ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells

Yutaka Masuda,* Hiroyuki Itabe,[1],*,† Miho Odaki,† Kotaro Hama,§ Yasuyuki Fujimoto,†
Masahiro Mori,† Naoko Sasabe,* Junken Aoki,§ Hiroyuki Arai,§ and Tatsuya Takano†

Department of Biological Chemistry,* School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan; Department of Molecular Pathology,† Faculty of Pharmaceutical Sciences, Teikyo University, 1091-1 Suarashi, Sagamiko, Tsukui, Kanagawa 199-0195, Japan; and Department of Health Chemistry,§ Faculty of Pharmaceutical Sciences, Tokyo University, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033

Abstract Adipose differentiation-related protein (ADRP) is a major protein associated with lipid droplets in various types of cells, including macrophage-derived foam cells and liver cells. However, the role of ADRP in the processes of formation and regression of these cells is not understood. When J774 murine macrophages were incubated with either VLDL or oleic acid, their content of both ADRP and triacylglycerol (TG) increased 3- to 4-fold. Induction of ADRP during TG accumulation was also observed in oleic acid-treated HuH-7 human liver cells. Addition of triacsin C, a potent inhibitor of acyl-CoA synthase, for 6 h decreased the amount of TG in VLDL-induced foam cells and oleic acid-treated liver cells; it decreased the amount of ADRP protein in parallel, indicating the amount of ADRP reduced during regression of the lipid-storing cells. Addition of a proteasome inhibitor during triacsin C treatment abolished the ADRP decrease and accumulated polyubiquitinated ADRP. In addition, the proteasome inhibitor reversed not only the degradation of ADRP but also TG reduction by triacsin C.█ These results suggest that cellular amounts of ADRP and TG regulate each other and that the ubiquitin-proteasome system is involved in degradation of ADRP during regression of lipid-storing cells.—Masuda, Y., H. Itabe, M. Odaki, K. Hama, Y. Fujimoto, M. Mori, N. Sasabe, J. Aoki, H. Arai, and T. Takano. **ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells.** *J. Lipid Res.* **2006.** 47: 87–98.

Supplementary key words lipid droplets • adipose differentiation-related protein • macrophages • liver cells • triacylglycerol • VLDL

The appearance of cytosolic lipid droplets is observed in many cell types in physiological and pathological conditions. Adipocytes, the major lipid-storing cells in mammals, are full of lipid droplets. Foam cells in atherosclerotic lesions and parenchymal cells in fatty liver are lipid droplet-containing cells associated with disease states. These cells accumulate massive amounts of cholesteryl ester and/or triacylglycerol (TG) as lipid droplets in their cytoplasmic space. Recently, we and others have shown that there is a distinct set of proteins specifically localized in lipid droplets (1–5), suggesting that the lipid droplet is an organized intracellular structure.

Adipose differentiation-related protein (ADRP), also called adipophilin, is known to be a lipid droplet-associating protein. ADRP was originally found as a major protein induced in the early stages of adipocyte differentiation (6), although it is now known that ADRP is expressed in a variety of tissues and cells (7–13) in addition to macrophage-derived foam cells in atherosclerotic lesions (14). ADRP is the most abundant protein in lipid droplets in hepatic cells (3) and macrophages (Y. Fujimoto, et al., unpublished data).

Expression of ADRP in macrophages during foam cell formation has been demonstrated by several studies. The gene-chip technique was used to show that ADRP is one of the most strongly induced genes in macrophages after incubation with oxidized LDL (OxLDL) (15). Addition of VLDL stimulated foam cell formation and ADRP mRNA expression in cultured macrophages (16, 17). Expression of ADRP mRNA in human atherosclerotic lesions has been demonstrated by in situ hybridization experiments (14). Overexpression of the ADRP gene in cultured cells resulted in increased fatty acid incorporation and accumu-

Abbreviations: AcLDL, acetylated LDL; ADRP, adipose differentiation-related protein; ALP, alkaline phosphatase; FC, free cholesterol; LPDS, lipoprotein-deficient serum; LPS, lipopolysaccharide; mAb, monoclonal antibody; OxLDL, oxidized LDL; pAb, polyclonal antibody; PNS, postnuclear supernatant; TC, total cholesterol; TG, triacylglycerol.
[1] To whom correspondence should be addressed.
   e-mail: h-itabe@pharm.showa-u.ac.jp

lation of lipid droplets in the cells (18, 19). In addition to its involvement in foam cell formation from macrophages, strong induction of ADRP expression occurs during lipid loading in hepatocytes (3, 4). These observations imply that ADRP facilitates lipid droplet formation, although the precise mechanism of this process has yet to be clarified.

