Dock Center Wellness in Fukuoka, Japan. We excluded 107 subjects on medications for dyslipidemia. Subjects diagnosed as having MetS at baseline received instruction for lifestyle modification and were referred to a clinic if they had untreated hypertension, diabetes or dyslipidemia. The study protocol was approved by the ethics committees of the related institutes.

3. Measurements

Waist circumference was measured at the level of the umbilicus. Blood pressure was measured at rest using an automatic sphygmomanometer. All blood samples were withdrawn after an overnight 10-h fast. Mean IMT was measured by ultrasonography (SDU-2200; Shimadzu, Kyoto, Japan) equipped with IMT measurement software (Intima-scope; Media Cross, Tokyo, Japan) [7].

4. Definitions of MetS

We diagnosed MetS using the International Diabetes Federation (IDF) definition for Japanese [8] that consists from waist circumference (WC, ≥90 cm for male and ≥80 cm for female), and the presence of two or more of the following risk factors: (1) blood pressure (BP), systolic ≥130 and/or diastolic ≥85 mmHg, (2) triglycerides (TG) ≥150 mg/dl, (3) HDL-cholesterol <40 mg/dl in men and <50 mg/dl in women, and (4) fasting plasma glucose (FPG) ≥100 mg/dl.

5. Statistical analyses

Student's *t*-tests were used to compare the mean basal IMT adjusted for age and the annual changes in IMT adjusted for age and basal IMT between the groups with or without MetS or the individual components of MetS. Multiple comparisons with Tukey's test were used to compare the mean IMT adjusted for age and the annual difference in IMT adjusted for age and basal IMT among four groups, namely subjects without risk factor and with 1, 2 and 3 or above risk factors of (RF0, RF1, RF2 and RF≥3, respectively) diagnosed at baseline. Subjects were regrouped based on the MetS diagnosis at the baseline and at the time of follow-up; MetS (baseline/follow-up): MetS (−/−), (−/+), (+/−) and (+/+). The statistical analyses were performed using JMP 7.0 software (SAS Institute, Cary, NC). Statistical significance was inferred at $P < 0.05$.

6. Results

At baseline, 108 (14.0%) males and 37 (7.3%) females had MetS. IMT was significantly greater in subjects of both sexes with MetS (Table 1). Some risk factor components of MetS were also significantly related to an increase in baseline IMT (WC, BP, TG and HDL in males and WC, BP, TG, HDL and FPG in females) after adjustment for age (Table 1). Increase in the number of risk factors (RF) including WC was also significantly related to an increase in baseline IMT in males and females (RF2 and RF≥3 group) (Table 1). We analyzed annual IMT changes in each subject after 1.2 ± 0.4 years follow-up. In males and females, annual IMT changes tended to be larger in subjects with the MetS compared with subjects without MetS, and in subjects with an increasing number of RF. This difference was statistically significant in females (MetS+ and RF≥3 group) (Fig. 1A). Among individual RF, WC in males (0.017 mm/year vs. 0.027 mm/year, $P < 0.05$), WC in females (0.004 mm/year vs. 0.012 mm/year, $P < 0.05$) and HDL in females (0.006 mm/year vs. 0.020 mm/year, $P < 0.01$) were related to larger annual IMT changes. These results indicate that MetS as well as an

Table 1 – Baseline IMT adjusted for age relates with MetS and its risk factors.

		Male			Female		
		N (%)	IMT (mm)	P	N (%)	IMT (mm)	P
MetS	−	661 (86)	0.601 ± 0.004		465 (93)	0.569 ± 0.003	
	+	108 (14)	0.631 ± 0.009	<0.001	37 (7)	0.633 ± 0.011	<0.001
WC	−	525 (68)	0.599 ± 0.004		335 (67)	0.566 ± 0.004	
	+	243 (32)	0.618 ± 0.006	0.005	167 (33)	0.589 ± 0.005	<0.001
BP	−	196 (25)	0.599 ± 0.004		441 (88)	0.567 ± 0.003	
	+	572 (75)	0.623 ± 0.007	0.001	61 (12)	0.618 ± 0.009	<0.001
TG	−	559 (73)	0.600 ± 0.004		467 (93)	0.571 ± 0.003	
	+	209 (27)	0.619 ± 0.006	0.009	35 (7)	0.607 ± 0.001	0.002
HDL	−	682 (89)	0.600 ± 0.003		453 (90)	0.569 ± 0.003	
	+	86 (11)	0.640 ± 0.010	<0.001	49 (10)	0.614 ± 0.009	<0.001
FPG	−	446 (58)	0.602 ± 0.005		442 (88)	0.571 ± 0.003	
	+	322 (42)	0.609 ± 0.005	0.282	60 (12)	0.594 ± 0.009	0.011
RF	0	208 (27)	0.589 ± 0.006		278 (54)	0.564 ± 0.004	
	1	255 (33)	0.598 ± 0.006		126 (25)	0.564 ± 0.006	
	2	172 (22)	0.610 ± 0.007	<0.05[a]	59 (12)	0.601 ± 0.008	<0.05[a]
	≥3	133 (17)	0.625 ± 0.008	<0.05[a]	39 (8)	0.629 ± 0.010	<0.05[a]

[a] vs. RF0 group.

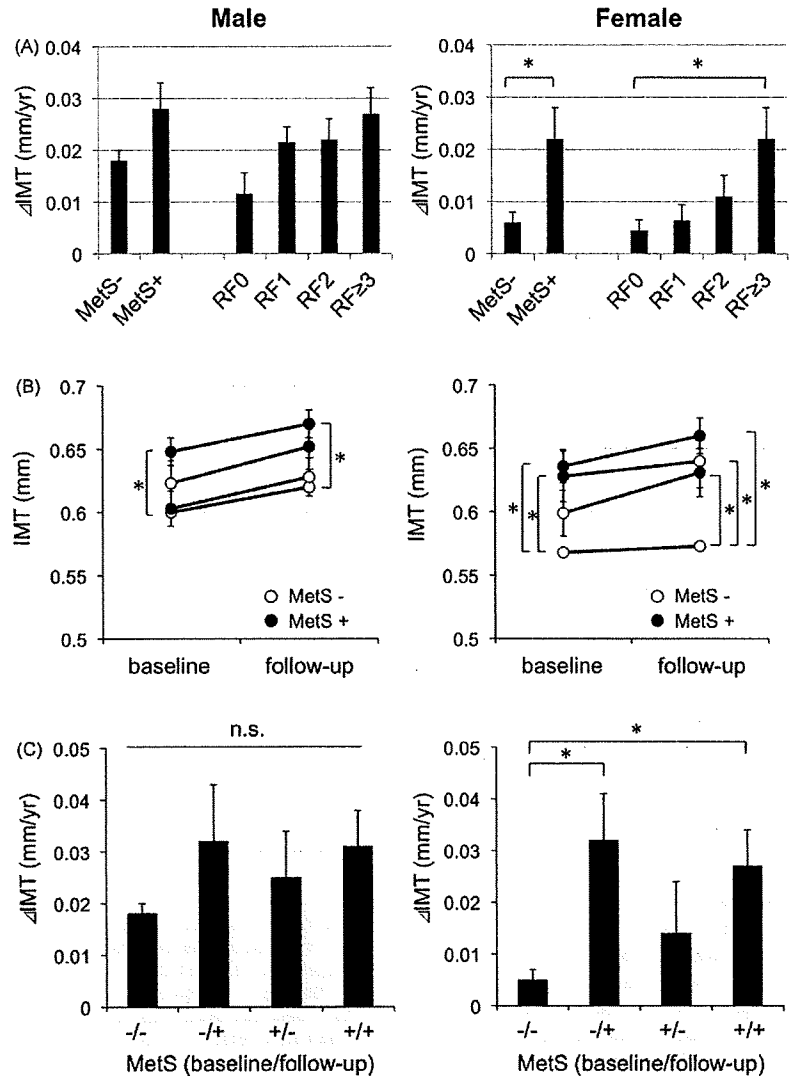

Fig. 1 – Impact of MetS and its components on mean and annual changes of IMT. (A) Annual changes of IMT after adjustment for age and basal IMT in the groups with or without MetS and the groups of accumulation risk number of the MetS at the base line in males (left) and females (right). Mean IMT after adjustment for age (B) and annual changes of IMT after adjustment for age and basal IMT (C) in the groups with or without MetS at the baseline and at the follow-up. Data are means ± SEM. *P < 0.05.

increased number of individual risk factors are associated with an increased IMT at baseline, and that MetS predicts annual increase in IMT particularly in females.

Next we analyzed baseline IMT and annual IMT changes based on a diagnosis of MetS at baseline and follow-up. In males, 635 (82.7%), 26 (3.4%), 40 (5.2%) and 68 (8.9%) subjects were diagnosed as MetS (–/–), (–/+), (+/–) and (+/+), respectively. Among the four groups, the IMT in the MetS (+/+) group was significantly greater than that in the MetS (–/–) group at baseline and at follow-up (Fig. 1B). In females, 452 (90.0%), 13 (2.6%), 11 (2.2%) and 26 (5.2%) subjects were diagnosed as MetS (–/–), (–/+), (+/–) and (+/+), respectively. IMT at baseline was significantly greater in the MetS (+/–) and MetS (+/+) groups than in the MetS (–/–) group (Fig. 1B). Similarly, IMT at follow-up was significantly greater in the MetS (–/+), MetS (+/–) and MetS (+/+) groups than in the MetS (–/–) group (Fig. 1B). Next, we assessed the annual IMT changes among these groups. In males, the annual IMT changes were not significantly different. In contrast, in females, the annual IMT change was significantly greater in the MetS (–/+) and MetS (+/+) group (Fig. 1C). Of interest, the annual increase in IMT in the MetS (+/–) females was not significantly different from that in the MetS (–/–) females, which may indicate a beneficial effect of medical intervention in MetS on IMT.

