Effects of Statins on Adipose Tissue Inflammation
Their Inhibitory Effect on MyD88-Independent IRF3/IFN-β Pathway in Macrophages

Manabu Abe, Morihiro Matsuda, Hironori Kobayashi, Yugo Miyata, Yuki Nakayama, Ryutaro Komuro, Atsunori Fukuhara, Iichiro Shimomura

Objectives—Macrophage-mediated chronic inflammation of adipose tissue is causally linked to insulin resistance in obesity. The beneficial effects of 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase inhibitors (statins) on glucose metabolism have been suggested, but the effects of these agents on adipose tissue inflammation are unclear. The aim of the present study is to define the effects of statins on adipose tissue inflammation and macrophages.

Methods and Results—Pravastatin or pitavastatin treatment of obese mice attenuated an increase in mRNA expressions of proinflammatory genes, including MCP1 and IL6, in adipose tissue. The supernatant of TLR4-stimulated RAW264 macrophages strongly induced the expression of these genes in 3T3-L1 adipocytes, which was inhibited by pretreatment of macrophages with either statin. Statins inhibited TLR4-mediated activation of interferon (IFN) regulatory factor (IRF)3 by either lipopolysaccharide (LPS) or palmitic acid, resulting in suppression of IFN-β expression, but not that of NF-κB or JNK. Moreover, statins strongly downregulated TLR3-mediated gene expressions by poly(I:C), but not TLR2-stimulation by zymosan A. Neutralization of IFN-β attenuated proinflammatory activities of the macrophage supernatant.

Conclusions—Statins partially attenuated the development of adipose tissue inflammation in obese mice, which might be associated with an inhibitory effect of statins on TLR4-triggered expression of IFN-β via MyD88-independent signaling pathway in macrophages. (Arterioscler Thromb Vasc Biol. 2008;28:871-877)

Key Words: statin ■ macrophage ■ adipocyte ■ inflammation ■ IFN-β

Statins, inhibitors of HMG-CoA reductase, are lipid-lowering agents that are widely used for treatment of hyperlipidemia. Clinical trials have demonstrated a marked reduction in cardiovascular mortality in patients treated with statins.1,2 Furthermore, recent studies have suggested that the beneficial effects of statins on the vascular wall may extend to mechanisms beyond lipid-lowering,3–6 such as improvement in endothelial function or reduction in proinflammatory actions of macrophages.3 In addition, the beneficial pleiotropic effects of statins on glucose metabolism have been suggested also.8–12 In the West of Scotland Coronary Prevention Study (WOSCOPS), patients on pravastatin had 30% lower incidence of new-onset diabetes than those on placebo.8 In obese rodents as well, we and others have demonstrated that statins suppressed the development of diabetes or increased insulin sensitivity.5,11,12 although other groups showed no or opposite effect for statins on glucose metabolism.13–17 As a potential mechanism of this action, we and others recently showed that pravastatin increased plasma adiponectin, an insulin-sensitizing factor derived from adipocytes, in human samples from WOSCOPS biobank9 and other subjects.10 However, in obese mice, pravastatin did not increase plasma adiponectin levels, whereas normal C57BL/6J mice showed an increase in plasma adiponectin levels after a longer treatment with pravastatin.9 On the other hand, an in vivo glucose transport assay showed that pravastatin upregulated glucose uptake in adipose tissue, but not in the liver or skeletal muscle.9 Insulin-stimulated glucose uptake was enhanced in primary adipocytes, but not in soleus muscles, isolated from pravastatin-treated obese mice.9 These data suggested that adipose tissue is involved in this effect of pravastatin, although the liver plays a major role in the cholesterol-lowering effect of this agent. However, the precise mechanism of this effect on adipose tissue has not been clarified.

