Pycnogenol, an extract from French maritime pine, suppresses Toll-like receptor 4-mediated expression of adipose differentiation-related protein in macrophages

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Running head: Pycnogenol suppresses the TLR4-mediated ADRP expression

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ABSTRACT

Adipose differentiation-related protein (ADRP) is highly expressed in macrophages and human atherosclerotic lesions. We demonstrated that Toll-like receptor (TLR) 4–mediated signals, which are involved in atherosclerosis formation, enhanced the expression of ADRP in macrophages. Lipopolysaccharide enhanced the ADRP expression in RAW264.7 cells or peritoneal macrophages from wild-type mice, but not in macrophages from TLR4-deficient mice. Actinomycin D almost completely abolished the LPS effect, while cycloheximide decreased the expression at 12 hr, indicating that the LPS-induced ADRP expression was stimulated at transcriptional level, and was also mediated by new protein synthesis. LPS enhanced the ADRP promoter activity, in part, by stimulating AP-1 binding to the Ets/AP-1 element. In addition, preceding the increase of the ADRP mRNA, LPS induced the expression of IL-6, IL-1α, and IFN-β mRNAs, all of which stimulated the ADRP expression. Antibodies against these cytokines or inhibitors of JNK and NF-κB suppressed the ADRP mRNA level. Thus, TLR4 signals stimulate the ADRP expression both in direct and indirect manners. Pycnogenol (PYC), an extract of French maritime pine, suppressed the expression of ADRP and the above mentioned cytokines. PYC suppressed the ADRP promoter activity and enhancer activity of AP-1 and NF-κB, while it did not affect the LPS-induced DNA binding of these factors. In conclusion, TLR4-mediated signals stimulate the ADRP expression in macrophages while PYC antagonizes this process. PYC, a widely used dietary supplement, might be useful for prevention of atherosclerosis.

Keywords: lipid droplet; atherosclerosis
INTRODUCTION

Most types of cells store lipid droplets in their cytoplasm (31). Adipocytes form lipid droplets during their differentiation and maturation, and these lipid droplets are thought to function as reservoirs of neutral lipids, i.e. energy storage (31). Such intracellular lipid droplet formation is closely related to various pathological events such as hepatic steatosis and atherosclerosis (31). A critical event in the development of atherosclerosis is a focal accumulation of lipid-laden foam cells derived from macrophages, smooth muscle cells and other vascular cells with subsequent fatty streak formation (25, 42). It has been disclosed that varieties of proteins exist on the surface of intracellular lipid droplets which participate in synthesis, storage, utilization and degradation of various lipids and miscellaneous function as well (31). Among such proteins, adipose differentiation-related protein (ADRP) is predominantly expressed in macrophages and foam cells at the site of the atherosclerotic lesion (23, 49), implicating that the expression of ADRP is closely related to the formation of atherosclerosis.

Forced expression of ADRP in cells enhances uptake of fatty acids and cholesterol (1, 13, 19, 24) and lipid droplet formation (19, 24). Its expression is stimulated by long-chain fatty acids (14) or very low density lipoprotein (6), oxidized low density lipoprotein (49) and several pharmacological stimuli including peroxisome proliferator-activated receptor (PPAR) ligands (2, 6, 8), cyclooxygenase inhibitors (53), etmoxir (46) and PMA (50). In a previous study, we elucidated, using a mouse RAW264.7 macrophage cell line, the molecular mechanisms of PMA-induced ADRP expression (50). We also demonstrated that ADRP is degraded by the proteasome pathway in the absence of lipids (50). Recent reports demonstrated that knocking-out or knocking-down of the ADRP gene prevented hepatic steatosis formation in mice (5, 18). Based on these observations, one can expect that the suppression of ADRP expression by certain medicines or food components might prevent the development of excessive lipid accumulation in
cells or tissues.

Macrophages are stimulated by pathogen-associated molecules by means of Toll-like receptors (43). Human and mouse TLRs consist of a large family with at least 13 members (3), which are known to recognize different molecules of microbial origin as well as a variety of endogenous ligands released during the process of diseases, including heat shock proteins (36, 51), extradomain A of fibronectin (35), hyaluronic acid fragments (47), soluble heparan sulfate (20) and oxidized LDL (27). Because of the broad capacity of ligands and the ubiquitous expression of TLRs, TLRs are involved in various phases of inflammatory and immune diseases (3). Activation of macrophages by TLR ligands such as bacterial LPS increased LDL uptake and cholesterol content, leading to foam cell formation (12, 21, 33). Activation of TLR2 and TLR4 stimulated formation of lipid bodies in leukocytes (9, 37). It was shown that LPS/TLR4 mediates lipid metabolism by influencing the expression of some lipid-associated genes; up-regulates sterol regulatory element binding protein-1 (SREBP-1) and adipose fatty acid-binding protein (aP2), while down-regulates scavenger receptor type B1 (SR-B1) and PPAR\(\alpha\) (10, 21, 22, 34). In addition, it was also demonstrated using apoE or LDL receptor knock-out mice that TLR signaling, at least via TLR4 and TLR2, plays a significant role in atherosclerogenesis (26, 39). It is not known, however, whether TLRs signals regulate ADRP expression, a possible key protein which contributes to foam cell formation in macrophages.