7. Discussion

In this study, subjects with MetS had significantly greater IMT compared with non-MetS subjects at baseline in both sexes as reported previously [1–6]. Each individual risk factor, high WC, high BP, high TG, low HDL-cholesterol and high FPG, was also related to greater IMT in both sexes except high FPG in males. Accumulation of 3 or more risk factors showed similar increase in baseline IMT (Table 1), which suggests a multiplier effect from risk factors with different atherogenic mechanisms. After the follow-up period, the annual increase in IMT was greater in the MetS, high WC, low HDL-cholesterol groups in females and in the high WC group in males. Interestingly, the annual IMT changes increased as the number of risk factors increased in both sexes and the changes were similar between MetS group and the group with 3 or above risk factors in both sexes (Fig. 1A). These results provide evidence that MetS and an increasing number of risk factors predict the future development of atherosclerosis particularly in female subjects in this short study period.

Of particular interest is that, despite the short follow-up period, new onset of MetS was related to a greater increase in IMT, whereas improving MetS status was related to a smaller increase in IMT (Fig. 1B and C). These findings suggest that the prevention or treatment of MetS may be beneficial to prevent future progression of atherosclerosis, particularly in female subjects. In the male subjects, the annual increase in IMT in the four groups showed a similar trend to that seen in females, however, the difference was not statistically significant. The baseline and annual increase in IMT in males were apparently greater than that in females, even in the absence of MetS, suggesting that male subjects may be exposed to other atherogenic risk factors, including hormonal differences, aside from components of the MetS. The sex difference observed in this study, as well in other reports, indicates that MetS was associated with a more pronounced adverse atherogenic risk in females than in males [9,10].

The limitations of this study are that most study subjects were employees of companies, so they do not represent the whole Japanese population; medical interventions for MetS subjects were not well controlled; and the study results were derived from observational findings obtained from annual medical checkups performed in consecutive years.

In conclusion, the present study showed that MetS and its individual components are related to increased IMT and that MetS predicts future progression of IMT particularly in females. Prospective studies are required to further examine this relationship and the effect of medical intervention for MetS to prevent atherosclerotic cardiovascular diseases.

Conflict of interest

The authors declare that they have no conflict of interest.

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Oleic acid-induced ADRP expression requires both AP-1 and PPAR response elements, and is reduced by Pycnogenol through mRNA degradation in NMuLi liver cells

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Fan B, Ikuyama S, Gu JQ, Wei P, Oyama J, Inoguchi T, Nishimura J. Oleic acid-induced ADRP expression requires both AP-1 and PPAR response elements, and is reduced by Pycnogenol through mRNA degradation in NMuLi liver cells. *Am J Physiol Endocrinol Metab* 297: E112–E123, 2009. First published April 21, 2009; doi:10.1152/ajpendo.00119.2009.—Fatty acids stimulate lipid accumulation in parallel with increased expression of adipose differentiation-related protein (ADRP) in liver cells. Although it is generally considered that the fatty acid effect on ADRP expression is mediated by peroxisome proliferator-activated receptors (PPARs), we identified here an additional molecular mechanism using the NMuLi mouse liver nonparenchymal cell line, which expresses PPARγ and δ but not α. Oleic acid (OA) and specific ligands for PPARγ and -δ stimulated ADRP expression as well as the −2,090-bp ADRP promoter activity which encompasses the PPAR response element (PPRE) adjacent to an Ets/activator protein (AP)-1 site. When the AP-1 site was mutated, OA failed to stimulate the activity despite the presence of the PPRE, whereas ligands for PPARγ and -δ did stimulate it and so did a PPARα ligand under the coexpression of PPARα. DNA binding of AP-1 was stimulated by OA but not by PPAR ligands. Because we previously demonstrated that Pycnogenol (PYC), a French maritime pine bark extract, suppressed ADRP expression in macrophages partly by suppression of AP-1 activity, we tested the effect of PYC on NMuLi cells. PYC reduced the OA-induced ADRP expression along with suppression of lipid droplet formation. However, PYC neither suppressed the OA-stimulated ADRP promoter activity nor DNA binding of AP-1 but, instead, reduced the ADRP mRNA half-life. All these results indicate that the effect of OA on ADRP expression requires AP-1 as well as PPRE, and PYC suppresses the ADRP expression in part by facilitating mRNA degradation. PYC, a widely used dietary supplement, could be beneficial for the prevention of excessive lipid accumulation such as hepatic steatosis.

lipid droplet; hepatic steatosis; adipose differentiation-related protein; activator protein-1; peroxisome proliferator-activated receptor

CYTOSOLIC LIPID DROPLETS are physiologically important because they have specific function in various cell types or tissues, for example, as an energy reservoir in adipocytes, sites of storage and biosynthesis of eicosanoids in leukocytes, and sites of production of pulmonary surfactants in pneumocytes (44). It has been demonstrated that excessive lipid accumulation, not only in an adipose tissue but also in various nonadipose tissues, is closely related to a variety of pathological conditions, including insulin resistance, type 2 diabetes mellitus, cardiovascular diseases, and fatty liver or nonalcoholic steatohepatitis (2, 59), all of which are emerging as important clinical ands socioeconomical worldwide problems. Mechanisms of intracellular lipid droplet formation have, therefore, increasingly attracted clinical and scientific interests.

A variety of proteins are associated with intracellular lipid droplets (10). Among them, PAT family proteins, which comprise perilipin, adipose differentiation-related protein (ADRP), TIP47, S3-12 and OXPAT/MLDP, are implicated in the formation, stabilization, and metabolism of lipid droplets (8, 37). ADRP was first identified in the early stages of adipocyte differentiation (33). Later studies revealed that ADRP does not directly induce adipogenesis but instead facilitates uptake of fatty acids or cholesterol (4, 22), whereas forced expression of ADRP also stimulates lipid droplet formation (31). In contrast to perilipin, whose expression is relatively limited in adipocytes and steroidgenic cells, ADRP is ubiquitously expressed in a variety of cells and is a specific marker of lipid accumulation (26).

The liver is a central organ for lipid metabolism, and excessive lipid accumulation in hepatocytes is generally associated with various metabolic abnormalities related to metabolic syndrome. In particular, fatty acids from several different sources, such as dietary fat, fatty acids released from adipose tissues, and de novo hepatic lipogenesis, facilitate lipid accumulation in hepatocytes in conjunction with oxidative stress or inflammatory stimuli (2, 59). ADRP expression is upregulated in hepatic steatosis in humans and mouse models (43). Considering that fatty acids act as ligands for all peroxisome proliferator-activated receptor (PPAR) subtypes (19, 34), although with different propensities for interaction (64), it is reasonable to hypothesize that the effect of fatty acids on ADRP expression is, at least in part, mediated by PPAR activation. Actually, a PPAR response element (PPRE) mediating the fatty acid effect was identified in the promoter region of mouse and human ADRP genes (13, 57). In our previous study (62), we demonstrated that oleic acid (OA)-induced enhancement of the mouse ADRP promoter activity required the upstream Ets/activator protein (AP)-1 element in RAW264.7 macrophage-like cells. This suggests that the PPRE in the ADRP promoter alone is likely to be insufficient for responding to OA, instead requiring the Ets/AP-1 element as well. This finding remains to be fully confirmed in other cell types.

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On the other hand, fatty liver formation was markedly prevented in mice whose ADRP expression was abrogated by gene knockout or antisense oligonucleotides (12, 30). Besides fatty liver, it has recently been reported that ADRP abrogation also protects from atherosclerosis development (48) and diet-induced insulin resistance (60). These findings strongly suggest that ADRP could be a promising target for the prevention of excessive lipid accumulation. In a previous study, we reported that Pycnogenol (PYC), an extract from French maritime pine bark, suppressed lipopolysaccharide (LPS)-induced ADRP expression in RAW264.7 cells (23). It is important to elucidate if PYC can suppress the ADRP expression and lipid droplet formation in liver cells, since this widely used dietary supplement could be a useful modality for prevention or treatment of hepatic steatosis.

In the present study, we aimed to further clarify the mechanism by which long-chain fatty acids, such as OA, simulate the ADRP expression in the NMuLi mouse hepatic nonparenchymal cell line. We also tested the effect of PYC on OA-induced ADRP expression and lipid droplet formation and disclosed a mechanism of action. We demonstrate here that OA-induced ADRP expression requires AP-1 function in addition to PPRE in the promoter, and PYC suppressed OA-induced ADRP expression along with lipid droplet formation, in part, through facilitating ADRP mRNA degradation.

MATERIALS AND METHODS

Cell culture. The mouse liver epithelial cell line NMuLi was obtained from Dainippon Sumitomo Pharma (Osaka, Japan). The cells were routinely cultured in 10-cm tissue culture dishes (Falcon 3003; Becton-Dickinson Labware, Franklin Lakes, NJ) in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% charcoal-treated FCS, 1% nonessential amino acids, and appropriate antibiotics. The charcoal treatment of the FCS did not adversely affect concentrations of fatty acids, triacylglyceride, and total cholesterol (data not shown). We preliminarily confirmed by trypan blue staining that concentrations of reagents we used in this study were those that did not affect cell viability.

Materials. Actinomycin D, GW-501516, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, astaxanthin, and curcumin were purchased from Sigma (St Louis, MI). Caproic acid and OA were purchased from Wako Pure Chemical Industries (Osaka, Japan), dissolved in water containing 5 mM BSA, and then diluted in the medium. Fenofibrate, bezafibrate, troglitazone, and pioglitazone were kindly provided by Kaken Pharmaceuticals (Tokyo, Japan), Kissei Pharmaceuticals (Matsumoto, Japan), Sankyo (Tokyo, Japan), and Takeda (Tokyo, Japan), respectively. All of these agents were dissolved in dimethyl sulfoxide. Antibodies to ADRP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from PROGEN Biotechnik (Heidelberg, Germany) and Ambion (Woodward Austin, TX), respectively. PYC was generously provided by Horphag Research (Geneva, Switzerland). Synthesized oligonucleotides were purchased from Roche Diagnostics (Tokyo, Japan). [α-^{32}P]dCTP (5,000 Ci/mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were purchased from GE Healthcare (Buckinghamshire, UK).

