 adipocytes. Data are $M \pm$SD. Sim: 10 μM of simvastatin, MV: 0.5 mM of mevalonate, GGPP: 50 μM of geranylgeranylpyrophoshate Sq: 10 μM of squalene, AG: 0.5 mM of aminoguanidine, NAME: 0.5 mM of nitro-L-arginine methyl ester, Y27632: 30 μM. * $p < 0.05$ vs Cont, † $p < 0.05$ vs LT, ‡ $p < 0.05$ vs LT + Sim (10).

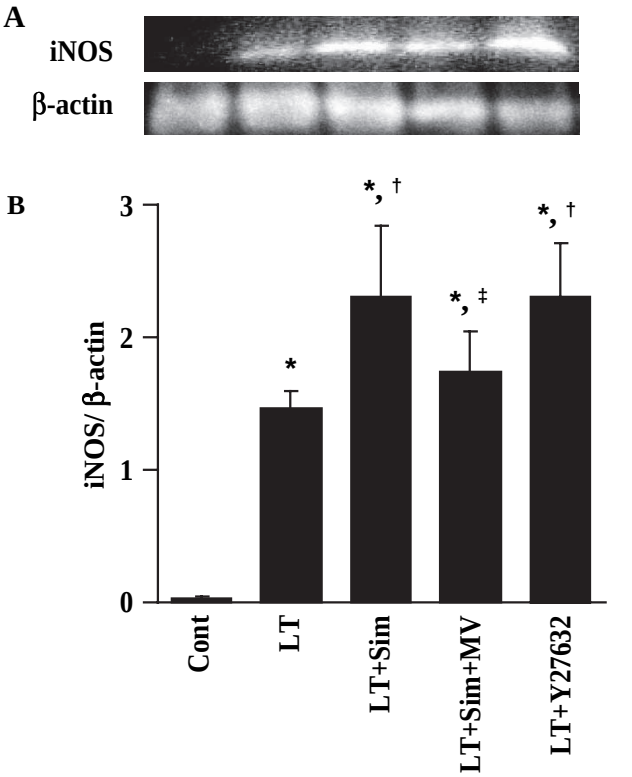

Figure 3. Effect of simvastatin on iNOS expression in 3T3-L1 adipocytes. The mRNA for iNOS was quantified by real time RT-PCR method in 3T3-L1 adipocytes. The mRNA was extracted 6 h after the final addition of the reagents and β-actin was used as the control. The PCR products were applied to agarose gel (2%) electrophoresis. Single band was depicted at 730 bp level for iNOS and at 452 bp level for β-actin (A). The data are expressed as $M \pm$ SD (for 3–5 observations; B). Abbreviations are the same as in Figure 2. * $p < 0.05$ vs Cont, † $p < 0.05$ vs LT, ‡ $p < 0.05$ vs LT + Sim (10).

RT-PCR. The PCR product for iNOS was not visible in the control lane, but in the lanes with LT-stimulation bands were detected at 730 bp level. Simvastatin enhanced the expression by 57%, while mevalonate inhibited it by 67% (Figure 3B). Y27632 induced iNOS mRNA to a similar extent to simvastatin. A band for β-actin as the control was seen at the 425 bp level in each lane.

Effect of simvastatin and pravastatin on the NF-κB activities in 3T3-L1 adipocytes

The NF-κB was activated markedly by LT-treatment (Figure 4). Addition of simvastatin (10 μM) or pravastatin (30 μM) further increased the activity by 56% and 37%, respectively ($p < 0.05$). Mevalonate and GGPP inhibited the effect of simvastatin by 97% and 81%, respectively ($p < 0.05$). Y 27632 enhanced the LT-mediated activation of NF-κB by 79% and Y27632 tended to further enhance the NF-κB level activated by LT with simvastatin.

Effect of statins on IL-6 production in LPS/ TNF-α-stimulated 3T3-L1 adipocytes

We investigated the effect of simvastatin and pravastatin on the production of IL-6, which was known to be regulated at the transcriptional level by NF-κB. Il-6 production was markedly increased by LT-stimulus and it was further enhanced dose-dependently by the addition of simvastatin to 178% of the LT level at simvastatin concentration of 10 μM (Figure 5). Mevalonate and GGPP inhibited the simvastatin-mediated increase down to the LT level. Y 27632 enhanced the LT-stimulus by 69%. Effect of 30 μM of pravastatin on IL-6 level appeared to be similar to that of 3 μM of simvastatin.

