

**Figure 4.** solLR11 enhances adhesion and migration of THP-1 macrophages. **A**, THP-1 macrophages were preincubated with 1  $\mu$ g/mL solLR11 in the presence or absence of neutralizing anti-uPAR antibodies. The cells were washed and then incubated on collagen- or fibronectin-coated plates. **B**, THP-1 macrophages were preincubated with 1  $\mu$ g/mL solLR11 in the presence or absence of neutralizing anti-uPAR, anti-LR11 (5-4-30-19-2, 1:2 dilution), anti-VLA-4 or anti-PSGL-1 antibody. The cells were washed and then incubated in the cultured SMCs. **C**, THP-1 monocytes were treated with 200 nM PMA in the presence or absence of 1  $\mu$ g/mL solLR11. Membranes were subjected to Western blotting with anti-VLA-4 antibody. **D**, Cells were preincubated with 1  $\mu$ g/mL solLR11 in the presence or absence of neutralizing anti-LR11 (5-4-30-19-2, 1:2 dilution) and anti-uPAR antibodies. The cells were washed, and then the PDGF-induced cell migration was measured using a micro-Boyden chamber. Data are expressed as mean  $\pm$  SD,  $n=4$  to 6 (\* $P<0.05$ , \*\* $P<0.01$ ).

bound form, solLR11's expression at an earlier stage is likely higher than that at late stages, because solLR11 was specifically expressed in rapidly proliferating SMCs in culture.<sup>11</sup> The macrophage infiltration into the intima and lipid accumulation was greatly decreased in LR11 knockout mice compared with those in wild-type mice (Figure 2). Because the expression of LR11 was barely detectable in monocytes/macrophages, we hypothesize that the soluble form of LR11 from intimal SMCs affects macrophage functions that facilitate progression of atherosclerosis, especially in early neointimal formation. With the preparation of recombinant solLR11, we were able to obtain experimental support for our above hypothesis concerning the role of solLR11 in macrophage function.

Macrophages express a variety of scavenger receptors which are involved in uptake of modified LDL and atherogenesis.<sup>2,26,27</sup> SR-A is highly expressed almost exclusively in differentiated macrophages, and is implicated in increased foam cell formation in atherogenesis.<sup>28,29</sup> We showed that solLR11 enhanced SR-A expression and DiI-AcLDL accumulation in THP-1 macrophages in vitro, suggesting a possible role of solLR11 in the formation of lipid-rich plaques. Furthermore, solLR11 significantly enhanced monocyte adhesion not only to extracellular matrices but also to the cultured SMCs in vitro. Increased adhesion and infiltration of circulating monocytes is believed to be the key event in early

stage of atherogenesis. Furthermore, the direct association between monocytes and SMCs is implicated in the prolonged retention of monocytes in atherosclerosis, and increases matrix metalloproteinase-1 production, possibly leading to the formation of unstable plaque.<sup>30</sup> Monocyte adhesion to SMCs is mediated, eg, by vascular cell adhesion molecule-1, and immunohistochemical analysis showed the abundant expression of vascular cell adhesion molecule-1 in SMCs in human atherosclerotic lesions.<sup>31</sup> PDGF-BB and angiotensin II are implicated in the enhanced binding of monocytes to cultured SMCs. Thus, solLR11 is probably involved in monocyte accumulation at activated areas in plaques at which SMCs actively migrate and proliferate, and prolongs on-site retention of macrophages.

solLR11 increased cell-surface uPAR levels in THP-1 monocytes/macrophages. Moreover, solLR11-enhanced lipid uptake, adhesion, and migration of THP-1 macrophages were almost completely blocked by neutralizing anti-uPAR as well as anti-LR11 antibodies. The increased expression of uPAR on monocytes/macrophages is implicated in the adhesion, differentiation, and increased metalloproteinase expression in the cells. Moreover, uPAR expression is increased in circulating monocytes in patients with acute myocardial infarction compared with that in patients with chronic stable angina.<sup>17</sup> LR11 upregulates cell surface uPAR levels in SMCs by inhibition of its catabolism, which is mediated by LRP1,

another member of the LDLR family.<sup>11</sup> LRP1 is also abundantly expressed in monocytes/macrophages;<sup>8</sup> hence, it is likely that LR11 regulates macrophage differentiation and lipid accumulation in plaques by increasing uPAR levels in monocytes/macrophages.

In summary, SMCs and macrophages coexist in plaques throughout the progressive stage of atherogenesis. SolLR11, which is secreted from activated SMCs in the intima, likely is a coregulator of scavenger receptor expression, lipid accumulation, adhesion, and migration of monocytes/macrophages at an early stage of neointimal formation. The uPAR-mediated effects were observed at the same concentration range (0.1 to 10  $\mu\text{g}/\text{mL}$ ) of recombinant solLR11 in cultured macrophages as that required for the migration of SMCs (Figure 1D). Although the pathophysiological concentrations of solLR11 in intima is difficult to determine, the increase in levels of intimal solLR11 in injured arteries, and the loss of infiltrated macrophages in LR11-KO mice strongly suggest that intimal SMCs locally secrete sufficient amounts of solLR11. Nevertheless, the elucidation of the significance of interactions of SMCs and macrophages involving solLR11 requires further analyses using various models for atherosclerosis. Clearly, the regulation of solLR11 function in the arterial wall is a promising target not only for such studies but also for therapeutic amelioration of atherosclerosis with unstable plaque.

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#### Disclosures

None.

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## Low-dose GH supplementation reduces the TLR2 and TNF- $\alpha$ expressions in visceral fat

Yoshitaka Kubota<sup>a</sup>, Hiroyuki Unoki<sup>b</sup>, Hideaki Bujo<sup>c,\*</sup>, Naoaki Rikihisa<sup>a</sup>, Akikazu Udagawa<sup>a</sup>, Shinya Yoshimoto<sup>a</sup>, Masaharu Ichinose<sup>a</sup>, Yasushi Saito<sup>d</sup>

<sup>a</sup> Department of Plastic and Reconstructive Surgery, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

<sup>b</sup> Department of Applied Translational Research, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

<sup>c</sup> Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

<sup>d</sup> Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

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### Abstract

The increased population of TLR2/TNF- $\alpha$  co-expressing adipocytes is associated with the development of insulin resistance. We have herein shown the significance of low-dose growth hormone (GH) supplementation for the regulation of TLR2 and TNF- $\alpha$  expressions in visceral fat using different kinds of mouse models fed with a high-fat diet. Low-dose GH supplementation reduced the increased population of TLR2/TNF- $\alpha$  co-expressing adipocytes in high-fat fed mice. The neutralization of IGF-1 abolished the effect of GH supplementation on the TLR2 expression using GH-overexpressing mice. IGF-1, but not GH, inhibited the FFA-induced TLR2 and TNF- $\alpha$  expression in 3T3-L1 cells. Finally, low-dose GH supplementation reduced the TLR2 expression without an obvious change in the visceral fat volume in ob/ob mice. These results indicate that low-dose GH supplementation possibly inhibits the high-fat induced change of the adipocytes to TLR2/TNF- $\alpha$  co-expressing cells through the action of IGF-1.

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A dysfunction of adipocytes leads to an accumulation of metabolic abnormalities, such as dyslipidemia, hypertension, and glucose intolerance [1]. This functional abnormality is characterized by a disturbance in the cytokine expressions of adipocytes, causing the development of insulin resistance, a pathogenesis of the metabolic syndrome [2]. However, the regulation of cytokine secretion from adipocytes accumulated in visceral regions has not yet been fully elucidated.

We have shown that cultured adipocytes implanted in mesenteric, but not in subcutaneous, regions induce tumor necrosis factor (TNF)- $\alpha$  secretion in mice [3]. The TNF- $\alpha$  expression of visceral adipocytes is accompanied with

toll-like receptor (TLR) 2 expression, and the population of TLR2/TNF- $\alpha$  co-expressing adipocytes is drastically induced in mice fed a high-fat diet [4]. These observations suggest that the identification of the regulator(s) for the occurrence of TLR2/TNF- $\alpha$  co-expressing adipocytes may provide a target for the amelioration of insulin resistance in the metabolic syndrome.

Insulin resistance accompanied with visceral fat accumulation is not only observed in the metabolic syndrome, but also in several hormonal disturbances. One such hormonal disturbance is growth hormone (GH) deficiency, which is frequently accompanied by reduced insulin sensitivity and accumulated visceral fat. Recent studies have shown the low-dose supplementation of GH to have a beneficial effect on the treatment of insulin resistance accompanied by aging and/or abdominal obesity, as well as GH deficiency itself. [5–7]. These studies have provided a novel

\* Corresponding author. Fax: +81 43 226 2095.

E-mail address: [hbujo@faculty.chiba-u.jp](mailto:hbujo@faculty.chiba-u.jp) (H. Bujo).

therapeutic possibility for GH in the regulation of insulin sensitivity. On the other hand, the beneficial effect of GH raises a complicated issue to be solved, specifically the development of acromegaly-associated glucose intolerance classically observed in association with GH excess. The obvious difference in the opposite effects of GH on the regulation of insulin sensitivity seems to be largely a function of the plasma GH concentration; low dose GH supplementation may be of benefit for GH deficiency and abdominal obesity, whereas excessive GH production results in pathological acromegaly. The aim of this study is to clarify the effect of low-dose GH supplementation on insulin resistance, particularly through the regulation of TLR2/TNF- $\alpha$  co-expressing adipocytes using cultured adipocytes and animal models of visceral fat accumulation.

