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**Fluvastatin inhibits mast cell degranulation without changing
the cytoplasmic Ca^{2+} level**

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Abstract

We evaluated the pharmacological effect of statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) on mast cell degranulation in RBL-2H3 cells. A hydrophilic statin (pravastatin) did not inhibit degranulation induced by dinitrophenol-human serum albumin (DNP-HSA); in contrast, lipophilic statins (simvastatin, fluvastatin and atorvastatin) inhibited DNP-HSA–induced degranulation in that order. The inhibitory effects were completely attenuated by simultaneous treatment with 100-1000 μ M mevalonic acid for 4 h. We used fluvastatin to clarify the mechanism of the statin-mediated inhibitory action of mast cell degranulation. Fluvastatin (3 μ M) had no effect on Ca^{2+} release from the endoplasmic reticulum or Ca^{2+} influx in the DNP-HSA- or thapsigargin-stimulated cells. Fluvastatin treatment also had no effect on the total granule content of the cell or sensitivity to DNP-HSA and IgE. Fluvastatin (3 μ M, 24 h treatment) also failed to affect the morphology, proliferation, and viability of RBL-2H3 cells. Geranylgeranyl transferase inhibitor, GGTI-286 (20 μ M), but not farnesyl transferase inhibitor, FPTIII (20 μ M), inhibited the DNP-HSA–induced degranulation. The GGTI-286–induced inhibitory action was not associated with a decrease in the cytoplasmic Ca^{2+} level. In conclusion, fluvastatin at a lower concentration range inhibited DNP-HSA–induced degranulation without affecting the cytoplasmic Ca^{2+} response and also without changing the maturation and proliferation of the mast cells. The statin-induced inhibitory action may be mediated by the suppression of geranylgeranyl transferase via the depletion of intracellular mevalonic acid .

Index words: mast cell, statin, fluvastatin, degranulation, geranylgeranylation, cytosolic Ca^{2+}

1. Introduction

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which is an upstream rate-limiting enzyme in the cholesterol synthesis pathway. They have been widely used for treating hypercholesterolaemia and reducing cardiovascular mortality and morbidity. Although all statins share a common mechanism of action, they differ in terms of their chemical structures, pharmacokinetic profiles, and lipid-modifying efficacy. The chemical structures of statins are correlated to their hydrophilicity, which influences their absorption, distribution, metabolism and excretion. Atorvastatin, simvastatin, lovastatin and fluvastatin are relatively lipophilic compounds, which are more susceptible to metabolism by the cytochrome P450 system. Pravastatin is relatively hydrophilic and is not significantly metabolized by cytochrome P450 enzymes (Greenwood et al., 2006).

Evidence from clinical studies (Albert et al., 2001; Joukhadar et al., 2001) and *in vitro* experiments (Crisby et al., 2001; Sakamoto et al., 2005) indicates that statins have additional anti-inflammatory and anti-immunoreactive properties unrelated to their cholesterol-lowering properties. Recently, it became evident that statins modulate immune responses; i.e., statin treatment has the potential to modify T-lymphocyte-driven disease by allosterically inhibiting the interaction between the cellular adhesion molecules, integrin lymphocyte function associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) (Weitz-Schmidt et al., 2001) and by decreasing the interferon-gamma (IFN- γ)-induced expression of the major histocompatibility complex-II (MHC-II) on antigen presenting cells (Kwak et al., 2000). Because of the diverse effects of statins on the immune system, there is considerable interest in their therapeutic potential for autoimmune diseases, such as rheumatoid

arthritis. Statins are particularly attractive therapeutic agents because their safety profile is generally good and they can be administered orally.

Statins also affect allergic reactions. Stanislaus et al. observed the amelioration of experimental allergic encephalomyelitis by lovastatin (Stanislaus, Neurosci Lett 1999). Mackay et al. reported an anti-inflammatory role for simvastatin in a murine model of allergic asthma (McKay et al., 2004). However, the molecular mechanisms responsible for these effects have not been fully elucidated. In addition to the *in vivo* studies of the effects of statins on allergies, there are reports of the effects of statin on mast cell degranulation *in vitro* (Deanin et al., 1991; Djouder et al., 2000; Graham et al., 1998). However, because these reports used high concentrations (10 μ M) of statin to clarify the inhibitory mechanism of mast cell degranulation, their pharmacological and clinical relevance should be reevaluated.

In the present study, we examined the effects of statins on the degranulation of mast cells, which are the key effector cells in allergic inflammation. Statins inhibit the production of mevalonic acid and its metabolites in the cholesterol biosynthesis pathway. We focused on the metabolites of mevalonic acid, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, because they function as adjuncts in the post-translational prenylation of cell-signaling proteins such as Ras and Rho (Zhang Annu. Rev. Biochem. 1996). Therefore, we also examined the effects of farnesyl transferase inhibitor and geranylgeranyl transferase inhibitor on mast cell degranulation. We found that clinically relevant, low concentrations of fluvastatin inhibit mast cell degranulation by inhibiting geranylgeranyl transferase via the depletion of intracellular mevalonic acid; the cytoplasmic Ca^{2+} response and the maturation and proliferation of mast cells were unaffected.

2. Materials and methods

2.1. Mast cells

RBL-2H3 cells (ATCC, Manassas, VA, U.S.A.) were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated overnight with 50 ng/ml anti-dinitrophenol IgE (anti-DNP IgE) before use.