Recent research has demonstrated that obesity gives rise to a state of chronic, low-grade inflammation that contributes to insulin resistance and type 2 diabetes.18,19 Macrophages accumulate in adipose tissue with increased body weight and their counts correlate with measures of insulin resistance.20,21 Macrophages in adipose tissue are a major source of proinflammatory cytokines such as tumor necrosis factor (TNF)-α.
that can block insulin action and adiponectin production in adipocytes, providing a potential link between inflammation and insulin resistance. \(^2^1\) Mice with target deletion in the gene for monocyte chemoattractant protein (MCP)-1 receptor, CCR2, exhibit a decrease in macrophage content and inflammation in adipose tissue and protection against high-fat diet–induced insulin resistance. \(^2^2\) Conversely, mice overexpressing MCP-1 have increased numbers of macrophages in adipose tissue, along with increased insulin resistance. \(^2^3,2^4\) These data suggest that adipose tissue macrophages play an important role in the development of obesity-associated insulin resistance. The exact mechanism responsible for activation of adipose tissue macrophages is not known at present. However, previous studies \(^2^5,2^6\) implicated toll-like receptor (TLR)4, a member of TLR family that plays a critical role in the innate immune system by activating proinflammatory signaling pathways. \(^2^7\) TLR4 is a well-characterized receptor that binds to bacterial LPS. \(^2^7\) In addition, recent reports have shown that “endogenous” lipids (saturated free fatty acids) can also activate TLR4, and TLR4 plays an important role in the pathogenesis of lipid-induced inflammation in adipose tissue and insulin resistance. \(^2^5,2^6\)

Statins reduce macrophage accumulation in atherosclerotic plaques and the levels of proinflammatory cytokines such as TNF-α and interleukin 6 (IL-6) in hypercholesterolemic individuals. \(^5,7\) However, the effects of statins on adipose tissue macrophages and inflammatory state in fat are unknown, and may be important in understanding the beneficial effects of statins on glucose metabolism. In the present study, we investigated the effects of statins on adipose tissue inflammation and TLR4-mediated signaling in macrophages.

**Materials and Methods**

Pitavastatin was a kind gift of Kowa Co (Tokyo, Japan). Pravastatin was a kind gift of Daiichi Sankyo Co (Tokyo, Japan). LPS was from Escherichia coli 0111:B4, palmitic acid, poly(I:C), zymosan A and bovine serum albumin (BSA) were purchased from Sigma. Palmitic acid was conjugated with BSA as described previously. \(^2^5\) Antibodies against signal transducer and activator of transcription (STAT)1, phospho-STAT1, Jun N-terminal kinase (JNK), and phospho-INF regulatory factor (IRF)3 were purchased from Cell Signaling Technology, anti-IRF3 antibody from Santa Cruz Biotechnology, and anti-IFN-β antibody from R&D system.

**Animals**

Male 5- to 6-week-old ob/ob mice were purchased from Charles River Labs, Yokohama, Japan. Female 5- to 6-week-old KKAy mice were purchased from CLEA Japan, Inc., Tokyo. All the mice were housed in a room maintained at 23°C with a fixed light/dark cycle. Pravastatin was given as 0.05% food admixture and pitavastatin was given as 0.003% food admixture for 4 weeks. Mice were fed with MF diet (Oriental Yeast). Blood samples from the tail vein were collected in capillary tubes coated with heparin. Blood glucose was measured using the glucose oxidase method (Wako Pure Chemical Industries). Triglyceride and free fatty acids were measured using Triglyceride E-test Wako and NEFA C-test Wako (Wako Pure Chemical Industries). Plasma insulin was measured using an enzyme-linked immunosorbent assay (ELISA) insulin kit (Mornaga). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University, Graduate School of Medicine.

**Cell Culture**

Mouse preadipocyte 3T3-L1 cells were maintained and differentiated into adipocytes as described previously. \(^9\) RAW264 cells were maintained in Minimum Essential Medium (MEM) containing 10% FBS. The cells were treated with statins under serum-free conditions for 24 hours and subsequently stimulated with 1 ng/mL LPS. RAW264-conditioned media were collected and filtered using a syringe filter with 0.45 μm pore. The culture media of RAW264 cells were analyzed for the amount of IL-6 protein using ELISA system.

**RNA Analysis**

Adipose tissue was homogenized in TRIzol reagent (Invitrogen), and total RNA was purified by RNeasy mini kit (Qiagen). Total RNA from cultured cells was purified using RNeasy mini kit. The cDNA was synthesized using Transcriptor cDNA synthesis kit (Roche Diagnostics). Real-time polymerase chain reaction (PCR) was performed on ABI7000 (Applied Biosystems) using the PowerSYBR Green PCR master mix (Roche Diagnostics) according to the protocol provided by the manufacturer. Sequences of primers used for real-time PCR are shown in supplemental Table I (available online at http://atvb.ahajournals.org). The mRNA expression is reported relative to that of 36B4.