ADRP is replaced by another lipid droplet-associating protein, perilipin A, during differentiation of adipocytes (7). Both ADRP and perilipin A localize on the surface of intracellular lipid droplets (8, 20), and they share a homologous sequence called the PAT domain, which is named after three lipid droplet-associating proteins, perilipin, adipophilin, and TIP-47. In mature adipocytes, perilipin A surrounds lipid droplets to stabilize the droplet structure and also regulates lipolysis by hormone-sensitive lipase. When adipocytes are stimulated with hormones such as adrenalin, perilipin A is phosphorylated by activated protein kinase A and the phosphorylated form of perilipin A allows hormone-sensitive lipase to access lipid molecules within the lipid droplets (21). Perilipin-null mice show extensive reduction in their adipose tissue mass because of elevated lipolysis (21–24). Perilipin A is expressed only in mature adipocytes and steroid hormone-producing cells, whereas ADRP mRNA is expressed in a variety of tissues (7, 8). Another important difference from perilipin A is that ADRP does not contain putative phosphorylation sites (2, 25). Thus, lipid metabolism in ADRP-coated lipid droplets is likely to be regulated by other regulatory mechanisms, although ADRP and perilipin A share similar properties for lipid droplet formation.

It is well known that foam cells can regress under certain conditions (26, 27). Cellular lipid storage is largely controlled by the balance between influx and efflux of lipids, and inducing the regression of lipid-storing cells may represent a valid therapeutic approach. In this study, we investigated what happens during regression of lipid droplets. To approach this issue, we used anti-ADRP antibodies to quantify the amount of ADRP protein expressed in macrophages and in liver cells in relation to their content of TG. We found that ADRP decreases in parallel with reduction in cellular TG and that the ubiquitin-proteasome system is involved in ADRP levels during regression of lipid-accumulating cells.

EXPERIMENTAL PROCEDURES

Lipoproteins

Human plasma was obtained from healthy volunteers by the use of heparinized syringes. LDL and VLDL were isolated from the plasma by step-wise ultracentrifugation from the plasma after adding 0.25 mM EDTA, as described previously (28). Acetylated LDL (AcLDL) was prepared according to the method of Basu et al. (29).

Macrophage foam cell formation and regression

Cells from the murine macrophage line J774.1 were cultured in RPMI1640 medium supplemented with 8% fetal bovine serum (FBS) as described previously (30). One day before the start

of the experiments, J774 cells (1×10^6 cells/dish) were cultured in 6 cm dishes (Falcon 3002) for 5 h, and then the medium was changed to RPMI1640 without FBS but supplemented with 10 µg/ml lipopolysaccharide (LPS) (DIFCO; Detroit, MI). Treatment with LPS causes the cells to differentiate into macrophage-like cells that spread on the plastic dishes and show vigorous phagocytic activity. On day 0, the medium was changed to RPMI1640 with 5% lipoprotein-deficient serum (LPDS) (Sigma) containing either 50 µg/ml human lipoproteins or 600 µM oleic acid, and the cells were incubated up to 3 days. The suspension of oleic acid sodium salt in BSA solution was prepared according to the method of Goldstein, Basu, and Brown (31). Free cholesterol (FC)-containing liposome (phosphatidylcholine-phosphatidylserine-dicetylphosphate-FC; 5:5:1:25) was prepared as described by Nishikawa et al. (32). To reduce TG levels in VLDL-induced foam cells, the J774 cells pretreated with VLDL for 3 days were further incubated with RPMI1640 with 5% LPDS, 25 mg/ml BSA, and 10 µg/ml triacsin C, which is an acyl-CoA synthase inhibitor (Kyowa Medex Co.; Tokyo, Japan) (33, 34). The cells were washed twice with cold phosphate-buffered saline (PBS), then homogenized in 0.4 ml of 5 mM Tricine-NaOH, pH 7.4, containing 250 mM sucrose, 0.75 mM EDTA, and protease inhibitors [10 µg/ml each of pepstatin A, chymostatin, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF)] using a teflon homogenizer. The total homogenate was centrifuged at 7,000 g for 10 min to remove cell debris and nuclei. The resulting supernatant was collected and used as post-nuclear supernatant (PNS) fraction.

Determination of murine ADRP

A rabbit polyclonal antibody (pAb) against murine ADRP was raised by immunizing Japanese White rabbits with the C-terminal peptide of murine ADRP (19 mer: CTEVNKASLKVQQSEVKAQ; the first amino acid residue was changed from serine to cystein to introduce a thiol group) conjugated to keyhole limpet hemocyanin using N-(m-maleimidobenzoyloxy)succinimde (Calbiochem) (35). The immunoglobulin fraction obtained from the antisera by ammonium sulfate precipitation was used as antibody. Although this pAb cross-reacts with keyhole limpet hemocyanin, Western blot analysis showed that ADRP is the only antigen detected in murine macrophage homogenates (see **Fig. 1B**). To study quantitative changes of ADRP during formation and regression of foam cells in murine macrophages, we developed an enzyme-linked immunosorbent assay (ELISA) procedure to measure mouse ADRP protein. Briefly, PNS fractions separated from macrophage homogenates (0.1–1 µg protein/well) were coated onto microtiter wells. The wells were blocked with Tris-buffered saline (TBS) containing 2% skimmed milk, and then anti-ADRP pAb (1:2,000 dilution) was added to the wells, followed by alkaline phosphatase (ALP)-conjugated anti-rabbit IgG (Chemicon International, Inc.; Temecula, CA), 1:8,000 dilution. ALP activity was visualized using p-nitrophenylphosphate as substrate. During this ELISA procedure, the C-terminal peptide of ADRP conjugated with BSA was used as a standard. Thus, we express the amount of ADRP as arbitrary units, where 1 unit of ADRP is equal to the amount of ADRP corresponding to 1 ng of BSA conjugated with the C-terminal peptide of ADRP.

