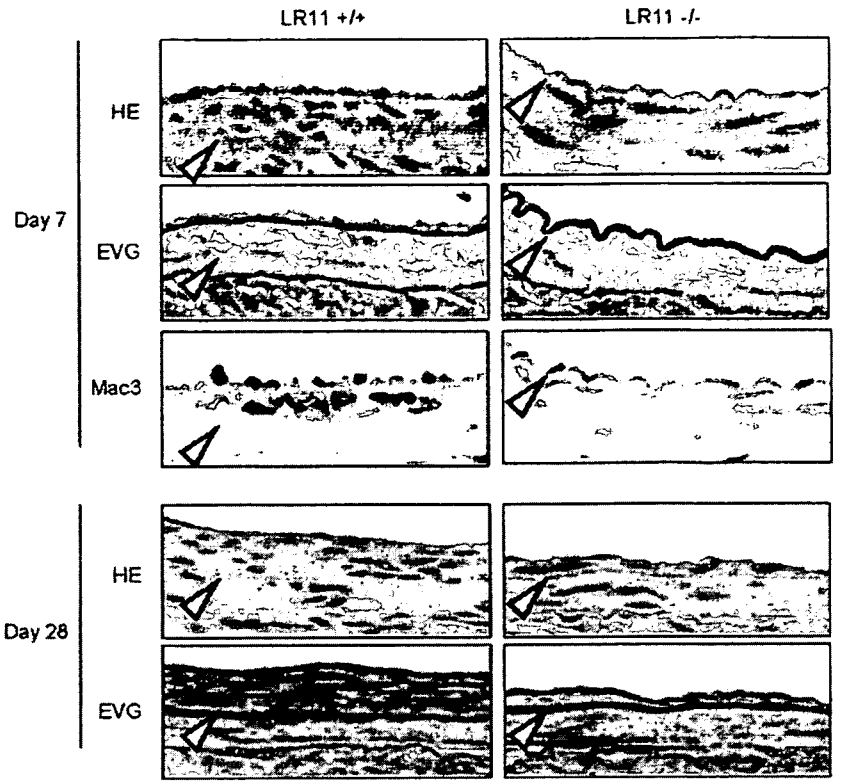


Figure 2. Reduced macrophage infiltration into intima of cuff-injured artery in LR11$^{-/-}$ mice. Sections of femoral artery obtained at day 7 and day 28 after cuff injury in LR11$^{+/+}$ and LR11$^{-/-}$ mice on a high-fat diet were subjected to histological analysis using hematoxylin & eosin (HE) and EVG staining, and to immunohistostaining with anti-Mac3 antibody. Arrowheads indicate the internal elastic layers.

SolLR11 Increases Scavenger Receptor Expression and Lipid Accumulation in THP-1 Macrophages

Because LR11KO mice showed reduced lipid-containing macrophages (Figure 2), we next investigated the effect of solLR11 on the regulation of scavenger receptor expression and lipid accumulation of THP-1 macrophages. THP-1 macrophages were cultured for 24 hours in the presence or absence of PMA and/or solLR11 at 1 μg/mL, followed by Western blot of plasma membrane preparations probed with anti-SR-A and anti-uPAR antibodies. Although solLR11 did not induce SR-A protein expression in the absence of PMA, it increased SR-A expression 2.8-fold in its presence (Figure 3A). The cell-surface level of uPAR was increased by solLR11, likely because of the solLR11-mediated stabilization of uPAR.[11] To test whether solLR11 affects lipid accumulation in macrophages, we evaluated DiI-AcLDL uptake in THP-1 macrophages (Figure 3B). In the undifferentiated THP-1 cells, there was no significant DiI-AcLDL uptake, and solLR11 did not affect DiI-AcLDL uptake (data not shown). However, in THP-1 macrophages, solLR11 at 1 to 100 μg/mL significantly increased DiI-AcLDL uptake (Figure 3C). Addition of neutralizing anti-LR11 or anti-uPAR antibodies almost totally inhibited the increase in DiI-AcLDL uptake by the cells (Figure 3D). These data indicate that solLR11 stimulates lipid uptake via SR-A, and that the accelerated lipid accumulation in macrophages may be attributable to the LR11-mediated upregulation of uPAR levels.

Recombinant SolLR11 Increases Adhesion and Migration of THP-1–Derived Macrophages

We next investigated the effect of solLR11 on the adhesion of THP-1–derived macrophages (THP-1 macrophages) in vitro using the recombinant protein. THP-1 cells were differentiated to macrophages by the treatment with 200 nM PMA for 24 hours, and then the cells were labeled with fluorescent dye Calcein-AM for quantitative analysis by the in vitro adhesion assay. SolLR11 at 1 μg/mL significantly increased the adhesion of THP-1 macrophages to collagen and fibronectin (Figure 4A) 1.8- and 2.1-fold, respectively. The neutralizing anti-LR11 antibody completely blocked solLR11-induced increase in adhesion. Next, we tested the effect of solLR11 on the adhesion of macrophages to SMCs, because of the drastic decrease in macrophage recruitment in intima of cuff-injured artery in LR11$^{-/-}$ mice, principally caused by proliferating SMCs. Pretreatment of THP-1 macrophages with 1 μg/mL solLR11 increased cell adhesion to cultured SMCs 1.6-fold (Figure 4B). The addition of neutralizing antibodies against VLA-4 and PSGL-1 completely inhibited the increased adhesion by solLR11, as observed with anti-LR11 or anti-uPAR antibodies. Thus, we analyzed the effect of solLR11 on the expression of adhesion molecules. SolLR11 enhanced the expression of cell-surface VLA-4 in the presence and absence of PMA (Figure 4C).

We next tested the effect of solLR11 on the migratory functions of THP-1 macrophages by using the Boyden chamber method. SolLR11 itself did not affect migration of THP-1 macrophages in vitro (data not shown). When cells were preincubated with 1 μg/mL solLR11 for 12 hours, PDGF-BB-induced migration of THP-1 macrophage was 2.0-fold greater than in the absence of solLR11 (Figure 4D). The stimulatory effect of solLR11 was decreased by addition of neutralizing anti-LR11 or anti-uPAR antibodies. These data indicate that solLR11 induces adhesion and migration

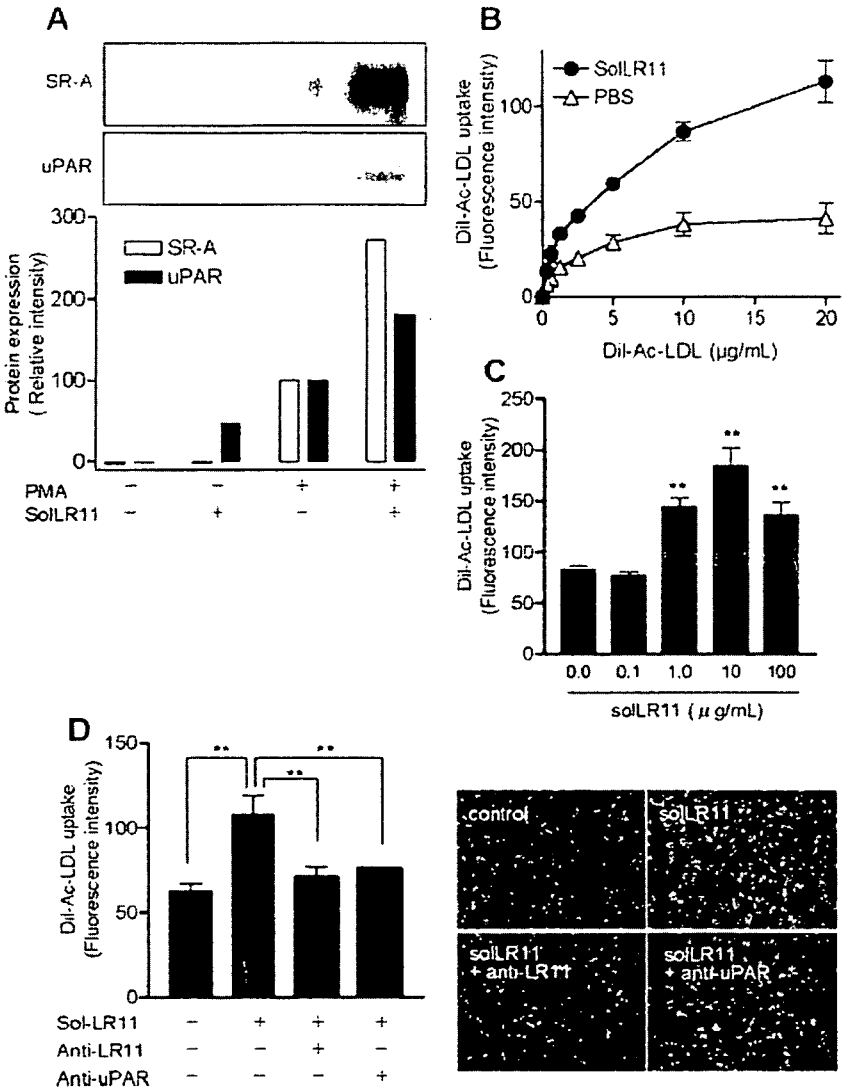


Figure 3. SolLR11 enhances cell-surface expression of SR-A and uPAR, and the uptake of modified LDL by THP-1 macrophages. A, Membranes of THP-1 monocytes or macrophages, prepared from cells obtained under the conditions indicated in the bottom panel, were subjected to Western blotting with anti–SR-A or anti-uPAR antibodies. B, THP-1 macrophages were preincubated with 1 μg/mL solLR11 (black circles) or phosphate-buffered saline (white triangles). Cells were washed and then incubated with the indicated concentrations of Dil-AcLDL in the presence or absence of excess amount of Ac-LDL. C, Dose-dependent effect of solLR11 on Dil-AcLDL uptake by THP-1 macrophages. D, The effects of anti-uPAR and anti-LR11 (5-4-30-19-2, 1:2 dilution) antibodies on solLR11-induced uptake of Dil-AcLDL (10 μg/mL) by THP-1 macrophages. DiAcLDL uptake was visualized by fluorescence microscopy and quantitative measurement was obtained using a fluorescence microplate reader. Data are expressed as mean±SD, n=6 (*$P<0.05$, **$P<0.01$).

activities of macrophages through uPAR-mediated pathways, possibly through increasing the levels of cell-surface adhesion molecules.