**Protein Analysis**

The cell lysates (10 μg protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed with target-specific antibodies. Nuclear factor-kappaB (NF-κB) activity in the cell lysates was determined by EZ-detect p50 assay kit (Endogen).

**Statistical Analysis**

All data were expressed as the mean±SEM. Differences between groups were examined for statistical significance using the Student t test. A probability value less than 0.05 denoted the presence of a statistically significant difference.

**Results**

**Statins Suppress Expression of Inflammatory Genes in Adipose Tissue of Obese Mice**

We administered 0.05% pravastatin or 0.003% pitavastatin as a food admixture to 5- to 6-week-old ob/ob mice for 4 weeks. Food intake or body weight was not altered by either statin treatment (supplemental Table II). After statin treatment, we measured mRNA expressions of several proinflammatory genes in epididymal and subcutaneous adipose tissues. Pravastatin or pitavastatin similarly attenuated the increases of IL-6 and MCP-1 mRNAs in epididymal adipose tissue in ob/ob mice (Figure 1A). Furthermore, these statin treatments showed a significant attenuation of the increase in TNF-α mRNA and a tendency to repress the increases of IL-6 and iNOS mRNAs in subcutaneous adipose tissue in ob/ob mice (Figure 1B). However, neither statin changed the mRNA expressions of F4/80 nor CD68, macrophage-restricted glycoproteins, \(^2^8,2^9\) in either depot of adipose tissue (Figure 1A and 1B), suggesting that the number of macrophages in adipose tissue was not altered by statin treatment. In this study, pravastatin or pitavastatin did not increase adiponectin mRNA in epididymal adipose tissue (Figure 1A) but slightly increased it in subcutaneous adipose tissue (Figure 1B), although the plasma adiponectin levels were not increased by either statin (data not shown), which is consistent with our previous report. \(^9\) Furthermore, pravastatin similarly suppressed inflammatory gene expressions in another obese...
model, KKAy mice (supplemental Figure I, available online at http://atvb.ahajournals.org).

Statins Suppress Macrophage-Mediated Inflammatory Response of Adipocytes

To investigate whether macrophages are involved in the anti-inflammatory effect of statins, we treated 3T3-L1 adipocytes with the conditioned medium (CM) of macrophage cell line RAW264 in the absence or presence of statins. LPS is a well-defined agonist of TLR4,27 a receptor known to mediate the activation of macrophages in obesity.25,26 The CM of LPS-stimulated RAW264 markedly increased mRNA expression levels of proinflammatory genes, such as MCP-1, iNOS, and IL-6, in differentiated 3T3-L1 adipocytes (Figure 2A), although direct treatment of 3T3-L1 adipocytes with LPS showed only a small change (supplemental Figure II). In this assay, pretreatment of RAW264 with pravastatin or pitavastatin significantly suppressed the induction of proinflammatory genes by CM of LPS-stimulated RAW264 in 3T3-L1 adipocytes (Figure 2A). On the other hand, pretreatment of 3T3-L1 adipocytes with either statin did not suppress the induction of proinflammatory genes by CM of LPS-stimulated RAW264 (Figure 2B). These results suggest that statins suppress the macrophage-mediated inflammatory response of adipocytes via primarily targeting macrophages rather than adipocytes.

Next, we examined the effect of statins on the mRNA expression of proinflammatory genes in RAW264. Pravastatin or pitavastatin significantly attenuated LPS-induced expression of proinflammatory genes including MCP-1, iNOS, IL-6, and TNF-α (Figure 3A). The effect of statins on the expression of TNF-α was relatively weak, compared with MCP-1 or IL-6 (Figure 3A). LPS-induced release of IL-6 protein was significantly decreased by statin treatment in parallel with the decrease in its mRNA expression (Figure 3B).