2.2. β -Hexosaminidase degranulation

We measured the release of β -hexosaminidase as an index of mast cell degranulation by using a previously described method (Oka et al., 2002). In brief, RBL-2H3 cells (2×10^5 cells/well) were washed with HEPES-buffered solution (125.4 mM NaCl, 11.5 mM glucose, 5.9 mM KCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 10 mM HEPES; pH 7.4). The IgE-sensitized cells were stimulated with various concentrations of dinitrophenol-human serum albumin (DNP-HSA) at 37°C for 15 min under gentle rotation. The supernatants were transferred to a 96-well plate. The rest of the supernatants were removed, and then Triton X-100 solution (0.5%) was added to the cells. The extracts were transferred to a 96-well plate. To each well, 1.3 mg/ml *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide in 0.04 M sodium citrate (pH 4.5) was added. The plates were incubated at 37°C for 1 h. Glycine (0.2 M; pH 10.0) was then added to each well. The absorbance at 405 nm (OD) of each well was measured with a multilabel counter (PerkinElmer Japan, Tokyo, Japan). The percentage of degranulation was calculated with the following formula: % Degranulation = $\frac{\text{OD}_{\text{supernatant}}}{(\text{OD}_{\text{supernatant}} + \text{OD}_{\text{Triton X-100}})} \times 100$

2.3. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by using fura-PE3/AM (TEF Laboratories, Austin, TX, U.S.A.) according to a method described previously (Oka et al., 2002). In brief, RBL-2H3 cells on glass coverslips in HEPES-buffered solution were loaded with 3 μ M fura-PE3/AM for 40 min in a dark room at 37°C. Every 3 s, the fluorescence of an image at 340 nm (F_{340}) was divided by the fluorescence of an image at 380 nm (F_{380}) to provide a resultant ratio (F_{340}/F_{380}) by means of a fluorescence imaging system (Hamamatsu Photonics, Hamamatsu, Japan). When the extracellular Ca^{2+} was removed, Ca^{2+} -free HEPES-buffered solution (containing 0.5 mM EGTA instead of 1.5 mM $CaCl_2$) was used. In the mast cells, the changes in $[Ca^{2+}]_i$ were oscillatory, asynchronous, and irregular and varied tremendously among cells. In this type of cell, measuring the area under the Δ ratio per time curve (area under the curve (AUC)) was effective for quantifying the response (Narenjkar et al., 1999): Δ ratio = ratio – ratio_{min}, where ratio_{min} is the ratio measured in the Ca^{2+} -free HEPES-buffered solution.

2.4. Immunostaining of tubulin and actin

To visualize the microtubule cytoskeleton, RBL-2H3 cells grown on glass coverslips were rinsed with HEPES-buffered solution at 37°C and then were fixed with 3.7% formaldehyde in HEPES-buffered solution for 10 min at 37°C. The fixed cells were permeabilized with 0.2% Triton X-100 for 15 s. Afterwards, the cells were incubated with a FITC-labeled mouse monoclonal anti-tubulin Ab at a 1/100 dilution for 30 min at 37°C. Filamentous actin was also stained with 4 U/ml FITC-labeled phalloidin (Molecular Probes, Eugene, OR, U.S.A.) for 30 min at 37°C. The images were captured by using a fluorescence imaging system (Planetron, Tokyo, Japan). The microscope was equipped with excitation (490 nm) and emission (520 nm) filters.

2.5. Cell viability

RBL-2H3 viability was assessed as a function of trypan blue exclusion (Black and Berenbaum, 1964). For trypan blue exclusion, cells were plated on 24-well plates and incubated with or without 3 μ M fluvastatin for 24 h. Then, cells were trypsinized and stained with trypan blue. Viable cells, which exclude dye, were counted, and viability was expressed as the percent of total cell number.

2.6. Chemicals

DMEM powder, FBS, anti-DNP IgE, Triton X-100, *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide, FITC-labeled mouse monoclonal anti-tubulin Ab, Evans blue, DNP-HSA, thapsigargin and ionomycin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Farnesylation transferase inhibitor III (FPTIII) and geranylgeranyl transferase inhibitor-286 (GGTI-286) were purchased from Calbiochem (Darmstadt, Germany).

2.7. Statistical analysis

The results of the experiments are expressed as means $\pm$ S.E.M. The unpaired Student's *t* test was used for comparisons between two groups, and ANOVA followed by Dunnett's test was used for comparisons between more than two groups. *P* values <0.05 were considered statistically significant.

3. Results

3.1. *Effects of statins on mast cell degranulation and counteraction by mevalonic acid*

We examined the effects of statins on DNP-HSA–induced degranulation of RBL2H3 mast cells sensitized with 50 ng/ml anti-DNP-IgE for 12 h. Intracellular β -hexosaminidase was released by DNP-HSA (0.1-100 ng/ml) stimulation for 15 min, and 10 ng/ml DNP-HSA–induced submaximal degranulation (data not shown). Pretreatment of the cells for 24 h with 3 μ M lipophilic statins (fluvastatin, simvastatin and atorvastatin), but not a hydrophobic statin (pravastatin), significantly inhibited the DNP-HSA–induced degranulation from RBL-2H3 mast cells (Fig. 1). Fluvastatin and simvastatin were more efficient than atorvastatin at reducing the degranulation. The inhibition was completely counteracted in a concentration-dependent manner by pre-treatment with mevalonic acid for 4 h (100-1000 μ M).