## Materials and methods

**Mice and blood samples.** Mice were obtained from Charles River Japan. All work was carried out according to the guidelines of the Animal Care Committees of Chiba University. The levels of plasma human growth hormone (Roche), mouse insulin-like growth factor (IGF)-1 (R&D Systems), and mouse insulin (Morinaga) were measured using ELISA kits. Insulin tolerance test was performed by intra-peritoneal injection of human insulin (Sigma–Aldrich) 0.5 or 2.0 U/kg body weight according to the mice models [8].

**Cell culture.** 3T3-L1 cells were from the American Type Culture Collection. The differentiation of preadipocytes to mature adipocytes was as described [8]. Cells were treated with DMEM supplemented with  $10^{-8}$  M hGH (Novo Nordisk Pharma) or  $10^{-8}$  M human IGF-1 (Jena Bioscience), with 2% free fatty acids (FFA)-free BSA (Sigma–Aldrich) overnight, and then with a fatty acid mixture composed of 500  $\mu$ M myristic acid and 500  $\mu$ M palmitic acid, with  $10^{-8}$  M hGH or  $10^{-8}$  M hIGF-1 in the presence of 2% FFA-free BSA at 37 °C for 8 h.

**Implantation of 3T3-L1 cells overexpressing hGH into BALB/c nude mice.** Human GH cDNA full clone was obtained by polymerase chain reaction (PCR) using human brain-derived cDNA pool using oligonucleotide primers specific for parts of human GH sequence (5'-GACGGC GATCGCCATGGGCTACAGGCTCCCGAC-3' and 5'-ATGCGT TTAACGAAGCCACAGCTGCCCTCCAC-3'). The cDNA fragment subcloned into pcDNA3.1/Hygro(-), were transfected into 3T3-L1 cells using GeneJammer Transfection Reagent (Stratagene). The cells stably expressing hGH and mock transfected cells by the transfection of pcDNA3.1/Hygro(-) without hGH cDNA were cloned as described [9]. The hGH production in the conditioned medium of 3T3-L1 cells overexpressing hGH was  $8.8 \pm 0.9$  ng/ $10^6$  cells/24 h, whereas it was not detectable in conditioned medium of the mock cells. 3T3-L1 cells overexpressing hGH, or the mock cells were suspended at  $4 \times 10^6$  cells/250  $\mu$ l in Matrigel (BD Bioscience) and injected subcutaneously in the back of male BALB/c nude mice (6-week old) as described [10]. The mice were fed with high fat diet from a week after the implantation. Insulin tolerance test were performed by using human insulin (Sigma–Aldrich) 0.5 U/kg, i.p. after fasting for more than 16 h.

**Isolation of single adipocytes and flow cytometry.** Mesenteric fat tissues or 3T3-L1 adipocytes were collected and digested at 37 °C for 60 min with 1 mg/ml type I collagenase (Nitta Gelatin). The digested tissue was centrifuged at 400 rpm for 4 min. The floating adipocyte fraction was prepared for flow cytometry analysis. Isolated adipocytes ( $1 \times 10^6$  cells) were analyzed with FACS Calibur flow cytometer (BD Bioscience) as described [4].

**Anti-IGF-1 antibody treatment in mice.** Goat polyclonal antibody against mouse IGF-1 (R&D Systems) or normal goat IgG (R&D Systems) was injected i.p. (0.1  $\mu$ g/g body weight) into male BALB/c nude mice (6-week old) at weekly intervals starting on the day of the implantation of the

established 3T3-L1 cells overexpressing hGH or the mock cells. The mice were started to be fed with high fat diet from a week after the implantation. At 4 weeks after the cell implantation, insulin tolerance test was performed by using human insulin 0.5 U/kg, i.p.

**RT-PCR.** Quantitative RT-PCR amplifications were performed using TaqMan Gene Expression Master Mix (Applied Biosystems) as described [8]. For TLR2 and TNF- $\alpha$  mRNA quantification, Real-time RT-PCR amplification were performed using TLR2 primers (Mm00442346\_m1, Applied Biosystems) and TNF- $\alpha$  primers (MA031450, Sigma Genosys). The quantification of given gene, expressed as relative mRNA level compared with a control, was calculated after normalization to 18s rRNA. All PCRs were performed in an ABI PRISM 7000 sequence system (PE Applied Biosystems.) [11].

**Fat volume measurement by computed tomography (CT).** From 12 weeks of age, male ob/ob mice were administered either hGH (0.5 mg/kg body weight/day) or equivalent volume of saline via mini-osmotic pumps for 4 weeks. There was no significant difference in body weight between the mice administered hGH and the mice administered PBS. The plasma hGH level was  $881 \pm 643$  pg/ml in the mice administered hGH. After fasting for overnight, abdominal CT was performed using GE Healthcare eXplore Locus MicroCT Scanner (GE Healthcare). Visceral and subcutaneous fat volume was calculated using GE Healthcare eXplore Locus Microview Software (ver 2.2) (GE Healthcare).

**Statistical analysis.** The results are shown as means  $\pm$  SD for each index. Statistical significance was determined by means of the Student's *t*-test or Dunnett's multiple range test followed by ANOVA among several groups. Statistical analyses were conducted by using SPSS software (version 13.0J; SPSS Inc.). All *P* values quoted are two-tailed. A *P*-value of  $<0.05$  was considered statistically significant.

## Results

### Low-dose GH supplementation reduces the number of TLR2/TNF- $\alpha$ co-expressing adipocytes in visceral fat

We have previously shown that high fat intake induces an increased number of TLR2/TNF- $\alpha$ -coexpressing adipocytes in mesenteric fat in mice [4]. In order to clarify the effect of low-dose GH supplementation on the increase in the population of TLR2/TNF- $\alpha$  co-expressing cells in the adipocytes of mesenteric fat, we performed a flow cytometry analysis of single adipocytes prepared from the mesenteric fat of high-fat fed mice after hGH administration for 2 weeks. There was no significant difference in body weight between the mice administered hGH (GH group) and the mice administered PBS (control group). The plasma hGH concentration in the GH group was  $160 \pm 86$  pg/ml, which is similar to the GH concentrations in previous studies using low dose GH supplementation [12] (Fig. 1A). The plasma IGF-1 concentrations were higher in the GH group in comparison to those in the control group (Fig. 1B). The blood glucose levels 30 min after insulin loading were decreased in the GH group in comparison to the control group (Fig. 1C). The TLR2 mRNA expression levels in mesenteric fat were significantly decreased in the GH group in comparison to those in the control group, suggesting the inhibitory effect of low-dose GH supplementation on TLR2 expression in visceral adipocytes (Fig. 1D). A flow cytometry analysis of single adipocytes prepared from mesenteric fat showed that the high-fat-induced increase in the population of TLR2/TNF- $\alpha$  co-expressing adipocytes was

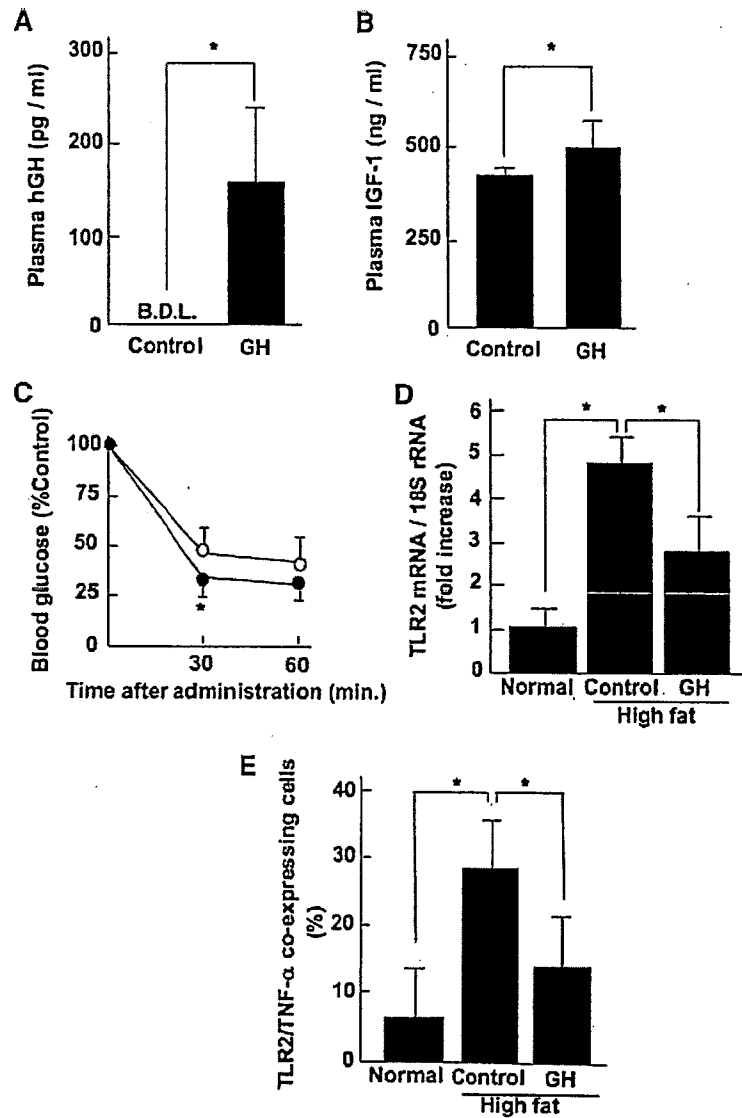


Fig. 1. The effects of low-dose hGH supplementation on reduced insulin sensitivity in high-fat fed mice. (A) The plasma hGH concentration in mice supplemented with hGH (GH) or PBS (control). Male C57BL/6J mice, which have been fed with a high-fat diet (60% fat) for 6 months, were supplemented with hGH (0.05 mg/kg/day) or PBS for 2 weeks. B.D.L., below the detection limit (less than 4 pg/ml).  $n = 8$ . \* $P < 0.05$  compared to the value of control. (B) The plasma IGF-1 levels in the mice supplemented with hGH (GH) or PBS (control).  $n = 8$ . \* $P < 0.05$  compared to the value of control. (C) Insulin tolerance test in the mice supplemented with hGH (●) or PBS alone (○). The blood glucose levels were monitored at 0, 30, and 60 min after injection of human insulin.  $n = 8$ . \* $P < 0.05$  compared to the value of the control. (D) The TLR2 mRNA expression in mesenteric fat tissues of the mice supplemented with hGH (GH) or PBS (control). Normal, mice fed with a normal diet. High fat, mice fed with a high-fat diet.  $n = 8$ . \* $P < 0.05$  compared to the value of the control. (E) Flow cytometric analyses of TLR2/TNF- $\alpha$  co-expressing adipocytes in the fat tissues of mice supplemented with hGH (GH) or PBS (control). Single adipocytes were prepared from mesenteric fat, and analyzed by FACS Calibur. The averaged populations of TLR2/TNF- $\alpha$  co-expressing adipocytes in the total cells (50,000 cells) were expressed ( $n = 8$ ).