We have been searching putative compounds which can suppress ADRP expression among a variety of widely used dietary supplements or health foods, because such agents could be useful for prevention of pathological conditions associated with excessive lipid accumulation. In the present study, we aimed to elucidate whether TLR4 signals are involved in ADRP expression in macrophages, and test the effect of Pycnogenol (PYC), an extract from the bark of French
maritime pine, on the ADRP expression. PYC has been used in traditional medicine by virtue of its anti-inflammatory effects, and nowadays, is widely used as a dietary supplement (7, 48). It has been proven that PYC has an anti-oxidant effect in vitro (38). Here, we have shown that LPS enhanced the ADRP expression in macrophages, and PYC effectively eliminated the LPS effects.
MATERIALS AND METHODS

Materials. LPS from *Escherichia coli* 0128:B12, actinomycine D, cycloheximide, pyrrolidinedithiocarbamate (PDTC), thioglycolate, mouse recombinant IL-1α, IL-6, TNF-α, IFN-α and IFN-β were purchased from Sigma (St. Louis, MO). Antibody to ADRP was purchased from PROGEN Biotechnik (Heidelberg, Germany). Antibodies to mouse IL-6, IL-1α, IFN-β, and normal rat IgG1, rat IgG2b, rabbit IgG were purchased from R&D systems (Minneapolis, MN). Antibody to β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pycnogenol (PYC) was generously provided by Horphag Research Ltd (Geneva, Switzerland), and dissolved in DMSO before use. Synthesized oligonucleotides were purchased from Roche Diagnostics (Tokyo, Japan). [α-32P]dCTP (5,000 Ci/mmol) and [γ-32P]ATP (5,000 Ci/mmol) were purchased from GE Healthcare (Buckinghamshire, England). DNA sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA). JNK inhibitor II (JNKi)/SP600125 were purchased from Calbiochem (La Jolla, CA).

Cell culture. The mouse RAW264.7 monocyte/macrophage–like cell line was provided by the RIKEN Cell Bank (RCB0535, Ibaraki, Japan). The cells were routinely cultured in 10 cm tissue culture dishes (Falcon 3003; Becton Dickinson Labware, Franklin Lakes, NJ) in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% charcoal-treated fetal calf serum (FCS), 1% non-essential amino acids and appropriate antibiotics (named complete medium). The FCS we used was proved to be the same in terms of the concentrations of fatty acids, triacylglyceride and total cholesterol with and without charcoal treatment.

Isolation of mouse peritoneal macrophages. Female C57BL/6N mice at the age of 5 months and age- and sex- matched Tlr4−/− mice raised on C57BL/6N background were kindly given by Dr. Shizuo Akira (Osaka University, Japan). All animal experiments were conducted in accordance
to the guidelines of Kyushu University Committee on Animal Research.

Three hr after intra-peritoneal injection of 3% thioglycolate solution, peritoneal cells were harvested from each mouse, and re-suspended in RPMI1640 medium containing 10% FCS, 1% non-essential amino acids and appropriate antibiotics, and ~2 × 10⁶ cells were plated in 6 cm dish (Nunc 150288, Nunc A/S, Roskide, Denmark). After incubation at 37°C for 2 hr, adherent cells were further cultured in the presence or absence of 100 ng/ml LPS for 24 hr, then total RNA was purified and subjected for RT-PCR as described later.

*Northern blot analysis.* RAW264.7 cells were plated at 1 × 10⁶ cells/dish in complete medium and then left for 24 h, and then the medium was changed to the same base medium with the addition of a test reagent(s), followed by culturing for an appropriate time. Total RNA was purified using a commercially available kit (Isogen; Nippon GENE, Tokyo, Japan). Full-length mouse ADRP cDNA was [α-32P]dCTP-labeled by a random priming method (Oligo labeling kit; GE Healthcare, Buckinghamshire, England) and used as the probe. Hybridization was performed according to the standard method (45); final washing was carried out at 65°C in 1 × SSPE (150 mM NaCl, 10 mM NaH2PO4 and 1 mM EDTA, pH 7.4) containing 0.5% SDS for 2 × 20 min.

*Construction of promoter reporter plasmids.* Construction of reporter plasmids containing the ADRP promoter region or its mutants was previously described (50). PGL3-Promoter plasmids containing an NF-κB sequence was also constructed as described (50), using oligonucleotides containing the sequence between -79 and -56 bp in the mouse IL-6 promoter region (5’-AAATGTGGGGATTTTCCCATGAGTCT-3’) and the NF-κB consensus sequence (5’-AGTTGAGGGGACTTCCAGG-3’). For all of the promoter constructs, the integrity of the total promoter region, the mutations, the copy number and direction were confirmed by sequencing.
**Transient transfection and luciferase assay.** Reporter plasmids were introduced into RAW264.7 cells using Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The cells (2 × 10^5 cells/well) were seeded in a 12-well cell culture cluster (Costar 3513; Corning Incorporated, Corning, NY) and grown in the complete medium for 24 h. Then the medium was changed to serum-depleted α-MEM, and the transfection mixture (Lipofectamine 2 µl and PLUS reagent 5 µl in a total volume of 50 µl/well) containing 1 µg reporter plasmid and 0.4 µg pRL-TK plasmid (Promega, Madison, WI) was added to the cells followed by incubation for 4 h. The cells were then washed with serum-depleted α-MEM, and cultured in normal α-MEM and a test reagent for an additional 24 h. Cell lysates were collected for a luciferase assay using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The transfection efficiency was normalized as to the Renilla luciferase activity expressed by pRL-TK. Each transfection experiment was performed in triplicate and repeated at least three times.