Progression and regression of lipid storage in hepatic cells

HuH-7 human hepatoma cells, purchased from Health Science Research Resources Bank, were cultured in DMEM medium supplemented with 10% FBS. HuH-7 cells (1×10^6 cells/dish) were cultured for 24 h in 6 cm dishes in medium containing 600 µM oleic acid to accelerate lipid accumulation in the cells (3). To regress the lipid-laden liver cells, HuH-7 cells pretreated with

A ADRP

B Western blot

C Neutral lipids

D TLC
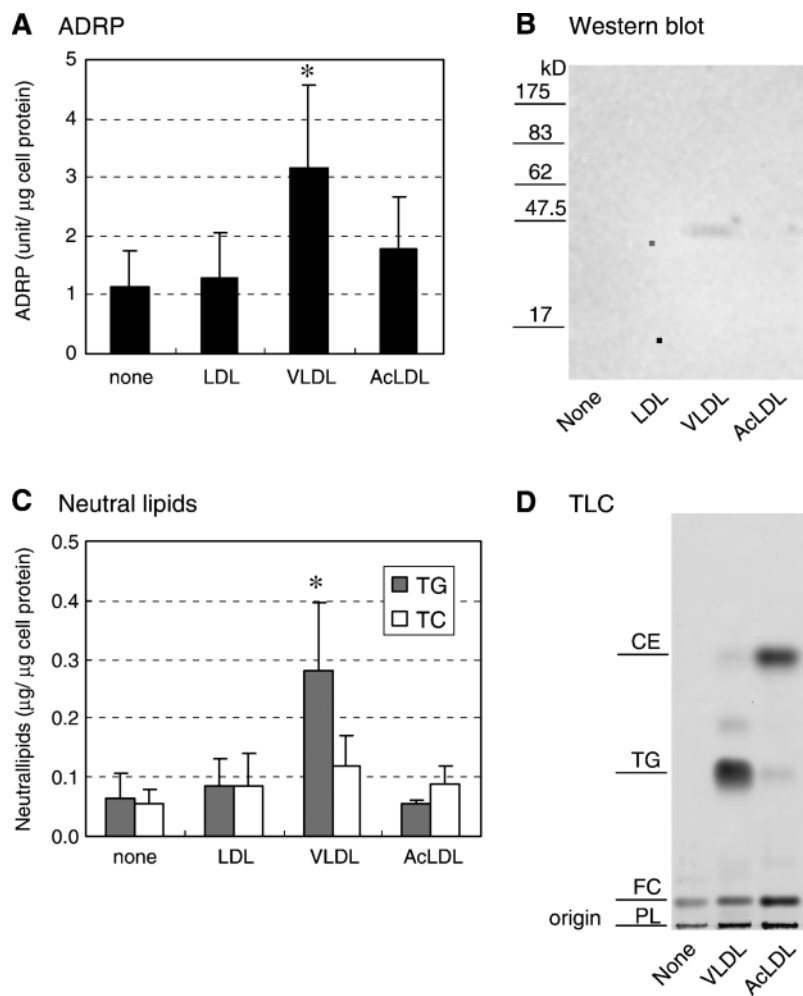
Fig. 1. VLDL increases the amounts of adipose differentiation-related protein (ADRP) and triacylglycerol (TG) in J774 macrophages. J774 macrophages (1 × 10⁶ cells/dish) pretreated overnight with lipopolysaccharide (LPS) (10 µg/ml) were incubated for 3 days with lipoproteins (50 µg protein/ml). The postnuclear supernatant (PNS) fractions were recovered from the cell homogenates. A: The amount of ADRP was measured by an enzyme-linked immunosorbent assay (ELISA) and is expressed as unit/µg of cell protein. B: The PNS fractions (20 µg protein each) were separated on SDS-PAGE and subjected to immunoblotting using anti-ADRP polyclonal antibody (pAb). C: The amounts of neutral lipids [TG and total cholesterol (TC)] in the PNS fraction were measured by enzymatic methods. D: Total lipids were extracted from the macrophages that had been incubated for 3 days with LDL or acetylated LDL or no lipoprotein. The cell lipids were separated on a thin-layer chromatograph developed with hexane-diethyl ether-acetic acid (80:20:1). The bands were visualized by soaking the plate in 3% copper actate-8% phosphoric acid, followed by heating on a hot plate. Values represent mean ± standard deviation (n = 4). *Data with statistical significance against control (none). $P < 0.05$.

oleic acid for 24 h were further incubated for up to 24 h with DMEM containing 5% LPDS, 25 mg/ml BSA, and 10 µg/ml triacsin C. To examine the contribution of the proteasome pathway to the decrease in ADRP in hepatic cells, oleic acid-induced lipid-laden HuH-7 cells were incubated with DMEM containing 5% LPDS, 25 mg/ml BSA, 10 µg/ml triacsin C, and the proteasome inhibitor MG132 (20 µM; Calbiochem). Cells were homogenized in a buffer containing 1% Triton X-100, and the homogenate was centrifuged at 15,000 rpm for 10 min at 4C; the resultant supernatant was subjected to further analysis by Western blotting and immunoprecipitation.