Northern blot analysis. NMuLi cells were plated at 1×10^6 cells/dish and cultivated for 24 h, and then the medium was changed to fresh medium containing the test reagent(s), followed by culturing for indicated times. Total RNA was purified using a commercially available kit (Isogen; Nippon GENE, Tokyo, Japan). Full-length mouse ADRP cDNA was [α-^{32}P]dCTP-labeled by a random priming method (Oligo Labeling kit; GE Healthcare) and used as a probe. Hybridization was performed according to the standard method (51); final washing was carried out at 65°C in 1× 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (pH 7.4) containing 0.5% SDS for 2 × 20 min. In addition, mRNA expression levels were also evaluated by the real-time PCR as described below, and the results are shown in bar graphs in the Figs. 1–8.

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% bromphenol blue) at 1:1 in volume. The proteins were heated at 95°C for 5 min, resolved by 10% SDS-PAGE, and electroblotted on 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 prestained standards (Bio-Rad). The membranes were blocked for 1 h at room temperature with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBS-Tween 20). Next, the membranes were incubated with guinea pig ADRP antibodies (0.5 μg/ml in PBS-Tween 20) for 1 h. After being washed four times (5 min each) with PBS-Tween 20, the membranes were incubated with an appropriate secondary IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS-Tween 20 (0.08 μg/ml) for 1 h, and then washed as above. The blots were developed using ECL Western blotting detection reagents (GE Healthcare) and exposed to Hyperfilm ECL (GE Healthcare) for ~15 min. Protein levels were evaluated using NIH Image, and the results are shown in bar graphs in Figs. 1–8.

Construction of promoter-reporter plasmids. Construction of reporter plasmids containing the ADRP promoter region or its mutants has been previously described (23, 62).

Transient transfection and luciferase assay. Reporter plasmids were introduced into NMuLi cells using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. Cells were seeded at 2×10^5 cells/well in a 12-well cell culture cluster (Costar 3513; Corning, Corning, NY) and grown for 24 h. Medium was then changed to serum-depleted DMEM, and the transfection mixture (2 μl lipofectamine and 5 μl PLUS reagent 5 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 and incubated for 3 h. The cells were then washed with serum-depleted DMEM and cultured in the DMEM containing the test reagent(s) for an additional 24 h. Cell lysates were collected, and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega). The transfection efficiency was normalized to the *Renilla* luciferase activity expressed by pRL-TK. In the experiments using PPARα coexpression, 0.5 μg of PPARα expression vector or a vacant vector (pCMX) was added to the transfection mixture. The PPARα expression vector was kindly provided by Dr. Yasutomi Kamei (Tokyo Medical and Dental University, Tokyo, Japan). Each transfection experiment was performed in triplicate and repeated at least three times.

Nuclear protein extracts. Nuclear protein extracts were prepared using a method described (55). NMuLi cells were seeded at 2×10^6 cells/dish and then cultured for 24 h. The medium was then changed to that containing a test reagent or a vehicle and cultured for an additional 1 h. When PYC effect was tested, cells were pretreated with PYC for 1 h before adding OA. The cells were washed with PBS, harvested by gentle scraping, and then resuspended in 5 volumes of *buffer* A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM EDTA) supplemented with 0.3 M sucrose and 0.5% Nonidet P-40. The cells were then homogenized by pipetting, and the suspension was layered on 1 ml *buffer* A containing 1.5 M sucrose, followed by centrifugation for 10 min (4°C, 15,000 *g*). After being washed 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 $MgCl_2$, 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 dithio-

theitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mg/ml pepstatin A, and 2 mg/ml leupeptin. The protein concentration was determined with a commercially available reagent (Bio-Rad) using BSA as a control.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed as previously described (23, 29, 62). A synthesized sense oligonucleotide was ^{32}P-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). NMuLi 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 $MgCl_2$, 1 mM DTT, 1 mM EDTA, 12.5% (vol/vol) glycerol, 0.1% Triton X-100, 8 μg/ml calf thymus DNA, and 50 mM PMSF. A radiolabeled probe (50,000 counts/min, ~1.0 ng DNA) was then added and incubated 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 Tris-borate-EDTA buffer. Thereafter, the gels were dried and subjected to autoradiography, and a specific protein-DNA complex was quatified by NIH Image if necessary.

Oil-red O staining. NMuLi cells were plated in a four-well chamber slide (Nalge Nunc International, Naperville, IL) at 1×10^5/chamber and cultured in the presence or absence of 50 μg/ml PYC for 1 h. The cells were then cultured in medium containing 200 μM OA in the presence or absence of PYC for 96 h. The cells were washed with PBS, fixed in 10% formaldehyde, and stained with the neutral

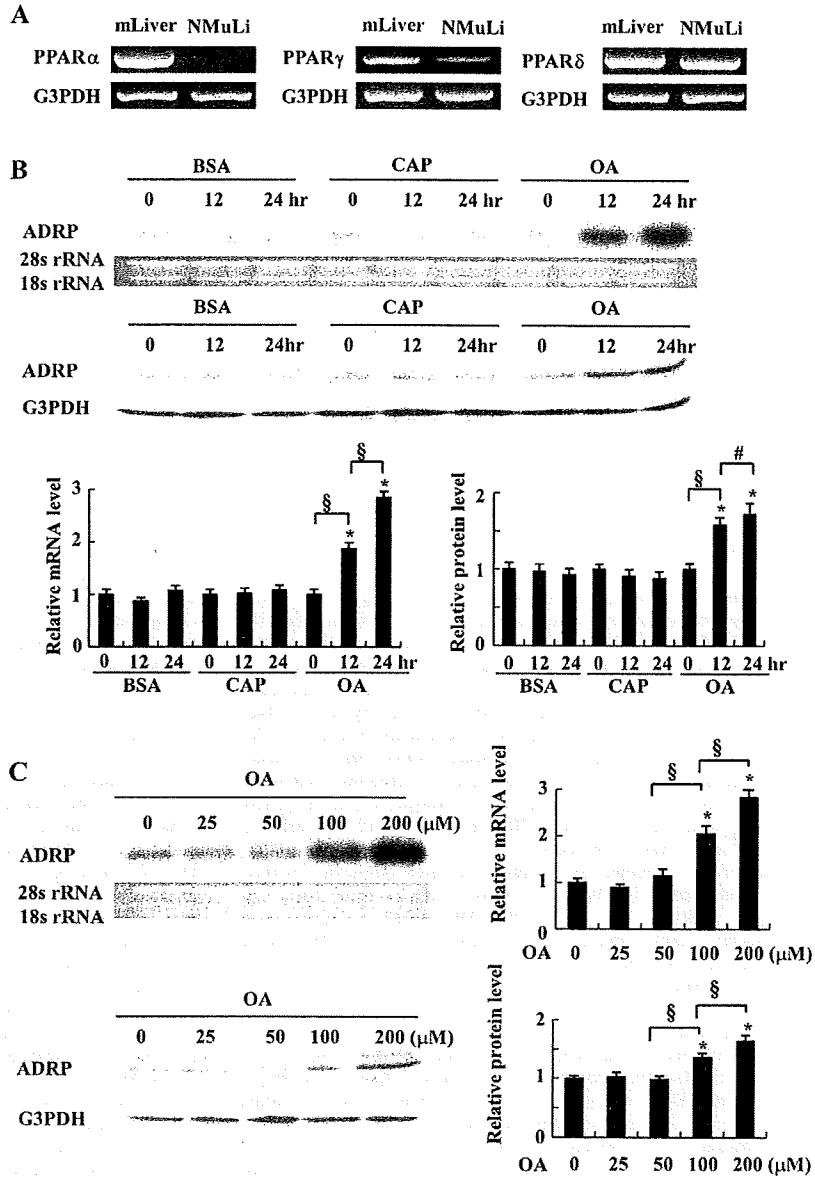

Fig. 1. The expression of adipose differentiation-related protein (ADRP) is enhanced by OA in NMuLi cells. *A*: NMuLi liver nonparenchymal cells express peroxisome proliferator-activated receptor (PPAR)-γ and -δ but not -α, whereas the liver of wild-type mouse (mLiver) expresses all PPARs. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression is shown as an internal control. *B*: oleic acid (OA) enhances ADRP mRNA (*top*) and protein (*bottom*) expression in a time-dependent manner. Cells were incubated with vehicle, 200 μM caproic acid (CAP), or 200 μM OA for 12 or 24 h. Representative results are shown. Relative expression levels of mRNA and protein obtained from 3 independent experiments are shown in bar graphs. For mRNA levels (*left*): $^*P < 0.01$ vs. control (BSA 0 h), $\S P < 0.01$; for protein levels (*right*): $^*P < 0.01$ vs. control (BSA 0 h), $\S P < 0.01$, and $\#P < 0.05$. *C*: OA stimulates ADRP mRNA (*top*) and protein (*bottom*) expression in a dose-dependent manner. Cells were incubated with vehicle or indicated concentrations of OA for 24 h. Ribosomal RNAs and G3PDH were used as a total RNA or protein-loading control. Representative results are shown. Relative expression levels of mRNA and protein obtained from 3 independent experiments are shown in bar graphs. For mRNA levels, $^*P < 0.01$ vs. control (0 μM), $\S P < 0.01$; for protein levels, $^*P < 0.05$ vs. control (0 μM), $\S P < 0.05$.

lipid dye Oil-red O (0.3% in 60% isopropanol), followed by extensive washes (38). Photomicrographs were generated using a digital camera (U-CMAD3; Olympus, Tokyo, Japan) connected to an Olympus BX51 microscope.

PCR and real-time RT-PCR. Single-stranded cDNA was synthesized with the ReverTra Ace-α kit (Toyobo, Osaka, Japan) using 0.5 μg of the total RNA. The PPAR expression was determined by PCR amplification of 35 cycles with annealing temperature at 60°C using the following primers: PPARα (forward: TGGACACAGAGAGCCCCATC, reverse: TCGTACACCAGCTTCAGCCG), PPARγ (forward: TGGCCATTGAGTGCCGAGTC, reverse: CGCCTTGGCTTTGGTCAGCG), and PPARδ (forward: GCTCAATGGGGGACCAGAAC, reverse: GCTTAGAGAAGGCCTTCAGG).