significantly and largely inhibited in the GH group in comparison to that in the control group (Fig. 1E). These results strongly suggest that low-dose GH supplementation reduces the increase in the population of TLR2/TNF- $\alpha$  co-expressing adipocytes in mesenteric fat, as well as reducing insulin resistance, in mice fed a high-fat diet.

*Neutralization of IGF-1 abolishes the effect of low-dose GH supplementation on the decrease in the number of TLR2/TNF- $\alpha$ -coexpressing adipocytes*

We next analyzed the effect of neutralization of IGF-1, an effector of GH actions for the regulation of insulin sensitiv-

ity, on the decrease in population of TLR2/TNF- $\alpha$  co-expressing adipocytes in visceral fat by low-dose GH supplementation. For this purpose, we established the hGH-expressing mice using cell transplantation methods as described [9]. The hGH-overexpressing 3T3-L1 preadipocytes were subcutaneously implanted into BALB/c nude mice (GH mice). The plasma GH concentrations increased 4 weeks after the implantation of hGH-overexpressing 3T3-L1 preadipocytes were significantly higher in the GH mice than those in the mock-implanted mice (mock mice) ( $322 \pm 165$  pg/ml vs  $136 \pm 128$  pg/ml,  $P < 0.05$ ). The plasma IGF-1 concentrations were significantly higher in the GH mice than in the mock mice ( $323 \pm 71$  ng/ml vs  $267 \pm 19$  ng/ml,  $P < 0.05$ ) (Fig. 2A). The blood glucose levels 30 min after insulin loading were significantly decreased in the GH mice in comparison to those in the mock mice (Fig. 2B). In accordance with the decreased insulin sensitivity, the plasma triglyceride levels were significantly lower in the GH mice in comparison to those in the mock mice

(Fig. 2C). The pretreatment of mice with anti-IGF-1 antibody cancelled the ameliorating effect of GH on the insulin resistance (Fig. 2B). The TLR2 mRNA expression levels of visceral fat were significantly lower in the GH mice than those in the mock mice, and anti-IGF-1 antibody treatment significantly increased the TLR2 mRNA expression levels to those expressed in the mock mice (Fig. 2D). Therefore, circulating IGF-1 is important for the effect of the low-dose GH supplementation on the high-fat-induced insulin resistance in mice.

*IGF-1, not GH, inhibits FFA-induced TLR2 and TNF- $\alpha$  gene expressions in 3T3-L1 adipocytes*

Two different mouse models fed with a high-fat diet showed that low-dose GH supplementation suppresses the population of TLR2/TNF- $\alpha$  co-expressing adipocytes in visceral fat, and possibly the amelioration by GH supplementation is mediated by the effects of increased plasma IGF-1.

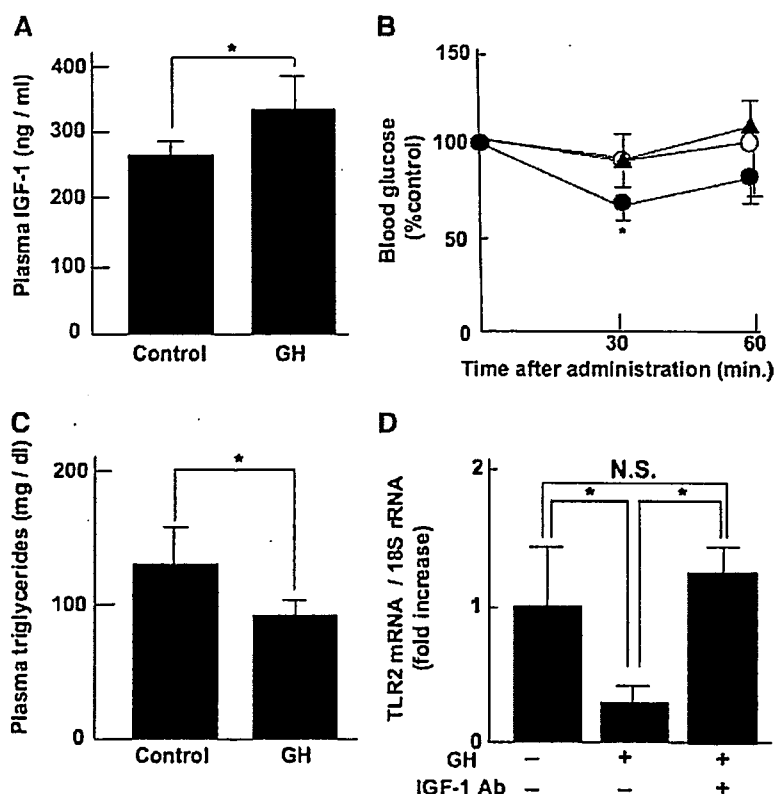


Fig. 2. Effects of IGF-1 neutralization on the actions of low-dose GH supplementation using cell transplantation models. Male BALB/c nude mouse was subcutaneously injected with  $10^6$  cells of hGH-overexpressing 3T3-L1 preadipocytes. A high-fat diet was started at a week after cell implantation, and continued for 3 weeks. (A) The plasma IGF-1 levels in the mice implanted with hGH-overexpressing cells (GH) or mock cells (Control).  $n = 6$ . \* $P < 0.05$  in comparison to the value of the control. (B) Insulin tolerance test in the mice implanted with hGH-overexpressing cells or with the mock cells (O). The mice implanted with hGH-overexpressing cells were injected with normal goat (●) or anti-mouse IGF-1 antibody (▲). Blood glucose levels were monitored at 0, 30, and 60 min after intraperitoneal insulin injection.  $n = 6$ . \* $P < 0.05$  compared to the value of the control. (C) The plasma triglyceride levels in the mice implanted with hGH-overexpressing cells (GH) or mock cells (control).  $n = 6$ . \* $P < 0.05$  compared to the value of the control. (D) TLR2 mRNA expression in mesenteric fat of the mice implanted with hGH-overexpressing cells or PBS alone. Anti-mouse IGF-1 antibody or normal goat IgG was injected after transplantation of hGH-overexpressing cells.  $n = 6$ . \* $P < 0.05$  compared to the value of the control. N.S., not significant.

Therefore, in order to know the role of IGF-1 in the regulation of TLR2/TNF- $\alpha$  co-expressing adipocytes, we analyzed the effects of IGF-1 on the TLR2 and TNF- $\alpha$  mRNA expressions in 3T3-L1 adipocytes (Fig. 3A). The TNF- $\alpha$  mRNA level was increased by the stimulation of a mixture of myristic and palmitic acids [4]. The incubation of 3T3-L1 cells with hGH did not inhibit the increased expression of TNF- $\alpha$  by FFAs. In contrast, IGF-1 completely inhibited the FFA-induced increase in TNF- $\alpha$  mRNA expression. Furthermore, IGF-1 almost inhibited all of the FFA-induced TLR2 mRNA expression in 3T3-L1 adipocytes. A flow cytometry analysis of single adipocytes prepared from 3T3-L1 adipocytes showed that the FFA-induced increase in the population of TLR2/TNF- $\alpha$  co-expressing adipocytes was largely inhibited by the incubation with IGF-1 (Fig. 3B). These results are in consistent with the observations made using *in vivo* models (see Figs. 1 and 2), thereby suggesting that IGF-1, not GH, reduces the number of TLR2/TNF- $\alpha$  co-expressing adipocytes in visceral fat.

*Low-dose GH supplementation reduces the TLR2 mRNA expression of visceral fat before an obvious change of fat volume in obese mice*

We finally examined the effect of low-dose GH supplementation on TLR2 mRNA expression in visceral fat in obese mice in order to know the relationship of TLR2

expression and fat volume in visceral fat. The plasma IGF-1 concentration significantly increased in ob/ob mice supplemented with low-dose GH (GH-ob) in comparison to ob/ob mice in the absence of supplementation (control-ob) (Fig. 4A). Measurements of the fat volume using a CT scan showed no significant difference in either the visceral or the subcutaneous fat volume between the GH-ob and the control-ob mice (Fig. 4B). In contrast, the TLR2 mRNA expression levels of visceral fat tissue were significantly decreased in the GH-ob mice in comparison to those in the control-ob mice (Fig. 4C). These results indicate that low-dose GH supplementation caused the decrease in TNF- $\alpha$  expression in the visceral fat before the obvious change in the visceral fat volume in the obese mice.