Western blotting

PNS fractions obtained from J774 cells were separated on SDS-PAGE using 10% polyacrylamide gel, then electrotransferred onto polyvinylidene difluoride membrane (Hybond-P*T*; Amersham). The membranes were blocked with TBS containing 5% skimmed milk and then incubated overnight with anti-ADRP pAb (1:1,000 dilution), after which the positive bands were visualized with the ALP-conjugated anti-rabbit IgG (1:8,000 dilution) and CSPD*T*, a substrate for ALP that generates chemiluminescence (Roche Diagnostics). The resulting chemiluminescence was detected with an imaging system (LumiVision*T*; Aisin Denshi Co., Japan).

Aliquots of supernatant recovered from the Triton X-100 homogenate of HuH-7 cells were separated on SDS-PAGE using 10% polyacrylamide gel, then electrotransferred onto nitrocellulose membranes (Protron BA-83; Schleicher and Schuell). The

membranes were blocked with TBS containing 3% BSA and then incubated for 1 h with anti-human ADRP monoclonal antibody (mAb) (American Research Products; Belmont, MA), 1:1,000 dilution, after which the positive bands were visualized with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:4,000 dilution) and an ECL Western blot detection kit (Perkin Elmer). The resulting chemiluminescence was detected by the use of X-ray film (RX-U; Fuji Film). The same membrane was reprobed with anti-GADPH mAb (MAB374; Chemicon International, Inc.).

Detection of ubiquitinated ADRP overexpressed in HuH-7 cells

cDNA for human ADRP was obtained from total RNA isolated from human leukemia HL60 cells by RT-PCR with primers that contained a 5′- ATG initiation codon and a 3′- TAA termination codon, namely, 5′-ATCTAGAATGGCATCCGTTGCAGTTGAT-CC-3′ and 5′-ATCTAGATTAATGAGTTTTATGCTCAGATCGC-3′, respectively. The PCR product was cloned into the XbaI site of the pFLAG-CMV-2 expression vector to create a construct that would express human ADRP protein tagged with FLAG at its N terminus. HuH-7 cells (1 × 10⁶ cells/dish) were transfected for 48 h either with empty vector (pFLAG-CMV-2) or with the expression plasmid for human ADRP (pFLAG-ADRP) by using TransIT transfection reagent according to the manufacturer's instructions. Transfected cells were then treated for 3 h with the proteasome inhibitor MG132 at 20 µM. Cells were washed twice with PBS, and then were lysed in a lysis buffer (50 mM Tris-

HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.5 mM PMSF). The lysate was centrifuged at 15,000 g for 15 min, and 200 μg of supernatant was incubated with anti-FLAG M2 affinity gel (Sigma) for 3 h. The gels were collected by centrifugation and washed three times with the lysis buffer. The proteins eluted with 0.1 M glycine-HCl (pH 3.5) from the gels were analyzed by immunoblotting either with a combination of an anti-multi ubiquitin mAb (clone FK2; MBL Co., Japan) and HRP-conjugated goat anti-murine kappa chain pAb (Southern Biotech; Birmingham, AL) or with the anti-ADRP mAb.

Real-time PCR

Total RNA from J774 cells (1×10^6 cells/dish) was extracted with ISOGEN (Nippongene; Toyama, Japan) and reverse transcribed with the SuperScript First-strand Synthesis System for RT-PCR (Invitrogen; Carlsbad, CA). Oligonucleotide primers for PCR were designed using Primer Express Software (Applied Biosystems; Foster City, CA). The sequences of the oligonucleotides used in PCR reactions were as follows: ADRP-forward 5′-GAAGAGAAGCATCGGCTACGA-3′, ADRP-reverse 5′-GTCAGG-TTGCGGGCGATA-3′, GAPDH-forward 5′-GCCAAGG TCATCCT-GACAACT-3′, and GAPDH-reverse 5′-GAGGGGCCATCCACA-GTCTT-3′. PCR reactions were performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The transcript number of GAPDH was quantified, and each sample was normalized on the basis of GAPDH content.

Thin-layer chromatography

After J774 macrophages had been incubated for 3 days with 50 μg/ml VLDL or AcLDL, total lipids were extracted with chloroform and methanol. The cellular neutral lipids were separated by thin-layer chromatography (TLC) (Kieselgel 60; Merck #5748) developed with hexane-diethyl ether-acetic acid (80:20:1). The bands were visualized by soaking the plate in 3% copper actate-8% phosphoric acid followed by heating on a hot plate (36).

Statistical analysis

Data are expressed as mean ± standard deviation. Results were analyzed using Student's t-test, and statistical significance for all comparisons was assigned at $P < 0.05$.

Other methods

The amounts of TG and total cholesterol (TC) were enzymatically determined using commercially available kits [Nescote TG-kit-GN (Azwell Inc.; Osaka, Japan) and Cholesterol E-test (Wako Pure Chemical Co.; Osaka, Japan), respectively]. Amounts of protein were determined using BCA Protein Assay reagent (Pierce; Rockford, IL) with BSA as standard.