**Nuclear protein extracts.** Nuclear protein extracts were prepared as previously described (17). RAW264.7 cells were seeded at 1 × 10^7 cells/dish and then cultured in the complete medium for 24 h. The medium was then changed to the complete medium containing a test reagent or vehicle, followed by culturing for 0.5 h. The cells were washed with PBS, harvested by gentle scraping, and then re-suspended in 5 volumes of buffer A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂ and 0.1 mM EDTA) supplemented with 0.3 M sucrose and 0.5% NP-40. The cells were then homogenized by pipetting, and the suspension was layered onto 1 ml buffer A containing 1.5 M sucrose, followed by centrifugation for 10 min (4°C, 15,000 g). After washing with buffer A, the precipitated nuclei were suspended in 50 µl buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂ and 0.2 mM EDTA) and then left on ice for
20 min. The mixture was centrifuged for 20 min (4°C, 15,000 g), and the resultant supernatant containing nuclear proteins was aliquoted, snap-frozen in liquid nitrogen and stored at -80°C until use (within 30 days). All solutions used were ice-cold and contained 0.5 mM DTT, 0.5 mM PMSF, 2 mg/ml pepstatin A and 2 mg/ml leupeptin. The protein concentration was determined with a commercially available reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as a control.

Electrophoretic mobility shift assay. EMSA was performed as previously described (17). A synthesized sense oligonucleotide was 32P-labeled with T4 polynucleotide kinase (Takara, Shiga, Japan), double-stranded with the unlabeled antisense-strand, and then purified using Chroma-Spin+TE–10 columns (Clontech, Palo Alto, CA). RAW264.7 nuclear extracts (10 µg) were first incubated in a 25 µl reaction volume for 20 min at 20°C with or without unlabeled competitor oligonucleotides (100-fold molar excess). The reaction buffer consisted of 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM MgCl2, 1 mM DTT, 1 mM EDTA, 12.5% (v/v) glycerol, 0.1% Triton X-100, 8 µg/ml calf thymus DNA and 50 mM PMSF. A radiolabeled probe (50,000 cpm, ~1.0 ng DNA) was then added followed by incubation for an additional 20 min at 20°C. DNA-protein complexes were analyzed on a 5% native polyacrylamide gel at 100 V for 3 h in TBE buffer. Thereafter, the gels were dried and subjected to autoradiography.

Western blot analysis. Whole cell extracts were prepared by directly dissolving the cells in 2×SDS loading buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 12% 2-mercaptoethanol and 2% bromophenol blue) at 1:1. The proteins were heated at 95°C for 5 min and then applied to 10% SDS-PAGE and electro-blotted onto a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan) for 1 h at 100 V with a wet blotting apparatus (Bio-Rad, Hercules, CA) in Tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol and
0.1% SDS). The transfer was monitored with Kaleidoscope pre-stained standards (Bio-Rad, Hercules, CA). The membranes were blocked for 1 h at room temperature with 5% non-fat milk in PBS containing 0.1% Tween20 (named PBS-Tween20). Next, the membranes were incubated with guinea pig ADRP antibodies (0.5 µg/ml in PBS-Tween20) for 1 h. After washing 4 times (5 min each) with PBS-Tween20, the membranes were incubated with an appropriate secondary IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS-Tween20 (0.08 µg/ml) for 1 h, and then washed as above. The blots were reacted with ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, England) and then exposed to Hyperfilm ECL (GE Healthcare, Buckinghamshire, England) for ~15 min. Three independent experiments were carried out and a representative example was shown.

**Real-time PCR and RT-PCR.** Total RNA was extracted using Isogen (Nippon GENE, Tokyo, Japan), according to the manufacture’s instructions. Single strand cDNA was synthesized with ReverTra Ace-α kit (Toyobo, Osaka, Japan) using 0.5 µg of the total RNA. Real-time PCR was performed with a SYBR Green method using the iCycler iQ multi-color real time PCR detection system (Bio-Rad, Hercules, CA) in 25 µl reactions [12.5 µl of 2 × iQ SYBR Green supermix (Bio-Rad, Hercules, CA), 320 nM each primer, 5 µl of 1:20 diluted cDNA]. Primers were synthesized at Roche Diagnostics (Tokyo, Japan). The primer sequences were: ADRP (sense: CTGTCTACCAAGCTCTGCTC, antisense: CGATGCTTCTCTCCACTCC), IL-1α (sense: GTTCTGCCATTCACCATCTC, antisense: GAATCTTTCCGTTGCTTGAC), IL-6 (sense: AGTTGCCCTCTGTGGACTGA, antisense: TCCACGATTTCCCAGAAAC), IFN-β (sense: TAAGCATCTCAGTCCCAAG, antisense: GGACATCTCCCAGTGACTC), GAPDH (sense: ACCACAGTCCATGCCATCAC, antisense: TCCACCACCTGTGGCTTA). PCR efficiencies for all reactions were more than 0.90. Quantitative PCR results were expressed as relative
induction fold toward the housekeeping gene GAPDH.

Conventional RT-PCR was performed using the GeneAmp PCR System 9600 (Applied Biosystems, Foster, CA) in 25 µl reactions (1 µM each primer, 1 unit TaKaRa LA Taq (Takara-Bio, Tokyo, Japan) and 1 µl cDNA) with 25 cycle amplification. The primers of ADRP and GAPDH were the same as above.