Real-time RT-PCR was performed with a SYBR Green method using the iCycler iQ multicolor real-time PCR detection system (Bio-Rad) 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]. Sequences of the primers were as follows: ADRP (forward: CTGTCTACCAAGCTCTGCTC, reverse: CGATGCTTCTCTTCCACTCC) and GAPDH (forward: ACCACAGTCCATGCCATCAC, reverse: TCCACCACCCTGTTGCTTA). PCR efficiencies for all reactions were >0.90. Quantitative PCR results were expressed as relative fold induction compared with the housekeeping gene GAPDH.

Statistical analysis. All data were presented as means ± SE. Statistical differences were determined by one-way ANOVA. A $P < 0.05$ was considered to be significant.

RESULTS

Long-chain fatty acids stimulate the expression of ADRP in the NMuLi hepatic nonparenchymal cell line. Several studies have demonstrated that long-chain fatty acids stimulate ADRP expression in different types of cells (21, 22, 52, 62), and they also act as ligands for all three PPAR subtypes (19, 34). We first determined the expression of PPAR subtypes in NMuLi cells used in this study. We found that this cell line expressed PPARγ and PPARδ but not PPARα (Fig. 1*A*). The expression pattern of these PPARs was not affected by the agents that we used in this study (data not shown). Although NMuLi is not of hepatic parenchymal origin, it has been used expediently as an alternative of a normal murine hepatocyte having some similar propensities to hepatic parenchymal cells (42). More importantly, we found it a merit that the cell line did not express PPARα because we could test an effect of exogenously introduced PPARα on ADRP promoter activity in the following analyses.

As in previous studies (21, 52, 62), the expression of ADRP mRNA and protein was increased significantly by OA (C-18) after 12 h incubation, but not by caproic acid (C-6) (Fig. 1*B*), and that the effect of OA was dose-dependent (Fig. 1*C*). We also confirmed that other long-chain fatty acids, such as palmitic acid (C-16), linoleic acid (C-18), and linolenic acid (C-18), enhanced the ADRP mRNA and protein levels (data not shown), and used only OA for further analyses.

The effect of OA on the ADRP expression was exerted at the transcriptional level, since the addition of actinomycin D completely abrogated OA-induced enhancement of ADRP mRNA expression (Fig. 2*A*). It has been demonstrated that triacsin C, a long-chain acyl-CoA synthetase inhibitor, inhibits synthesis of triacylglycerol and cholesteryl esters, thereby blocking foam cell formation in macrophages (45). We, therefore, tested the effect of triacsin C on OA-induced expression of ADRP mRNA. As shown in Fig. 2*B*, the stimulatory effect

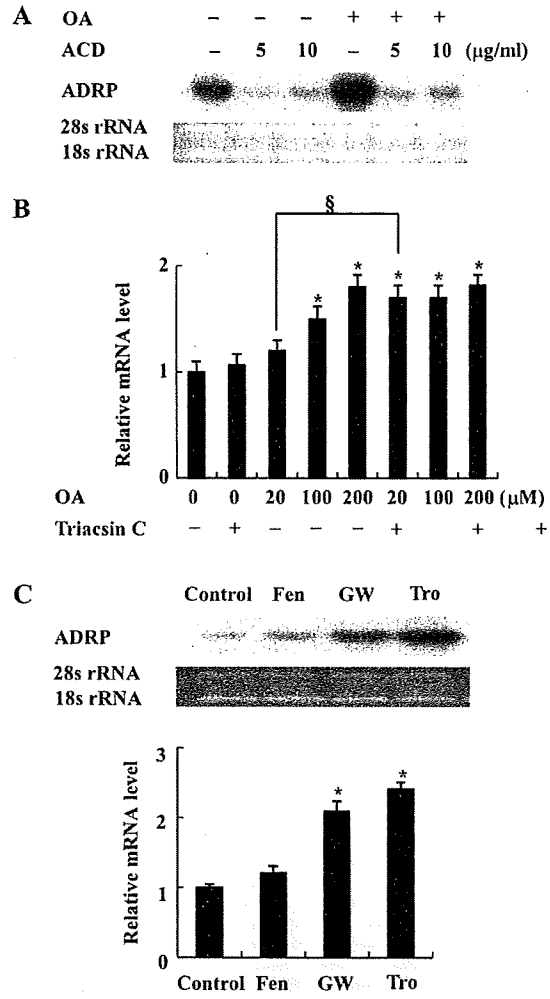

Fig. 2. OA stimulates ADRP expression at the transcriptional level and is independent of triglyceride synthesis. *A*: actinomycin D (ACD) suppresses basal and OA-induced ADRP mRNA expression. NMuLi cells were preincubated with 5 or 10 μg/ml ACD for 1 h and then stimulated by 200 μM OA for 24 h. Ribosomal RNAs were used as a total RNA loading control. *B*: triacsin C, an acyl-CoA synthase inhibitor, does not inhibit OA-induced ADRP expression. Cells were preincubated with or without 4.8 μM triacsin C for 1 h and then stimulated by OA for 24 h. The mRNA levels were evaluated by real-time PCR. Data represent means ± SE of 3 independent experiments. *$P < 0.05$ vs. control (without OA) and §$P < 0.05$. *C*: ADRP expression is stimulated by PPARγ (troglitazone, Tro) and PPARδ (GW-501516, GW) ligands but not by a PPARα ligand (fenofibrate, Fen) in NMuLi cells. Ribosomal RNAs were used as a total RNA loading control. Representative data (*top*) and the mRNA levels evaluated by real-time PCR from 3 independent experiments (*bottom*) are shown. *$P < 0.01$ vs. control.

of low-dose OA (20 μM) was significantly potentiated in the presence of triacsin C. Because a higher dose of OA (100 and 200 μM) stimulated high mRNA expression possibly at maximum level, the additive effect of triacsin C was not evident. This result suggested that OA itself, but not triacylglycerol, stimulates the mRNA expression. We confirmed that the PPARγ ligands troglitazone (Fig. 2*C*), pioglitazone, and 15-

deoxy-$\Delta^{12,14}$-prostaglandin J_2 (not shown) and a PPARδ ligand, GW-501516 (Fig. 2C), enhanced ADRP mRNA expression. As expected, PPARα ligands, fenofibrate (Fig. 2*C*) and bezafibrate (data not shown), did not significantly stimulate the expression. Taken together with previous reports proving that fatty acids are potential ligands for PPARs (19, 34), these results suggest that OA stimulates ADRP expression in part through PPARγ and -δ activation in NMuLi cells.

Functional AP-1 site is requisite for OA action. Next, we investigated the effect of OA on the mouse ADRP promoter activity. The −2,090-bp promoter region of the mouse ADRP gene encompasses a canonical PPRE, starting at −2,001 bp (13, 62). In addition, we demonstrated that an Ets/AP-1 composite element exists at −2,047 bp, which is important for phorbol myristate ester (PMA)- and LPS-induced enhancement of ADRP expression in macrophages (23, 62). The promoter region encompassing these elements is highly conserved between mouse and human ADRP genes (Fig. 3*A*). As shown in Fig. 3*B*, activity of the −2,090-bp ADRP promoter was enhanced by OA, whereas caproic acid had no effect, consistent with the effects on the mRNA expression.

In a previous study, we raised the possibility of functional interaction between Ets/AP-1 and PPRE in response to PMA and OA in RAW264.7 cells (62). We, therefore, tested the OA-induced promoter activity, using a series of deleted or mutated promoters derived from the wild-type −2,090-bp promoter. By deletion of all three elements (−1,889 bp), the basal promoter activity was reduced significantly, and the OA-induced enhancement was completely abolished (Fig. 3*C*). Basal activity of the PPRE mutation (Pmut) was almost comparable to the −2,090-bp promoter, whereas OA failed to stimulate, actually suppressed, the Pmut promoter activity (Fig. 3*C*), indicating that OA acts through the PPRE. The basal activity of promoters containing either AP-1 or Ets mutations (Amut or Emut) was reduced, suggesting that these elements might be important for basal promoter activity. Interestingly, OA-induced enhancement was almost completely diminished in the Amut promoter despite the presence of an intact PPRE, whereas OA stimulated the Emut promoter to the wild-type levels (Fig. 3*D*). These results indicate that AP-1, in addition to the PPRE, is indispensable for OA action on ADRP expression in this cell line.

We further tested whether intact AP-1 function is also required for the action of synthesized ligands specific for PPAR subtypes. Like OA, a PPARγ ligand (troglitazone) and a PPARδ ligand (GW-501516) stimulated activity of the wild-type −2,090-bp and Emut promoters but not −1,889-bp and Pmut promoters. In contrast to OA, however, these compounds also stimulated Amut promoter activity (Fig. 4, *A* and *B*). This finding was duplicated by a PPARα ligand (fenofibrate) when PPARα was coexpressed in the cells (Fig. 4*C*). These results indicate that, while specific ligands for all three PPAR subtypes and OA could stimulate ADRP promoter activity through PPRE, only OA requires intact AP-1 function.