Statins Inhibit MyD88-Independent IRF3/IFN-β Pathway of Macrophages

To elucidate the mechanism of the anti-inflammatory actions of statins, we investigated several kinds of intracellular signaling pathways. LPS induced phosphorylation of STAT1 and JNK, and activation of NF-κB (Figure 4A and 4B). Pretreatment with pravastatin or pitavastatin significantly inhibited the LPS-induced phosphorylation of STAT1 but not that of JNK or the activity of NF-κB (Figure 4A and 4B).
IRF3. On the other hand, stimulation of TLR3 triggers dependent and -independent pathways, and the latter activates phosphorylation of IRF3 (Figure 4E) and mRNA expression of IFN-β, indicating that the inhibitory effect of statins on TLR1 phosphorylation could be mediated by suppression of IFN-β production in RAW264. LPS-induced mRNA expression of proinflammatory genes including MCP-1, iNOS, and IL-6 was significantly decreased by neutralizing IFN-β in RAW264 whereas the effect on TNF-α was relatively small (Figure 5B). Conversely, recombinant IFN-β protein induced these mRNA expressions (supplemental Figure III). These findings indicate that IFN-β actions are involved in the TLR4-stimulated expression of proinflammatory genes, and that the inhibitory effect of statins on IFN-β expression should contribute to the antiinflammatory effects of these agents. Furthermore, neutralization of IFN-β significantly suppressed the proinflammatory activity of the CM of LPS-stimulated RAW264 to increase the expression of MCP-1 and iNOS in 3T3-L1 adipocytes (Figure 5C), suggesting that IFN-β-mediated autoactivation of macrophages could contribute to the macrophage-mediated proinflammatory gene expressions of adipocytes.

**Discussion**

In the present study, we found that statin treatment partially decreased inflammatory gene expressions in adipose tissue of obese mice. Recently, adipose tissue macrophages have been implicated in chronic, low-grade inflammation of adipose tissue in obesity, which is causally linked to insulin resistance. Therefore, we hypothesized that statins might suppress the proinflammatory activity of adipose tissue macrophages. To test this hypothesis, we conducted in vitro experiments in which we treated macrophages with statins and assessed the proinflammatory activity of the macrophage supernatant. The supernatant of TLR4-stimulated macrophages strongly upregulated mRNAs of MCP-1, iNOS, and IL-6 in adipocytes. Statins inhibited these mRNA changes in adipocytes when we pretreated macrophages with statins, but not when we pretreated adipocytes directly with statins, suggesting that macrophages are probably the initial target rather than adipocytes of statins to exert this antiinflammatory effect.

TLRs recognize a variety of microbial components and play a critical role in the innate immune system by activating proinflammatory signaling pathways. TLR4 is a well-characterized receptor that binds to bacterial LPS. In addition, recent reports have shown that “endogenous” lipids (a number of saturated free fatty acids) can also activate TLR4, and that TLR4 plays an important role in the pathogenesis of lipid-induced inflammation in adipose tissue and insulin resistance.

On binding of these ligands to TLR4, a
Toll/IL-1 receptor (TIR) domain-containing adaptor molecule, MyD88, is recruited to the receptor, leading to activation of downstream signaling cascades, including JNK and NF-κB pathway. In addition to this MyD88-dependent pathway, TLR4 uses a MyD88-independent signaling pathway. In this pathway, TIR domain-containing adaptor inducing IFN-β (TRIF) plays an essential role in activating downstream signaling cascades on TLR4 stimulation, leading to the activation of IRF3 which transactivates IFN-β promoter, and the late phase activation of NF-κB. In the present study, we demonstrated that statins strongly inhibited TLR4-mediated activation of IRF3, resulting in suppression of IFN-β expression and subsequent autocrine/paracrine action of IFN-β including STAT1 phosphorylation and induction of proinflammatory genes in macrophages. Moreover, statins strongly downregulated TLR3-mediated inflammatory gene expressions by poly(I:C), but not TLR2-mediated inflammatory gene expressions by zymosan A. Stimulation of TLR3 triggers downstream signals using TRIF-mediated MyD88-independent pathway but not MyD88-dependent pathway, leading to the activation of IFN-β promoter, and in contrast, TLR2 uses only MyD88-dependent pathway. Taken together, these results suggest that statins inhibit TRIF-mediated MyD88-independent pathway but not MyD88-dependent pathway. However, as the in vivo IFN-β mRNA levels were not high enough to quantify accurately in adipose tissue, questions still remain as to whether IFN-β is involved in the pathogenesis of obesity-associated diseases and whether statins suppress IFN-β expression in the adipose tissue of obese mice.