Statistical analysis. All data were presented as the means ± SE. Statistical differences were determined by one-way analysis of variance (ANOVA). A $P < 0.05$ was considered significant.
RESULTS

*TLR4-mediated pathway is involved in the stimulation of ADRP expression in macrophages.* Since the TLR4 signal was shown to play a role in the development of atherosclerosis (26, 39), we first examined if LPS, a TLR4 ligand, could stimulate the expression of ADRP in RAW264.7 cells. While ADRP mRNA is constitutively expressed in this cell line (50), LPS further stimulated the expression in a time- and concentration-dependent manner (Fig. 1, A and B). Western blot analysis confirmed that LPS increased the ADRP protein level as well (Fig. 1C). While the mRNA increased by ~10 folds, the protein level increased by ~2 folds in the complete medium (Fig. 1, A and C). This discrepancy between mRNA and protein levels could be currently explained by rapid proteasomal degradation of ADRP protein in the absence of lipid in the medium (50, 52). Thus, even if the transcription of ADRP gene is stimulated, its protein level seems to be settled by the amount of lipids available for the cells. To confirm that LPS stimulates the ADRP expression thought TLR4, we investigated the effect of LPS using TLR4-deficient primary macrophages. While the expression of ADRP mRNA was enhanced by LPS in wild type macrophages, it was not stimulated in TLR4-deficient macrophages (Fig. 1D), indicating that TLR4 pathway is involved in the ADRP expression.

Simultaneous addition of actinomycin D to the cells effectively suppressed the LPS-induced ADRP mRNA (Fig. 1E, lane 6 and 7) and protein (Fig. 1E, lane 15 and 16) expression, indicating that LPS enhanced the ADRP expression directly at the transcriptional level. On the other hand, the mRNA and protein expression was not suppressed at 6 h by simultaneous addition of cycloheximide, while it was apparently suppressed at 12 h (Fig. 1E, lane 5 vs. 9, and lane 14 vs. 18). This finding was further confirmed by the result that longer pretreatment with cycloheximide evidently suppressed the mRNA expression (data not shown), indicating that LPS-induced ADRP expression is also mediated by the new protein synthesis. These findings are understandable
because TLR4 stimulation causes diverse intracellular signal transduction pathways (3).

*Direct pathway involved in LPS-induced ADRP expression.* TLR4-mediated signals involve the activation of transcription factor AP-1 in macrophages (3). In the previous study (50), we demonstrated that there is an Ets/AP-1 composite element in the region between –2090 and –2005 bp of the mouse ADRP promoter, which mediates PMA-induced ADRP expression. This element is recognized co-operatively by AP-1 and PU.1 in macrophages (50). Therefore, we presumed that this Ets/AP-1 element could mediate the LPS-induced direct effect on the ADRP promoter activity. LPS significantly stimulated the activity of the –2090 bp promoter which contains the Ets/AP-1 site, approximately 3 times that of the control, while LPS-induced activity decreased to 55–63% that of –2090 bp promoter when the Ets/AP-1 site was deleted (-2005 bp) or a mutation was introduced into one or both of these elements (A mut, E mut, AE mut) (Fig. 2A). The LPS-induced, Ets/AP-1-mediated increase in promoter activity was again evident when the site was ligated to a heterologous SV40 core promoter (Fig. 2B).

In the EMSA using the Ets/AP-1 site as a probe, nuclear extracts from RAW264.7 cells treated with LPS for 0.5 h formed two specific complexes A and B (Fig. 2C). Formation of both bands was inhibited by the wild type sequence, as well as the mixture of AP-1 and PU.1 consensus oligonucleotides (Fig. 2C, lane 2 and 8). Complex A formation was inhibited by oligonucleotides containing the intact AP-1 sequence (Fig. 2C, lane 4 and 6), while complex B formation was inhibited by oligonucleotides containing the intact Ets sequence (Fig. 2C, lane 3 and 7). This result is consistent with our previous report (50) and indicates that complexes A and B were formed by AP-1 and PU.1, respectively. It is noted that LPS stimulation only increased AP-1 binding, but not PU.1 binding (Fig. 2C, lane 9 vs. 10).

All these results indicate that LPS, a TLR4 ligand, stimulates the ADRP mRNA expression by activating the AP-1 binding activity to the Ets/AP-1 element in the ADRP promoter. However,
there may be other cis-elements on the ADRP promoter which mediate the LPS effect, since the promoter activity was still stimulated by LPS even in the promoters whose Ets/AP-1 site was deleted or mutated. This possibility remains to be clarified.

*Indirect pathway involved in LPS-induced ADRP expression.* The LPS/TLR4 system induces production of many kinds of cytokines, such as IL-6, IL-1α and IFN-β (3). Therefore, we hypothesized that the ADRP expression is also stimulated through the LPS-induced production of cytokines. Northern blot analysis showed that the expression of ADRP mRNA was enhanced in RAW264.7 cells by IL-6, IL-1α and IFN-β, but not by TNF-α and IFN-α (Fig. 3A). These cytokines increased the ADRP mRNA in a concentration-dependent manner (Fig. 3B). LPS induced the expression of IL-6, IL-1α, and IFN-β mRNA, and induction of these cytokines preceded the increase of ADRP mRNA (Fig. 3C).

In the presence of antibodies against IL-6, IL-1α, and IFN-β in the medium, the LPS-induced ADRP mRNA expression was suppressed to 69%, 85% and 89% of that of the control IgG, respectively. The mRNA level was suppressed to 35% of that of the control IgG when all three antibodies were added simultaneously (Fig. 4A). Since AP-1 and NF-κB are the most important transcription factors involved in the TLR4-mediated regulation of these three cytokines, we further tested whether inhibitors of AP-1 and NF-κB could suppress the expression of ADRP mRNA. Pretreatment with a JNK inhibitor (SP600125) or an NF-κB inhibitor (PDTC) suppressed the LPS-induced ADRP mRNA expression (Fig. 4B). These results suggest that the induction of ADRP expression by LPS is also mediated in part by the autocrine mechanism of IL-6, IL-1α and IFN-β.