3.2. *Effects of fluvastatin on cell morphology, maturation, proliferation and viability of mast cells*

Next, we used fluvastatin to clarify the mechanisms used by lipophilic statins to inhibit degranulation. Fluvastatin inhibited the DNP-HSA–induced degranulation in a concentration-dependent manner (0.5-10 μ M), in which a 24-h treatment with 3 μ M fluvastatin showed submaximal inhibition (Fig. 2A). We further investigated the time-dependency of the inhibitory action of fluvastatin on mast cell degranulation by using a 5- μ M concentration (Fig. 2B). The results indicated that fluvastatin inhibited the mast cell degranulation in a time-dependent manner, and maximum inhibitory action was observed after 24 h treatment. Three μ M fluvastatin also inhibited the mast cell degranulation with a similar time dependency (data not shown, n=4). We then investigated the effects of the statin on RBL-2H3 mast cell morphology, maturation,

proliferation, and viability. We compared morphological changes in RBL-2H3 mast cells before and after treatment with 3 μ M fluvastatin for 24 h, and we found no apparent effects on the cell shape and the tubulin networks (Fig. 2B). In contrast, in the RBL-2H3 mast cells treated with 10 μ M fluvastatin, the cell shape became rounded, and the mesh-like tubulin formations disappeared. Peripheral actin filament formation did not change in the presence of 1 μ M fluvastatin for 24 h (data not shown).

DNP-HSA (1-100 ng/ml) induced a concentration-dependent degranulation of mast cells that were sensitized by 50 ng/ml anti-DNP-IgE (Fig. 3A); the maximum degranulation was induced by 100 ng/ml DNP-HSA. Fluvastatin (3 μ M) significantly inhibited mast cell degranulation at each antigen concentration range. However, even in the presence of fluvastatin, 100 ng/ml DNP-HSA induced a maximum degranulation. In addition, we normalized the concentration response curve of DNP-HSA in the presence or absence of fluvastatin (inset figure of Fig. 3A). The results indicated that the concentration response curve of DNP-HSA did not change in the presence of fluvastatin, indicating that fluvastatin does not alter the sensitivity of the cell to antigen.

Anti-DNP-IgE induced mast cell degranulation in a concentration-dependent manner (Fig. 3B). When mast cells were stimulated with 10 ng/ml DNP-HSA for 15 min, 10 ng/ml anti-DNP-IgE induced a submaximum degranulation, and 50-100 ng/ml anti-DNP-IgE induced a maximum degranulation. Fluvastatin (3 μ M) significantly inhibited degranulation at each concentration of anti-DNP-IgE. In addition, we compared the response curve of IgE normalized by the maximum response in the presence or absence of fluvastatin (inset figure of Fig. 3B). However, we failed to observe any change in the sensitivity of the mast cells to anti-DNP-IgE.

We investigated the effect of fluvastatin on the granule content per cell by

measuring the total β -hexosaminidase content, and we found that fluvastatin did not change the total β -hexosaminidase content (Fig. 4A). Figure 4B shows the effect of fluvastatin on total cell number during the incubation period with fluvastatin (3 μ M) for 24 h. Fluvastatin provoked no conformational changes in RBL-2H3 mast cells (as shown in Fig. 2B) and did not change the total cell number, which is an indicator of proliferative ability. Other statins, pravastatin, simvastatin and atorvastatin, also did not affect the total granule content and proliferative activity of mast cells (data not shown).

We further investigated the effect of fluvastatin on mast cell viability as shown in Table 1. Although Graham et al. reported that statin induced apoptosis in mast cells (Graham et al., 1998), fluvastatin did not induce cell death under our experimental conditions.

3.3. Effect of fluvastatin on DNP-HSA- or thapsigargin-induced increment of $[Ca^{2+}]_i$ in mast cells

An increase in $[Ca^{2+}]_i$ is a prerequisite for mast cell degranulation; therefore, we investigated whether or not fluvastatin affects $[Ca^{2+}]_i$ signaling. In RBL-2H3 mast cells in the absence of external Ca^{2+} , DNP-HSA (10 ng/ml) evoked a transient increase in $[Ca^{2+}]_i$ by Ca^{2+} release from the endoplasmic reticulum via the high affinity receptor for the Fc region of anti-specific IgE (Fc ϵ RI) stimulation. Sequential addition of external Ca^{2+} (1.5 mM) induced a sustained increase in $[Ca^{2+}]_i$ by Ca^{2+} influx through the plasma membrane, and the sustained increment of $[Ca^{2+}]_i$ was maintained for at least 10 min. In the cells pretreated with 3 μ M fluvastatin for 24 h, the DNP-HSA-induced Ca^{2+} release and Ca^{2+} influx were comparable to those in control cells (Fig. 5A). Fig. 5B shows the quantitative results of the Ca^{2+} release and Ca^{2+} influx by calculating the AUC of the $[Ca^{2+}]_i$ movement. Fluvastatin did not change the AUC of the

DNP-HSA–induced change in $[Ca^{2+}]_i$. We further tried to investigate the effect of a higher concentration (10 μ M) of fluvastatin on $[Ca^{2+}]_i$ movement stimulated with antigen. However, treatment with 10 μ M fluvastatin for 24h induces cell shape change of RBL-2H3 cells, and the cells were detached from the bottom of the culture dish. Therefore, we could not measure the $[Ca^{2+}]_i$ transient stimulated by 10 μ M fluvastatin. We further examined the effect of fluvastatin on non-IgE-mediated mast cell degranulation with $[Ca^{2+}]_i$ increment. An inhibitor of the endoplasmic reticulum Ca^{2+} ATPase, thapsigargin, induces Ca^{2+} depletion of the endoplasmic reticulum and causes capacitative Ca^{2+} entry, which induces mast cell degranulation without antigen/IgE (Parekh and Penner, 1997; Thastrup et al., 1990). We measured both mast cell degranulation and $[Ca^{2+}]_i$ transient in thapsigargin-stimulated mast cells in the presence or absence of fluvastatin. Fluvastatin significantly inhibited mast cell degranulation in a concentration-dependent manner (Fig. 6A). However, fluvastatin had no effect on Ca^{2+} release and Ca^{2+} influx induced by thapsigargin (Fig. 6B), similar to the case of antigen/IgE stimulation.