### Discussion

An abnormal expression of cytokines in adipocytes, particularly in the visceral regions, causes the onset of metabolic syndrome through the development of insulin resistance [2]. We have shown that TNF- $\alpha$  expression is induced in adipocytes accumulated in the visceral, and not in the subcutaneous, regions, using a cell transplantation model [3]. The TNF- $\alpha$  expression in the visceral fat is closely associated with the increased population of TLR2/TNF- $\alpha$  co-expressing adipocytes in response to a high-fat intake [4]. The identification of the TLR2/TNF-

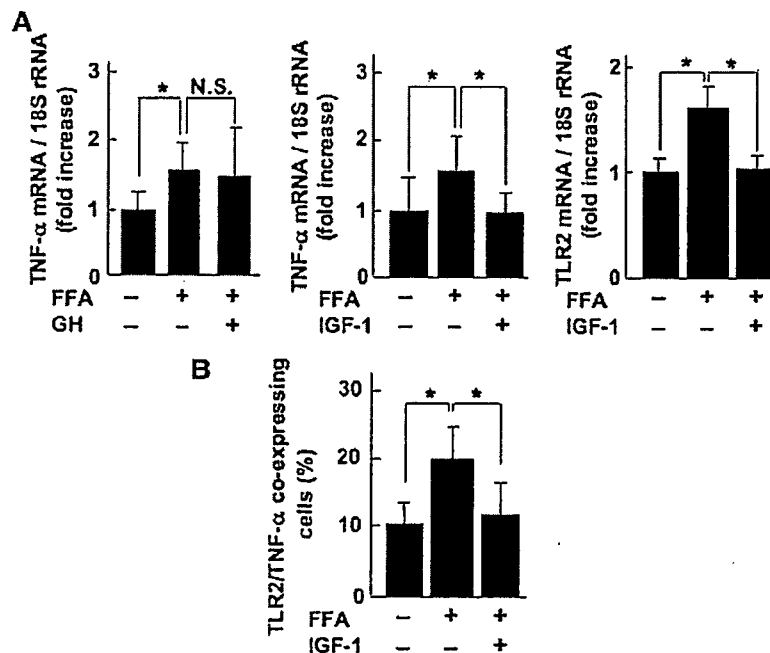


Fig. 3. Effects of GH or IGF-1 on FFA-induced TLR2 and TNF-expressions in 3T3-L1 adipocytes. (A) Serum-starved 3T3-L1 adipocytes treated with 1 mM FFA in the presence or absence of hGH or IGF-1 for 8 h. Quantitative RT-PCR was used to measure the expression level of TNF- $\alpha$  gene or TLR2 gene  $n = 6$ . \* $P < 0.05$ . (B) Flow cytometric analyses of TLR2/TNF- $\alpha$  co-expressing adipocytes in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes treated with 1 mM FFA in the presence or absence of IGF-1 for 8 h, and analyzed by FACS Calibur. The averaged populations of TLR2/TNF- $\alpha$  co-expressing adipocytes in the total cells (20,000 cells) were expressed ( $n = 3$ ). \* $P < 0.05$ .

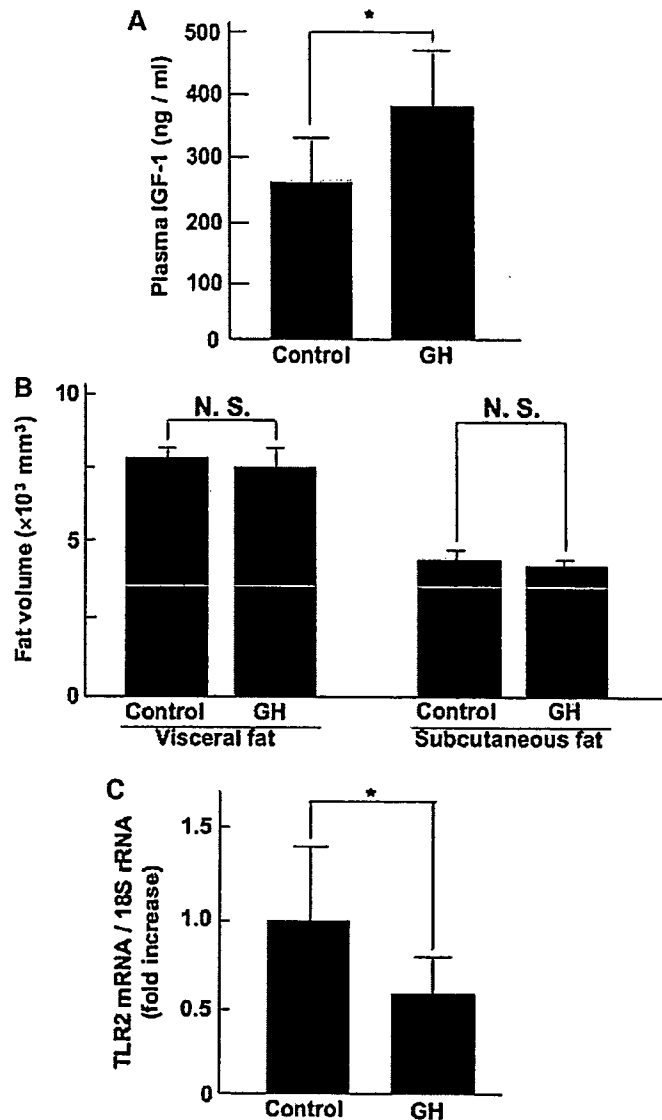


Fig. 4. Effects of low-dose hGH supplementation on the fat volume and TLR2 mRNA expression of visceral fat. Male ob/ob mice were supplemented with hGH (0.5 mg/kg/day) (GH) or PBS (Control) for 4 weeks. (A) The plasma mouse IGF-1 concentration was measured.  $n = 7$ . \* $P < 0.05$ . (B) Visceral or subcutaneous fat volumes at 4 weeks after administration of hGH or PBS alone was measured by using CT.  $n = 7$ . \* $P < 0.05$ . (C) The TLR2 mRNA expression levels in mesenteric fat tissue were measured by RT-PCR.  $n = 7$ . \* $P < 0.05$ .

$\alpha$  co-expressing adipocytes as a regulator of TNF- $\alpha$  expression in the visceral fat suggested that the regulation of the occurrence of pathogenic adipocytes in the visceral fat is important for the improvement of TNF- $\alpha$ -mediated insulin resistance.

Low-dose GH supplementation research has recently focused on the regulation of insulin resistance accompanied by visceral obesity. Yuen et al. found that low-dose GH therapy (0.1 mg/day) improved insulin sensitivity in GH-deficient adults and also notably in subjects with the metabolic syndrome [7]. Johansson showed that GH treatment

of obese men reduces the abdominal fat mass, and improved the accompanied metabolic abnormalities [6]. These clinical studies indicate that low-dose GH supplementation is potentially beneficial for metabolic abnormalities accompanied by visceral obesity, in contrast to the glucose intolerance due to the GH overproduction in acromegaly. In this context, there are relevant studies regarding the heterogeneous effect of GH on metabolic abnormalities using animal models [13,14]. Based on this background, we performed this study in order to clarify the mechanism for the effect of low-dose GH supplementation on insulin resis-



tance, particularly through the regulation of the population of TLR2/TNF- $\alpha$  co-expressing adipocytes, which has been shown to be related to high-fat-induced insulin resistance [4]. A flow cytometry analysis clearly showed that continuous low-dose GH supplementation reduced the population of TLR2/TNF- $\alpha$  co-expressing adipocytes in visceral regions, and improved insulin resistance. These results using high-fat fed mice are inconsistent with the above clinical observations in obese subjects [6]. We then studied the mechanism of low-dose GH supplementation-mediated inhibition of high-fat induced TLR2 and TNF- $\alpha$  expressions in visceral fat using another model. The GH continuously supplemented from the subcutaneously implanted cells reduced the high-fat induced insulin resistance, and the effect was abolished by the neutralization of IGF-1, a mediator of GH action [15]. The cancellation of GH-mediated action was also observed in the inhibition of TLR2 expression in the visceral fat. Thus, our study showed that IGF-1 was a key molecule in the low-dose GH supplementation for the regulation of TLR2 and TNF- $\alpha$  expressions in visceral fat. The results obtained from cultured adipocytes supported the role of IGF-1 in the effect of low-dose GH supplementation.

The effect of IGF-1 on apoptosis and adipogenesis have been shown in primary cultured adipocytes [16,17]. Our results suggested that TLR2 is one of the genes regulated by IGF-1 in 3T3-L1 cells. The induction of TLR2 expression in high-fat intake could be protected by low-dose GH supplementation through the effect of IGF-1 on visceral adipocytes. The study using ob/ob mice suggested that the effect of IGF-1 on the suppression of TLR2 expression is not necessarily linked to the changes in visceral fat volume. The identification of IGF-1 as a regulator of TLR2 mRNA expression in adipocytes may contribute to the elucidation of the heterogeneous functions of GH in various metabolic states. Recent clinical trials suggested that the effects of low-dose GH supplementation are mediated by its ability to increase IGF-1 without the induction of lipolysis [18]. The studies of IGF-1-mediated function on visceral adipocytes may be important for the further therapeutic application of low-dose GH (or IGF-1) supplementation in patients with metabolic syndrome and insulin resistance.