Discussion

In this study, we have shown that LR11 is secreted in a soluble form from intimal SMCs in a balloon injury model, and that LR11-deficient mice show drastically decreased lipid-accumulating macrophages in early intimal formation after cuff injury in mice on a high-fat diet. Functional analysis of recombinant solLR11 demonstrated that solLR11 can regulate the functions of THP-1 macrophages toward foam cell formation, such as lipid incorporation, adhesion, and migration. The inducing effect on foam cell formation of solLR11 was almost abolished by functional neutralization of solLR11 or of its target protein, uPAR. Based on these results, we propose a new role of intimal SMCs in the regulation of monocyte/macrophage functions involving the secretion of soluble LR11.

Although LR11 was originally identified as a type I transmembrane protein, significant amounts of LR11 are shed from cultured SMCs, IMR32 and BON cells, and hydra as a soluble form of the large extracellular domain cleaved off by metalloprotease.[11,15,24] In CHO cells, it was demonstrated that tumor necrosis factor-α convertase is responsible for the proteolytic cleavage of LR11.[14,25] However, the physiological function of solLR11 is still poorly understood because of the lack of availability of recombinant protein. We have reported that solLR11, secreted from cultured cells as well as the membrane-bound form,[11] enhance SMC migration, and that the expression of solLR11 largely depends on the differentiation stage of SMCs. The medial contractile type does not express solLR11, whereas the intimal synthetic type does, consistent with the expression of embryonic myosin isoform SMemb.[16] These data suggest that LR11-expressing cells likely perform diverse functions via secretion of soluble LR11 and/or expression of membrane-bound LR11, respectively.

We detected solLR11 protein by Western blot of thickened intima obtained 14 days after balloon injury (when neointimal formation is almost accomplished). Although the level of solLR11 expression was lower than that of the membrane-

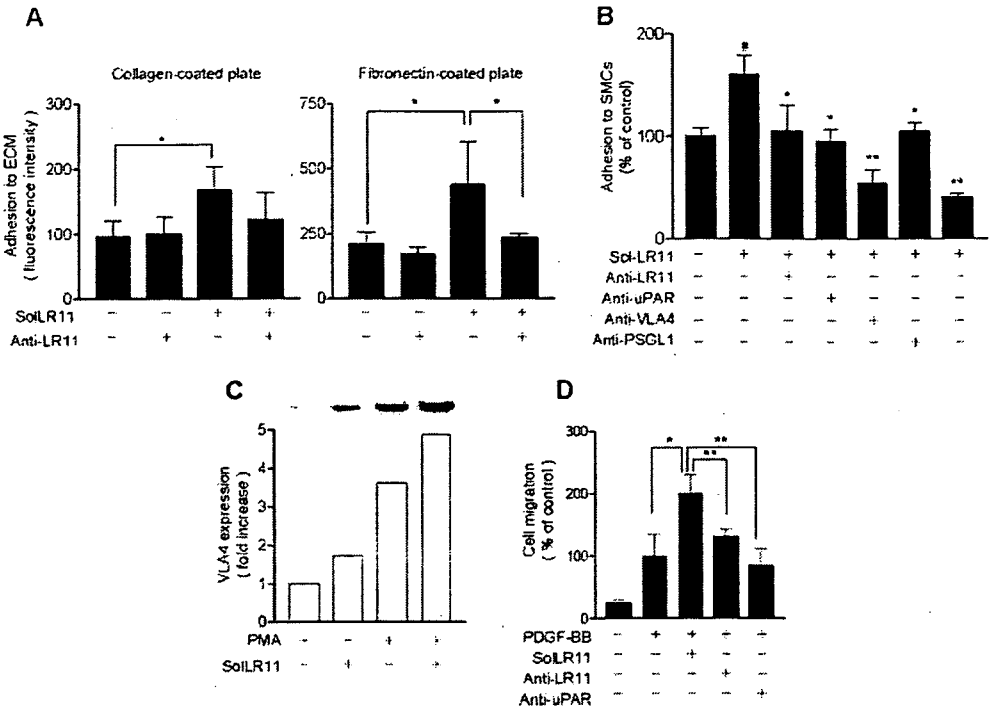


Figure 4. SolLR11 enhances adhesion and migration of THP-1 macrophages. A, THP-1 macrophages were preincubated with 1 μg/mL solLR11 in the presence or absence of neutralizing anti-uPAR antibodies. The cells were washed and then incubated on collagen- or fibronectin-coated plates. B, THP-1 macrophages were preincubated with 1 μg/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 μg/mL solLR11. Membranes were subjected to Western blotting with anti–VLA-4 antibody. D, Cells were preincubated with 1 μg/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±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.[11] 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.[2,26,27] SR-A is highly expressed almost exclusively in differentiated macrophages, and is implicated in increased foam cell formation in atherogenesis.[28,29] 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.[30] 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.[31] 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.[17] LR11 upregulates cell surface uPAR levels in SMCs by inhibition of its catabolism, which is mediated by LRP1,

another member of the LDLR family.[11] LRP1 is also abundantly expressed in monocytes/macrophages;[8] 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 μg/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.

Sources of Funding

These studies were supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to Y.S. and H.B., and from the Austrian Science Foundation to W.J.S. (F-0608).

Disclosures

None.