3.4. Effects of GGTI-286 and FPTIII on DNP-HSA–induced degranulation and $[Ca^{2+}]_i$ movement

As shown in Fig. 1, the statin-mediated inhibitory action of mast cell degranulation was reversed by the recruitment of mevalonic acid. mevalonic acid is further metabolized to farnesyl pyrophosphate and then to geranylgeranyl pyrophosphate, which is utilized for post-translational modification of proteins including small GTP-binding proteins (Konstantinopoulos et al., 2007; McTaggart, 2006). Therefore, we investigated the effects of FPTIII and GGTI-286 on mast cell degranulation. RBL-2H3 cells were pre-treated with 20 μ M GGTI-286 or FPTIII for 24

h, and then we measured DNP-HSA–induced degranulation for 15 min. GGTI-286, but not FPTIII, significantly inhibited the DNP-HSA–induced degranulation (Fig. 7A). We next investigated the effects of these inhibitors on DNP-HSA–induced $[Ca^{2+}]_i$ increment. FPTIII as well as GGTI-286 did not alter the increment of $[Ca^{2+}]_i$ induced by DNP-HSA (Fig. 7B).

4. Discussion

Statins are classified as lipophilic statins, such as fluvastatin and lovastatin, and hydrophilic statins, such as pravastatin. Lovastatin inhibits degranulation in RBL-2H3 mast cells (Deanin et al., 1991; Graham et al., 1998; Shakarjian et al., 1993). In the present study, we compared the effectiveness of fluvastatin, pravastatin, simvastatin and atorvastatin on the degranulation of RBL-2H3 mast cells, and we found that fluvastatin and simvastatin had stronger effects on degranulation (Fig. 1). In contrast, pravastatin had almost no effect on degranulation. The difference in the effectiveness of these four statins might depend on their hydrophilicity; i.e., pravastatin might not go through the cell surface membrane because of its hydrophilicity. Atorvastatin had a strong effect on the degranulation of human mast cells *in vitro* (Krauth et al., 2006). While atorvastatin is known as very effective and thus it is widely used in clinical practice (Colhoun et al., 2004), our data indicates that atorvastatin is not as effective in RBL-2H3 mast cells. Differences in the effectiveness of this agent in different mast cells might be attributable to the mast cell subtypes and/or species differences. Further investigation is required to clarify this point.

Ca^{2+} signaling is a prerequisite for the degranulation of mast cells (Galli et al., 2005). Previous reports indicated that lovastatin inhibits mast cell degranulation by inhibiting the inositol triphosphate turnover followed by the Ca^{2+} influx (Deanin et al., 1991; Djouder et al., 2000; Graham et al., 1998). In these reports, the authors used a relatively high concentration of lovastatin (10 μM) to more clearly demonstrate the inhibitory mechanisms for mast cell degranulation. They found that 24-h treatment with 10 μM lovastatin induces cell shape change and inhibits cell proliferation in RBL-2H3 mast cells (Deanin et al., 1991; Graham et al., 1998). In addition, 48-h treatment with 10

μM lovastatin induced apoptosis. In the present study, we carefully determined the margin of submaximal concentration of fluvastatin on mast cell degranulation. We found that 24-h treatment with 3 μM fluvastatin effectively inhibited mast cell degranulation without changing the shape, proliferation or cytosolic Ca^{2+} level in RBL-2H3 mast cells, suggesting the possibility that low concentrations of lipophilic statins inhibit mast cell degranulation without changing the Ca^{2+} movement. We also confirmed that a 24-h treatment with 3 μM fluvastatin did not induce cell death in RBL-2H3 mast cells, as monitored by trypan blue staining. However, Graham also reported that a 24-h treatment with 10 μM lovastatin did not induce apoptosis in RBL-2H3 mast cells. Therefore, it is possible to induce cell death when cells are treated with 10 μM fluvastatin over 48 h. At higher concentrations of statin, statin may influence various cell functions unrelated to mast cell degranulation.

We next examined the inhibitory mechanisms of fluvastatin on mast cell degranulation. Under our experimental conditions, 3 μM fluvastatin did not change Ca^{2+} release or influx. One possible mechanism is that statins would change cell maturation, such as granule content and IgE and/or antigen sensitivity of $\text{Fc}\epsilon\text{RI}$ receptors in inducing degranulation. However, 3 μM fluvastatin did not change granule content, as measured by total β -hexosaminidase, or IgE and/or antigen sensitivity for mast cell degranulation (as shown in Fig.3 and 4). In addition, fluvastatin significantly inhibited mast cell degranulation via endoplasmic reticulum Ca^{2+} depletion without antigen/IgE (as shown in Fig. 6). These data strongly support that fluvastatin treatment did not influence mast cell maturation including granule formation and the functional activity of the $\text{Fc}\epsilon\text{RI}$ receptor.