#### Acknowledgments

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## Effect of PPAR $\alpha$ activation of macrophages on the secretion of inflammatory cytokines in cultured adipocytes

Kentaro Murakami <sup>a</sup>, Hideaki Bujo <sup>b,\*</sup>, Hiroyuki Unoki <sup>c</sup>, Yasushi Saito <sup>a</sup>

<sup>a</sup> Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

<sup>b</sup> Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

<sup>c</sup> Division of Applied Translational Research, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

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### Abstract

The relationship between adipocytes and infiltrated macrophages in fat tissue is important for the pathogenesis of insulin resistance through the activation of cytokines. Peroxisome proliferator-activated receptors (PPARs) play a role in the regulation of cytokine secretion in these cells. We studied the effect of the PPAR $\alpha$  activation of macrophages on the modulation of the tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) expression in adipocytes using a cell culture system. A conditioned medium of lipopolysaccharide (LPS)-stimulated RAW264.7 cells, a macrophage cell line, induced the level of TNF $\alpha$  mRNA in 3T3-L1 adipocytes. This effect was inhibited by the addition of neutralizing antibody against interleukin 6 (IL-6) in the conditioned medium or the preincubation of RAW264.7 cells with a specific PPAR $\alpha$  agonist, K-111 (2,2-dichloro-12-(4-chlorophenyl)dodecanoic acid). K-111 reduced both the IL-6 production and mRNA expression in RAW264.7 cells, and its effect was stronger than that of rosiglitazone, a PPAR $\gamma$  agonist. The activation of the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) pathway and nuclear factor kappa B (NF- $\kappa$ B) subunits of p65 was significantly inhibited by K-111. The blocking of IL-6 production through the SAPK/JNK pathway or by transfection with siRNA specific for IL-6 abolished the inhibitory effect of K-111 on the TNF $\alpha$  expression in the 3T3-L1 adipocytes. As a result, the IL-6 produced by RAW264.7 cells is an inducer of TNF $\alpha$  expression in 3T3-L1 adipocytes, and the IL-6 secretion is inhibited by the activation of PPAR $\alpha$ . The PPAR $\alpha$  activators may suppress the pathogenetical secretion of TNF $\alpha$  in the adipocytes through the functional modulation of the infiltrated macrophages.

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**Keywords:** Adipocyte; PPAR $\alpha$ ; Macrophage; Fat tissue; IL-6; TNF $\alpha$

### 1. Introduction

Insulin resistance is linked to a wide array of metabolic disorders leading to atherosclerosis, such as hypertension, dyslipidemia, or disturbed glucose tolerance (Ginsberg, 2000; Hayden and Reaven, 2000; Reaven, 1995). A cluster of these abnormalities is now recognized as metabolic syndrome (Report of a WHO Consultation, 1999; Expert Panel on Detection, 2001). We have previously shown that visceral accumulation of adipose tissue, and not subcutaneous accumulation, causes systemic insulin resistance through the increased tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) secretion from adipocytes using a cell-transplanted model (Shibasaki et al., 2002). Resistin is also

another possible molecule, which regulates the systemic insulin sensitivity, possibly through the TNF $\alpha$  activation in the model mice (Kitagawa et al., 2004). In this context, the accumulated visceral fat secretes other cytokines, such as Vascular Endothelial Growth Factor (VEGF) (Miyazawa-Hoshimoto et al., 2005). As a result, the cytokine secretion of adipocytes accumulated in the visceral area seems to play an important role in the pathogenesis of insulin resistance and the related vascular diseases in humans. Recent transcriptional profiling experiments using animal models have pointed to a striking regulation of the inflammatory cytokines in adipose tissue, thus suggesting that macrophage infiltration into adipose tissue could be integral to these pathogenic changes (Weisberg et al., 2003; Xu et al., 2003). Interleukin 6 (IL-6) is one of the inflammatory cytokines which link both adipocytes and macrophages. The expression of IL-6 in fat tissue is elevated in individuals demonstrating

\* Corresponding author. Tel.: +81 43 226 2091; fax: +81 43 226 2095.  
E-mail address: [hbujo@faculty.chiba-u.jp](mailto:hbujo@faculty.chiba-u.jp) (H. Bujo).

obesity with insulin resistance (Mohamed-Ali et al., 1998, 1997; Vozarova et al., 2001; Straub et al., 2000; Fernandez-Real et al., 2001).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, which belong to the nuclear receptor family. PPAR $\gamma$  plays a pivotal function in the differentiation of adipocytes (Adams et al., 1997; Rosen and Spiegelman, 2001). In fact, chronic treatment with PPAR $\gamma$  activators improves the degree of glucose homeostasis by increasing the insulin sensitivity in various animal models of obesity and diabetes as well as in humans (Miyazaki et al., 2001; Hirose et al., 2002). However, a previous study showed that PPAR $\gamma$  agonists do not obviously suppress the IL-6 production in macrophage (Thieringer et al., 2000). PPAR $\alpha$  was first identified for its role in the regulation of both the lipid and carbohydrate metabolisms, and subsequent data have also demonstrated that it exhibits a potent anti-inflammatory activity (Sheu et al., 2002; Delerive et al., 1999). Therefore, in this study we analyzed the effect of a PPAR $\alpha$  agonist on the inflammatory cytokine expressions in adipocytes through the macrophage-derived IL-6 pathway using cultured cells, in order to elucidate the possible involvement of PPAR $\alpha$  activation in the pathological link between adipocytes and infiltrated macrophages in fat tissue.

## 2. Materials and methods

### 2.1. Materials

K-111 (2,2-dichloro-12-(4-chlorophenyl)dodecanoic acid, purity 99%), was synthesized at the Research Laboratories of Kowa company (Tokyo, Japan). Rosiglitazone was given from Takeda Pharmaceutical company (Osaka, Japan). SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-one, purity 98%) was purchased from BIOMOL international L.P. (Plymouth Meeting, PA, USA) and these samples were used as solution of various concentrations in dimethyl sulfoxide (DMSO) purchased from SIGMA-Aldrich Company Ltd. (St. Louis, MO, USA). The final concentrations of DMSO were less than 0.1% where the cell viability was not affected. RAW264.7 cells and 3T3-L1 cells were obtained from ATCC (Dainippon Pharmaceutical, Osaka, Japan). DMEM containing 10 mM glucose (DMEM-L) medium and DMEM containing 25 mM glucose (DMEM-H) medium were obtained from SIGMA-Chemicals (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gemini-Bio Products (Woodland, CA, USA). Lipopolysaccharide (LPS: *Escherichia coli* 0127 B8) was purchased from SIGMA-Chemicals (St. Louis, MO, USA). IL-6 Enzyme-Linked Immunosorbent Assay (ELISA) kits and Anti-mouse IL-6 neutralizing antibody were obtained from R&D systems (Minneapolis, MN, USA).

### 2.2. Cell culture

RAW264.7 cells were maintained in DMEM-L medium supplemented with 10% FBS, and gentamicin sulfate (20 mg/ml) at 37 °C under humidified 5% CO<sub>2</sub>/95% air. 3T3-L1 adipocytes

were differentiated while referring to the method described previously (Rubin et al., 1977). Briefly, preadipocytes were grown to confluence after which they were cultured for 3 days in DMEM-H, 10% FBS, and antibiotics (culture medium) further supplemented with 10  $\mu$ g/ml insulin, 0.5 mM isobutylmethyl-xanthine and 0.25  $\mu$ M dexametazone.

The cells had accumulated fat droplets after an additional 3 days in the culture medium with 5  $\mu$ g/ml insulin followed by 3–6 days in culture medium. All stimulations were carried out in DMEM-H without any additions.

### 2.3. Preparation of RAW264.7-conditioned medium (CM)

RAW264.7 cells were cultured in DMEM-L with 10% FBS in 5% CO<sub>2</sub>/95% humidified air at 37 °C. The cells were treated with or without K-111 (30  $\mu$ M) and/or SP600125 (10  $\mu$ M) and then were incubated for 18 h, followed by the addition of LPS (1  $\mu$ g/ml). After 8 h, the medium was changed with or without K-111 (30  $\mu$ M) and/or SP600125 (10  $\mu$ M). After 18 or 36 h, the medium was collected and centrifuged at 300  $\times$ g for 5 min. The supernatant was concentrated by Centoricon (MILLIPORE Corporation, Billerica, MA, USA), and then was sterilized by filtrating through a 0.22  $\mu$ m filter, and used as RAW264.7-CM.

### 2.4. Cytokine production assay using ELISA

RAW264.7 cells suspended in DMEM-L with 10% FBS were placed in multi-well culture plates, and treated with K-111 (30  $\mu$ M), rosiglitazone (30  $\mu$ M) or SP600125 (10  $\mu$ M) followed by the addition of LPS (1  $\mu$ g/ml). The mixture was incubated at 37 °C for 8 h. The culture medium was then subjected to centrifugation at 300  $\times$ g for 5 min, and the concentrations of the cytokines in the supernatant were measured using commercial ELISA kits according to the manufacturer's instructions.

### 2.5. Measurement of mRNA levels using quantitative real-time reverse transcriptase-polymerase chain reaction

IL-6 and TNF $\alpha$  mRNA expression was determined by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from RAW264.7 cells or 3T3-L1 adipocytes with ISOGEN (Nippon gene, Tokyo, Japan) and 1  $\mu$ g RNA was reverse transcribed. The amplification of each target cDNA was performed with TaqMan PCR reagent kits in the ABI PRISM 7700 sequence detection system according to the protocols provided by the manufacturer (PE Applied Biosystems, Foster City, CA). The primer/probe sets of IL-6 and TNF $\alpha$  were purchased from the manufacturer (PE Applied Biosystems, Foster City, CA), and then were used for the amplification step. IL-6 and TNF $\alpha$  mRNA expressions were calculated relative to 18S ribosomal RNA (rRNA).