References

1. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med.* 1999; 340:115–126.
2. Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: beyond lipid uptake. *Arterioscler Thromb Vasc Biol.* 2006;26:1702–1711.
3. Kranzhofer R, Schmidt J, Pfeiffer CA, Hagl S, Libby P, Kubler W. Angiotensin induces inflammatory activation of human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1999;19:1623–1629.
4. Tao Z, Smart FW, Figueroa JE, Glancy DL, Vijayagopal P. Elevated expression of proteoglycans in proliferating vascular smooth muscle cells. *Atherosclerosis.* 1997;135:171–179.
5. Bujo H, Saito Y. Modulation of smooth muscle cell migration by members of the low-density lipoprotein receptor family. *Arterioscler Thromb Vasc Biol.* 2006;26:1246–1252.
6. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. *Science.* 2003; 300:329–332.
7. Webb DJ, Nguyen DH, Gonias SL. Extracellular signal-regulated kinase functions in the urokinase receptor-dependent pathway by which neutralization of low density lipoprotein receptor–related protein promotes fibrosarcoma cell migration and matrigel invasion. *J Cell Sci.* 2000;113: 123–134.
8. Llorente-Cortes V, Badimon L. LDL receptor-related protein and the vascular wall: implications for atherothrombosis. *Arterioscler Thromb Vasc Biol.* 2005;25:497–504.
9. Herz J, Hui DY. Lipoprotein receptors in the vascular wall. *Curr Opin Lipidol.* 2004;15:175–181.
10. Tanaga K, Bujo H, Zhu Y, Kanaki T, Hirayama S, Takahashi K, Inoue M, Mikami K, Schneider WJ, Saito Y. LRP1B attenuates the migration of smooth muscle cells by reducing membrane localization of urokinase and PDGF receptors. *Arterioscler Thromb Vasc Biol.* 2004;24:1422–1428.
11. Zhu Y, Bujo H, Yamazaki H, Ohwaki K, Jiang M, Hirayama S, Kanaki T, Shibasaki M, Takahashi K, Schneider WJ, Saito Y. LR11, an LDL receptor gene family member, is a novel regulator of smooth muscle cell migration. *Circ Res.* 2004;94:752–758.
12. Kanaki T, Bujo H, Hirayama S, Ishii I, Morisaki N, Schneider WJ, Saito Y. Expression of LR11, a mosaic LDL receptor family member, is markedly increased in atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* 1999;19:2687–2695.
13. Zhu Y, Bujo H, Yamazaki H, Hirayama S, Kanaki T, Takahashi K, Shibasaki M, Schneider WJ, Saito Y. Enhanced expression of the LDL receptor family member LR11 increases migration of smooth muscle cells in vitro. *Circulation.* 2002;105:1830–1836.
14. Hermey G, Sjogaard SS, Petersen CM, Nykjaer A, Gliemann J. Tumour necrosis factor alpha-converting enzyme mediates ectodomain shedding of Vps10p-domain receptor family members. *Biochem J.* 2006;395:285–293.
15. Hampe W, Riedel IB, Lintzel J, Bader CO, Franke I, Schaller HC. Ectodomain shedding, translocation and synthesis of SorLA are stimulated by its ligand head activator. *J Cell Sci.* 2000;113:4475–4485.
16. Jiang M, Bujo H, Zhu Y, Yamazaki H, Hirayama S, Kanaki T, Shibasaki M, Takahashi K, Schneider WJ, Saito Y. Pitavastatin attenuates the PDGF-induced LR11/uPA receptor-mediated migration of smooth muscle cells. *Biochem Biophys Res Commun.* 2006;348:1367–1377.
17. May AE, Schmidt R, Kanse SM, Chavakis T, Stephens RW, Schomig A, Preissner KT, Neumann FJ. Urokinase receptor surface expression regulates monocyte adhesion in acute myocardial infarction. *Blood.* 2002; 100:3611–3617.
18. Gu JM, Johns A, Morser J, Dole WP, Greaves DR, Deng GG. Urokinase plasminogen activator receptor promotes macrophage infiltration into the vascular wall of ApoE deficient mice. *J Cell Physiol.* 2005;204:73–82.
19. Cozen AE, Moriwaki H, Kremen M, DeYoung MB, Dichek HL, Slezicki KI, Young SG, Veniant M, Dichek DA. Macrophage-targeted overexpression of urokinase causes accelerated atherosclerosis, coronary artery occlusions, and premature death. *Circulation.* 2004;109:2129–2135.
20. McMurray HF, Parrott DP, Bowyer DE. A standardised method of culturing aortic explants, suitable for the study of factors affecting the phenotypic modulation, migration and proliferation of aortic smooth muscle cells. *Atherosclerosis.* 1991;86:227–237.
21. Minamino T, Kurihara H, Takahashi M, Shimada K, Maemura K, Oda H, Ishikawa T, Uchiyama T, Tanzawa K, Yazaki Y. Endothelin-converting enzyme expression in the rat vascular injury model and human coronary atherosclerosis. *Circulation.* 1997;95:221–230.
22. Yamazaki H, Bujo H, Kusunoki J, Seimiya K, Kanaki T, Morisaki N, Schneider WJ, Saito Y. Elements of neural adhesion molecules and a yeast vacuolar protein sorting receptor are present in a novel mammalian low density lipoprotein receptor family member. *J Biol Chem.* 1996;271: 24761–24768.
23. Trigwell S, Wood L, Jones P. Soluble urokinase receptor promotes cell adhesion and requires tyrosine-92 for activation of p56/59(hck). *Biochem Biophys Res Commun.* 2000;278:440–446.
24. Hampe W, Urny J, Franke I, Hoffmeister-Ullerich SA, Herrmann D, Petersen CM, Lohmann J, Schaller HC. A head-activator binding protein is present in hydra in a soluble and a membrane-anchored form. *Development.* 1999;126:4077–4086.
25. Bohm C, Seibel NM, Henkel B, Steiner H, Haass C, Hampe W. SorLA signaling by regulated intramembrane proteolysis. *J Biol Chem.* 2006; 281:14547–14553.
26. Boyle JJ. Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. *Curr Vasc Pharmacol.* 2005;3:63–68.
27. Bobryshev YV. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron.* 2006;37:208–222.
28. de Winther MP, van Dijk KW, van Vlijmen BJ, Gijbels MJ, Heus JJ, Wijers ER, van den Bos AC, Breuer M, Frants RR, Havekes LM, Hofker MH. Macrophage specific overexpression of the human macrophage scavenger receptor in transgenic mice, using a 180-kb yeast artificial chromosome, leads to enhanced foam cell formation of isolated peritoneal macrophages. *Atherosclerosis.* 1999;147:339–347.
29. Linton MF, Fazio S. Class A scavenger receptors, macrophages, and atherosclerosis. *Curr Opin Lipidol.* 2001;12:489–495.
30. Cai Q, Lanting L, Natarajan R. Interaction of monocytes with vascular smooth muscle cells regulates monocyte survival and differentiation through distinct pathways. *Arterioscler Thromb Vasc Biol.* 2004;24:2263–2270.
31. O'Brien KD, McDonald TO, Chait A, Allen MD, Alpers CE. Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. *Circulation.* 1996;93:672–682.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 368 (2008) 81–87
BBRC
www.elsevier.com/locate/ybbrc

Low-dose GH supplementation reduces the TLR2 and TNF-α expressions in visceral fat

Yoshitaka Kubota [a], Hiroyuki Unoki [b], Hideaki Bujo [c,*], Naoaki Rikihisa [a], Akikazu Udagawa [a], Shinya Yoshimoto [a], Masaharu Ichinose [a], Yasushi Saito [d]

[a] *Department of Plastic and Reconstructive Surgery, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[b] *Department of Applied Translational Research, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[c] *Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[d] *Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*

Received 3 January 2008
Available online 15 January 2008

Abstract

The increased population of TLR2/TNF-α 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-α 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-α 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-α 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-α co-expressing cells through the action of IGF-1.

Keywords: Growth hormone; Toll-like receptor 2; Adipocyte; Visceral fat; Insulin-like growth factor-1

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)-α secretion in mice [3]. The TNF-α expression of visceral adipocytes is accompanied with toll-like receptor (TLR) 2 expression, and the population of TLR2/TNF-α 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-α 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 (H. Bujo).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.01.035

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-α 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 μM myristic acid and 500 μM 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 GATCGCCATGGGCTACAGGCTCCCGGAC-3′ and 5′-ATGCGT TTAAACGAAGCCACAGCTGCCCTCCAC-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 ± 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 × 10^6 cells/250 μl 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 × 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 μg/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-α mRNA quantification, Real-time RT-PCR amplification were performed using TLR2 primers (Mm00442346_m1, Applied Biosystems) and TNF-α 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 ± 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 Lucus Microview Software (ver 2.2) (GE Healthcare).

Statistical analysis. The results are shown as means ± 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-α co-expressing adipocytes in visceral fat

We have previously shown that high fat intake induces an increased number of TLR2/TNF-α-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-α 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 ± 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-α co-expressing adipocytes was

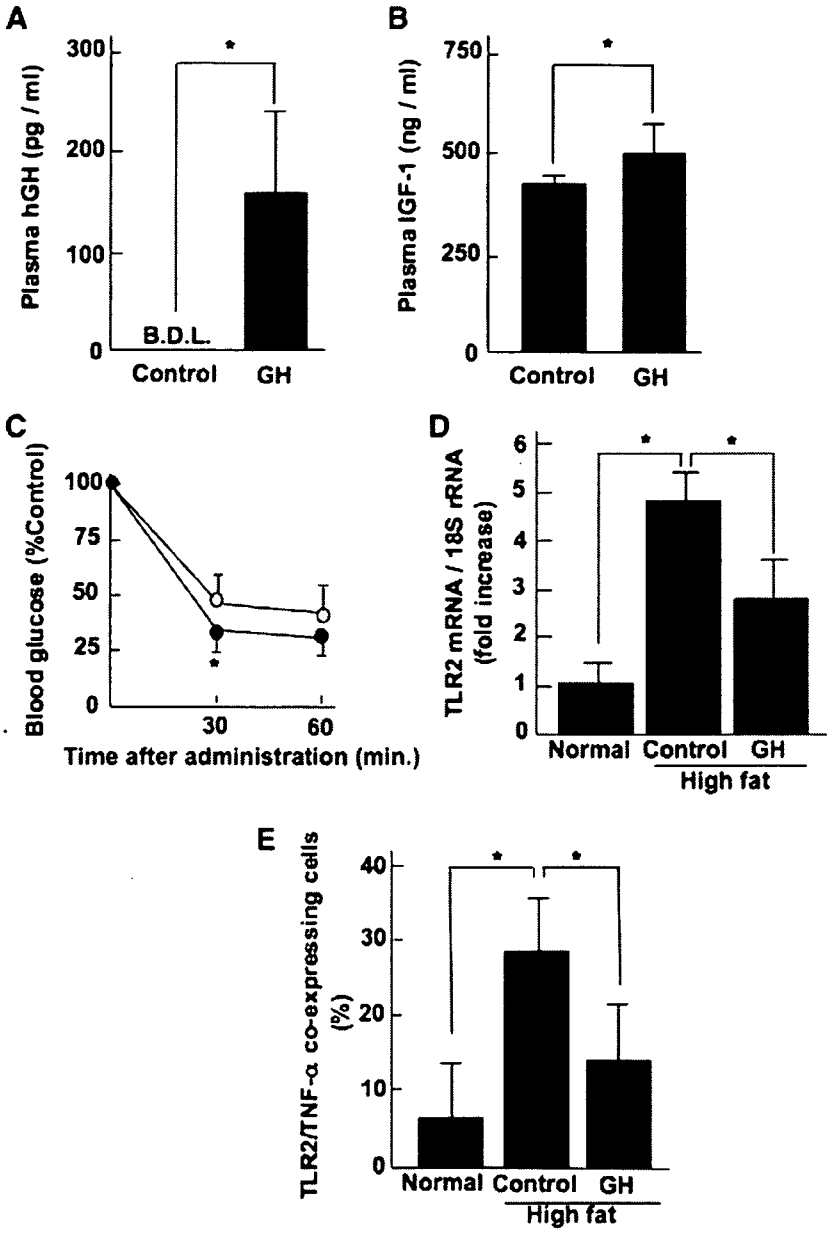


Fig. 1. The effects of low-dose hGH supplementation on reduced insulin sensitivity in high-fat fed mice. (A) The plasma hGH concentration in mice supplemented with hGH (GH) or PBS (control). Male C57BL/6J mice, which have been fed with a high-fat diet (60% fat) for 6 months, were supplemented with hGH (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-α 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-α 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-α 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-α-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-α 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 ± 165 pg/ml vs 136 ± 128 pg/ml, $P < 0.05$). The plasma IGF-1 concentrations were significantly higher in the GH mice than in the mock mice (323 ± 71 ng/ml vs 267 ± 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-α 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-α co-expressing adipocytes in visceral fat, and possibly the amelioration by GH supplementation is mediated by the effects of increased plasma IGF-1.