The inhibition of degranulation mediated by fluvastatin was reversed by

mevalonic acid (Fig. 1), as has been reported previously (Deanin et al., 1991; Djouder et al., 2000; Shakarjian et al., 1993). However, it was reported that dolichol and cholesterol, which are synthesized from products of the isoprenoid pathway, did not restore the inhibition of degranulation induced by statins (Deanin et al., 1991). These findings implicate geranylgeranyl pyrophosphate and/or farnesyl pyrophosphate, but not the end-product (cholesterol), in mast cell degranulation. Protein prenylation plays crucial roles in the regulation of cellular functions (Greenwood et al., 2006; Konstantinopoulos et al., 2007). Therefore, we studied the effect of GGTI-286 (Lerner et al., 1995) and FPTIII (Byk et al., 1997; Kelly et al., 1998), which selectively inhibit geranylgeranylation and farnesylation, respectively, on mast cell degranulation. GGTI-286 but not FPTIII significantly inhibited antigen-induced degranulation. In addition, the inhibition of mast cell degranulation by GGTI-286 was not associated with decreased cytosolic Ca^{2+} levels, as was also the case with fluvastatin. Thus, the fluvastatin-induced inhibitory effect on mast cell degranulation may be mediated by geranylgeranylation rather than farnesylation. These results are consistent with a previous report by Graham et al. (Graham et al., 1998). Farnesylation may be more important in cytokine production and the arachidonic acid pathway in mast cells (Kawakami and Galli, 2002). In the present study, FPTIII slightly augmented mast cell degranulation, which is consistent with the results of Graham et al (Graham et al., 1998). These observations indicate the possibility that farnesylated protein(s) may be involved in the suppression of degranulation. Further investigations are necessary to clarify this point.

Some evidence indicates that small GTP-binding proteins are involved in the mast cell degranulation process (Kawakami and Galli, 2002). Most recently, it has been

reported that N-Ras and RhoA are required for mast cell degranulation (Liu et al., 2007; Nishida et al., 2005). The FcεRI-mediated signaling pathway can be dissected into Ca^{2+} -dependent and Ca^{2+} -independent pathways. The Lyn/Syc/LAT pathways activate PLCγ, which in turn induces Ca^{2+} release followed by Ca^{2+} influx through store-operated Ca^{2+} entry channels (Nishida et al., 2005; Oka et al., 2005); this Ca^{2+} -dependent pathway is involved in the granule-membrane fusion process in which Rac and Cdc42 might play an important role. In contrast to the Ca^{2+} dependent pathways, Fyn/Gab2/PI3K/RhoA signaling changes microtubule formation, which is important for granule translocation in a Ca^{2+} -independent manner (Nishida et al., 2005). Another small GTP-binding protein, N-RAS, can modulate PI3K activation to activate mast cell degranulation. Because N-RAS and RhoA can be geranylgeranylated by geranylgeranyl transferase (Konstantinopoulos et al., 2007), it is possible that statins may inhibit N-RAS and RhoA by inhibiting the geranylgeranylation of these small GTP-binding proteins without changing the cytoplasmic Ca^{2+} level.

On the other hand, Djouder et al. reported that *Clostridium sordellii* lethal toxin, which glycosylates Rac and Cdc42 but not Rho, inhibited the release of β-hexosaminidase induced by FcεRI receptor stimulation in RBL-2H3 mast cells (Djouder et al., 2000). The lethal toxin also inhibited Ca^{2+} mobilization and the Ca^{2+} current in antigen-stimulated mast cells. In addition, a higher concentration (10 μM) of lovastatin also inhibited mast cell degranulation in accordance with the decrease in cytosolic Ca^{2+} levels (Deanin et al., 1991; Graham et al., 1998). Taken together, these results indicate that a higher concentration of statin may affect Ca^{2+} mobilization, possibly through G proteins such as Rac and Cdc42. Further study is necessary to clarify this point.

In conclusion, fluvastatin, one of the lipophilic statins, but not pravastatin, a hydrophilic statin, inhibits DNP-HSA-induced degranulation. The inhibitory mechanism of fluvastatin for mast cell degranulation may be mediated by the inhibition of geranylgeranyl transferase via the depletion of intracellular MEVALONIC ACID, without affecting the cytoplasmic Ca^{2+} response stimulated by DNP-HSA and also without changing the maturation and proliferation of mast cells. Further examinations regarding the effects of low concentrations of other lipophilic statins on mast cell Ca^{2+} homeostasis are necessary to generalize the results of the current study.

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Figure legend

Fig. 1 Effect of statins on DNP-HSA–induced degranulation. RBL-2H3 cells were preincubated with or without 3 μ M fluvastatin (A), simvastatin (B), atorvastatin (C) and pravastatin (D) for 24 h in the presence or absence of mevalonic acid (0.1, 0.3 and 1 mM) for the last 4 h followed by the addition of DNP-HSA (10 ng/ml) for 15 min to induce degranulation. The percentages of released β -hexosaminidase were calculated against the total amount of β -hexosaminidase. Results are expressed as the mean $\pm$ S.E.M. of 6 experiments. ## $P < 0.01$ as compared to the DNP-HSA–treated groups (cross-hatching bars); ** $P < 0.01$ as compared to the DNP-HSA–treated and statin-treated groups (upward-hatching bars).

Fig. 2 Concentration-dependent inhibition of degranulation and change in cell morphology induced by fluvastatin in RBL-2H3 mast cells. (A) RBL-2H3 cells were preincubated with or without 0.5-10 μ M fluvastatin for 24 h followed by the addition of DNP-HSA (10 ng/ml) for 15 min to induce mast cell degranulation. The percentages of released β -hexosaminidase were calculated against the total amount of β -hexosaminidase. Results are expressed as the mean $\pm$ S.E.M. of 6 experiments. ** $P < 0.01$. (B) RBL-2H3 cells were preincubated with 3 μ M fluvastatin for 6 h to 24 h followed by the addition of DNP-HSA (10 ng/ml) for 15 min to induce degranulation. Results are expressed as the mean $\pm$ S.E.M. of 4 experiments. ** $P < 0.01$. (C) RBL-2H3 cells were preincubated with or without 3 or 10 μ M fluvastatin for 24 h. The cells were labeled for tubulin.