*PYC suppresses LPS-induced ADRP mRNA expression.* In an effort for searching substances which can suppress the ADRP expression, here, we focused on PYC, an extract from French
maritime pine bark, which was shown to have a potent antioxidant effect (38). As shown in Fig. 5A, PYC suppressed the LPS-induced ADRP mRNA expression in a dose-dependent manner in RAW264.7 cells.

To pursue the mechanism underlying the PYC effect, we first tested the effect of PYC on ADRP promoter activity. PYC (50 µg/ml) significantly suppressed the LPS-induced enhancement of the -2090 bp promoter activity, but not that of the -2005 bp promoter, while PYC itself did not influence the promoter activity (Fig. 5B). The activity of the -2090 bp promoter was suppressed by PYC in a concentration dependent manner (Fig. 5C). In addition, PYC also abolished the LPS-induced enhancement of Ets/AP-1-SV40 core promoter activity (Fig. 5D). These results indicate that PYC inhibits the LPS-induced ADRP promoter activity possibly in part through affecting the enhancer activity of Ets/AP-1. Unexpectedly, however, we found that PYC did not affect the LPS-induced DNA binding activity of AP-1 to the Ets/AP-1 element (Fig. 5E).

There could be another possible mechanism to explain the PYC effect on the ADRP expression by influencing the cytokine production. PYC significantly reduced the LPS-induced mRNA expression of IL-6, IL-1α and IFN-β in a dose-dependent manner (Fig. 6A). To determine whether PYC affects transcriptional activity through a NF-κB signal, we constructed luciferase reporter plasmids containing the NF-κB consensus sequence or NF-κB element in the mouse IL-6 promoter ligated to a SV40 core promoter. PYC significantly suppressed the enhancer activity of NF-κB induced by LPS (Fig. 6, B and C). LPS increased the NF-κB binding to the site (Fig. 7, lane 1 vs. 2), while PYC did not affect the LPS-induced DNA binding activity of NF-κB (Fig. 7, lane 6 vs. 7).

Collectively, all these results indicate that PYC suppressed the LPS-induced ADRP expression in RAW264.7 cells, not only through suppression of the direct pathway acting on the ADRP
promoter but also via suppression of the indirect pathway mediated by production of inflammatory cytokines. The point at which PYC suppresses the promoter activity of ADRP or cytokine genes, however, seems not to be at the step of transcription factor binding, but rather at the transactivation process including recruitment or activation of cofactors. This possibility is under further investigation.
DISCUSSION

Despite the wide tissue distribution of ADRP, knowledge about the regulatory mechanism of its expression remains limited. Incubation of adipogenic cells \textit{in vitro} by long-chain fatty acids increases ADRP expression (14). Oxidized LDL and VLDL increase ADRP expression in macrophages (6, 49). Synthetic ligands for PPARα/β also enhance ADRP expression in hepatocytes and macrophages (6, 8). We previously disclosed the molecular mechanism of the PMA-stimulated ADRP expression in macrophages (50). In atherosclerotic lesions the high expression of ADRP in macrophages was shown (23, 49). TLR4-mediated signaling in macrophages was also shown to play an important role in atherosclerosis formation (26, 39). A recent \textit{in vivo} study demonstrated that a single injection of LPS induced transient ADRP expression and lipid accumulation in liver in mouse model (34), it did not, however, define the molecular mechanism. In the present study, we demonstrated that TLR4-mediated signals induced the ADRP expression in macrophages and disclosed in part its molecular basis. In addition and more importantly, we showed that this increase is effectively inhibited by PYC, a widely used dietary supplement.

Two major intracellular signaling pathways following TLR4 activation are myeloid differentiation factor 88 (MyD88)-dependent (11) and MyD88-independent pathways (16). The activation of the MyD88-dependent pathway results in IKK and JNK activation, and subsequently triggers the activation of Rel family transcription factor NF-κB and several members of AP-1 transcription factors, which finally regulate the expression of pro-inflammatory genes. In the MyD88-independent pathway, TIR domain-containing adaptor inducing IFN-β (TRIF) mediates the activation of interferon regulatory factor 3 (IRF3) and a late phase activation of NF-κB, which then activates a large set of antiviral genes including IFN-α and
IFN-β. Based on the complexity of TLR4-mediated signaling, we speculated that the LPS-induced enhancement of ADRP expression could be mediated through both direct and indirect pathways. Thus, we presumed that the TLR4 signal directly activates ADRP promoter activity and also stimulates cytokine production which in turn enhances the ADRP expression, both of which are mediated by AP-1 and NF-κB transcription factors.

In our previous study, we identified the Ets/AP-1 composite element which is conjointly recognized by AP-1 and PU.1 in macrophages (50). We also showed that it is a key element for PPAR-mediated ADRP expression (50). Here, we demonstrated that the Ets/AP-1 element is involved in LPS-induced ADRP expression. LPS activated the -2090 bp promoter encompassing the intact Ets/AP-1 element, and enhanced AP-1, but not PU.1, binding to the site was shown in EMSA. The constitutive binding of PU.1 was, however, indispensable for LPS-induced ADRP expression, since the mutation in the Ets site significantly decreased the LPS-induced promoter activity. This finding is consistent with our previous result using PMA stimulation (50). On the other hand, even the -2005 bp promoter which lacks the Ets/AP-1 element was still significantly stimulated by LPS as compared to the promoter-less control plasmid, suggesting that there exists other possible elements involved in LPS stimulation. This possibility is now under investigation. Nevertheless, this finding clearly indicates that the promoter activity of the ADRP gene is directly stimulated by LPS in macrophages.