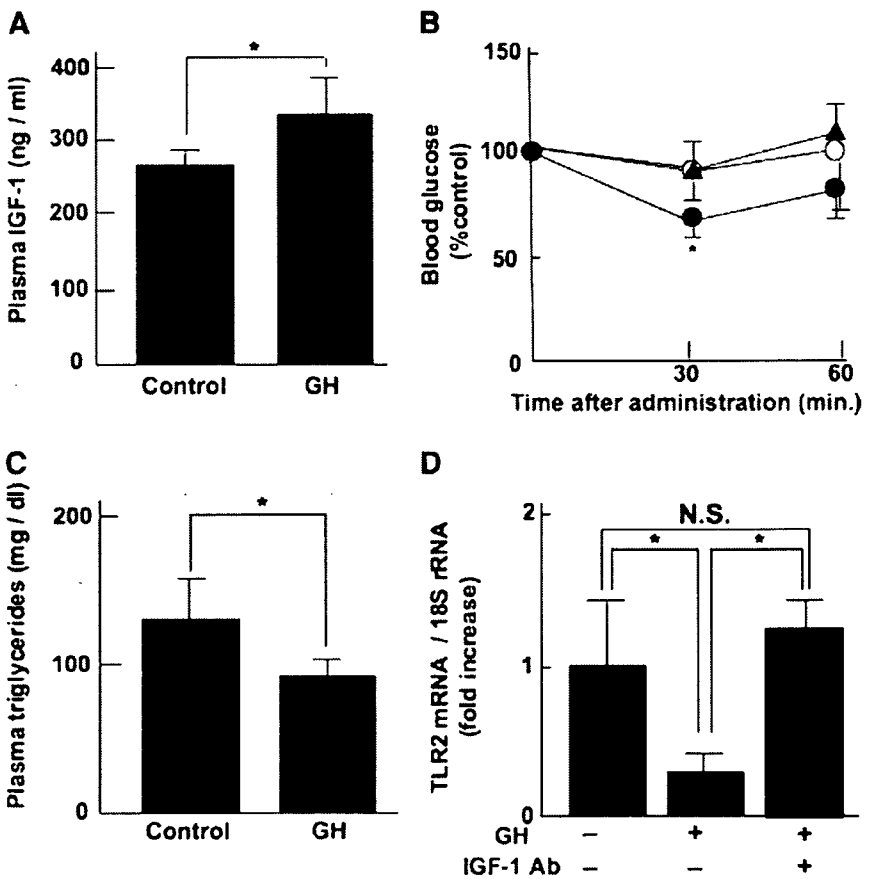


Fig. 2. Effects of IGF-1 neutralization on the actions of low-dose GH supplementation using cell transplantation models. Male BALB/c nude mouse was subcutaneously injected with 10^6 cells of hGH-overexpressing 3T3-L1 preadipocytes. A high-fat diet was started at a week after cell implantation, and continued 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 (○). 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-α co-expressing adipocytes, we analyzed the effects of IGF-1 on the TLR2 and TNF-α mRNA expressions in 3T3-L1 adipocytes (Fig. 3A). The TNF-α 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-α by FFAs. In contrast, IGF-1 completely inhibited the FFA-induced increase in TNF-α 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-α 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-α 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-α 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-α expression is induced in adipocytes accumulated in the visceral, and not in the subcutaneous, regions, using a cell transplantation model [3]. The TNF-α expression in the visceral fat is closely associated with the increased population of TLR2/TNF-α co-expressing adipocytes in response to a high-fat intake [4]. The identification of the TLR2/TNF-

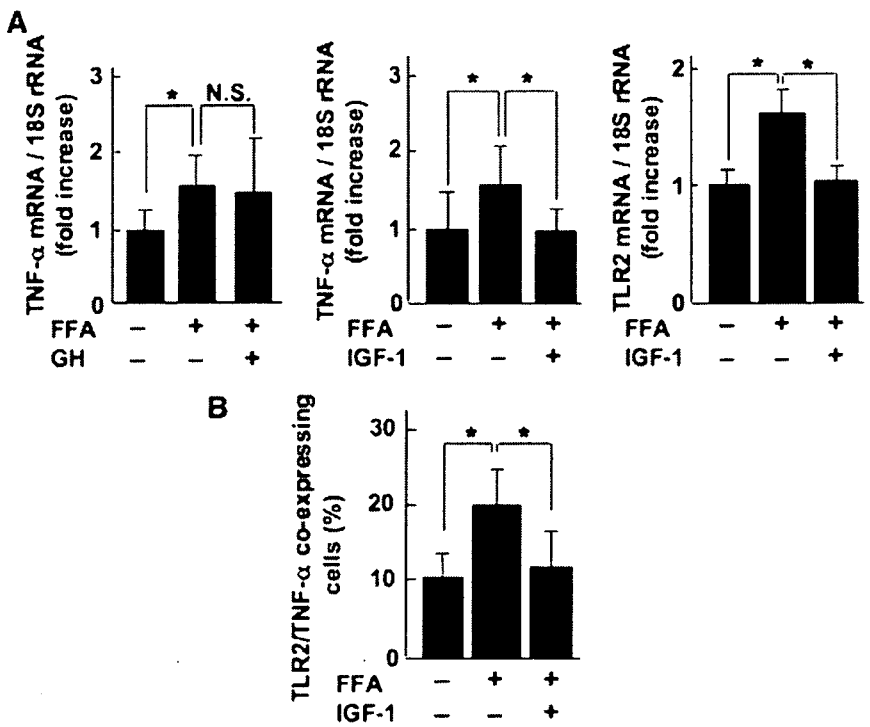


Fig. 3. Effects of GH or IGF-1 on FFA-induced TLR2 and TNF-expressions in 3T3-L1 adipocytes. (A) Serum-starved 3T3-L1 adipocytes treated with 1 mM FFA in the presence or absence of hGH or IGF-1 for 8 h. Quantitative RT-PCR was used to measure the expression level of TNF-α gene or TLR2 gene $n = 6$. $^*P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

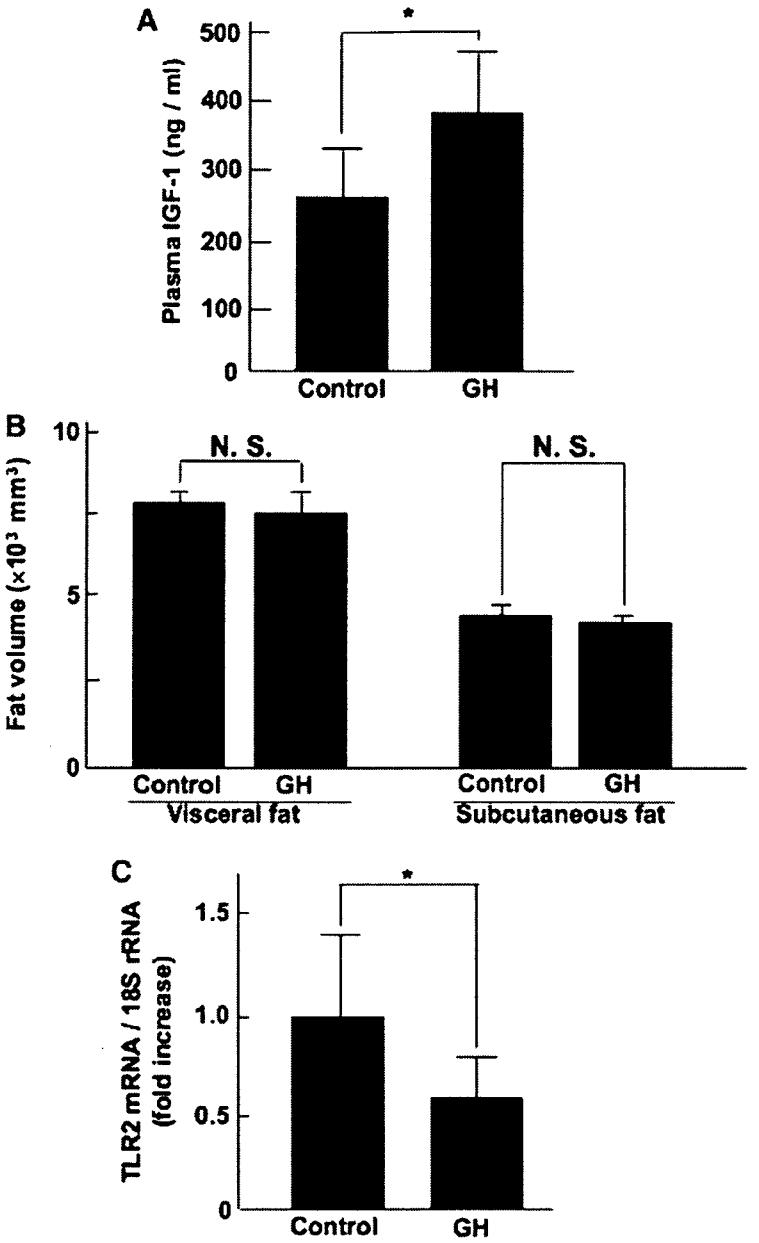


Fig. 4. Effects of low-dose hGH supplementation on the fat volume and TLR2 mRNA expression of visceral fat. Male ob/ob mice were supplemented with hGH (0.5 mg/kg/day) (GH) or PBS (Control) for 4 weeks. (A) The plasma mouse IGF-1 concentration was measured. $n = 7$. $^*P < 0.05$. (B) Visceral or subcutaneous 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. $n = 7$. $^*P < 0.05$.