RESULTS

Induction of ADRP protein in TG-accumulating cells

Cells from the murine macrophage cell line J774 were incubated for 3 days with 50 μg/ml of various lipoproteins. As shown in Fig. 1A, VLDL was the most effective inducer of ADRP under these conditions. Cells treated with VLDL contained three times the amount of ADRP as cells incubated without lipoproteins, whereas incubation of the cells with AcLDL increased the amount of ADRP by only 50%. A Western blot analysis confirmed accumulation of ADRP in cells treated with VLDL (Fig. 1B). The amounts

of the neutral lipids TG and TC in these PNS fractions were also determined (Fig. 1C). VLDL induced 4- and 2-fold increases in TG and TC, respectively, whereas LDL and AcLDL increased the amount of neutral lipids only slightly. Lipids extracted from the PNS fractions of cells treated with VLDL, AcLDL, or without lipoproteins were analyzed by TLC (Fig. 1D). VLDL-treated cells accumulated a large amount of TG, whereas AcLDL-treated cells showed an increase in CE but not in TG.

It is well known that LDL, VLDL, and AcLDL are taken up by different receptors. To assess whether such lipoprotein receptors are involved in ADRP induction in macrophages, lipid-laden cells were prepared without the addition of lipoproteins. Incubation of J774 cells with 600 μM of oleic acid for 3 days increased cellular TG approximately 8-fold, and the amount of ADRP in these oleic acid-treated cells was significantly higher than that in nontreated cells **(Fig. 2)**, suggesting that lipoprotein receptors are not directly involved in ADRP induction in macrophages. When macrophages were incubated with FC-containing liposomes, in which the amount of TC was comparable to that observed in oleate-treated cells but TG accumulated moderately, ADRP protein was induced also in the liposome-treated cells. These results suggest that receptor-mediated processes of lipoprotein uptake are not necessary for ADRP induction, and that induction of ADRP corresponds better to TG accumulation than to that of cholesterol.

We observed good correlation between the amounts of ADRP and TG in macrophage PNS fractions of VLDL-treated macrophages. J774 macrophages were incubated for up to 3 days with 50 μg/ml of VLDL, and the amounts of ADRP and TG accumulated in the PNS fractions were monitored. ADRP accumulated after 2 days of incubation, and the increase in TG seems to proceed prior to the increase in ADRP **(Fig. 3A)**. When macrophages were incubated for 3 days with various amounts of VLDL, ADRP and TG accumulated in a dose-dependent manner (Fig. 3B).

We have previously shown that ADRP is the most abundant lipid droplet-associated protein in HuH-7 human hepatic cells, and that the amount of ADRP is greatly increased when the cells have been incubated with oleic acid (3). When the HuH-7 cells have been incubated for up to 24 h with 600 μM oleic acid, expression of ADRP protein increases in a time-dependent manner **(Fig. 4A)**. As in macrophages, the increase in ADRP protein in HuH-7 cells is closely correlated with the amount of TG accumulated in the cells (Fig. 4B).

### Effect of TG reduction on ADRP protein in lipid-storing cells

It is well known that lipid-laden foam cells can be induced to regress under various conditions (26, 27, 35), and we investigated how ADRP contributes to reduction of the preformed lipid droplets during this process of regression. Our approach was to reduce the amount of TG in foam cells by inducing consumption of the stored lipid and to follow metabolic changes in ADRP in these cells. J774 macrophages were incubated for 3 days with
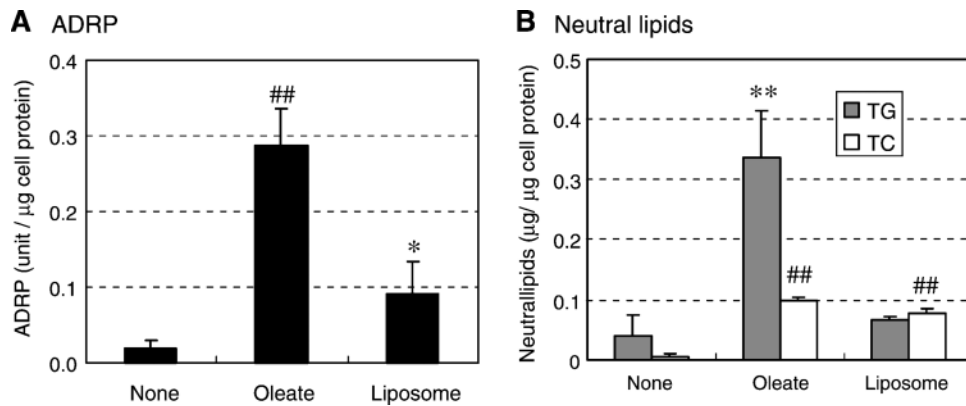
Fig. 2. Addition of oleic acid to J774 macrophages also increases the amounts of ADRP and TG. J774 macrophages pretreated with LPS (1×10^6 cells/dish) were incubated for 3 days with either oleic acid (600 μM) or free cholesterol (FC)-containing liposome (phosphatidylcholine-phosphatidylserine-dicetylphosphate-FC (5:5:1:25; 50 μM for FC), or without lipids, as control. The PNS fraction was recovered from the cell homogenates. A: The amounts of ADRP in the PNS fractions were measured by ELISA. B: The amounts of neutral lipids (TG and TC) in the PNS fraction were measured by enzymatic methods. ##, *,**Data with statistical significance against control (no addition). *P < 0.05; **P < 0.005; ##P < 0.001.