Our data indicated that statins inhibited the phosphorylation of IRF3 and the following IFN-β expression in macrophages. IRF3 induces not only the transcription of IFN-β but also that of IFNα. In our study, statins suppressed the LPS-induced mRNA expression of IFNα as well (supplemental Figure IV). IFNα and IFN-β share much homology and transduce signals through the same receptor complex with

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**Figure 4.** Pitavastatin (pit) or pravastatin (pra)-pretreated RAW264 were stimulated with LPS (A–D), palmitic acid (C16) (E, F), poly(I:C) (G), or zymosan A (H). Phosphorylated and total STAT1, JNK, IRF3 proteins (A, D, E), NF-κB activities (B), mRNAs of the indicated genes (C, F–H). *P<0.05, #P<0.01 vs without statin. (For detailed figure legend, please see http://atvb.ahajournals.org).
similar effects.\textsuperscript{37} Indeed, statins strongly reduced MCP-1, iNOS, IL-6 mRNA levels in macrophages, but neutralization of IFN-\(\beta\) or the addition of recombinant IFN-\(\beta\) had no such strong effect. These data suggest that the effect on IFN\(\alpha\) might explain, at least in part, the inconsistency in the anti-inflammatory effects between statins and neutralization of IFN-\(\beta\), although further analyses would be required to confirm this conjecture.

Several studies have suggested that the pleiotropic effects of statins mediate the inhibition of HMG CoA reductase and subsequent inhibition of isoprenylation of crucial signaling molecules.\textsuperscript{4} However, we could not conclude whether mevalonate itself strongly inhibited LPS-induced expression of IFN-\(\beta\). Further studies are required to elucidate the precise mechanism.

In our in vivo study, pravastatin or pitavastatin decreased triglyceride or free fatty acids in the plasma of ob/ob mice (supplemental Table II). Recently, Jula et al reported that simvastatin decreased proportions from the total fatty acids of palmitic acid by 2.0\%, and increased the sum of long-chain polyunsaturated fatty acids (PUFAs) by 9.0\% in the serum of hypercholesterolemic men.\textsuperscript{38} It has been reported that palmitic acid is a potential TLR4 ligand and that PUFAs antagonize this effect.\textsuperscript{39,40} According to these data, there is a possibility that statin-induced changes in the fatty acid composition might also be involved in the suppression of inflammatory gene expressions. However, such suppression of inflammatory gene expressions was observed in adipose tissue, but not in the liver or skeletal muscle (supplemental Figure V). In obese model mice, inflammation is exaggerated mainly in adipose tissue, not in liver nor skeletal muscle\textsuperscript{21} (supplemental Figure V), which may partly explain why the anti-inflammatory effects of statins are relatively apparent in adipose tissue. Moreover, in the previous study, pravastatin enhanced insulin-induced glucose uptake only in adipose tissue, but not in the liver or skeletal muscle.\textsuperscript{9} These findings suggest that this antiinflammatory effect of statin in adipose tissue may be locally linked to its effect on glucose metabolism.

In summary, the present study showed that statins inhibited MyD88-independent IRF3/IFN-\(\beta\) pathway in macrophages. Inhibition of TLR4-mediated IFN-\(\beta\) expression by statins might be associated with the attenuation of chronic inflammation of adipose tissue, which could be linked to the suppression of the progression of insulin resistance in obese mice, although further in vivo analyses would be required to confirm these conjectures.

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**Disclosures**

M.A. is an employee of Daiichi-Sankyo Co. and was a visiting investigator in Osaka University when studies in this article were performed.

**References**


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### Supplemental Table I. Sequences of primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
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| IL-6   | 5’-ACAACCACGGCCTTCCTACTT-3’  
         | 5’-CACGATTTCCCCAGAGAACATGTG-3’ |
| MCP-1  | 5’-CCACTCACCCTGCTGCTACTCAT-3’  
         | 5’-TGTTGATCCTCTTTGTAGCTCTCC-3’ |
| iNOS   | 5’- CAGCTGGGCTGTACAAACCTT-3’  
         | 5’-CATTGGAAGTGAAGCGTTTCG-3’ |
| F4/80  | 5’-GCCTGGACACTGTGGGTTTCT-3’  
         | 5’-TGGGCAATGGCCTTTGAAG-3’ |
| TNF-α  | 5’-TGTGCTCAGAGCTTTCAACAAC-3’  
         | 5’-GCCCATTTGAGTCCTTGATG-3’ |
| IFNβ   | 5’-AGCAGCTGAATGGAAAGATCAAC-3’  
         | 5’-GGATGGCAAAGGCAGTGAAC-3’ |
| 36B4   | 5’-GCTCCAAGCAGATGCAGCA-3’  
         | 5’-CCGGATATGAGGCAGCAG-3’ |
**Supplemental Table II.** Characteristics of ob/ob mice presented in Figure. 1