α co-expressing adipocytes as a regulator of TNF-α 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-α-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 heterogenous 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-α 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-α 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-α 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-α 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 heterogenous 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

We thank for Drs. K. Aragane and J. Kusunoki (Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd) for their assistance of fat volume measurement. These studies were supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology to N.R., Y.S., and H.B.

References

[1] S.M. Grundy, Metabolic syndrome: a multiplex cardiovascular risk factor, J. Clin. Endocrinol. Metab. 92 (2007) 399–404.

[2] L. Hutley, J.B. Prins, Fat as an endocrine organ: relationship to the metabolic syndrome, Am. J. Med. Sci. 330 (2005) 280–289.

[3] M. Shibasaki, K. Takahashi, T. Itou, S. Miyazawa, M. Ito, J. Kobayashi, H. Bujo, Y. Saito, Alterations of insulin sensitivity by the implantation of 3T3-L1 cells in nude mice. A role for TNF-alpha? Diabetologia 45 (2002) 518–526.

[4] K. Murakami, H. Bujo, H. Unoki, Y. Saito, High fat intake induces a population of adipocytes to co-express TLR2 and TNFalpha in mice with insulin resistance, Biochem. Biophys. Res. Commun. 354 (2007) 727–734.

[5] C.W. Ahn, C.S. Kim, J.H. Nam, H.J. Kim, J.S. Nam, J.S. Park, E.S. Kang, B.S. Cha, S.K. Lim, K.R. Kim, H.C. Lee, K.B. Huh, Effects of growth hormone on insulin resistance and atherosclerotic risk factors in obese type 2 diabetic patients with poor glycaemic control, Clin. Endocrinol. (Oxf.) 64 (2006) 444–449.

[6] G. Johannsson, P. Marin, L. Lonn, M. Ottosson, K. Stenlof, P. Bjorntorp, L. Sjostrom, B.A. Bengtsson, Growth hormone treatment of abdominally obese men reduces abdominal fat mass, improves glucose and lipoprotein metabolism, and reduces diastolic blood pressure, J. Clin. Endocrinol. Metab. 82 (1997) 727–734.

[7] K.C. Yuen, D.B. Dunger, Persisting effects on fasting glucose levels and insulin sensitivity after 6 months of discontinuation of a very low-dose GH therapy in adults with severe GH deficiency, Clin. Endocrinol. (Oxf.) 64 (2006) 549–555.

[8] T. Hirata, H. Unoki, H. Bujo, K. Ueno, Y. Saito, Activation of diacylglycerol O-acyltransferase 1 gene results in increased tumor necrosis factor-alpha gene expression in 3T3-L1 adipocytes, FEBS Lett. 580 (2006) 5117–5121.

[9] M. Shibasaki, K. Takahashi, T. Itou, H. Bujo, Y. Saito, A PPAR agonist improves TNF-alpha-induced insulin resistance of adipose tissue in mice, Biochem. Biophys. Res. Commun. 309 (2003) 419–424.

[10] M. Ito, H. Bujo, K. Takahashi, T. Arai, I. Tanaka, Y. Saito, Implantation of primary cultured adipocytes that secrete insulin modifies blood glucose levels in diabetic mice, Diabetologia 48 (2005) 1614–1620.

[11] H. Unoki, H. Bujo, S. Yamagishi, M. Takeuchi, T. Imaizumi, Y. Saito, Advanced glycation end products attenuate cellular insulin sensitivity by increasing the generation of intracellular reactive oxygen species in adipocytes, Diabetes Res. Clin. Pract. 76 (2007) 236–244.

[12] H.S. Kooistra, G. Voorhout, P.J. Selman, A. Rijnberk, Progestin-induced growth hormone (GH) production in the treatment of dogs with congenital GH deficiency, Domest. Anim. Endocrinol. 15 (1998) 93–102.

[13] M.A. Salem, Effects of the amino-terminal portion of human growth hormone on glucose clearance and metabolism in normal, diabetic, hypophysectomized, and diabetic-hypophysectomized rats, Endocrinology 123 (1988) 1565–1576.

[14] M.A. Heffernan, A.W. Thorburn, B. Fam, R. Summers, B. Conway-Campbell, M.J. Waters, F.M. Ng, Increase of fat oxidation and weight loss in obese mice caused by chronic treatment with human growth hormone or a modified C-terminal fragment, Int. J. Obes. Relat. Metab. Disord. 25 (2001) 1442–1449.

[15] E. Corpas, S.M. Harman, M.R. Blackman, Human growth hormone and human aging, Endocr. Rev. 14 (1993) 20–39.

[16] M.N. Dieudonne, R. Pecquery, M.C. Leneveu, Y. Giudicelli, Opposite effects of androgens and estrogens on adipogenesis in rat preadipocytes: evidence for sex and site-related specificities and possible involvement of insulin-like growth factor 1 receptor and peroxisome proliferator-activated receptor gamma2, Endocrinology 141 (2000) 649–656.

[17] P. Fischer-Posovszky, H. Tornqvist, K.M. Debatin, M. Wabitsch, Inhibition of death-receptor mediated apoptosis in human adipocytes by the insulin-like growth factor I (IGF-I)/IGF-I receptor autocrine circuit, Endocrinology 145 (2004) 1849–1859.

[18] K.C. Yuen, D.B. Dunger, Therapeutic aspects of growth hormone and insulin-like growth factor-I treatment on visceral fat and insulin sensitivity in adults, Diabetes Obes. Metab. 9 (2007) 11–22.

Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 561 (2007) 206–213
ejp
www.elsevier.com/locate/ejphar

Effect of PPARα activation of macrophages on the secretion of inflammatory cytokines in cultured adipocytes

Kentaro Murakami [a], Hideaki Bujo [b,*], Hiroyuki Unoki [c], Yasushi Saito [a]

[a] Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan
[b] Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
[c] Division of Applied Translational Research, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

Received 8 June 2006; received in revised form 20 December 2006; accepted 21 December 2006
Available online 20 January 2007

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 PPARα activation of macrophages on the modulation of the tumor necrosis factor α (TNFα) 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 TNFα 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 PPARα 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 PPARγ agonist. The activation of the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) pathway and nuclear factor kappa B (NF-κB) 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 TNFα expression in the 3T3-L1 adipocytes. As a result, the IL-6 produced by RAW264.7 cells is an inducer of TNFα expression in 3T3-L1 adipocytes, and the IL-6 secretion is inhibited by the activation of PPARα. The PPARα activators may suppress the pathogenetical secretion of TNFα in the adipocytes through the functional modulation of the infiltrated macrophages.

Keywords: Adipocyte; PPARα; Macrophage; Fat tissue; IL-6; TNFα

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 α (TNFα) 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 TNFα 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 (H. Bujo).

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.12.037

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. PPARγ plays a pivotal function in the differentiation of adipocytes (Adams et al., 1997; Rosen and Spiegelman, 2001). In fact, chronic treatment with PPARγ 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 PPARγ agonists do not obviously suppress the IL-6 production in macrophage (Thieringer et al., 2000). PPARα 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 PPARα 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 PPARα activation in the pathogenetical 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(2*H*)-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_2/95% 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 μg/ml insulin, 0.5 mM isobutylmethylxanthine and 0.25 μM dexamethazone.

The cells had accumulated fat droplets after an additional 3 days in the culture medium with 5 μg/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_2/95% humidified air at 37 °C. The cells were treated with or without K-111 (30 μM) and/or SP600125 (10 μM) and then were incubated for 18 h, followed by the addition of LPS (1 μg/ml). After 8 h, the medium was changed with or without K-111 (30 μM) and/or SP600125 (10 μM). After 18 or 36 h, the medium was collected and centrifuged at 300 ×*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 μm 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 μM), rosiglitazone (30 μM) or SP600125 (10 μM) followed by the addition of LPS (1 μg/ml). The mixture was incubated at 37 °C for 8 h. The culture medium was then subjected to centrifugation at 300 ×*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 TNFα 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 μg 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 TNFα were purchased from the manufacturer (PE Applied Biosystems, Foster City, CA), and then were used for the amplification step. IL-6 and TNFα 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 μM). After 30 min, each well

was treated with LPS (1 μg/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 μM). After 18 h, each well was treated with LPS (1 μg/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-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-κB) p65, or total ERK1/2, p38MAPK, SAPK/JNK and IκBα 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 IκBα and phosphorylated NF-κB p65.