50 μg/ml of VLDL to prepare foam cells, and then the cells were cultured for 2 days with a medium containing 5% LPDS, without the addition of any lipoprotein. As shown in **Fig. 5A**, during 2 days of culture without lipids,

the foam cells lost 50% of their TG and the amount of ADRP decreased by about 30%. In another set of experiments, the VLDL-induced foam cells were incubated with a medium containing 10 μg/ml triacsin C, a potent
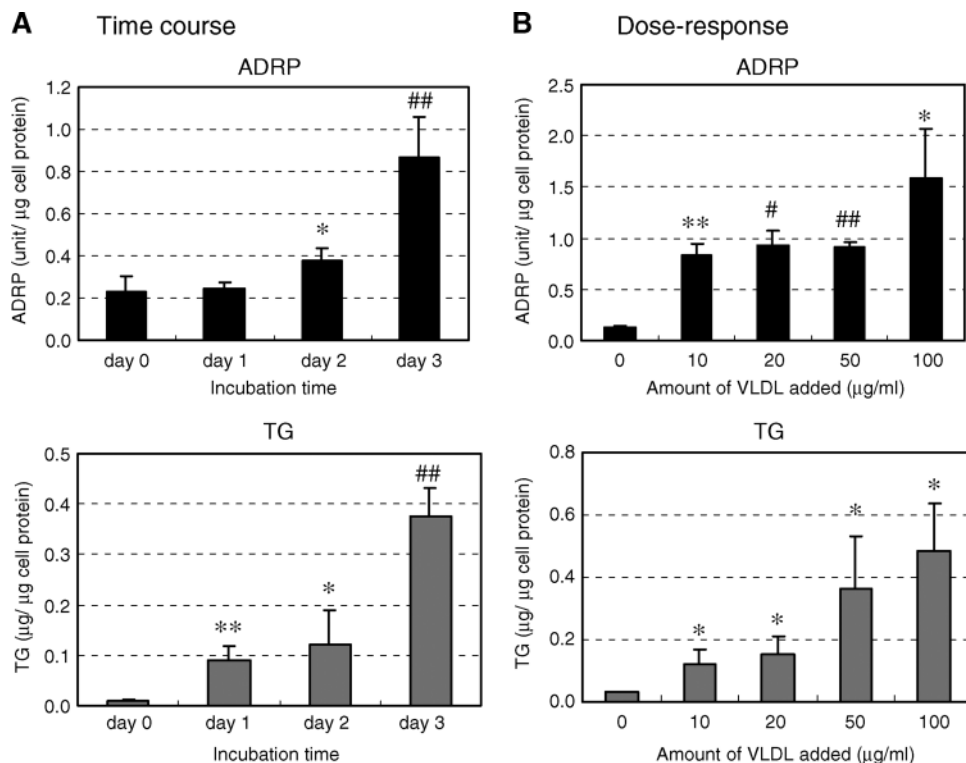
Fig. 3. VLDL increases the amounts of ADRP and TG in a time- and dose-dependent manner. A: J774 macrophages pretreated with LPS (1×10^6 cells/dish) were incubated for up to 3 days with VLDL (50 μg protein/ml). The amounts of ADRP and TG in the PNS fraction were measured as described in the legend to Fig. 2. B: J774 macrophages pretreated with LPS (1×10^6 cells/dish) were incubated for 3 days with various concentrations of VLDL. The amounts of ADRP and TG in the PNS fraction were measured. Values represent mean ± standard deviation (n = 4). *, **, #, ## Data with statistical significance against control (day 0 or 0 μg/ml VLDL). *P < 0.05; #P < 0.01; **P <0.005; ##P < 0.001.

A ADRP

B TG
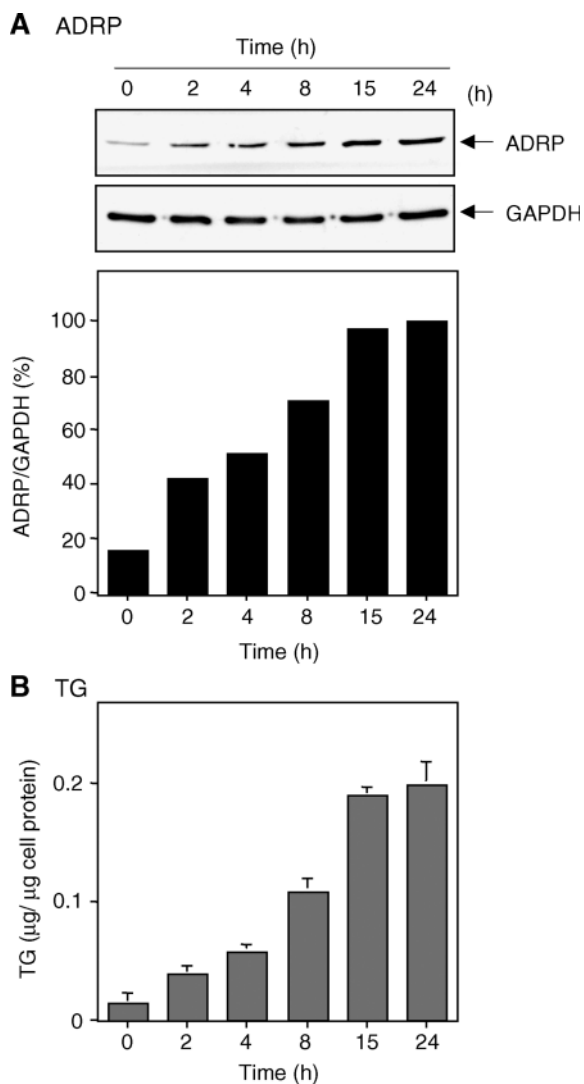
Fig. 4. Induction of ADRP during TG accumulation in HuH-7 human hepatic cells. HuH-7 cells (1×10^6 cells/dish) were incubated for up to 24 h in medium containing 600 µM oleic acid. Cell lysates prepared in a lysis buffer containing 1% Triton X-100 were subjected to immunoblotting with anti-human ADRP monoclonal antibody. As a loading control, GAPDH was also detected on the same membrane. The intensity of the bands was quantified with NIH Image, and the amount of ADRP relative to GAPDH was calculated (A). Under the same experimental conditions, the amounts of TG in the cell lysates were measured using the enzymatic method (B). Error bars represent mean values ± standard deviation of triplicates.