Discussion

The effect of statins on NF-κB activity and NO production via iNOS has been studied previously in various cell types. However, there are controversial reports concerning the regulation of iNOS expression by statins. The present study revealed that statins enhanced LPS and cytokines induced NO production by stimulating iNOS gene expression at mRNA level via the NF-κB dependent pathway. 3T3-L1 mature adipocytes had a signal pathway from HMG-CoA to small GTP-binding proteins and statins enhanced NF-κB activity and iNOS expression by inhibiting that pathway.

Satins inhibit the iNOS expression in astrocytes, microglia, macrophages, embryonic cardiac cells and

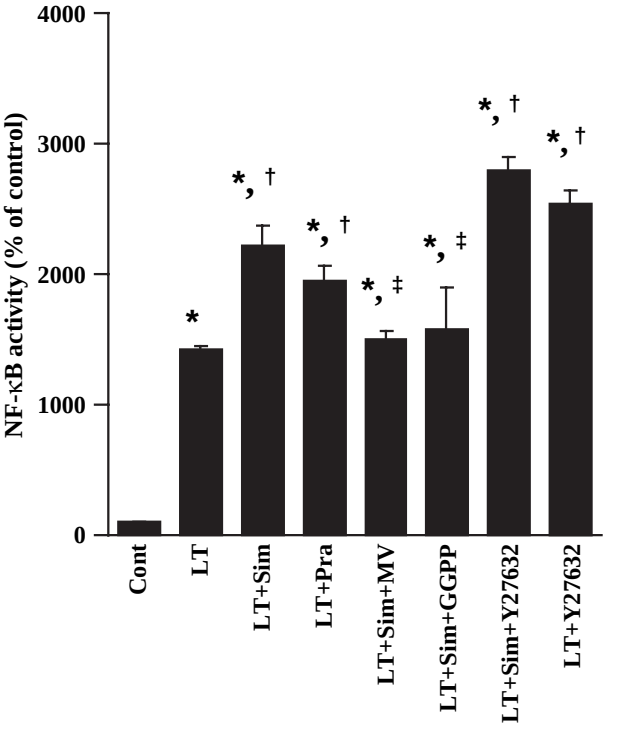

Figure 4. Effect of simvastatin and pravastatin on the NF-κB activities in 3T3-L1 adipocytes. The nuclear fraction of 3T3-L1 adipocytes was extracted and assayed for free NF-κB level by ELISA. The activity was expressed as percentage of the controls ($n=3$). Abbreviations are the same as in Figure 2. Pra: 30 μM of pravastatin. * $p<0.05$ vs Cont, † $p<0.05$ vs LT, ‡ $p<0.05$ vs LT+ Sim (10).

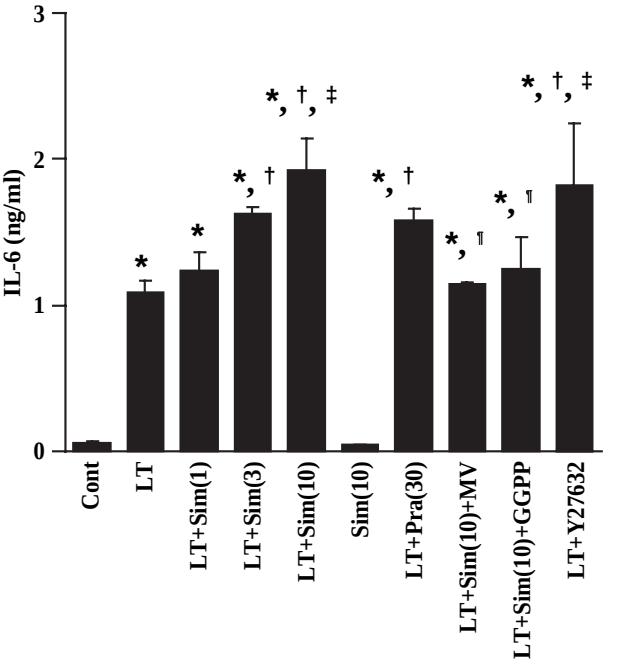

Figure 5. Effect of simvastatin and pravastatin on IL-6 production in LT-stimulated 3T3-L1 adipocytes. IL-6 concentration was assayed in culture medium 24 h after the final addition of the reagents to 3T3-L1 adipocytes. The data are standardized for total protein content of the cells and expressed as $M\pm$SD ($n=5-6$). Figures in the parentheses indicate the concentrations of statins. Abbreviations are the same as in Figure 2. * $p<0.05$ vs Cont, † $p<0.05$ vs LT, ‡ $p<0.05$ vs LT+Sim (1), ¶ $p<0.05$ vs LT+Sim (10).