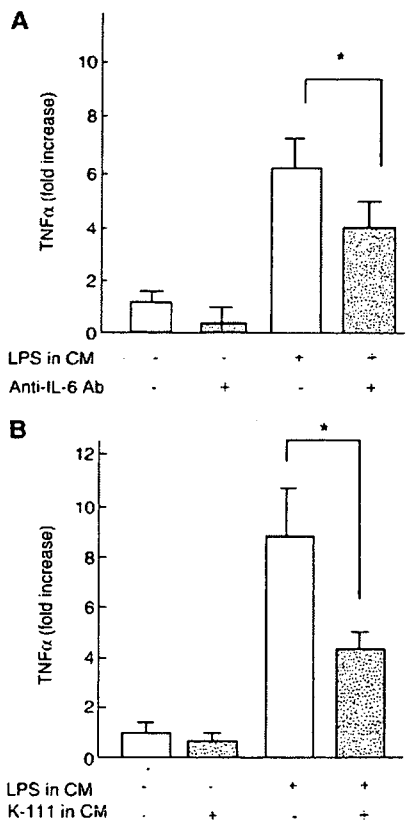


Fig. 1. Effect of K-111 on the potency of RAW264.7 cells of the conditioned medium for the TNFα mRNA expression in 3T3-L1 cells. A. 3T3-L1 adipocytes were treated with a conditioned medium of RAW264.7 cells, which were added until they reached 10%, in the absence or presence of IL-6-neutralizing antibodies 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 TNFα. Levels of TNFα mRNA expression in each treatment are presented as a fold increase of that in untreated cells. $^{*}P<0.05$ ($n=4$–5).

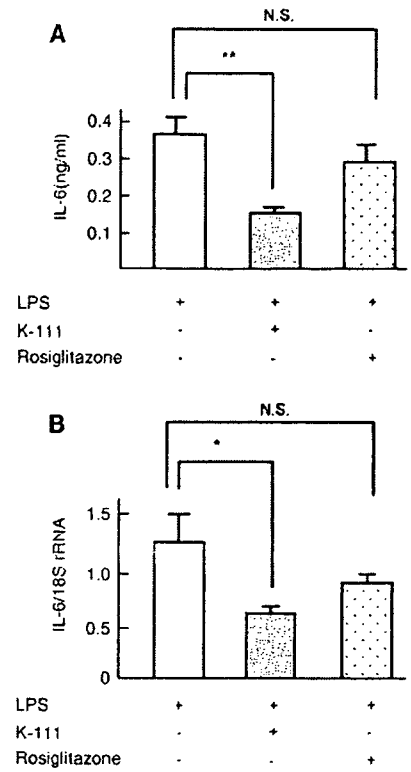


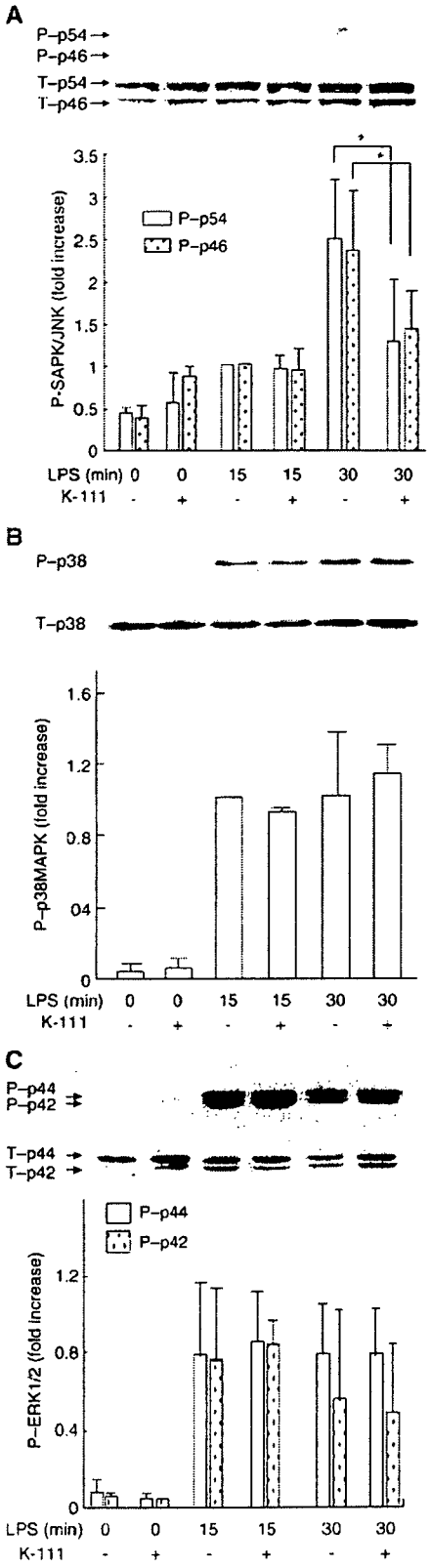
Fig. 2. Effect of K-111 on the secretion and gene expression of IL-6 in RAW264.7 cells. A. RAW264.7 cells were pretreated with or without 30 μM of K-111 or rosiglitazone for 18 h before the addition of LPS (1 μg/ml). The concentrations of IL-6 in the culture supernatants 8 h after stimulation by LPS 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.

2.8. siRNA transfection

RAW264.7cells 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±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 PPARα agonist, K-111, on the TNFα expression in 3T3-L1 adipocytes through the action for RAW264.7 cells

In order to determine the effect of the PPARα 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 TNFα 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 TNFα mRNA in 3T3-L1 adipocytes to be significantly induced with the conditioned medium of RAW264.7 cells (Fig. 1A). The mRNA level of TNFα was increased by 6-fold of the control. The increased level of TNFα mRNA was significantly inhibited in the presence of neutralizing antibody against IL-6 (1 μg/ml). These results indicated that IL-6, which is secreted from RAW264.7 cells, is a major inducer of the TNFα expression in 3T3-L1 adipocytes.

We next analyzed the effect of CM of RAW264.7 cells pretreated with a specific PPARα agonist, K-111 on the TNFα expression in 3T3-L1 adipocytes (Fig. 1B). Pretreatment of CM with K-111 reduced its induction activity for TNFα mRNA expression in 3T3-L1 cells. As a result, the PPARα activation was found to cause a decrease in the TNFα 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 PPARγ agonist. The results of ELISA showed that K-111 (30 μM) significantly reduced the IL-6 production after stimulation with LPS (1 μg/ml) in RAW264.7 cells (Fig. 2A). On the other hand, rosiglitazone (30 μM) 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 μg/ml) in the presence of K-111 using real-time RT-PCR. K-111 (30 μM) 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 μM of K-111 for 18 h before the addition of LPS (1 μg/ml). Samples (each containing 7.5 μg 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 PPARα 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 PPARα 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.

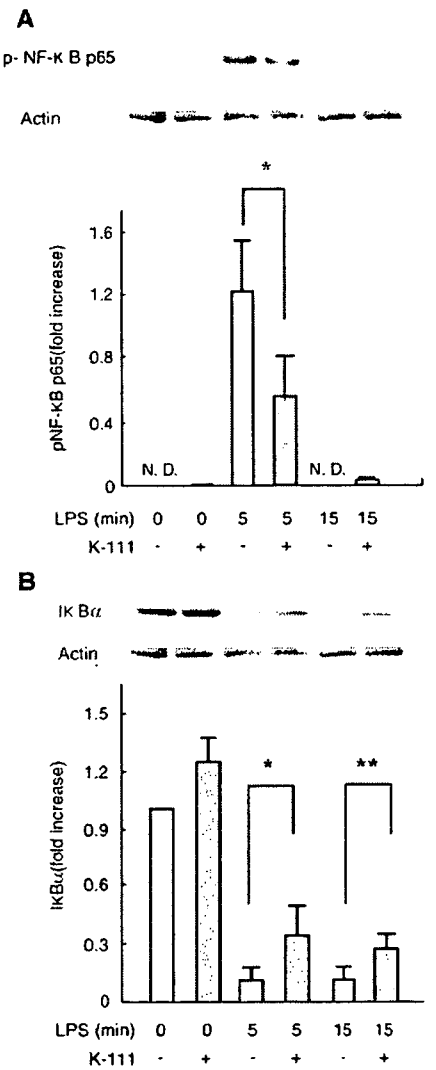


Fig. 4. The effect of K-111 on the LPS-induced NF-κB activation in RAW264.7 cells. RAW264.7 cells were preincubated at 37 °C for 18 h with or without K-111 (30 μM), and then were treated with 1 μg/ml of LPS for 0–15 min. Next, cytosol extracts (each containing 10 μg of protein) were prepared and analyzed by Western blotting using an antibody which specifically recognizes phospho(p)-NF-κB p65 (A). The cytosol extracts (each containing 10 μg of protein) were also analyzed by Western blotting using an antibody which specifically recognizes IκBα (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 μg/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 potently activated at 30 min (maximum at 15 min). Pretreatment of the cell with 30 μM 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-κB activation in RAW264.7 cells

The IL-6 expression is known to be mediated by NF-κB 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-κB in RAW264.7 cells. The effect of K-111 on LPS-induced activation of NF-κB was evaluated by Western blot analysis using the cytosol extracts from RAW264.7 cells. RAW264.7 cells were preincubated with 30 μM of K-111, and then with LPS (1 μg/ml) for 0–30 min. LPS significantly induced the phospho(p)-NF-κB 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 IκBα degradation, since the NF-κB activation is regulated by the steady-state levels of its inhibitor IκBα protein. RAW264.7 cells were pretreated with K-111 (30 μM) for 18 h prior to stimulation with LPS (1 μg/ml). Thereafter, the cytoplasmic IκBα levels were analyzed by Western blot analysis. Stimulation with LPS decreased the IκBα signal intensity within 15 min (Fig. 4B), and re-appeared at 30 min (data not shown). The IκBα 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-κB activation.