<table>
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<th>Control</th>
<th>Prava 0.05%</th>
<th>Pitava 0.003%</th>
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<td>Final Body Weight (g)</td>
<td>53.6±0.79</td>
<td>51.2±0.43</td>
<td>52.0±0.59</td>
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<td>Body Weight Gain (g)</td>
<td>13.3±0.51</td>
<td>12.1±0.26</td>
<td>12.8±0.21</td>
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<td>Food intake (mg/g BW/day)</td>
<td>73.1±0.11</td>
<td>71.6±0.06</td>
<td>73.9±0.08</td>
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<td>Plasma Glucose (mg/dl)</td>
<td>176.6±11.4</td>
<td>157.3±6.6</td>
<td>156.5±11.8</td>
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<tr>
<td>Plasma insulin (ng/ml)</td>
<td>15.9±1.65</td>
<td>19.4±2.78</td>
<td>15.3±0.75</td>
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<tr>
<td>Plasma Total Cholesterol (mg/dl)</td>
<td>203.0±4.53</td>
<td>215.4±5.85</td>
<td>183.4±8.17</td>
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<tr>
<td>Plasma Triglyceride (mg/dl)</td>
<td>95.3±4.11</td>
<td>68.8±2.36*</td>
<td>67.3±2.77*</td>
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<tr>
<td>Plasma FFA (mEq/l)</td>
<td>1.37±0.04</td>
<td>1.26±0.07</td>
<td>1.16±0.07*</td>
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</tbody>
</table>

Data are mean ± SEM. *; p<0.001, #; p<0.05
Supplemental Figure I

Female 5-week old KKAy mice were treated with control (n=5) or 0.05% pravastatin (prava, n=4) as a food mixture for 4 weeks. mRNA expression levels of indicated genes were quantified by real-time RT-PCR in parametrial adipose tissue. Results are presented as % of control. Values are mean ± SEM. #p<0.01, *p<0.05 vs. control.
Differentiated 3T3-L1 adipocytes were treated with the conditioned medium (CM) of LPS (1 ng/ml)-treated RAW264 cells, or LPS alone by the same dose of remaining LPS in CM of RAW264 cells, and measured mRNA expression of MCP-1 by real time RT-PCR as described in Methods. MCP-1 mRNA was normalized by that of 36B4. The data are expressed as fold change compared to Non-treat group. Values are mean ± SEM.
Supplemental Figure III

RAW264 cells were treated with 100 ng/ml recombinant human IFNβ for 8 hours. mRNA expressions of indicated genes were quantified by real-time RT-PCR as described in Methods. White bar=non-treated, black bar=IFNβ-treated. mRNAs of indicated genes were normalized by that of 36B4. The data are expressed as fold change compared to non-treated group. Values are mean ± SEM. *: p<0.05, #: p<0.01 vs. non-treated.
Supplemental Figure IV

RAW264 cells were treated with vehicle, 100 μM pravastatin (prava) or 1 μM pitavastatin (pitava) for 24 hours and subsequently stimulated by vehicle or 1 ng/ml LPS for 8 hours. mRNA expressions of IFNα were quantified by real-time RT-PCR as described in Methods, using the following primers; 5'-GCTAGGCTCTGTGCTTTCCTGAT-3' and 5'-AGGTCACATCCTAGAGAGCAGGTT-3'. White bar=non-treated, black bar=LPS-treated. mRNAs of indicated genes were normalized by that of 36B4. The data are expressed as fold change compared to non-treated group. Values are mean ± SEM.
Supplemental Figure V

Male 5~6 weeks old ob/ob mice were treated with pravastatin as a 0.05% food admixture or pitavastatin as a 0.003% food admixture for 4 weeks. (A,B) the mRNA expression levels of IL-6 and MCP-1 were quantified by real-time RT-PCR in epididymal adipose tissue, liver and skeletal muscle. A, Comparison of mRNA expression levels among adipose tissue, liver and skeletal muscle in C57BL/6J (lean, n=6) and ob/ob mice (ob, n=6). B, Effects of pravastatin (prava) and pitavastatin (pitava) on mRNA expressions of these genes in skeletal muscle and liver in ob/ob mice (ob+prava, n=6; ob+pitava, n=6). Results are presented as % change compared to control (contr, n=6). Values are mean ± SEM.
Supplemental Materials