This was investigated further using an EMSA. Use of an oligonucleotide probe containing the Ets/AP-1 element exhibited double complexes (complex A) by NMuLi nuclear extracts (Fig. 5*A*). Complex A formation was effectively inhibited by the addition of oligonucleotide competitors containing wild-type sequence (self), Emut, and AP-1 consensus sequence, but not by competitors containing Amut, double mutations in Ets/AP-1 sequence, and PU.1 consensus sequence. This result

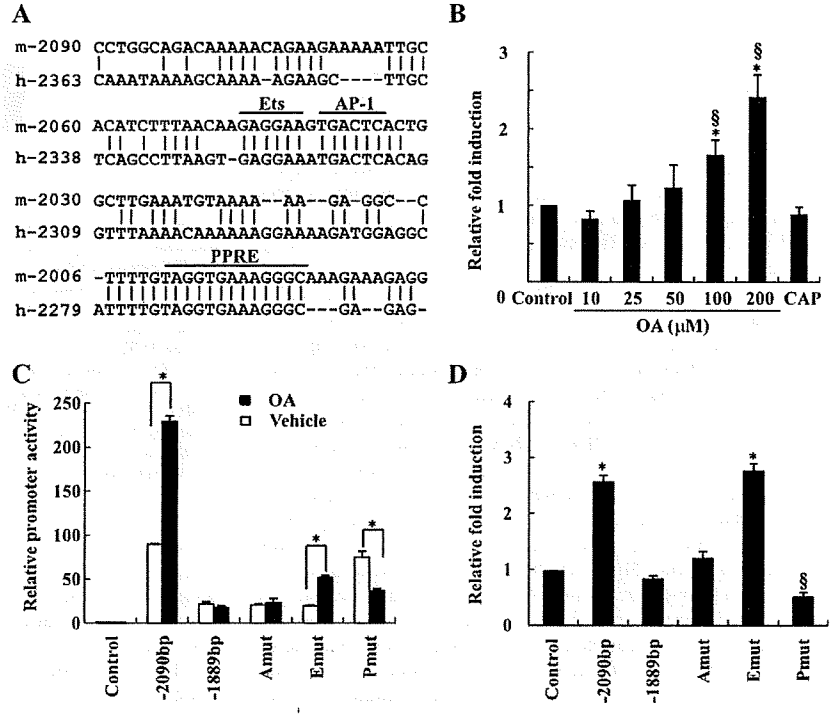

Fig. 3. OA-induced enhancement of the ADRP promoter activity requires activator protein (AP)-1 in addition to PPAR response element (PPRE). *A*: promoter region containing an Ets/AP-1 site and a PPRE shows high homology between mouse (m) and human (h) ADRP genes. Mouse genomic sequence was from GenBank accession no. AB207137. Human ADRP promoter sequence was identified in the human genome database (Contig NT037733.1, chromosome 9p21.3) probed by human adipophilin coding sequence (GenBank accession no. NM001122). *B*: OA stimulates activity of the −2,090-bp promoter but caproic acid does not. The activity of the −2,090-bp promoter without stimulation (control) is designated as 1 for comparison. $*P < 0.05$ vs. control and $§P < 0.05$ vs. caproic acid. *C*: effect of OA on activity of the −2,090-bp promoter and promoters containing a deletion or a mutation. Amut, Emut, and Pmut are the promoters containing site-directed mutations in the AP-1 site, Ets site, and PPRE of the −2,090-bp promoter, respectively (62). Activity of the promoter-less plasmid (pGL3) in the absence of OA is designated as 1 for comparison. Means ± SE of 3 independent experiments are shown. $*P < 0.05$. *D*: relative fold induction (ratio of the activity in the presence vs. in the absence of OA) converted from the data in *C*. The value of the promoterless control is designated as 1 for comparison. Note that OA does not stimulate the Amut promoter activity. $*P < 0.05$ (increased) vs. control and $§P < 0.05$ (decreased) vs. control.

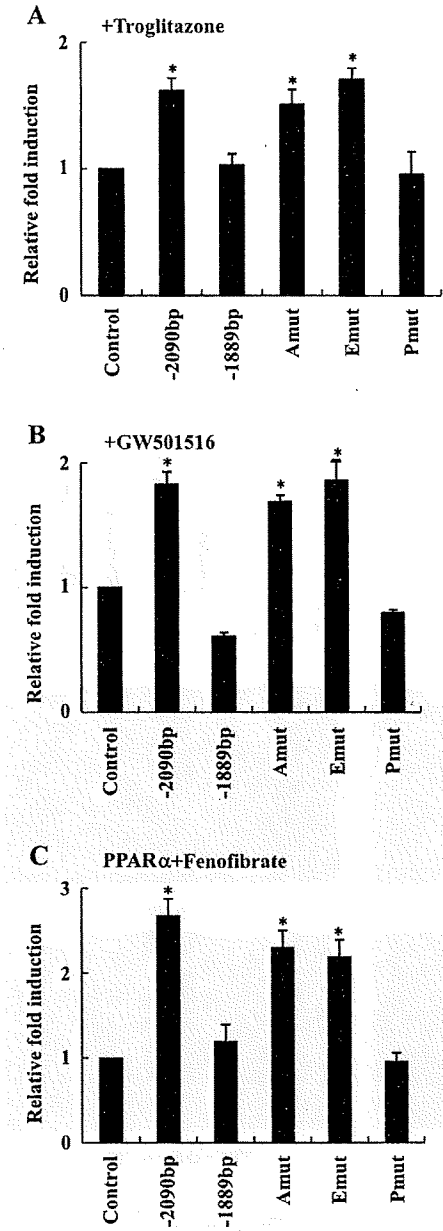

Fig. 4. Specific ligands for PPARs do not stimulate the promoter containing the PPRE mutation but do stimulate the promoters containing the Ets/AP-1 mutation. PPARγ ligand (*A*) and PPARδ ligand (*B*) stimulate the activity of the Amut and Emut promoters but not the Pmut promoter. Under the coexpression of PPARα, the PPARα ligand also stimulates the activity of the Amut and Emut promoters but not the Pmut promoter (*C*). Concentration of each ligand was 10 μM. Data were analyzed as in Fig. 3*D*. Means ± SE of 3 independent experiments are shown. $^*P < 0.05$ vs. control (pGL3).

indicates that the Ets/AP-1 site is recognized only by the AP-1 family of transcription factors in NMuLi cells, unlike RAW264.7 cells in which AP-1 and PU.1 conjointly bind (23, 62). DNA binding of AP-1 family proteins was enhanced by OA but not by caproic acid and synthesized PPAR ligands (Fig. 5*B*). This finding further supported the involvement of AP-1 in the OA effect.

Collectively, these results indicate that the Ets/AP-1 element is important for the basal promoter activity, and more importantly that OA stimulates ADRP expression by acting through the PPRE in collaboration with the AP-1 site in the promoter in NMuLi cells. The Ets sequence also seems to be involved in basal promoter activity, although we could not identify protein binding to this site by EMSA. This problem requires further investigation.

PYC suppresses OA-induced ADRP expression and lipid droplet formation in NMuLi cells. Previously, we demonstrated that PYC suppressed ADRP expression in RAW264.7 cells (23). We, therefore, tested the effect of PYC on ADRP expression and intracellular lipid droplet formation in NMuLi cells. PYC significantly suppressed the OA-induced increase in ADRP mRNA and protein levels in a dose-dependent manner, whereas it did not affect basal ADRP expression levels (Fig. 6*A*). PYC (50 μg/ml) almost completely inhibited OA-induced ADRP expression. OA markedly induced intracellular lipid accumulation, which was clearly visualized by Oil-red O

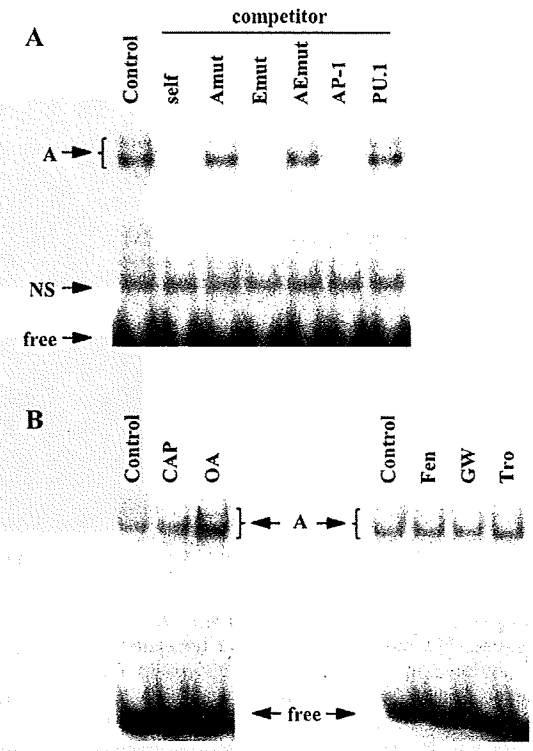

Fig. 5. OA stimulates DNA binding of AP-1. *A*: oligonucleotide containing the Ets/AP-1 element of the ADRP promoter was used as a probe. Wild-type sequence (self); oligonucleotides containing an AP-1 mutation (Amut), Ets mutation (Emut), both AP-1 and Ets mutations (AEmut); and oligonucleotides containing AP-1 and PU.1 consensus sequences were used as competitors. The result indicates that complex A (A) is formed by the AP-1 family transcription factors in NMuLi nuclear extracts. *B*: OA stimulates DNA binding of AP-1, but caproic acid as well as PPAR ligands, such as fenofibrate, GW-501516, and troglitazone, do not. NS, nonspecific band.