human vascular endothelial cells [12–15], but enhance it in vascular smooth muscle cells (VSMC) [16,17] and transformed brain cell lines [18]. In the cell types in which stains inhibit the iNOS expression, statins enhance the expression of IκB-α, thereby blocking NF-κB activation [12–15]. On the other hand, the iNOS induction by statins in VSMC is reported to be mediated by the F-actin cytoskeleton disruption secondary to Rho kinase inhibition but not by NF-κB activation [16,17]. In transformed brain cell lines, inhibition of Rho A leads to an increase in I-κB kinase activity and to NF-κB activation [18]. Y 27632, which decreases Rho level and inhibits Rho kinase activity, enhances NO production in both cases [16–18]. Thus, the effect of statins on NF-κB activation appears to vary from cell to cell but it is consistent that the inhibition of small G protein signal plays a role in the regulation of iNOS [17,18]. It has recently been reported that 3T3-L1 pre-adipocytes act as macrophage-like cells [22]. As described above, statins inhibit the iNOS expression in macrophages [12,13]. We used fully differentiated 3T3-L1 adipocytes in this study. Adipocytes may change the character including some signal transduction pathways during the differentiation process from pre-adipocytes.

The activation of NF-κB in adipocytes is regulated by several different mechanisms. We previously reported that both activation of PPAR-γ by a thiazolidine derivative and PKA activation by cAMP inhibited the NF-κB activity and iNOS expression in 3T3-L1 adipocytes [5,19]. The mechanism of NF-κB activation by small G protein signalling in adipocytes remains unclear. In this study, simvastatin activated NF-κB and this activation was offset by the addition of mevalonate and GGPP but not by squalene. In addition, the inactivation of Rho kinase signal may contribute to these changes because Y27632 could mimic the stains' effect. These results imply that the effect of statins on NF-κB activation and iNOS induction are mediated by the inhibition of the pathway playing between mevalonate and squalene, namely the signal from isoprenoid to small G proteins. The opposing effect of statin between mature adipocytes and other cells may reflect the fact that different signal transduction pathways may operate between those cells.

Adipose tissue has been proven to be not merely a site for energy storage but also the largest endocrine organ that secretes various physiologically active substances, collectively called adipocytokines [23]. Production of PAI-1, an important mediator of

atherosclerosis, is reported to be increased by activation of NF-κB [7,24]. Goto et al. [25] reported that Y 27632 increased the expression of PAI-1 in 3T3-L1 adipocytes. The small G protein signal probably plays a role in inhibiting the NF-κB signal in adipocytes.

Another finding of this study is that potency of simvastatin appeared to be more than 10-fold of that of pravastatin in terms of activation for productions of NO and IL-6. Pharmacological potency of simvastatin for inhibition of cholesterol synthesis is roughly 3 times that of pravastatin in rat hapatocyte primary culture, but the potency of pravastatin is 1/1000 that of simvastatin in fibroblasts [26]. The difference between pravastatin and other statins, simvastatin, lovastatin and atorvastatin, etc., may be related to the former's hydrophilic nature and lack of specific carrier for pravastatin on extra-hepatic cell membranes, making diffuse of pravastatin through the plasma membrane difficult. The specific carrier for pravastatin may exist only in hepatocytes [27]. Therefore, the action of hydrophilic statins is more selective to hepatocytes than lipophilic ones [28] and the pleiotropic effects to other cells may be weak. In fact, simvastatin, but not pravastatin, lowered monocyte tissue factor activity that contributes to blood coagulation *in vivo* [29].

Rho kinase regulates the insulin signalling positively [30] and its inactivation by statins may decrease insulin sensitivity in adipocytes. In addition, excessive production of NO by iNOS in adipocytes may result in insulin resistance [20]. In this study, we also found that stains and Y27632 increased IL-6, which induces insulin resistance. These effects in adipocytes observed here may be related to the adverse effect of statins.

There are several reports on the detrimental effects of lipophilic statins on glycemic control in patients with type 2 diabetes mellitus (DM) [31,32]. Lovastatin lowered glucose utilization by inhibiting the translocation of glucose transporter-4 in 3T3-L1 adipocytes [33] and glycemic control deteriorated by administration of atorvastatin in rats with streptozotocin-induced DM [34]. On the other hand, both lipophilic and hydrophilic statins are reported not to exacerbate glucose tolerance and insulin secretion in diabetic GK rats [35]. In general, the lipid-lowering effect of statins is beneficial for preventing atherosclerosis in both diabetic and non-diabetic patients with hyperlipidemia. A large clinical trial (ASCOT) has provided evidence that atorvastatin, a strong lipophilic statin, reduces cardiovascular events even in patients with type 2 DM [36]. Paolisso et al. [37] have reported that simvastatin and atorvastatin are useful not only for controlling dyslipidemia but also for improving metabolic control in type 2 DM patients. The effect of stains on glucose tolerance needs to be elucidated in further studies.

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