3.5. Blocking of SAPK/JNK pathway abolishes the effect of K-111 on the IL-6 secretion of RAW264.7 and TNFα 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 TNFα 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 TNFα expression in RAW264.7 cells and 3T3-L1 adipocytes, respectively (Fig. 5). For this purpose, RAW264.7 cells were pretreated with 10 μM SP600125, a specific SAPK/JNK inhibitor (Bennett et al., 2001; Han et al., 2001), before the K-111 treatment. SP600125 significantly decreased the IL-6 production after stimulation with LPS (1 μg/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 TNFα expression in 3T3-L1 adipocytes. The pretreatment of CM with SP600125 significantly reduced the LPS-induced level of the TNFα 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 TNFα expression in 3T3-L1 adipocytes (Fig. 5B). Furthermore, the

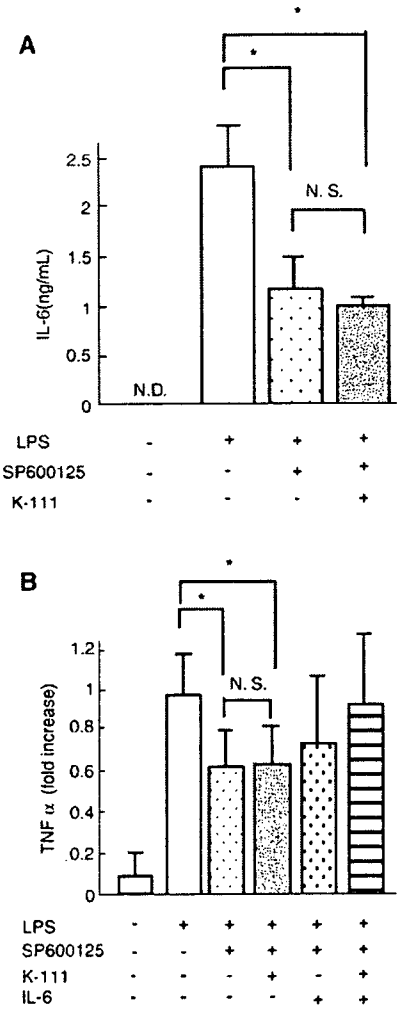


Fig. 5. Effect of blocking of SAPK/JNK pathway on the K-111 induced inhibition for IL-6 secretion in RAW264.7 cells and TNFα expression in 3T3-L1 adipocytes. A. RAW264.7 cells were pretreated with or without 10 μM SP600125 in the presence or absence of 30 μM K-111 for 18 h before the addition of LPS (1 μg/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 TNFα. Levels of TNFα 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 ($n=5$).

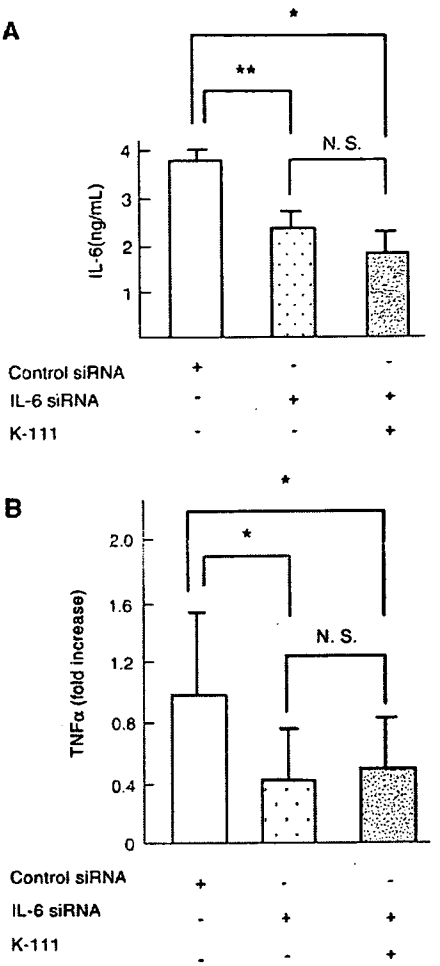


Fig. 6. The effect of the IL-6 gene knockdown using the specific siRNA in RAW264.7 cells on the inhibitory action of K-111 for the TNFα expression in 3T3-L1 adipocytes. RAW264.7 cells were transfected with siRNA to IL-6 or control siRNA. Transfected cells were incubated with 30 μM K-111 for 18 h before the addition of LPS (1 μg/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. TNFα 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 TNFα. Levels of TNFα 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 TNFα 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 TNFα 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 TNFαexpression 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 TNFα expression in 3T3-L1 adipocytes. The TNFα 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 TNFα 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 TNFα 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 TNFα 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 TNFα expression in 3T3-L1 adipocytes induced by the CM of activated macrophage. K-111, a potent PPARα 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-κB translocation in the cells. These effects were evident in comparison to those of rosiglitazone, a selective PPARγ 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 TNFα 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-κB pathways, thereby reducing the TNFα secretion in 3T3-L1 adipocytes. The PPARα 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-κB (Sancéau et al., 1995). To characterize the mechanism of the inhibitory effect of PPARα 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 PPARα also suppressed the phosphorylation of NF-κB subunits of p65, and the degradation of IκBα was stimulated by LPS. These results indicate that PPARα activation probably suppresses the activation of NF-κB by the inhibition of the degradation of IκBα protein. The PPARα ligands were demonstrated to inhibit the IL-1β-induced IL-6 secretion, thereby inducing the IκBα mRNA and protein expression in smooth muscle cells and hepatocytes (Delerive et al., 1999, 2000). IκBα protein induction mainly occurs in the nucleus, which may reduce the NF-κB-binding activity (Delerive et al., 2000). In this study, the PPARα 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 PPARα-deficient mice (Delerive et al., 1999). PPARα-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 PPARα activation to be obvious in comparison to those of PPARγ agonist, rosiglitazone. K-111 was recently characterized by Meyer et al. (1999) as a potent PPARα activator, without the activation of PPARγ. 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 PPARγ agonists do not obviously suppress IL-6 in macrophage (Thieringer et al., 2000). As a result, PPARα 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 TNFα mRNA, and the bioactive peptide secreted causes decreased insulin sensitivity in muscle (Shibasaki et al., 2002).

In summary, we showed that the activation of PPARα inhibited the expression of TNFα in 3T3-L1 adipocytes by suppressing IL-6 production in macrophages using a culture system. Our findings therefore suggest that PPARα 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 PPARα regulation in fat tissue.

References

Adams, M., Montague, C.T., Prins, J.B., Holder, J.C., Smith, S.A., Sanders, L., Digby, J.E., Sewter, C.P., Lazar, M.A., Chatterjee, V.K., O'Rahilly, S., 1997. Activators of peroxisome proliferators activated receptor gamma have depot specific effects on human preadipocyte differentiation. J. Clin. Invest. 100, 3149–3153.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., Anderson, D.W., 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc. Natl. Acad. Sci. U. S. A. 98, 13681–13686.

Delerive, P., De Bosscher, K., Besnard, S., Vanden Berghe, W., Peters, J.M., Gonzalez, F.J., Fruchart, J.C., Tedgui, A., Haegeman, G., Staels, B., 1999. PPARα negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-κB and AP-1. J. Biol. Chem. 274, 32048–32054.

Delerive, P., Gervois, P., Fruchart, J.C., Staels, B., 2000. Induction of IkBα expression as a mechanism contributing to the anti-inflammatory activities of PPARα activators. J. Biol. Chem. 275, 36703–36707.

Expert panel on detection, evaluation and treatment of high blood cholesterol in adults, 2001. Executive Summary of the Third Report of the National

Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA 285, 2486–2497.

Fernandez-Real, J.M., Vayreda, M., Richart, C., Gutierrez, C., Broch, M., Vendrell, J., Ricart, W., 2001. Circulating interleukin 6 levels, blood pressure, and insulin sensitivity in apparently healthy men and women. J. Clin. Endocrinol. Metab. 86, 1154–1159.

Ginsberg, H.N., 2000. Insulin resistance and cardiovascular disease. J. Clin. Invest. 106, 453–458.

Han, Z., Boyle, D.L., Chang, L., Bennett, B., Karin, M., Yang, L., Manning, A.M., Firestein, G.S., 2001. c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. J. Clin. Invest. 108, 73–81.

Hayden, J.M., Reaven, P.D., 2000. Cardiovascular disease in diabetes mellitus type 2: a potential role for novel cardiovascular risk factors. Curr. Opin. Lipidol. 11, 519–528.

Hirose, H., Kawai, T., Yamamoto, Y., Taniyama, M., Tomita, M., Matsubara, K., Okazaki, Y., Ishii, T., Oguma, Y., Takei, I., Saruta, T., 2002. Effects of pioglitazone on metabolic parameters, body fat distribution, and serum adiponectin levels in Japanese male patients with type 2 diabetes. Metabolism 51, 314–317.