Fig. 3 Effect of fluvastatin on DNP-HSA or IgE sensitivity for mast cell degranulation. (A) RBL-2H3 cells were preincubated with (Fluvastatin) or without (Control) 3 μ M fluvastatin for 24 h, followed by the addition of 1-1000 ng/ml

DNP-HSA for 15 min to induce secretion. Inset figure indicates the relative figure of (A) by normalizing with each the maximum value as 100%. (B) IgE (0-100 ng/ml)-sensitized RBL-2H3 cells were preincubated with (Fluvastatin) or without (Control) 3 μ M fluvastatin for 24 h followed by the addition of 10 ng/ml DNP-HSA for 15 min to induce secretion. The percentages of released β -hexosaminidase were calculated against the total amount of β -hexosaminidase. Results are expressed as the mean $\pm$ S.E.M. of 6-12 experiments. Inset figure indicates the relative figure of (B) by normalizing with each the maximum value as 100%.

Fig. 4 Effect of fluvastatin on granule content or cell proliferation in RBL-2H3 mast cells. RBL-2H3 cells were preincubated with (Fluvastatin) or without (Control) 3 μ M fluvastatin for 24 h. Next, the number of cells in each well were counted (B), and Triton X-100 solution (0.5%) was added to the cells to measure granule content. Results are expressed as the mean $\pm$ S.E.M. of 4-8 experiments. n.s., not significantly different.

Fig. 5 Effect of fluvastatin on $[Ca^{2+}]_i$ elicited by antigen in mast cells. (A) RBL-2H3 cells were preincubated with (Fluvastatin) or without (Control) 3 μ M fluvastatin for 24 h, and then fura-PE3 was loaded. After stimulation with DNP-HSA (10 ng/ml⁻¹) for 3 min in the absence of Ca^{2+} (with 0.5 mM EGTA), 1.5 mM Ca^{2+} was added to the medium without EGTA. Typical recordings are shown in panel A. (B) AUC for 1 min in the absence of Ca^{2+} . (C) AUC for 5 min after the addition of 1.5 mM Ca^{2+} . Results are expressed as mean $\pm$ S.E.M. of 144 cells in 4 independent experiments. n.s., not significantly different.

Fig. 6 Effects of fluvastatin on mast cell degranulation and $[Ca^{2+}]_i$ movement elicited by thapsigargin.

(A) RBL-2H3 cells were preincubated with or without 0.1-3 μ M fluvastatin

followed by the addition of 1 μM thapsigargin for 15 min to induce degranulation. (Band C) RBL-2H3 cells preincubated with or without 3 μM fluvastatin. After stimulation with 1 μM thapsigargin for 3 min in the absence of Ca^{2+} with 0.5 mM EGTA to evoke Ca^{2+} release, external CaCl_2 (1.5 mM) was added to the medium to induce Ca^{2+} entry via capacitative Ca^{2+} entry. (B) AUC for 3 min in the absence of Ca^{2+} (indicated Ca^{2+} release) and (C) AUC for 5 min after the addition of 1.5 mM Ca^{2+} (indicated Ca^{2+} influx). Results are expressed as mean $\pm$ S.E.M. of 120-122 cells in 4 independent experiments. n.s., not significantly different.

Fig. 7 Effect of FPTIII and GGTI-286 on DNP-HSA-induced degranulation and the increase in $[\text{Ca}^{2+}]_i$. (A) IgE-sensitized RBL-2H3 cells were preincubated with or without (Control) 20 μM GGTI-286 and 20 μM FPTIII for 24 h. Cells were then stimulated with 10 ng/ml DNP-HSA for 15 min. Representative results of 3 separate experiments (n=12) are shown. (B) IgE-sensitized RBL-2H3 cells were preincubated with or without (Control) 20 μM GGTI-286 and 20 μM FPTIII for 24 h, and then fura-PE3 was loaded. Cells were stimulated with DNP-HSA (10 ng/ml) for 5 min in the presence of 1.5 mM Ca^{2+} . The AUC for 5 min after the addition of DNP-HSA is shown. Results are expressed as means $\pm$ S.E.M.. ** $P < 0.01$; n.s., not significantly different.

Table.1 Effect of fluvastatin on cell viability in RBL-2H3 mast cells.

RBL-2H3 cells were preincubated with or without 1 to 10 μM fluvastatin for 12 and 24 h. Then, cells were trypsinized, stained with trypan blue and number of cells which exclude dye was counted. Percentages of viable cells were shown. Results are expressed as the mean $\pm$ S.E.M. of 8 experiments. -; not determined.