Our present study also demonstrated that the ADRP expression is secondarily stimulated by inflammatory cytokines which are induced by LPS. We have obtained several lines of evidence which support this conclusion as follows. First, pretreatment of the cells with cycloheximide reduced the LPS-induced ADRP expression, suggesting a requirement of new protein synthesis. Second, LPS induced the production of IL-6, IL-1α and IFN-β, and these cytokines significantly
stimulated the ADRP expression in macrophages. Third, the cytokine induction by LPS preceded the increase in ADRP mRNA. Forth, inhibitors of JNK and NF-κB, which block the TLR4 signaling pathway and cytokine production, reduced the LPS-induced ADRP expression. And lastly, specific antibodies against IL-6, IL-1α and IFN-β significantly suppressed the LPS-induced ADRP expression. Taken together, these results suggest that induction of the ADRP expression by LPS is, in part, mediated by the production of IL-6, IL-1α, and IFN-β.

Various in vitro studies have shown that forced expression of ADRP enhances lipid droplet formation and lipid accumulation in several cell lines (19, 24). In addition to atherosclerotic lesions (23, 49), the ADRP expression is increased in fatty liver in human and mouse models (30). On the other hand, ADRP deficient mice, produced by gene knock-out (5) or antisense oligonucleotide technology (18), did not acquire diet-induced hepatic steatosis formation. These lines of evidence strongly raise the possibility that ADRP could be a putative molecular target for treatment or prevention of pathological conditions associated with excessive intracellular lipid accumulation, because ADRP is apparently one of the key molecules for lipid droplet formation. Based on this hypothesis, we have been screening compounds among dietary supplements which can suppress the ADRP expression.

PYC is an extract from the bark of French maritime pine and has been used as a historical medicinal material for the treatment of scurvy, skin wounds and sores (4). PYC is a mixture of phenols, polyphenols, taxifolin and condensed flavonoids (40), and is known that it has a strong antioxidant effect (38). Nowadays, PYC is widely used in general populations as a dietary supplement beneficial for various conditions despite the lack of sufficient knowledge about the molecular basis of its action. Nevertheless, to date several studies have described some biological actions of PYC. In human umbilical vascular endothelial cells (HUVEC), PYC suppressed
TNF-α-induced VCAM-1 and ICAM-1 expression, which play a critical role in atherosclerosis, inflammation, ischemic vascular disorders, and cancer metastasis (40). In keratinocytes, PYC inhibits UV radiation-induced NF-κB-dependent gene expression (44). Very preliminary studies have shown that PYC induced lipolysis and inhibited lipogenesis in 3T3 L-1 cells without any defined mechanisms (28).

Here, we demonstrated that PYC inhibited the LPS-induced ADRP expression in macrophages in vitro, and PYC suppressed the dual pathways involved in LPS action on the ADRP expression. Thus, PYC directly suppressed the LPS-induced enhancement of the ADRP promoter activity. PYC also suppressed the expression of IL-6, IL-1α and IFN-β genes and in turn, suppressed the ADRP expression. The underlying mechanism of both effects seems to be inhibition of enhancer activity of AP-1 and NF-κB, which are supported by the following findings. First, PYC suppressed the activity of a -2090 bp promoter which encompasses the Ets/AP-1 site, but not the -2005 bp promoter. Second, PYC suppressed the LPS-induced activity of heterologous promoters ligated with either the Ets/AP-1 site or the NF-κB site. Contrary to our expectations, however, we found that treatment of the cells with PYC did not inhibit the DNA binding activity of both AP-1 and NF-κB stimulated by LPS. Based on the literature published to date, the effect of PYC on the DNA binding activity of AP-1 and NF-κB seems to be contradictory. Cho et al (7) showed that PYC suppressed the LPS-induced DNA binding activity of AP-1 in RAW264.7 cells. Peng et al (40) also showed that PYC suppressed the TNF-α-induced DNA binding of NF-κB in HUVEC cells. On the other hand, Saliou C et al (44) showed that PYC reduced the transactivation capacity of NF-κB, but not its DNA binding activity in keratinocytes. Similar results were also shown using flavonoids, apigenin and genistein, in endothelial cells and in a monocytic cell line, U937 (15, 32).
Despite the above controversy, our data clearly showed that PYC suppressed the AP-1 or NF-κB-dependent promoter activity irrespective of the DNA binding. These transcription factors are redox-sensitive and oxidative stress activates the transcriptional activity. Recent reports have shown that curcumin, a well known naturally occurring flavonoid, can act as an oxygen radical and hydroxyl radical scavenger and inhibit NF-κB expression/activation (41). Anti-oxidant substances may inhibit histone acetyltransferase (HAT) activity of co-factors such as CREB-binding protein (CBP)/p300, and/or may activate histone deacetylase activity (41). Actually, curcumin has recently been shown to inhibit p300/HAT activity (29). Therefore, we speculate that PYC possibly acts epigenetically to suppress AP-1- or NF-κB-regulated genes through the similar manner to curcumin. We are currently pursuing this possibility.