inhibitor of acyl-CoA synthase, and 25 mg/ml BSA, together with 5% LPDS (Fig. 5B) (34). The addition of triacsin C and BSA reduced the amount of TG in the cells by 55%, and the amount of ADRP protein decreased by about 45% (Fig. 5B). Although the reduction of ADRP by removing VLDL or by triacsin C treatment did not reach statistical significance, these results suggest that the decrease in TG mass could cause the decrease in ADRP.

As in macrophages, the decrease in ADRP protein in HuH-7 cells was closely correlated with the decrease of TG mass in the cells **(Fig. 6A)**. HuH-7 cells were incubated

for 24 h with 600 µM oleic acid, and then treated for up to 24 h with a new medium containing triacsin C, but without oleic acid. The amount of ADRP protein in HuH-7 cells at time 0 was 5-fold larger than that in control cells, because the ADRP had already been induced with oleic acid (Fig. 6A). During the subsequent incubation without lipid supply, the amount of ADRP decreased in parallel with the decrease in cellular TG (Fig. 6B). In total, these results indicate that the close correlation between cellular TG storage and the amount of ADRP is not unique to macrophage-derived foam cells.

Induction of ADRP mRNA during accumulation of TG

Because the addition of VLDL to the macrophages increased the amount of ADRP protein, changes in the amount of ADRP mRNA were examined by real-time PCR. J774 macrophages were treated with VLDL for 2 days, 1 day less than the usual incubation period, and then cDNA was prepared from the cells. As shown in **Fig. 7A**, the ADRP mRNA level was increased 7-fold by the treatment with VLDL, whereas neither LDL nor AcLDL affected ADRP mRNA levels. These results are in full agreement with our earlier observation that VLDL, but not LDL or AcLDL, induces ADRP protein (Fig. 1).

When the VLDL-induced foam cells were induced to regress by treatment with triacsin C for 6 h, the level of ADRP mRNA was reduced by 60%, compared with the cells before the addition of triacsin C, although the difference did not reach statistical significance (Fig. 7B). It is unlikely that the rapid reduction of ADRP protein in the lipid-storing cells was caused by the reduced mRNA level alone. Note that a VLDL-induced increase in ADRP protein required 3 days of incubation with VLDL, even though levels of TG and of ADRP mRNA were already increased by day 2 (see Figs. 3 and 7A). These results suggest that ADRP is actively degraded during the regression process.