Kitagawa, Y., Bujo, H., Takahashi, K., Shibasaki, M., Ishikawa, K., Yagui, K., Hashimoto, N., Noda, K., Nakamura, T., Yano, S., Saito, Y., 2004. Impaired glucose tolerance is accompanied by decreased insulin sensitivity in tissues of mice implanted with cells that overexpress resistin. Diabetologia 47, 1847–1853.

Meyer, K., Volkl, A., Endele, R., Kuhnle, H.F., Pill, J., 1999. Species differences in induction of hepatic enzymes by BM 17.0744, an activator of peroxisome proliferator-activated receptor alpha (PPARα). Arch. Toxicol. 73, 440–450.

Miyazawa-Hoshimoto, S., Takahashi, K., Bujo, H., Hashimoto, N., Yagui, K., Saito, Y., 2005. Roles of degree of fat deposition and its localization on VEGF expression in adipocyte. Am. J. Physiol., Endocrinol Metabol. 288, E1128–E1136.

Miyazaki, Y., Glass, L., Triplitt, C., Matsuda, M., Cusi, K., Mahankali, A., Mahankali, S., Mandarino, L.J., DeFronzo, R.A., 2001. Effect of rosiglitazone on glucose and non-esterified fatty acid metabolism in Type II diabetic patients. Diabetologia 44, 2210–2219.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D.R., Miles, J.M., Yudkin, J.S., Klein, S., Coppack, S.W., 1997. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J. Clin. Endocrinol. Metab. 82, 4196–4200.

Mohamed-Ali, V., Pinkney, J.H., Coppack, S.W., 1998. Adipose tissue as an endocrine and paracrine organ. Int. J. Obes. 22, 1145–1158.

Pill, J., Kuhnle, H.F., 1999. BM 17.0744: a structurally new antidiabetic compound with insulin-sensitizing and lipid-lowering activity. Metabolism 48, 34–40.

Poynter, M.E., Daynes, R.A., 1998. Peroxisome proliferator-activated receptor α activation modulates cellular redox status, represses nuclear factor-κB signalling, and reduces inflammatory cytokine production in aging. J. Biol. Chem. 273, 32833–32841.

Report of a WHO Consultation. Definition, diagnosis and classification of diabetes mellitus and its complications, 1999. Part 1 Diagnosis and classification of diabetes mellitus. World Health Organization, Department of Noncommunicable Disease Surveillance, Geneva.

Reaven, G.M., 1995. Pathophysiology of insulin resistance in human disease. Physiol. Rev. 75, 473–486.

Rosen, E.D., Spiegelman, B.M., 2001. PPAR: a nuclear regulator of metabolism, differentiation, and cell growth. J. Biol. Chem. 276, 37731–37734.

Rubin, C.S., Lai, E., Rosen, O.M., 1977. Acquisition of increased hormone sensitivity during in vitro adipocyte development. J. Biol. Chem. 252, 3554–3557.

Sancéau, J., Kaisho, T., Hirano, T., Wietzerbin, J.J., Kaisho, T., 1995. Triggering of the human interleukin-6 gene by interferon-γ and tumor necrosis factor-α in monocytic cells involves cooperation between interferon regulatory factor-1, NFκB, and Sp1 transcription factors. J. Biol. Chem. 270, 27920–27931.

Schafer, S.A., Hansen, B.C., Volkl, A., Fahimi, H.D., Pill, J., 2004. Biochemical and morphological effects of K-111, a peroxisome proliferator-activated receptor (PPAR)α activator, in non-human primates. Biochem. Pharmacol. 68, 239–251.

Sheu, M.Y., Fowler, A.J., Kao, J., Schmuth, M., Schoonjans, K., Auwerx, J., Fluhr, J.W., Man, M.Q., Elias, P.M., Feingold, K.R., 2002. Topical peroxisome proliferators activated receptor-alpha activators reduce inflammation in irritant and allergic contact dermatitis models. J. Invest. Dermatol. 118, 94–101.

Shibasaki, M., Takahashi, K., Itou, T., Miyazawa, S., Ito, M., Kobayashi, J., Bujo, H., Saito, Y., 2002. Alterations of insulin sensitivity by the implantation of 3T3-L1 cells in nude mice. A role for TNF-alpha? Diabetologia 45, 518–526.

Straub, R.H., Hense, H.W., Andus, T., Scholmerich, J., Riegger, G.A., Schunkert, H., 2000. Hormone replacement therapy and interrelation between serum interleukin-6 and body mass index in postmenopausal women: a population-based study. J. Clin. Endocrinol. Metab. 85, 1340–1344.

Thieringer, R., Fenyk-Melody, J.E., Le Grand, C.B., Shelton, B.A., Detmers, P.A., Somers, E.P., Carbin, L., Moller, D.E., Wright, S.D., Berger, J., 2000. Activation of peroxisome proliferator-activated receptor gamma does not inhibit IL-6 or TNF-alpha responses of macrophages to lipopolysaccharide in vitro or in vivo. J. Immunol. 164, 1046–1054.

Tuyt, L.M., Dokter, W.H., Birkenkamp, K., Koopmans, S.B., Lummen, C., Kruijer, W., Vellenga, E., 1999. Extracellular-regulated kinase 1/2, Jun N-terminal kinase, and c-Jun are involved in NF-kappa B-dependent IL-6 expression in human monocytes. J. Immunol. 162, 4893–4902.

Vozarova, B., Weyer, C., Hanson, K., Tataranni, P.A., Bogardus, C., Pratley, R.E., 2001. Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. Obes. Res. 9, 414–417.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., Ferrante Jr., A.W., 2003. Obesity is associated with macrophage accumulation in adipose tissue. J. Clin. Invest. 112, 1796–1808.

Wellen, K.E., Hotamisligil, G.S., 2003. Obesity-induced inflammatory changes in adipose tissue. J. Clin. Invest. 112, 1785–1788.

Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J.S., Tartaglia, L.A., Chen, H., 2003. Chronic inflammation in fat plays a crucial role in the development of obesity related insulin resistance. J. Clin. Invest. 112, 1821–1830.

Original Article

Polymorphisms of Apolipoprotein E and Methylenetetrahydrofolate Reductase in the Japanese Population

Hidenori Arai[1], Akira Yamamoto[2], Yuji Matsuzawa[3], Yasushi Saito[4], Nobuhiro Yamada[5], Shinichi Oikawa[6], Hiroshi Mabuchi[7], Tamio Teramoto[8], Jun Sasaki[9], Noriaki Nakaya[10], Hiroshige Itakura[11], Yuichi Ishikawa[12], Yasuyoshi Ouchi[13], Hiroshi Horibe[14], Tohru Egashira[15], Hiroaki Hattori[15], and Toru Kita[16]

[1]Department of Geriatric Medicine, Kyoto University Graduate School of Medicine, Japan.
[2]National Cardiovascular Center, Japan.
[3]Sumitomo Hospital, Japan.
[4]Department of Clinical Cell Biology and Medicine, Chiba University, Japan.
[5]Department of Internal Medicine, University of Tsukuba, Japan.
[6]Department of Internal Medicine, Nippon Medical School, Japan.
[7]Department of Laboratory Science, Kanazawa University, Japan.
[8]Department of Internal Medicine, Teikyo University, Japan.
[9]International University of Health and Welfare, Japan.
[10]Fussa Hospital, Japan.
[11]Ibaraki Christian University, Japan.
[12]Faculty of Health Sciences, Kobe University, Japan.
[13]Department of Geriatric Medicine, University of Tokyo, Japan.
[14]Keisen Clinic, Japan.
[15]Department of Advanced Technology and Development, BML, Inc., Japan.
[16]Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Japan.

Aim: The aim of this study is to analyze the effect of apolipoprotein E (apo E) and methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms on serum lipid and homocysteine levels in the general Japanese population.
Methods: We analyzed the polymorphisms in individuals randomly selected from among participants of Serum Lipid Survey 2000.
Results: The frequency of the $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ alleles of *APOE* was 4.2, 85.3, and 10.5%, respectively. Individuals with the genotype $\varepsilon 4/\varepsilon 4$ had the highest total and low-density lipoprotein (LDL) cholesterol levels, while those with $\varepsilon 2/\varepsilon 2$ had the lowest. Individuals with the $\varepsilon 2/\varepsilon 2$ and $\varepsilon 2/\varepsilon 4$ genotypes had higher remnant-like particles (RLP)-cholesterol levels than those with $\varepsilon 2\varepsilon 3$, $\varepsilon 3\varepsilon 3$, and $\varepsilon 3\varepsilon 4$. There was a trend for individuals with the $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ genotypes to have higher triglyceride levels, although the difference was not significant. The presence of the T allele in a *MTHFR* polymorphism (C667T) was associated with higher homocysteine levels, which is more prominent in men than in women.
Conclusion: Thus in our large-scale analysis we have shown that RLP-cholesterol is better associated with *APOE* genotype than triglyceride and the effect of the T allele on *MTHFR* polymorphism (C667T) homocysteine levels is more prominent in men than in women among Japanese.

J Atheroscler Thromb, 2007; 14:167-171.

Key words; Hyperlipidemia, Polymorphism, Apolipoprotein E, *MTHFR*, Homocysteine

Address for correspondence: Hidenori Arai, Department of Geriatric Medicine, Kyoto University School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: harai@kuhp.kyoto-u.ac.jp
Received: January 22, 2007
Accepted for publication: March 14, 2007