	Control	Fluvastatin (1 μ M)	Fluvastatin (3 μ M)	Fluvastatin (10 μ M)
0 h	— 96.26+1.88 —	— - —	— - —	— - —
12 h	-91.67+1.49	-93.19+1.91	-93.36+1.05	-95.92+1.23

Figure 1

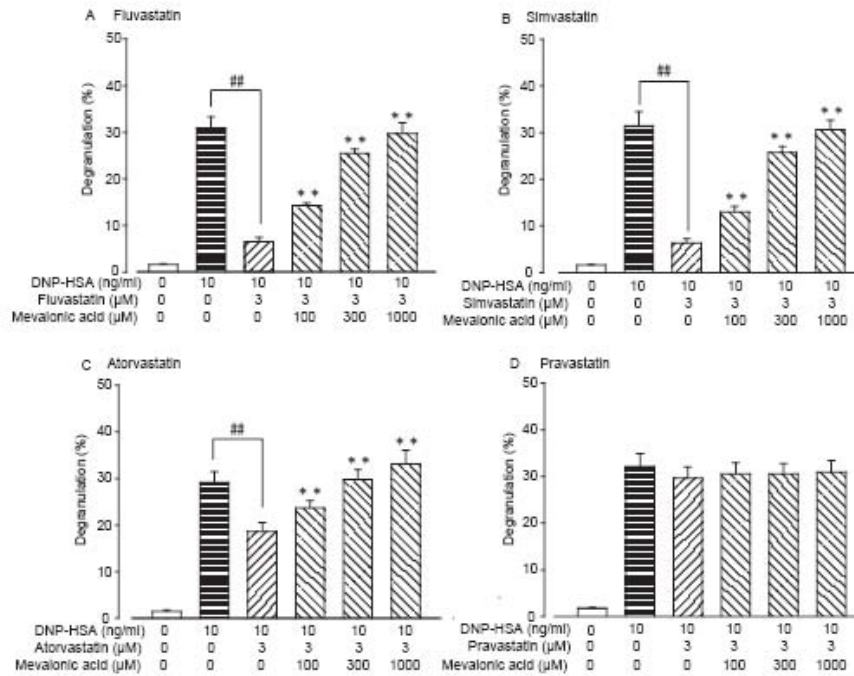


Figure 2

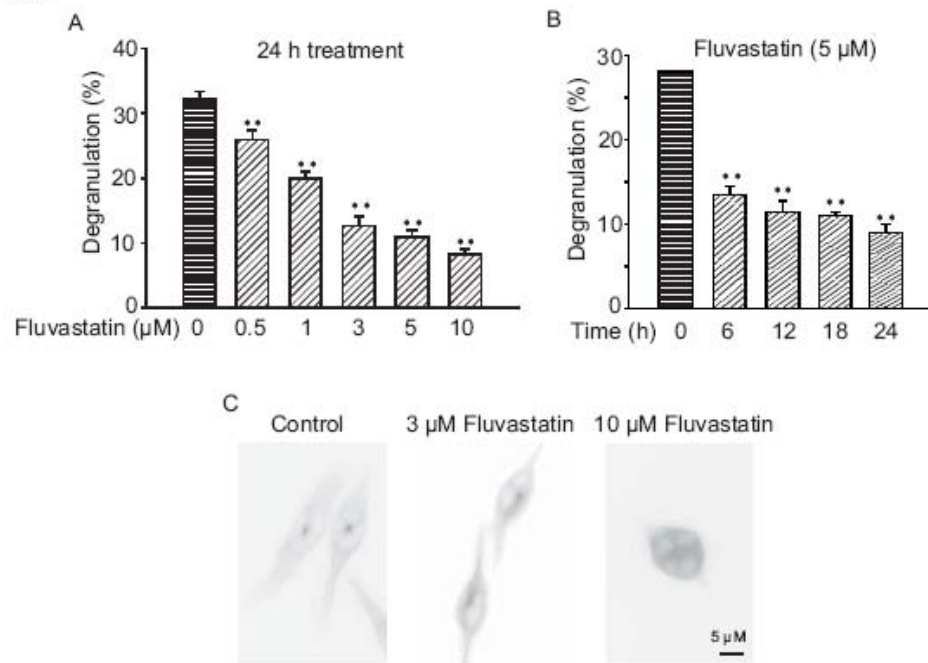