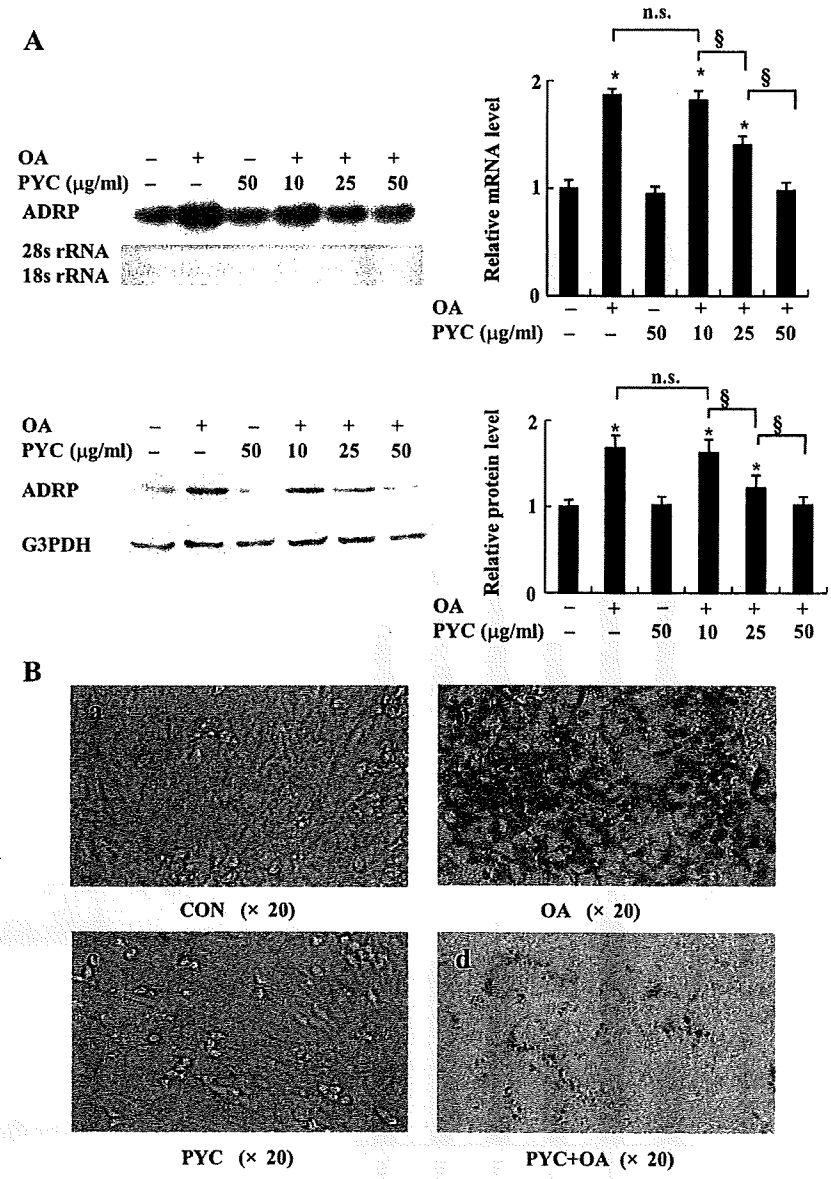

Fig. 6. Pycnogenol (PYC) suppresses OA-induced ADRP expression and lipid droplet formation in NMuLi cells. *A*: PYC suppresses OA-induced ADRP mRNA and protein levels in a dose-dependent manner. Note that PYC does not affect the constitutive expression of ADRP. Cells were preincubated with dimethyl sulfoxide (DMSO; vehicle) or PYC for 1 h and then stimulated with 200 μM OA for 24 h. Ribosomal RNAs and G3PDH were used as total RNA or protein loading control, respectively. Representative data are shown. Relative mRNA and protein levels obtained from 3 independent experiments are shown in bar graphs. For mRNA levels (*top*), $^*P < 0.01$ vs. control and $\S P < 0.01$; for protein levels (*bottom*), $^*P < 0.05$ vs. control and $\S P < 0.05$, n.s., Not significant. *B*: PYC suppresses OA-induced lipid droplet formation. Oil-red O staining of the cells is shown. Cells were preincubated with DMSO (vehicle) or 50 μg/ml PYC for 1 h, and then 200 μM OA or vehicle was added and incubated for 96 h. *a* and *c*: Without OA stimulation; *b* and *d*: with OA stimulation. CON, control.

staining (Fig. 6*B*, *a* vs. *b*). Consistent with the effect on ADRP expression, OA-induced lipid droplet formation was remarkably inhibited in the presence of 50 μg/ml PYC with no apparent change in cell viability (Fig. 6*B*, *b* vs. *d*). The suppressive effect of PYC on OA-induced ADRP expression was likely to be specific, because the antioxidants, curcumin and astaxanthin (25, 27), failed to antagonize the OA effect on mRNA expression (Fig. 7).

PYC suppresses ADRP expression by facilitating the mRNA degradation. Previously, we demonstrated that PYC suppresses the enhancer activity of AP-1 and nuclear factor (NF)-κB, thereby leading to the suppression of LPS-induced ADRP expression in RAW264.7 cells (23). Specifically, LPS-stimulated activity of the −2,090-bp promoter was suppressed by PYC in RAW264.7 cells (23). Because of the importance of AP-1 function in OA-induced activation, we investigated the effect of PYC on the ADRP promoter and found that OA-stimulated activity of the −2,090-bp promoter was not suppressed at all in NMuLi cells (Fig. 8*A*). In addition, OA-induced enhancement of AP-1 binding to the Ets/AP-1 element was not inhibited by PYC (Fig. 8*B*).

To clarify the mechanism by which PYC suppresses the OA-induced ADRP mRNA expression, we presumed that a posttranscriptional mechanism, such as mRNA degradation, might be involved. Therefore, we tested whether PYC might shorten the half-life of ADRP mRNA. NMuLi cells were

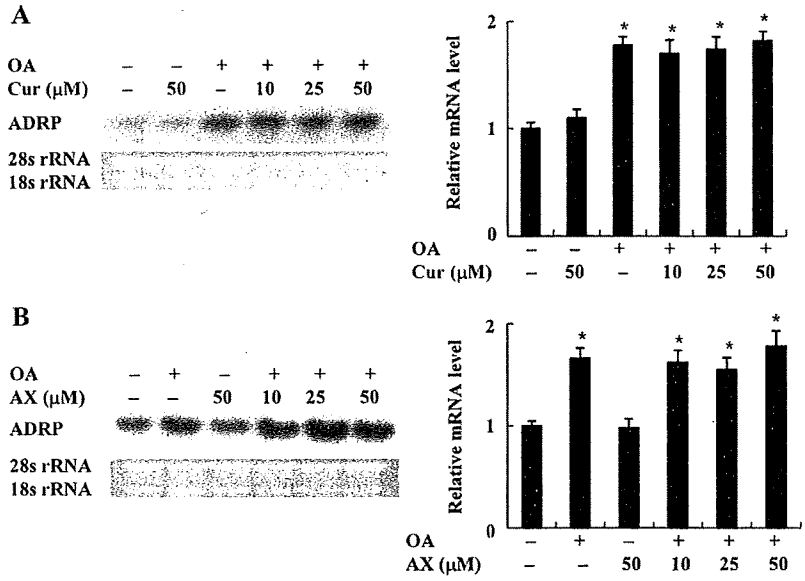

Fig. 7. Antioxidant substances do not necessarily suppress OA-induced ADRP expression. Curcumin (Cur) (*A*) and astaxanthin (AX) (*B*), well-known dietary supplements having an antioxidant effect, did not affect OA-induced ADRP expression. Ribosomal RNAs were used as a total RNA loading control. Representative data and the mRNA levels evaluated by real-time PCR from 3 independent experiments (bar graphs) are shown. $^*P < 0.01$ vs. control.

cultured in the presence or absence of 50 μg/ml PYC for 1 h and then incubated with OA in the presence of 5 μg/ml actinomycin D. Here we added OA in the medium since OA itself might affect the mRNA stability. The ADRP mRNA level was measured up to 12 h after stimulation. When PYC was present in the medium, a faster decrease in ADRP mRNA levels was observed when compared with the absence of PYC (Fig. 8*C*). GAPDH mRNA as a control was not affected by PYC (data not shown). These results were confirmed by quantitative PCR (Fig. 8*D*). The estimated half-life of the ADRP mRNA was ~6 h in the presence of PYC and 11 h in the absence of PYC.

All of these results indicate that PYC suppresses OA-induced ADRP expression and lipid droplet formation in NMuLi cells. The mechanism involved is, in part, facilitation of ADRP mRNA degradation. The precise molecular mechanism of mRNA degradation still remains to be elucidated.

DISCUSSION

ADRP is a ubiquitously and constitutively expressed protein (9) that is rapidly degraded via the proteasome in the absence of lipids (39, 62, 63). A number of studies demonstrated high expression levels of ADRP in various pathological conditions in humans and animal models, including steatohepatitis or fatty liver (43), atherosclerosis (36), and diabetic nephropathy (40). Although it has been shown that several pathological or pharmacological stimuli enhance ADRP expression, the precise molecular mechanism regulating the expression is not fully elucidated. In previous studies, we and others demonstrated that an inflammatory signal, such as PMA or LPS, stimulates ADRP expression in macrophages (14, 23, 62), hepatocytes (46), and keratinocytes (15). We identified the Ets/AP-1 composite element in the mouse ADRP promoter and proved that it mediates, in part, the action of PMA or LPS, which enhances ADRP expression in RAW264.7 cells (23, 62).

Another important regulatory mechanism of the ADRP gene is a PPAR-mediated pathway that plays a central role in lipid homeostasis in various tissues. A number of reports demonstrated that specific ligands for PPARα (16, 18), PPARγ (7, 24, 52), and PPARδ (13, 49, 53, 61) upregulate the expression of ADRP, and functional PPRE was identified in human and mouse ADRP genes (13, 57, 62). In addition to specific PPAR ligands, it was shown that long-chain fatty acids stimulated ADRP expression in various cells (21, 58, 62). Because long-chain fatty acids act as ligands for all PPAR subtypes (19, 34), it is understandable that the effects of fatty acids are mediated through PPAR activation. Specifically, it was shown that the activity of ADRP promoters of human (57) and mouse (13) origin, both of which encompass the PPRE, is stimulated by OA and lipoprotein lipase-hydrolyzed very low-density lipoprotein, respectively. Here, we have demonstrated that OA and specific ligands for PPARγ and -δ enhanced ADRP expression and ADRP promoter activity in NMuLi hepatic nonparenchymal cells, which express PPARγ and PPARδ but not PPARα. Under the coexpression of PPARα, however, a PPARα ligand stimulated ADRP promoter activity in this cell line. Furthermore, all of these specific ligands and OA failed to stimulate activity of the promoter containing a PPRE mutation. These findings confirm an indispensable role of PPARs and PPRE in the regulation of ADRP gene.