Detailed Figure Legends

Figure 1. Effects of pravastatin or pitavastatin on adipose tissue in ob/ob mice.
Male 5~6 weeks old ob/ob mice were treated with pravastatin as a 0.05% food admixture or pitavastatin as a 0.003% food admixture for 4 weeks. The mRNA expression levels of the indicated genes were quantified by real-time RT-PCR in epididymal adipose tissue (A) and subcutaneous adipose tissue (B) of C57BL/6J (lean, n=6), ob/ob (ob, n=6), and ob/ob mice treated with pravastatin (ob+prava, n=6) or pitavastatin (ob+pitava, n=6). Results are presented as relative mRNA levels (%) compared to lean (adiponectin), or ob (other genes). * p<0.05, # p<0.01 vs. ob. Values are mean ± SEM.

Figure 2. Statins suppress macrophage-mediated inflammatory response of adipocytes.
A, RAW264 cells were treated with vehicle, 100 μM pravastatin (pra) or 1 μM pitavastatin (pit) for 24 hours and subsequently stimulated by vehicle or 1 ng/ml LPS for 8 hours. Differentiated 3T3-L1 cells were cultured in the conditioned media (CM) of RAW264 for 24 hours. B, Differentiated 3T3-L1 adipocytes were treated with 100 μM pravastatin (pra) or 1 μM pitavastatin (pit) for 24 hours and subsequently cultured in the CM of LPS-stimulated RAW264 cells for 8 hours. mRNA expression levels of the indicated genes in 3T3-L1 cells were quantified by real-time RT-PCR. Results are presented as
relative mRNA change (%) compared with CM of LPS-stimulated RAW264 without statin-pretreatment (CM_LPS) (* p<0.01). Values are mean ± SEM (n=3).

**Figure 3. Statins repress proinflammatory activity of RAW264 cells.**
A, RAW264 cells were treated under the same conditions as Figure 2A. mRNA expression levels of the indicated genes in RAW264 cells were quantified by real-time RT-PCR. Results are presented as relative mRNA change (%) compared with LPS alone. B, IL-6 protein in the CM of RAW264 cells was quantified using ELISA system. Values are mean ± SEM (n=3). * p<0.01 vs. LPS alone.

**Figure 4. Statins repress MyD88-independent signaling pathway in RAW264 cells.**
RAW264 cells were pre-treated with vehicle, 1 μM pitavastatin (pit) or 100 μM pravastatin (pra) for 24 hours. The cells were then stimulated by 1 ng/ml LPS for the indicated time period (A,D) or 8 hours (B,C), by 500 μM palmitic acid (C16) for 8 hours (E,F), by 100 μg/ml poly(I:C) for 8 hours (G), or by 20 μg/ml zymosan A for 8 hours (H). A, The cell lysates were subjected to SDS-PAGE, and then immunoblotted with antibodies against phospho- and total STAT1, and phospho- and total JNK. B, NF-κB activities in the cell lysates were determined. C, IFNβ mRNA expression in RAW264 was quantified by real-time RT-PCR. D,E, The cell lysates were immunoblotted with antibodies against phospho- and total IRF3. F,G,H, mRNA expression levels of the indicated genes were quantified by real-time RT-PCR. Results are presented as relative mRNA change (%) compared with LPS alone (C), C16 alone (F), poly(I:C) alone (G), or zymosan A alone (H) (* p<0.05, # p<0.01). Values are mean ± SEM (n=3).
Figure 5. Effects of neutralization of IFNβ on RAW264 and 3T3-L1 cells.

RAW264 cells were incubated with 1 ng/ml LPS in the presence of a neutralizing antibody against IFNβ or non-immune rabbit IgG for 8 hours. A, The cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies against phospho-and total STAT1. B, mRNA expression levels of the indicated genes were quantified by real-time RT-PCR (n=3 or 6 from three experiments). C, Differentiated 3T3-L1 adipocytes were cultured in the conditioned media of RAW264 cells (RAW_CM) prepared under the indicated conditions for 24 hours. mRNA expression levels of MCP-1 and iNOS in 3T3-L1 cells were quantified by real-time RT-PCR (n=6). B,C, Results are presented as relative mRNA change (%) compared with LPS alone (* p<0.05, # p<0.01). Values are mean ± SEM.