In summary, we have described, for the first time, the TLR4-mediated ADRP gene expression in macrophages. And more importantly, we have shown that PYC, a widely used dietary supplement, suppresses LPS-induced ADRP gene expression. Based on our findings, we would suggest that PYC could be useful as a preventive or therapeutic agent for diseases caused by excessive lipid accumulation such as atherosclerosis, obesity and hepatic steatosis. We are planning to test this possibility in human or mouse models.
REFERENCES


8. Dalen KT, Ulven SM, Arntsen BM, Solaas K, Nebb HI. PPARa activators and fasting induce the expression of adipose differentiation-related protein in liver. *J Lipid Res* 47:


40. **Peng Q, Wei Z, Lan BHS.** PYC inhibits tumor necrosis factor-α-induced nuclear factor
kappa B activation and adhesion molecule expression in human vascular endothelial cells. 


Figure Legends

Fig. 1. TLR4 mediates the expression of ADRP in RAW264.7 cells. A: RAW264.7 cells were incubated with 100 ng/ml LPS or vehicle for 0-24 h, and then the expression of ADRP mRNA was assessed by Northern blot analysis. B: RAW264.7 cells were incubated for 24 h with 0.01-100 ng/ml LPS, and then the expression of ADRP mRNA was assessed by Northern blot analysis. C: (left panel) The ADRP protein level was assessed by Western blot analysis in RAW264.7 cells treated with 0.01-100 ng/ml LPS for 24 h. (right panel) ADRP protein levels were quantified by NIH Image. The data represent the means ± SE of three independent experiments. *P < 0.05 vs. 0 ng/ml. D: Peritoneal macrophages from wild type (WT) (n=3) and TLR4-deficient (KO) (n=2) mice were treated with or without 100 ng/ml LPS for 24 hr, and then the expression of mRNA for ADRP was analyzed by RT-PCR. E: RAW264.7 cells were incubated with DMSO (vehicle), 2.5 µg/ml actinomycin D (AcD) or 10 µg/ml cycloheximide (CHX) with or without 100 ng/ml LPS for 6 h and 12 h. The expression of ADRP mRNA and protein was assessed by Northern blot (left panel) and Western blot (right panel) analyses. 28S and 18S ribosomal RNAs were used as a total RNA loading control. β-actin was used as a total protein loading control.

Fig. 2. LPS enhances the ADRP promoter activity. A: RAW264.7 cells were transfected with wild type and mutant ADRP promoter reporter constructs as well as the promoter-less control vector for 4 h, and then stimulated with 100 ng/ml LPS for an additional 24 h. Sequences of A mut, E mut, and AE mut promoters were described in ref. 50. Nucleotide sequence of the whole promoter region is available in the DDBJ database under accession number AB203137. B: RAW264.7 cells were transfected with the constructs containing one or two copies of the
Ets/AP-1 element in the SV40 core promoter as well as the control vector, and then stimulated with 100 ng/ml LPS for an additional 24 h. Luciferase activities were measured, and the results were indicated as the relative fold induction of each promoter. The value for the promoter-less control vector is arbitrarily designated as 1. The results are shown as the means ± SE of three independent experiments (*$P < 0.05$ vs. control, $§P < 0.05$ vs. LPS). C: LPS increases DNA-protein complex formation with Ets/AP-1 element. (left panel) The nuclear extracts were prepared from RAW264.7 cells treated with 100 ng/ml LPS for 30 minutes. DNA-protein complexes were competed with a 100-molar excess of indicated competitors. (right panel) Nuclear extracts from RAW264.7 cells treated with 100 ng/ml LPS for 30 minutes, or non-treated cells were used. EMSA was performed using ADRP oligonucleotide containing the Ets/AP-1 element as a probe. The representative results from three independent experiments are shown.

Fig. 3. Inflammatory cytokines stimulates the ADRP expression in RAW264.7 cells. A: RAW264.7 cells were incubated for 24 h with 100 ng/ml IL-1α, IL-6, TNF-α, and 100 U/ml IFN-α, IFN-β, as indicated. The expression of ADRP mRNA was assessed by Northern blot analysis. 28S and 18S ribosomal RNAs were used as a total RNA loading control. B: RAW264.7 cells were incubated with 0.1-100 ng/ml IL-1α or IL-6, 1-1000 U/ml IFN-β for 24 h respectively. The level of ADRP mRNA was determined by real-time PCR. The results are shown as the mean ± SE of three independent experiments (*$P < 0.05$ vs. 0 ng/ml). C: RAW264.7 cells were incubated with 100 ng/ml LPS for 0-9 h. The level of mRNA for IL-6, IL-1α, IFN-β and ADRP was determined by real-time PCR. The results are shown as the means ± SE of three independent experiments (*$P < 0.05$ vs. 0 h for cytokines, $§P < 0.05$ vs. 0 h for ADRP).
Fig. 4. Specific antibodies against cytokines or inhibitors of JNK and NF-κB suppress the LPS-induced ADRP expression. A: RAW264.7 cells were pretreated with control immunoglobulin (Ig) or specific antibodies to mouse IL-6 (25 µg/ml), IL-1α (30 µg/ml), IFN-β (2 × 10³ U/ml) for 1 hr, then, stimulated with 100 ng/ml LPS for 24 hr. Concentration of each antibody was the minimal dose which exhibited the maximal effect in preliminary experiments (data not shown). The expression of ADRP mRNA was assessed by real-time PCR. Control immunoglobulin did not affect the LPS effect and increment of the ADRP mRNA by LPS is arbitrarily designated as 100%. The results are shown as means ± SE of three independent experiments (*P < 0.05 vs. LPS; §P < 0.05 vs. IL-6 ab + IL-1α ab + IFN-β ab). B: RAW264.7 cells were pretreated with DMSO (vehicle) or JNKI (upper panel) or PDTC (lower panel) at indicated concentrations for 1 h, and then stimulated with 100 ng/ml LPS for 24 h. The ADRP mRNA was assessed by Northern blot analysis. A representative result from three independent experiments is shown.