We found, however, an important difference between effects of OA and specific PPAR ligands on ADRP promoter activity. Specific PPAR ligands stimulated the promoter activity containing an AP-1 mutation with an intact PPRE (Amut), whereas OA did not. This finding was preliminarily implicated in our previous report (62). In the process of characterizing the Ets/AP-1 element of the mouse ADRP promoter in RAW264.7 cells, we found that the synergistic effect of OA and PMA was abolished when the Ets/AP-1 site was mutated, even though the promoter contained an intact PPRE. As shown by EMSA, the

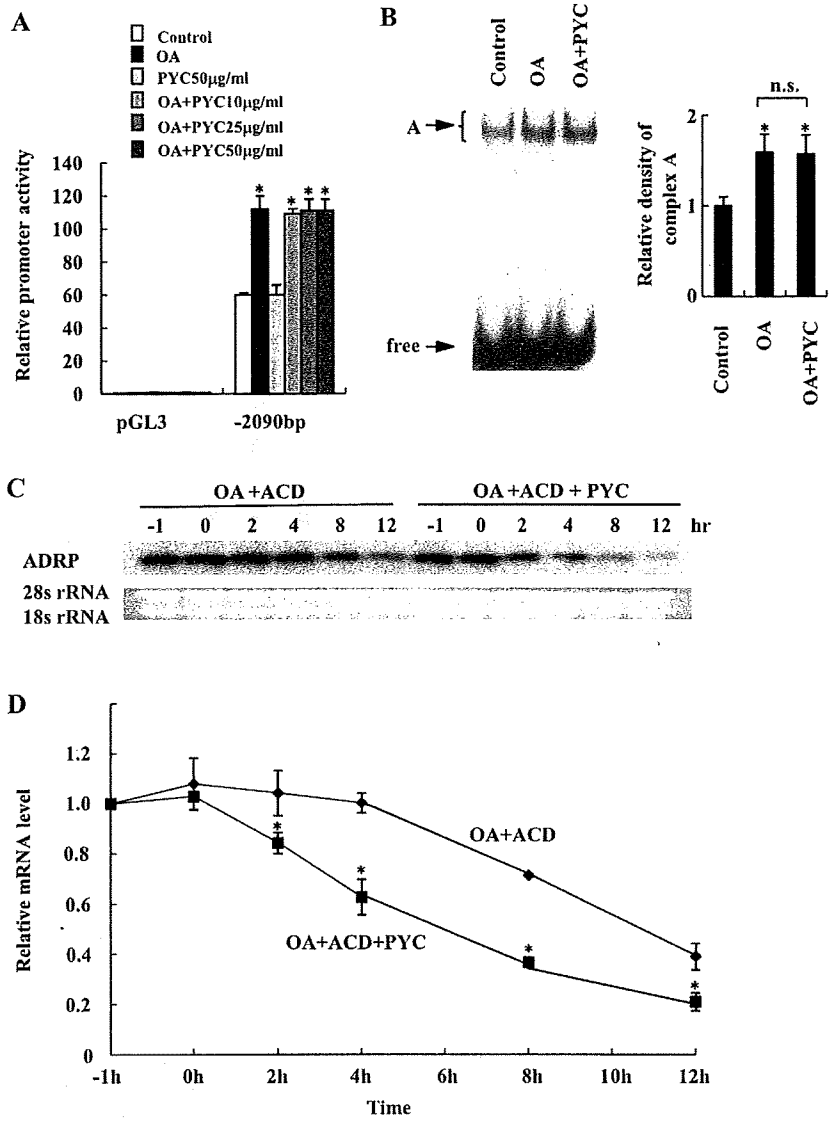

Fig. 8. PYC does not affect the ADRP promoter activity, but it facilitates ADRP mRNA degradation. *A*: activity of the −2,090-bp promoter was stimulated by 200 μM OA, but it was not suppressed by increasing concentrations of PYC. Results are shown as means ± SE of 3 independent experiments. *P <0.05 vs. control (vehicle). *B*: PYC does not inhibit OA-stimulated DNA binding of AP-1 in EMSA. Relative density of complex A quantified by NIH Image is shown in a bar graph. The value of the control is designated as 1 for comparison. Data from 3 independent experiments were analyzed. *P < 0.01 vs. control. *C*: Northern blot analysis shows that ADRP mRNA levels decline faster in the presence of PYC than in the absence of PYC. Cells were preincubated with DMSO (vehicle) or 50 μg/ml PYC for 1 h and then cultured in medium containing 200 μM OA and ACD with or without PYC for 24 h. A representative result of 3 independent experiments is shown. *D*: ADRP mRNA levels shown in *C* were quantified using real-time PCR. Data are means ± SE of 3 independent experiments. The mRNA level at −1 h, which was equivalent for both groups, is designated as 1 for comparison. *P < 0.05 vs. without PYC.

Ets/AP-1 site was recognized only by the AP-1 family of transcription factors but not the Ets family proteins such as PU.1 in NMuLi cells. Therefore, it seems reasonable that only the AP-1 mutation abolished the OA-induced activation of the promoter. DNA binding of AP-1 was enhanced by OA but not by specific PPAR ligands and caproic acid. It was shown that long-chain fatty acids, but not short-chain fatty acids, activate membrane receptors such as GPR40 and GPR120 (11, 28, 32). Recently, it has been reported that OA induces AP-1-DNA binding activity through Src kinase-dependent extracellular signal-regulated kinase (ERK) 1/2 activation in breast cancer cells (54). It could be surmised that this kind of membrane receptor-mediated mechanism might also be responsible for the ADRP gene regulation by OA, since our preliminary experiments showed that a Src kinase inhibitor suppressed ADRP mRNA expression (data not shown).

Thus, we have shown here that functional AP-1 activity is indispensable for PPAR-mediated ADRP expression by OA but not by specific PPAR ligands. Although responsiveness of the human or mouse ADRP gene to fatty acids was demonstrated in previous reports (13, 57), the Ets/AP-1 site was involved in the promoter region but not modified in their experiments. As we proposed (62), the Ets/AP-1 element and PPRE may form a functional complex as a lipid sensor in the ADRP promoter. We surmise that possible factors connecting these two elements might involve the transcriptional coactivator CBP/p300. CBP/p300 was shown to be indispensable for PPARγ-mediated adipocyte differentiation (56). OA, which

stimulates AP-1 activity, could efficiently recruit CBP/p300 (3, 5), and induce interaction with PPARγ-interacting protein (PRIP) or PRIP-interacting protein with methyltransferase domain (41, 50), in turn, enhancing to PPAR-mediated actions. This possibility is currently under investigation.

PYC is a natural extract from French maritime pine bark and widely used as a dietary supplement or a herbal drug. The main constituents are monomeric phenolic compounds (catechin, epicatechin, and taxifolin) and condensed flavonoids (procyanidines and proanthocyanidines) (47). We showed here that PYC suppressed OA-induced ADRP expression in parallel with lipid droplet formation in NMuLi cells. In our previous report, we demonstrated that PYC suppressed enhancer activity of AP-1 and NF-κB, thereby suppressing ADRP expression in RAW264.7 cells by directly acting on its promoter and via the inhibition of inflammatory cytokine production (23). Considering the functional importance of AP-1 in mediating the OA effect, we had presumed that PYC would suppress the −2,090-bp ADRP promoter activity, which was significantly suppressed by PYC in RAW264.7 cells. Contrary to our expectation, PYC did not suppress ADRP promoter activity at all. Instead, we found that PYC shortened the half-life of ADRP mRNA. Although dominant biological effects of PYC have been attributed to its antioxidant property (20, 47), it seems that the effect on the ADRP expression could not be necessarily ascribed to it. In this study and our previous report (23), we demonstrated that DNA binding of AP-1 or NF-κB, typical redox-sensitive transcription factors, was not inhibited by PYC. In addition, curcumin or astaxanthin, which are well-known antioxidant substances (25, 27), failed to antagonize the OA-induced increase of ADRP mRNA. One of the major posttranscriptional regulatory mechanisms of gene expression is degradation of mRNAs (17). Many kinds of stabilizing or destabilizing factors, which bind to AU-rich stretches of 3′-untranslated region (UTR) of mRNAs, have been identified (6). Berberine, a well-accepted herbal medicine in China, modulates mRNA stability. It activates the mitogen/extracellular-regulated kinase 1-ERK pathway and stabilizes low-density lipoprotein (LDL) receptor mRNA, resulting in a serum LDL-cholesterol lowering effect (1, 35). Although the presence of AU-rich stretches in 3′-UTR in ADRP mRNA are yet to be identified, it is possible that PYC antagonizes the effect of OA through modification of stabilizing or destabilizing factors that regulate the ADRP mRNA stability.

In conclusion, we have demonstrated here, using the NMuLi mouse hepatic nonparenchymal cell line, that the OA-induced ADRP expression requires not only a PPRE but also an AP-1 site on its promoter, and this mechanism is distinct from that of specific PPAR ligands. In addition, we demonstrated that PYC suppressed ADRP expression and lipid accumulation by facilitating ADRP mRNA degradation. This study is the first to report showing the efficient effect of PYC on intracellular lipid accumulation. PYC, a widely used dietary supplement, could be a unique tool for the prevention or therapy of excessive lipid accumulation such as fatty liver disease.