Figure 3

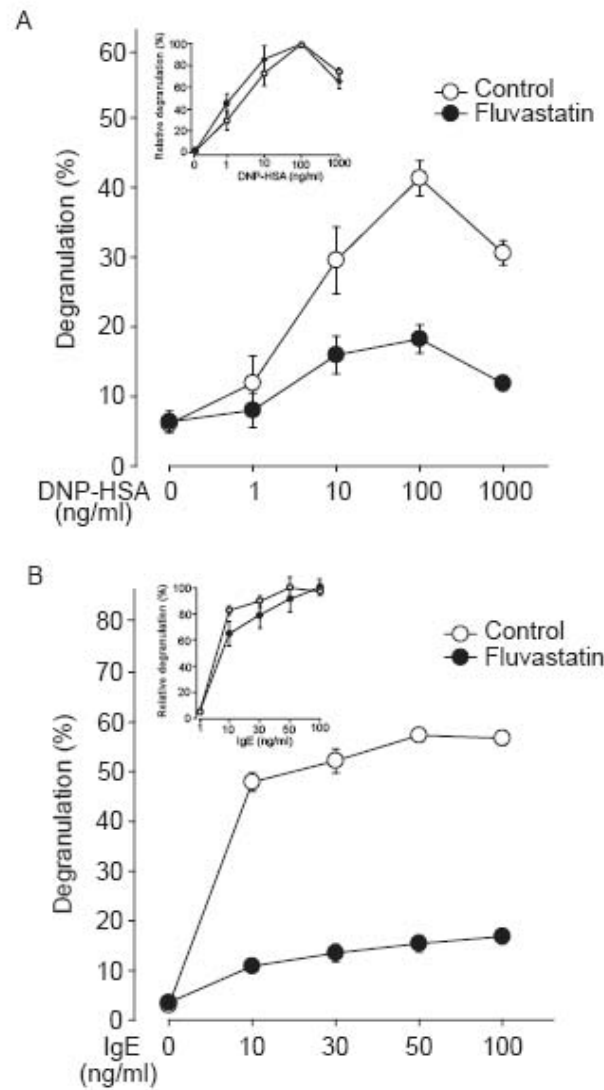


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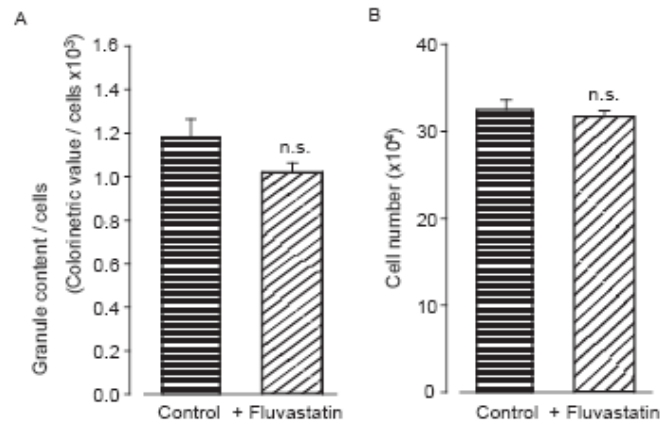


Figure 6

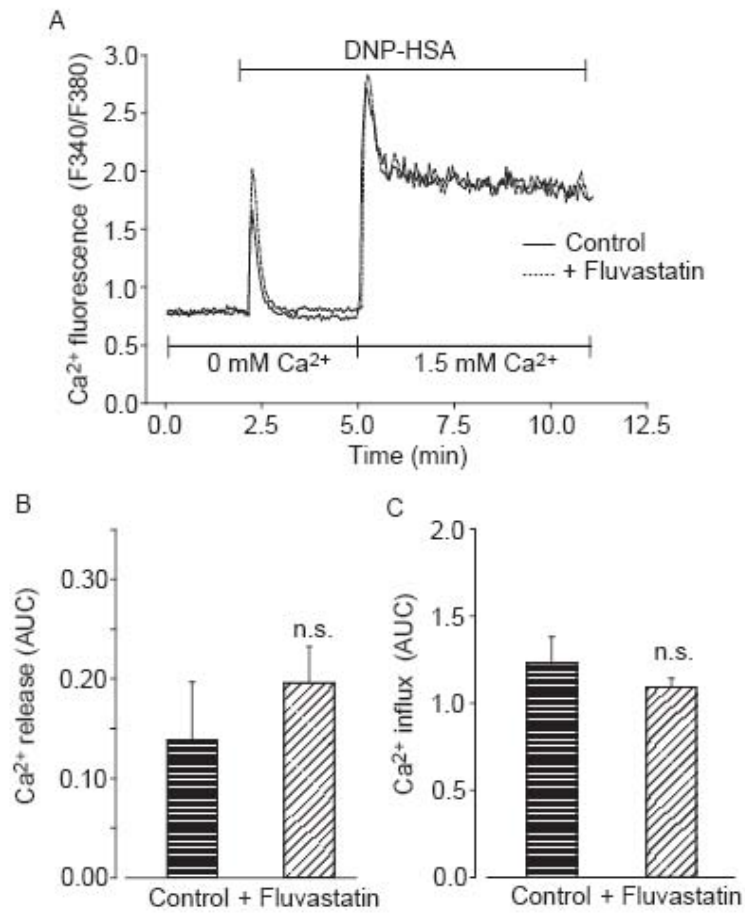


Figure 8

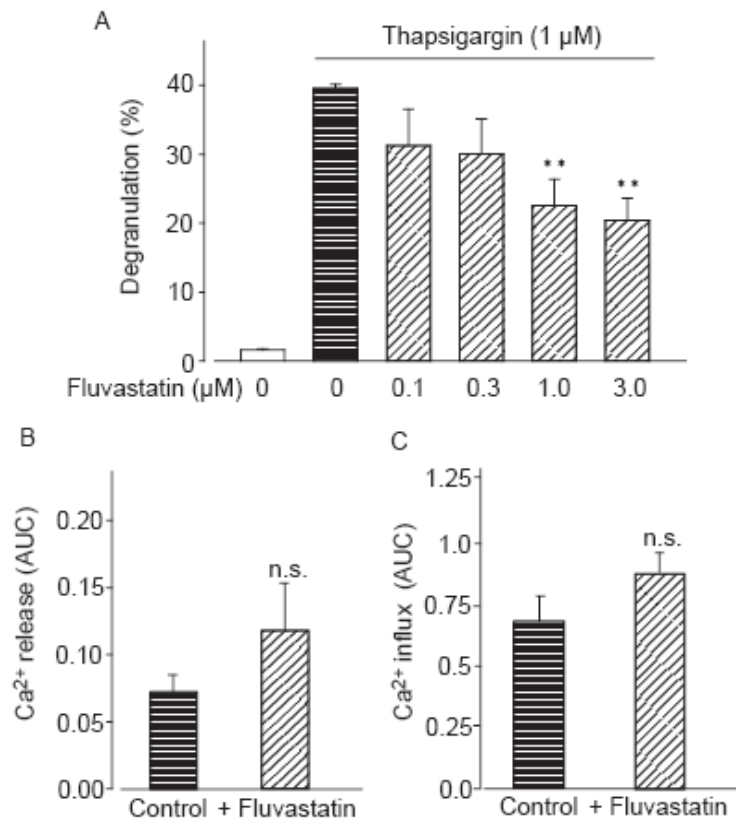


Figure 7