Fig. 5. PYC suppresses the LPS-induced ADRP expression in RAW264.7 cells. A: RAW264.7 cells were pretreated with DMSO (vehicle) or 10-50 µg/ml PYC for 1 h, and then stimulated with 100 ng/ml LPS for 24 h. The ADRP mRNA was assessed by Northern blot analysis. B: RAW264.7 cells were transfected with -2090 bp promoter, -2005 bp promoter or promoter-less control vector for 4 h, and then exposed to 50 µg/ml PYC for 1 h prior to 100 ng/ml LPS stimulation. C: RAW264.7 cells were transfected with -2090 bp promoter for 4 h, and then exposed to 10-50 µg/ml PYC for 1 h prior to 100 ng/ml LPS stimulation. D: RAW264.7 cells were transfected with PGL3-Promoter (SV40 core promoter) or the promoters containing 1 copy or 2 copies of Ets/AP-1 element for 4 h, and then exposed to 50 µg/ml PYC for 1 h prior to 100
ng/ml LPS stimulation. The luciferase activity was measured at 24 h after LPS stimulation. The value for the control vector was arbitrarily designated as 1. Data are shown as means ± SE from three independent experiments (*$P < 0.05$ vs. LPS). E: EMSA was performed using nuclear extracts from RAW264.7 cells treated with LPS or LPS plus PYC, or vehicle. The probe was the same as that in Fig. 3. A representative result from three independent experiments is shown.

Fig. 6. PYC suppresses the LPS-induced cytokine expression and NF-κB enhancer activity. A: RAW264.7 cells were incubated with 10-50 µg/ml PYC or DMSO (vehicle) for 1 h, and then stimulated with 100 ng/ml LPS for 3 h. The levels of mRNA for IL-6, IL-1α and IFN-β were determined by real-time PCR. The results are shown as the means ± SE of three independent experiments (*$P < 0.05$ vs. LPS alone). B and C: The constructs containing an NF-κB consensus sequence (B) or an NF-κB element in the mouse IL-6 promoter region (C) were transfected into RAW264.7 cells for 4 h, and pretreated with PYC for 1 h, then followed by challenge with 100 ng/ml LPS for 24 h. *$P < 0.05$. 

Fig. 7. PYC does not affect LPS-induced DNA binding of NF-κB. (left panel) Nuclear extracts from RAW264.7 cells treated with LPS or vehicle were used. DNA-protein complexes were competed with a 100-molar excess of unlabeled NF-κB consensus oligonucleotide (NF-κB) or NF-κB mutant oligonucleotide (NF-κB mut), indicating that the upper band was formed by NFκB. (right panel) Nuclear extracts from RAW264.7 cells treated with vehicle, LPS or LPS plus PYC were used. EMSA was performed using the NF-κB consensus oligonucleotide as a probe. A representative result from three independent experiments is shown.
**Fig. 1**

A. Western blot analysis showing the expression levels of ADRP, 28s rRNA, and 18s rRNA under control (CON) and LPS (100 ng/ml) treatment at different time points (0, 6, 12, 24 hr).

B. Western blot analysis showing the expression levels of ADRP, 28s rRNA, and 18s rRNA under different concentrations of LPS (0, 0.01, 0.1, 1, 10, 100 ng/ml).

C. Western blot analysis showing the expression levels of ADRP and β-actin under different concentrations of LPS (0, 0.01, 0.1, 1, 10, 100 ng/ml).

D. Western blot analysis showing the expression levels of GAPDH, ADRP, and β-actin under LPS treatment (+) and control (-) conditions in WT-1, WT-2, WT-3, KO-1, KO-2.

E. Western blot analysis showing the expression levels of ADRP, 28s rRNA, and 18s rRNA under DMSO, LPS, LPS+AeD, LPS+CHX treatment at different time points (0, 6, 12 hr).

*Significant difference compared to control.
Complex-A (AP-1)
Complex-B (PU.1)

Fig. 2
### A

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### B

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### C

**IL-6, IL-1α, and ADRP mRNA induction**

**IFN-β mRNA induction**

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</table>

**Fig.3**
**A**

![Bar chart showing ADRP mRNA levels.](image)

- **LPS**: + + + + +
- **Control Ig**: + - - - -
- **IL-6 ab**: - + - - +
- **IL-1α ab**: - - + - +
- **IFN-β ab**: - - - + +

**B**

![Western blot images showing ADRP, 28s rRNA, and 18s rRNA.](image)

- **LPS**: - + - - - + + +
- **JNKI (μM)**: - - 0.5 5 50 0.5 5 50
- **LPS**: - + - - - + + +
- **PDTC (μM)**: - - 25 50 75 100 25 50 75 100

Fig. 4
Fig. 5
**Fig. 6**

A. Relative IL-6 mRNA levels after treatment with LPS and PYC

B. Relative IFN-β mRNA levels after treatment with LPS and PYC

C. Relative IL-1α mRNA levels after treatment with LPS and PYC

Table of PYC concentrations:

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* Indicates significant differences.
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Fig. 7