### 2.6. Preparation of cytosol extracts

RAW264.7 cells suspended in DMEM-L with 10% FBS were placed in 6-well plates (2 ml/well), and incubated at 37 °C in the absence or presence of K-111 (30  $\mu$ M). After 30 min, each well

was treated with LPS (1  $\mu\text{g/ml}$ ) for the times indicated in each figure. The cells were washed with ice-cold phosphate-buffered saline (PBS) and collected on ice. Cytosol extracts from these cells were isolated by NE-PER Nuclear and Cytoplasmic extraction reagents (PIERCE Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions.

### 2.7. Western blot analysis

RAW264.7 cells suspended in DMEM-L with 10% FBS were placed in 6-well plates (2 ml/well), and incubated at 37 °C in the absence or presence of K-111 (30  $\mu\text{M}$ ). After 18 h, each well was treated with LPS (1  $\mu\text{g/ml}$ ) for the times indicated in each figure. The cells were washed with ice-cold PBS and lysed in radio-immunoprecipitation assay (RIPA) buffer (whole cell lysates).

Western blot analysis was conducted essentially as previously described using ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Phosphorylated extracellular signal-

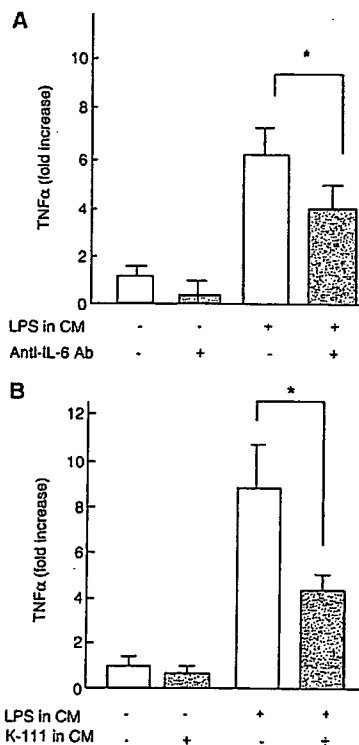


Fig. 1. Effect of K-111 on the potency of RAW264.7 cells of the conditioned medium for the TNF $\alpha$  mRNA expression in 3T3-L1 cells. A. 3T3-L1 adipocytes were treated with a conditioned medium of RAW264.7 cells, which were added until they reached 10%, in the absence or presence of IL-6-neutralizing antibodies for 48 h. B. 3T3-L1 adipocytes were treated with conditioned medium of RAW264.7 cells with or without K-111, which were added until they reached 10% for 48 h. Isolated total RNA was transcribed into cDNA, and then was analyzed by real-time RT-PCR using specific primers for TNF $\alpha$ . Levels of TNF $\alpha$  mRNA expression in each treatment are presented as a fold increase of that in untreated cells. \* $P < 0.05$  ( $n = 4-5$ ).

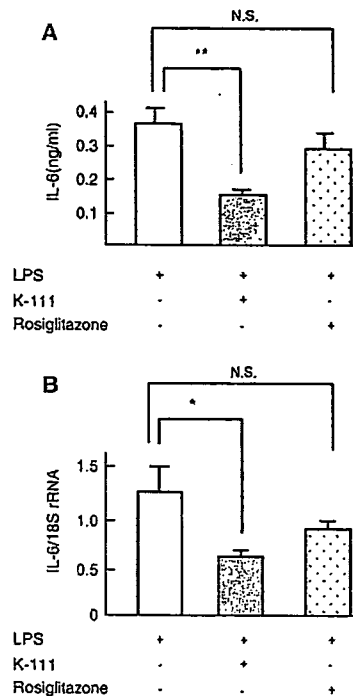


Fig. 2. Effect of K-111 on the secretion and gene expression of IL-6 in RAW264.7 cells. A. RAW264.7 cells were pretreated with or without 30  $\mu\text{M}$  of K-111 or rosiglitazone for 18 h before the addition of LPS (1  $\mu\text{g/ml}$ ). The concentrations of IL-6 in the culture supernatants 8 h after stimulation by LPS were measured using ELISA. B. Total RNA as isolated 8 h after stimulation by LPS, and transcribed into cDNA. IL-6 mRNA level was analyzed using real-time RT-PCR. \* $P < 0.05$ , \*\* $P < 0.01$  ( $n = 3-5$ ). N.S., not significant.

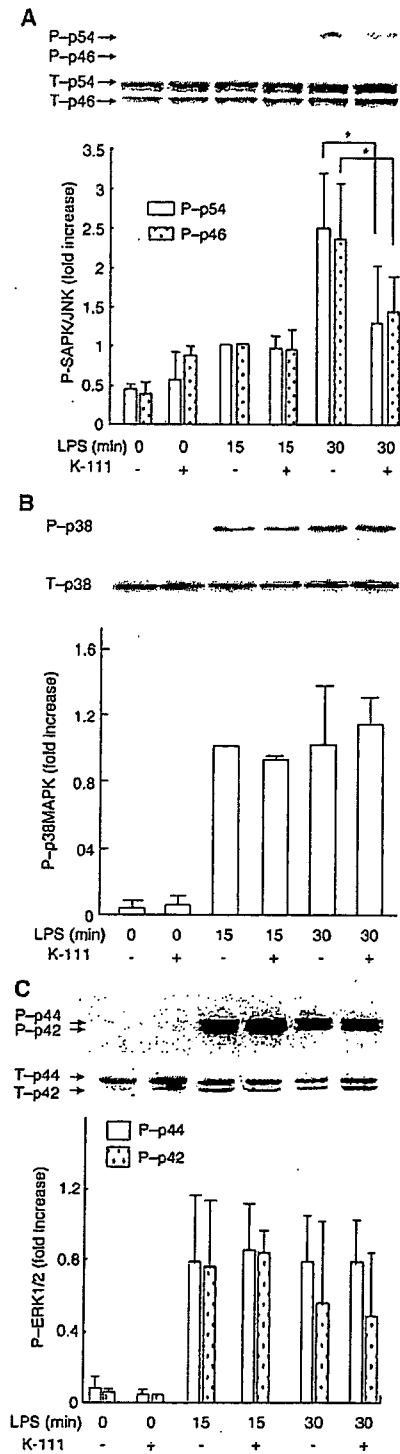
regulated kinase (ERK) 1/2 (p44/42 mitogen-activated protein kinase (MAPK)), p38MAPK, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) and nuclear factor kappa B (NF- $\kappa$ B) p65, or total ERK1/2, p38MAPK, SAPK/JNK and I $\kappa$ B $\alpha$  were detected using rabbit polyclonal antibodies from Cell Signaling Technology (Beverly, MA). Whole cell lysates were used for the assay of phosphorylated ERK1/2, p38MAPK and SAPK/JNK and cytosol extracts were used for the assay of I $\kappa$ B $\alpha$  and phosphorylated NF- $\kappa$ B p65.

### 2.8. siRNA transfection

RAW264.7 cells were transfected with 5 nM siRNA to IL-6 or All Star Negative Control siRNA (Qiagen, Germany) using Hiperfect transfection reagent (Qiagen) as described by the manufacturer. The treated cells were then subjected to the following experiments.

### 2.9. Statistical analysis

The results are shown as the means  $\pm$  S.D. Differences between various treatments were analyzed by unpaired Student's *t* tests with *P* values  $< 0.05$  considered to be significant.



### 3. Results

#### 3.1. Effect of a specific PPAR $\alpha$ agonist, K-111, on the TNF $\alpha$ expression in 3T3-L1 adipocytes through the action of RAW264.7 cells

In order to determine the effect of the PPAR $\alpha$  activation of macrophages for the regulation of cytokine secretion from adipocytes through IL-6 secretion from macrophages, we studied the effect of a conditioned medium with RAW264.7, a macrophage cell line, on the TNF $\alpha$  expression in cultured adipocytes, 3T3-L1 cells. For this aim, 3T3-L1 adipocytes were incubated with or without 10% (v/v) LPS-stimulated RAW264.7-CM for 48 h. Real-time RT-PCR showed the expression of TNF $\alpha$  mRNA in 3T3-L1 adipocytes to be significantly induced with the conditioned medium of RAW264.7 cells (Fig. 1A). The mRNA level of TNF $\alpha$  was increased by 6-fold of the control. The increased level of TNF $\alpha$  mRNA was significantly inhibited in the presence of neutralizing antibody against IL-6 (1  $\mu$ g/ml). These results indicated that IL-6, which is secreted from RAW264.7 cells, is a major inducer of the TNF $\alpha$  expression in 3T3-L1 adipocytes.

We next analyzed the effect of CM of RAW264.7 cells pretreated with a specific PPAR $\alpha$  agonist, K-111 on the TNF $\alpha$  expression in 3T3-L1 adipocytes (Fig. 1B). Pretreatment of CM with K-111 reduced its induction activity for TNF $\alpha$  mRNA expression in 3T3-L1 cells. As a result, the PPAR $\alpha$  activation was found to cause a decrease in the TNF $\alpha$  expression in 3T3-L1 adipocytes, which was induced by the LPS-stimulated RAW264.7-CM.