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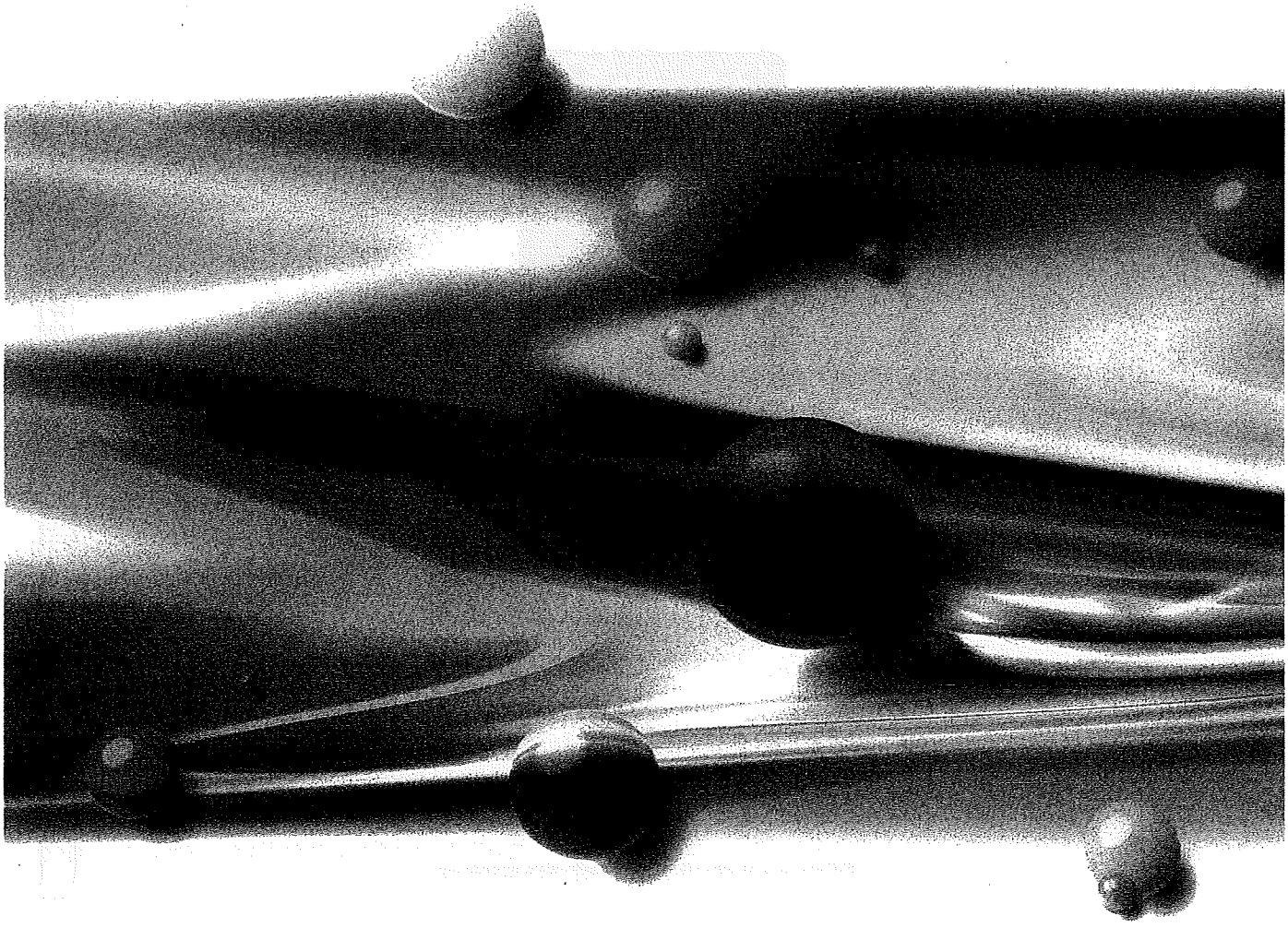
編集　臨床血圧脈波研究会



MEDICAL VIEW

High prevalence of peripheral arterial disease diagnosed by low ankle-brachial index in Japanese patients with diabetes: the Kyushu Prevention Study for Atherosclerosis.

Maeda Y, Inoguchi T, Tsubouchi H, Sawada F, Sasaki S, Fujii M, Saito R, Yanase T, Shimabukuro M, Nawata H, Takayanagi R.
Diabetes Res Clin Pract 2008; 82: 378-82. PMID: 18930561

ABI低値により診断した日本人糖尿病患者における末梢性動脈疾患の有病率 ～九州動脈硬化研究より～

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井口登與志／坪内博孝／澤田布美／佐々木修二／藤井雅一／西藤亮子／柳瀬敏彦／島袋充生／名和田新／高柳涼一

背 景

糖尿病患者において末梢動脈障害（PAD）の有病率が高いことが知られているが、最近ではPADは心血管疾患の予測因子となることも示されている。また、PADの人種差については、以前よりアジア人では白人や黒人と比して有病率が低いと報告されているが、わが国における糖尿病患者のPAD有病率を調査した大規模臨床研究は少ない。1983年の研究では日本人糖尿病患者におけるPAD有病率は1.9%と低率であったが、当時は下肢動脈の触診と間欠的跛行などの自覚症状によってPADの診断を行うのが一般的であった。近年ではankle-brachial index（ABI）を用いたPADのスクリーニング法が確立し、ABI自動測定装置も普及している。

目 的

日本人糖尿病患者3,906人におけるPADの有病率と危険因子について、ABIを用いて検討した。

方法・対象

九州動脈硬化研究に登録されている糖尿病患者3,906人（平均年齢60.8歳、65歳以上の高齢患者1,612人を含む）を対象とした。form PWV/ABI（オムロンコーリン社製）を用いてABIを測定し、左右低いほうのABIが0.9未満の症例をPADと定義した。神経障害は糖尿病専門医、網膜症は眼科専門医がそれぞれ診断し、冠動脈疾患は冠動脈造影などで有意狭窄を認めた症例、脳血管疾患は画像所見を伴う症候性脳梗塞と定義した。高血圧は収縮期血圧＞140mmHg、拡張期血圧＞90mmHg、脂質異常症は総コレステロール＞220mg/dL、中性脂肪＞150mg/dL、またはそれぞれの治療歴で定義した。喫煙は現在の喫煙歴と定義した。検査値の比較にはYate補正χ二乗検定を用いた。また、ロジスティック回帰モデルを用いて各危険因子（HbA1c、性別、BMI、喫煙、年齢、高血圧、脂質異常症、冠動脈疾患、脳血管障害、糖尿病神経障害、糖尿病網膜症、糖尿病腎症）とPADとの相関を検討した。

結 果

表1に臨床的特徴を示した。全糖尿病患者のPAD（ABI＜0.9）有病率は7.6%であった。PAD有病率に明らかな性差はなく（男性7.3%、女性8.1%）、65歳以上の高齢者（12.7%）では、65歳未満の患者（4.0%）と比べて有意に高かった。末梢動脈の石灰化が疑われるABI 1.4以上の高値症例も全患者の0.6%存在した。一方、今回ABI低値（0.9未満）を指摘された症例のうち、測定前にすでにPADと診断されていた割合（既診断率）は24.4%と非常に低かった。同様に、適応疾患を問わず抗凝固療法を受けている患者の割合（既治療率）も45.1%と5割未満にとどまった。ABI階層別の検討では、既診断率、既治療率は重症例ほど高くなる傾向にはあったが、最重症虚血肢が疑われるABI 0.4未満の症例においても既診断率は5割、既治療率は約6割にとどまっていた。また、**表2**に示すとおり、ロジスティック回帰モデルを用いた多変量解析では、年齢、高血圧、冠動脈疾患、および蛋白尿とPADに強い相関が認められた。

考 察

今回示された日本人糖尿病患者におけるPAD有病率は、1983年の報告（1.9%）と比して高率であった。この相違の原因は、一つは近年における日本人の食習

慣の変化や運動量の減少などのライフスタイルを反映して糖尿病患者のPAD罹患率そのものが増加している可能性が考えられる。またもう一つには、この20年間でのPAD診断方法の進歩があげられる。同様の診断基準で行われた報告によれば日本人一般住民のPAD有病率は2.7%で、本研究での糖尿病患者におけるPAD有病率は一般住民の約3倍となる。このように糖尿病診療におけるABIを用いたスクリーニングは非常に有用であると考えられるが、本研究で示された既診断率の低さから、いまだにABIが臨床の場で十分には活用されていない可能性が伺える。PAD発症の危険因子に関しては、男性のほうが女性よりもPAD発症のリスクが高いという報告もあるが、本研究をはじめとしたいくつかの研究では糖尿病患者におけるPADの有病率に明らかな性差は認められていない。また、喫煙はPADを含むアテローム性動脈硬化疾患の最も強い危険因子の1つであるが、本研究では喫煙歴とPADに相関を認めなかった。今回の喫煙歴の定義が現在の喫煙のみで過去の喫煙歴を含んでいなかったためと考えられるが、わが国における喫煙率の低下が関与している可能性も否定はできない。HbA1cと脂質異常症もPADと有意な相関関係を示さなかった。断面調査の限界ともいえるが、ワンポイントのみのHbA1c値や脂質プロファイルには長期の疾患コントロールが反映されないためと考えられた。

結　論

日本人糖尿病患者におけるPAD有病率は高い一方で、既診断率は低率にとどまっており、未治療の患者が多く残されていると考えられる。ABIによるPADの早期診断・早期治療が、糖尿病患者におけるQOLと予後の改善に大きく寄与しうると考えられる。

表1 臨床的特徴

	全患者(n=3,906)	65歳以上の高齢者(n=1,612)
ABI	1.07±0.13	1.05±0.15
年齢	60.8±11.9	71.8±5.4
BMI (kg/m²)	24.6±4.1	23.9±3.5
喫煙 (%)	900(23.0)	240(14.9)
HbA1c (%)	7.8±2.1	7.7±1.9
高血圧 (%)	2,013(51.5)	989(61.4)
脂質異常症 (%)	1,832(46.9)	797(49.4)
冠動脈疾患 (%)	581(14.9)	377(23.4)
脳血管障害 (%)	351(9.0)	214(13.3)
糖尿病神経障害 (%)	1,029(26.4)	524(32.6)
糖尿病網膜症 (%)	1,079(27.6)	510(31.6)
蛋白尿 (%)	572/3,003(19.1)	269/1,176(22.9)

平均値±標準偏差、または実数 (%)。

表2 PADの危険因子 (ロジスティック回帰分析)

危険因子	オッズ比(95%信頼区間)	p値
年齢 (10歳加齢につき)	1.51(1.31-1.74)	<0.0001
性別/男性	1.00(0.75-1.34)	0.9934
BMI (1kg/m²増加につき)	1.00(0.96-1.03)	0.809
喫煙 (%)	1.04(0.73-1.50)	0.8259
HbA1c (1%増加につき)	0.99(0.92-1.06)	0.6626
高血圧	1.50(1.10-2.03)	0.0097
脂質異常症	0.97(0.73-1.29)	0.8369
冠動脈疾患	2.41(1.78-3.26)	<0.0001
脳血管障害	1.37(0.94-2.00)	0.0971
糖尿病神経障害	1.85(1.39-2.47)	<0.0001
糖尿病網膜症	1.09(0.80-1.48)	0.596
蛋白尿	2.31(1.69-3.18)	<0.0001

コメディカル・研修医・一般臨床医のための

糖尿病治療ハンドブック

― 基本的な考え方とその実践・心理的アプローチ ―

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