#### 3.2. K-111 inhibits IL-6 transcription and secretion in LPS-stimulated RAW264.7 cells

Based on the obtained results, we next studied the effect of K-111 on the IL-6 transcription and secretion in the cultured macrophages, and compared our findings with those for rosiglitazone, a potent PPAR $\gamma$  agonist. The results of ELISA showed that K-111 (30  $\mu$ M) significantly reduced the IL-6 production after stimulation with LPS (1  $\mu$ g/ml) in RAW264.7 cells (Fig. 2A). On the other hand, rosiglitazone (30  $\mu$ M) did not show a significant decrease in the secreted IL-6 level, and this finding was consistent with that of a previous report (Thieringer et al., 2000). Therefore, in order to determine whether the effect is caused at the transcription level of the IL-6 gene, the mRNA level was analyzed after the exposure of LPS (1  $\mu$ g/ml) in the presence of K-111 using real-time RT-PCR. K-111 (30  $\mu$ M) significantly reduced the IL-6 mRNA level (Fig. 2B). These

Fig. 3. Effect of K-111 on the MAPK activation in RAW264.7 cells. RAW264.7 cells were pretreated with or without 30  $\mu$ M of K-111 for 18 h before the addition of LPS (1  $\mu$ g/ml). Samples (each containing 7.5  $\mu$ g of protein) were analyzed by Western blotting using antibodies against phospho(p)-SAPK/JNK or total (T)-SAPK/JNK (A), phospho(p)-p38 or total (T)-p38 (B), and phospho(p)-ERK1/2 (p44/42 MAPK) or total (T)-ERK1/2(p44/42 MAPK) (C), respectively. Each signal was scanned and calculated as a fold increase of the signal in the stimulation of LPS for 15 min. \* $P$ <0.05 ( $n$ =3–4).

results show that PPAR $\alpha$  activation inhibits IL-6 at the transcriptional and secretional levels in cultured macrophages.

### 3.3. K-111 suppresses the activation of SAPK/JNK in RAW264.7 cells

To elucidate the reason why the PPAR $\alpha$  activation of macrophage suppresses the IL-6 secretion in cultured macrophage, we analyzed the effect of K-111 on the phosphorylation of ERK1/2, SAPK/JNK, and p38MAPK in RAW264.7 cells.

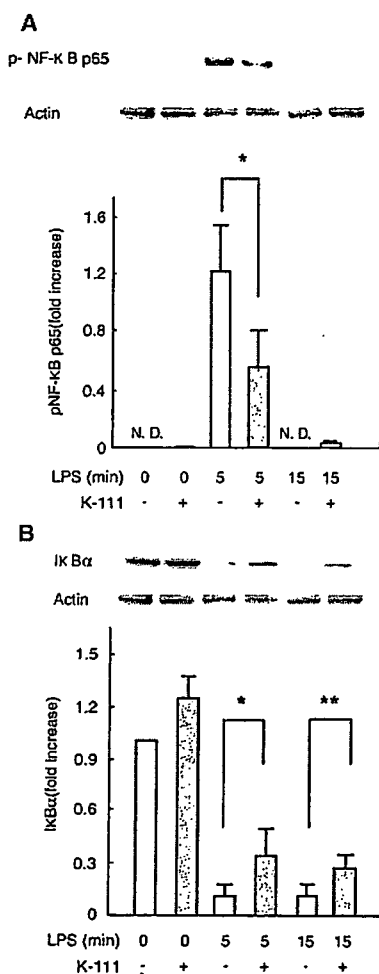


Fig. 4. The effect of K-111 on the LPS-induced NF- $\kappa$ B activation in RAW264.7 cells. RAW264.7 cells were preincubated at 37 °C for 18 h with or without K-111 (30  $\mu$ M), and then were treated with 1  $\mu$ g/ml of LPS for 0–15 min. Next, cytosol extracts (each containing 10  $\mu$ g of protein) were prepared and analyzed by Western blotting using an antibody which specifically recognizes phospho(p)-NF- $\kappa$ B p65 (A). The cytosol extracts (each containing 10  $\mu$ g of protein) were also analyzed by Western blotting using an antibody which specifically recognizes I $\kappa$ B $\alpha$  (B). Western blotting using an antibody against Actin was used as control. Each signal was scanned and calculated as a fold increase of the signal in the stimulation of LPS for 5 min (A) or for 0 min (B). \* $P$ <0.05, \*\* $P$ <0.01 ( $n$ =3). N.D., not detected.

These intracellular pathways have been shown to be involved in the transcription of inflammatory cytokines. The kinetics of MAPK activation stimulated with 1  $\mu$ g/ml LPS in subconfluent RAW264.7 cells was examined using a Western blot analysis (Fig. 3). SAPK/JNK and p38 were activated by LPS, thus reaching their maximal activities within 30 min, and ERK1/2 was also potentially activated at 30 min (maximum at 15 min). Pretreatment of the cell with 30  $\mu$ M of K-111 for 18 h significantly inhibited the LPS-induced phosphorylation of SAPK/JNK (Fig. 3A). The phosphorylation of p38MAPK and ERK1/2 was not significantly inhibited (Fig. 3B and C). These results suggest that the inhibitory effect of K-111 on the activation of IL-6 secretion in RAW264.7 cells is possibly caused by the suppression of the SAPK/JNK pathway.

### 3.4. K-111 inhibits NF- $\kappa$ B activation in RAW264.7 cells

The IL-6 expression is known to be mediated by NF- $\kappa$ B activation followed by signals through the SAPK/JNK pathway in human monocytes (Tuyt et al., 1999). Therefore, the suppression of the SAPK/JNK pathway, and IL-6 mRNA and protein expression by K-111 suggests that the decrease in IL-6 mRNA is mediated by the deactivation of NF- $\kappa$ B in RAW264.7 cells. The effect of K-111 on LPS-induced activation of NF- $\kappa$ B was evaluated by Western blot analysis using the cytosol extracts from RAW264.7 cells. RAW264.7 cells were preincubated with 30  $\mu$ M of K-111, and then with LPS (1  $\mu$ g/ml) for 0–30 min. LPS significantly induced the phospho(p)-NF- $\kappa$ B subunits of p65 in cytosol within 5 min, and then the induction was inhibited in the presence of K-111 (Fig. 4A). We next analyzed the effect of K-111 on the LPS-induced I $\kappa$ B $\alpha$  degradation, since the NF- $\kappa$ B activation is regulated by the steady-state levels of its inhibitor I $\kappa$ B $\alpha$  protein. RAW264.7 cells were pretreated with K-111 (30  $\mu$ M) for 18 h prior to stimulation with LPS (1  $\mu$ g/ml). Thereafter, the cytoplasmic I $\kappa$ B $\alpha$  levels were analyzed by Western blot analysis. Stimulation with LPS decreased the I $\kappa$ B $\alpha$  signal intensity within 15 min (Fig. 4B), and re-appeared at 30 min (data not shown). The I $\kappa$ B $\alpha$  degradation level decreased in the presence of K-111 for 15 min. The effect of K-111 on IL-6 transcription in macrophage is thus possibly mediated by the inhibition of NF- $\kappa$ B activation.

### 3.5. Blocking of SAPK/JNK pathway abolishes the effect of K-111 on the IL-6 secretion of RAW264.7 and TNF $\alpha$ expression of 3T3-L1 adipocytes, respectively

In order to know the role of IL-6 secretion mediated by SAPK/JNK pathway in the action of K-111 in activated macrophages on the following inhibition of TNF $\alpha$  expression in adipocytes, we analyzed the effect of blocking of SAPK/JNK pathway on the inhibition of K-111 for the IL-6 production and TNF $\alpha$  expression in RAW264.7 cells and 3T3-L1 adipocytes, respectively (Fig. 5). For this purpose, RAW264.7 cells were pretreated with 10  $\mu$ M SP600125, a specific SAPK/JNK inhibitor (Beunet et al., 2001; Han et al., 2001), before the K-111 treatment. SP600125 significantly decreased the IL-6 production after stimulation with LPS (1  $\mu$ g/ml). The treatment

of K-111 in the pretreated RAW264.7 cells did not show any significant change in the level of IL-6 production in SP600125-treated RAW264.7 cells (Fig. 5A). We then analyzed the effects of CM of LPS-stimulated RAW264.7 cells pretreated by SP600125 on the TNF $\alpha$  expression in 3T3-L1 adipocytes. The pretreatment of CM with SP600125 significantly reduced the LPS-induced level of the TNF $\alpha$  mRNA expression in 3T3-L1 adipocytes. The treatment of K-111 on the SP600125 pretreatment of RAW264.7 cells did not show a significant change in the inhibitory effect on the TNF $\alpha$  expression in 3T3-L1 adipocytes (Fig. 5B). Furthermore, the

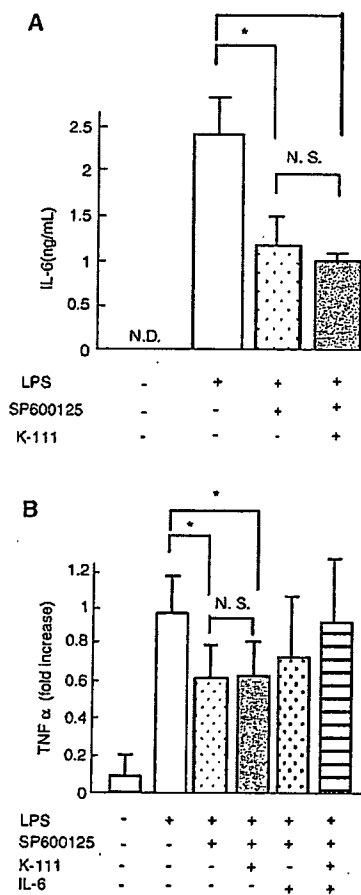


Fig. 5. Effect of blocking of SAPK/JNK pathway on the K-111 induced inhibition for IL-6 secretion in RAW264.7 cells and TNF $\alpha$  expression in 3T3-L1 adipocytes. A. RAW264.7 cells were pretreated with or without 10  $\mu$ M SP600125 in the presence or absence of 30  $\mu$ M K-111 for 18 h before the addition of LPS (1  $\mu$ g/ml). The concentrations of IL-6 in the culture supernatants 24 h after stimulation by LPS were measured using ELISA. \* $P$ <0.05. N.S., not significant ( $n$ =3). N.D., not detected. B. 3T3-L1 adipocytes were treated with CM of RAW264.7 cells at 10% of medium for 48 h. Isolated total RNA was transcribed into cDNA, and analyzed by real-time RT-PCR using specific primers for TNF $\alpha$ . Levels of TNF $\alpha$  mRNA expression in each treatment are presented as a fold increase of the signal of the LPS-stimulated RAW264.7-CM-treated cells. \* $P$ <0.05. N.S., not significant ( $n$ =5).

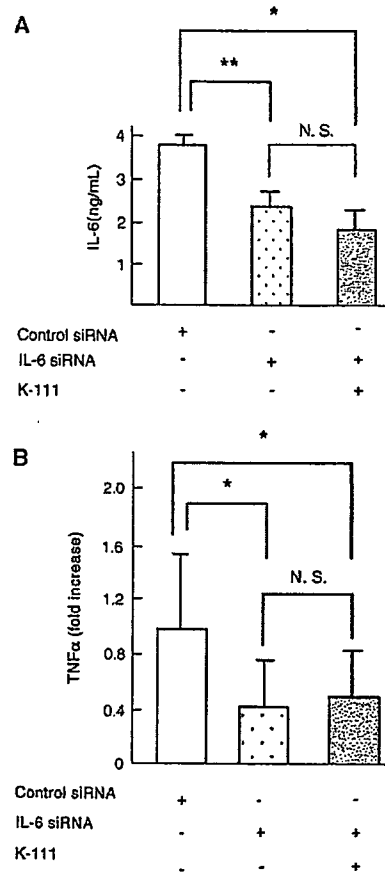


Fig. 6. The effect of the IL-6 gene knockdown using the specific siRNA in RAW264.7 cells on the inhibitory action of K-111 for the TNF $\alpha$  expression in 3T3-L1 adipocytes. RAW264.7 cells were transfected with siRNA to IL-6 or control siRNA. Transfected cells were incubated with 30  $\mu$ M K-111 for 18 h before the addition of LPS (1  $\mu$ g/ml). A. The IL-6 concentration in the culture supernatants incubated for 36 h after stimulation with LPS for 8 h. \* $P$ <0.05, \*\* $P$ <0.01. N.S., not significant ( $n$ =3). B. TNF $\alpha$  mRNA levels in 3T3-L1 adipocytes incubated with CM of RAW264.7 cells at 10% of medium for 48 h. Total RNA was transcribed into cDNA, and analyzed by real-time RT-PCR using specific primers for TNF $\alpha$ . Levels of TNF $\alpha$  mRNA expression are presented as fold increases of that in 3T3-L1 cells incubated with the CM of RAW264.7 cells transfected with control siRNA. \* $P$ <0.05. N.S., not significant ( $n$ =6–10).

decreased mRNA level of TNF $\alpha$  recovered after the addition of recombinant IL-6 in the medium of 3T3-L1 adipocytes.

### 3.6. The knockdown of IL-6 gene in RAW264.7 cells abolishes the effect of K-111 on the TNF $\alpha$ expression in 3T3-L1 adipocytes

We finally knockdowned IL-6 gene using its specific siRNA in order to clarify the role of IL-6 gene expression in RAW264.7 cells in the induction of TNF $\alpha$  expression in 3T3-L1 adipocytes. The transfection with IL-6 siRNA significantly reduced the levels of LPS-induced IL-6 secretion compared to that with control siRNA. Treatment of K-111 had no significant effect on LPS-induced IL-6 production in the IL-6 knockdown cells

(Fig. 6A). We therefore analyzed the effect of CM of LPS-stimulated RAW264.7 cells transfected with IL-6 siRNA on the TNF $\alpha$  expression in 3T3-L1 adipocytes. The TNF $\alpha$  expression induced by CM of IL-6 knockdown cells was significantly decreased compared to the expression level by the CM of control cells (Fig. 6B). K-111 did not change the TNF $\alpha$  expression level induced by the CM of IL-6 knockdown RAW264.7 cells. These results indicate that the effect of K-111 on the inhibition of TNF $\alpha$  expression in 3T3-L1 adipocytes is mediated by the decreased in IL-6 gene expression in RAW264.7 cells.

#### 4. Discussion

Our study using the activated (LPS-stimulated) macrophage cell line, RAW264.7, showed the conditioned medium of activated macrophages to increase the level of TNF $\alpha$  mRNA in 3T3-L1 adipocytes, and this effect was largely dependent on the IL-6 secretion from the activated macrophages. The preincubation of LPS-stimulated RAW264.7-CM with K-111 suppressed the TNF $\alpha$  expression in 3T3-L1 adipocytes induced by the CM of activated macrophage. K-111, a potent PPAR $\alpha$  agonist, inhibited the transcription and subsequent production of IL-6. This effect was caused by the suppression of the activation of SAPK/JNK and the NF- $\kappa$ B translocation in the cells. These effects were evident in comparison to those of rosiglitazone, a selective PPAR $\gamma$  agonist. The blocking of IL-6 production by the inhibition of the SAPK/JNK pathway or siRNA specific for the IL-6 gene abolished the inhibitory effect of K-111 on the TNF $\alpha$  expression in 3T3-L1 adipocytes. These results using cultured macrophages and adipocytes indicate that K-111 suppresses the IL-6 secretion in macrophages through both the SAPK/JNK and NF- $\kappa$ B pathways, thereby reducing the TNF $\alpha$  secretion in 3T3-L1 adipocytes. The PPAR $\alpha$  agonists are thus suggested to be able to modulate the interaction of macrophages and adipocytes in visceral fat, which have been proposed to cause systemic insulin resistance through the activation of cytokines derived from visceral fat.

In macrophages, the biosynthesis of cytokines is regulated at multiple levels, thus involving a multitude of signal transduction pathways. LPS binds to LPS-binding protein (LBP), which activates the MAPKs (ERK1/2, p38 and SAPK/JNK) signals, and then the transcription factors, NF- $\kappa$ B (Sancéau et al., 1995). To characterize the mechanism of the inhibitory effect of PPAR $\alpha$  activation on IL-6 production, we analyzed the effect of K-111 on the activation of MAPKs by LPS. K-111 inhibited the LPS-induced JNK activation, but not the activation of ERK1/2 and p38. The activation of PPAR $\alpha$  also suppressed the phosphorylation of NF- $\kappa$ B subunits of p65, and the degradation of I $\kappa$ B $\alpha$  was stimulated by LPS. These results indicate that PPAR $\alpha$  activation probably suppresses the activation of NF- $\kappa$ B by the inhibition of the degradation of I $\kappa$ B $\alpha$  protein. The PPAR $\alpha$  ligands were demonstrated to inhibit the IL-1 $\beta$ -induced IL-6 secretion, thereby inducing the I $\kappa$ B $\alpha$  mRNA and protein expression in smooth muscle cells and hepatocytes (Delerive et al., 1999, 2000). I $\kappa$ B $\alpha$  protein induction mainly occurs in the nucleus, which may reduce the NF- $\kappa$ B-binding activity

(Delerive et al., 2000). In this study, the PPAR $\alpha$  activation was thought to suppress IL-6 in macrophages through a similar mechanism. In agreement with our observations, fibrates are thus not considered to affect the LPS-induced IL-6 transcription in PPAR $\alpha$ -deficient mice (Delerive et al., 1999). PPAR $\alpha$ -deficient splenocytes produced, in response to LPS stimulation, two to three times more IL-6 than the splenocytes from wild-type mice (Poynter and Daynes, 1998). Furthermore, our results showed the effects of PPAR $\alpha$  activation to be obvious in comparison to those of PPAR $\gamma$  agonist, rosiglitazone. K-111 was recently characterized by Meyer et al. (1999) as a potent PPAR $\alpha$  activator, without the activation of PPAR $\gamma$ . An anti-diabetic potency with insulin sensitizing and lipid-lowering activities was demonstrated in rodent models of type 2 diabetes (Pill and Kuhnle, 1999). Furthermore, K-111 reduced hyperinsulinaemia without changing the blood glucose levels in obese rhesus monkeys (Schafer et al., 2004). A previous study also showed that PPAR $\gamma$  agonists do not obviously suppress IL-6 in macrophage (Thieringer et al., 2000). As a result, PPAR $\alpha$  may play a role in the inflammatory response in fat tissues. K-111, therefore, seems to also show a significant suppression of the inflammatory effect induced by LPS in macrophages. Many cytokines and other factors are produced and released by the fat tissue, and this observation has recently been recognized as the chronic inflammatory state of visceral obesity (Wellen and Hotamisligil, 2003). The cells implanted into visceral, and not subcutaneous, areas express TNF $\alpha$  mRNA, and the bioactive peptide secreted causes decreased insulin sensitivity in muscle (Shibasaki et al., 2002).

In summary, we showed that the activation of PPAR $\alpha$  inhibited the expression of TNF $\alpha$  in 3T3-L1 adipocytes by suppressing IL-6 production in macrophages using a culture system. Our findings therefore suggest that PPAR $\alpha$  activation may improve insulin resistance through the inhibitory effect on the interaction between macrophages and adipocytes in visceral fat tissue in humans. These results obtained using cultured cells need to be further analyzed in other systems to elucidate the significance of PPAR $\alpha$  regulation in fat tissue.

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