Ubiquitin-mediated degradation of ADRP in HuH-7 cells

To investigate whether the rapid decrease in ADRP during regression of intracellular lipid storage is proteasome dependent, HuH-7 cells pretreated with 600 µM oleic acid for 24 h were incubated for a further 15 h with a new medium containing the proteasome inhibitor MG132 in the absence of a lipid source. Incubation of the cells in the absence of a lipid source reduced the amount of ADRP by 50%, but the decrease in ADRP was inhibited in a dose-dependent manner by coincubation of the cells with 20 µM MG132 (**Fig. 8A,** B). The addition of proteasome inhibitor blocked the decrease in cellular TG as well as the decrease in ADRP, suggesting that ADRP could prevent TG in lipid droplets from hydrolyzing. Proteasomal inhibitors also prevented ADRP decrease when VLDL-induced macrophage-derived foam cells were treated with triacsin C for 6 h (data not shown). The proteolytic activity in lysosomes is unlikely to contribute to the decrease of ADRP protein, because ammonium chloride, which increases lysosomal pH, did not prevent ADRP in HuH-7 from decreasing upon removal of oleic acid (Fig. 8C, D). These results sug-
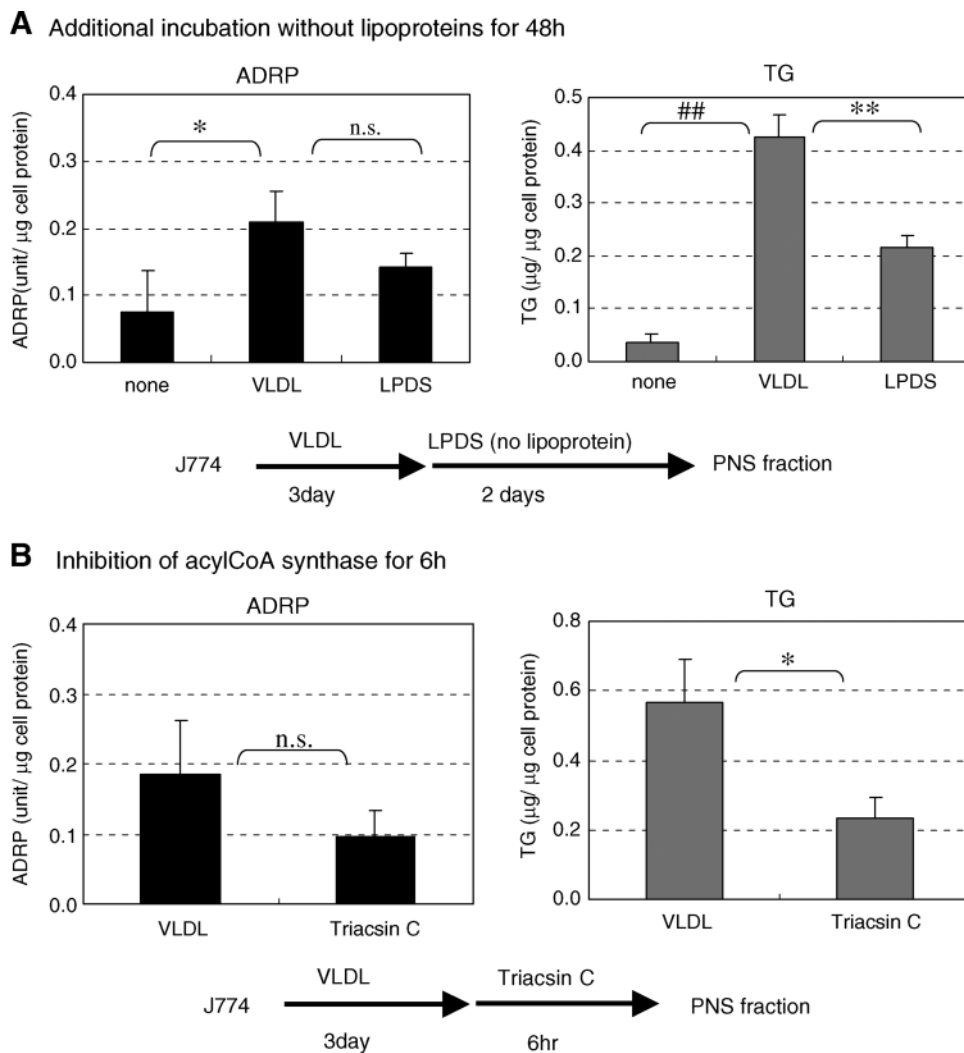
Fig. 5. ADRP protein decreases during regression of foam cells. A: J774 macrophage cells pretreated with LPS (1 × 10^6 cells/dish) were incubated for 3 days with VLDL (50 μg protein/ml). Then the cells were cultured for 2 days with a new medium containing 5% lipoprotein-deficient serum (LPDS) but not lipoprotein. B: VLDL-induced foam cells (1 × 10^6 cells/dish) were incubated for a further 6 h after changing the medium to a new one containing 5% LPDS, 10 μg/ml triacsin C, and 25 mg/ml BSA. The amounts of ADRP and TG in the PNS fraction were measured. Values represent mean ± standard deviation (n = 3). *$P < 0.05$; **$P < 0.005$; ##$P < 0.001$; n.s., not significant.

gest that proteasome is involved in the reduction of ADRP protein during reduction of cellular TG, and also suggest that keeping levels of ADRP could modulate the loss of TG.

We tried pull-down experiments to confirm the presence of ubiquitinated ADRP in the HuH-7 cells, but the anti-human ADRP mAb used in this study was not suitable for immunoprecipitation. Therefore, our approach was to express a FLAG-tagged ADRP protein in HuH-7 cells, yielding cells containing excess amounts of ADRP protein without the accumulation of neutral lipids. After incubation of HuH-7 cells with the expression vector for human ADRP tagged with FLAG sequence for 48 h, the cells were incubated with MG132 for 3 h. Both FLAG-ADRP and native ADRP were recovered from the TG-enriched fraction after centrifugation of cell lysates (data not shown). FLAG-

ADRP was immunoprecipitated with anti-FLAG affinity gels, and then ubiquitinated proteins were detected with anti-multi ubiquitin mAb. Ubiquitin-positive bands larger than 60 kDa increased only when the cells were treated with GM132 **(Fig. 9A)**. Reprobing the membrane with anti-ADRP mAb revealed that this short incubation with MG132 did not significantly reduce the amount of ADRP (Fig. 9B). These results indicate that ubiquitin-mediated proteolysis is a regulatory mechanism that reduces the amount of cellular ADRP in lipid-poor conditions.

DISCUSSION

Recent studies, including ours, have shown that several specific proteins are localized on intracellular lipid drop-
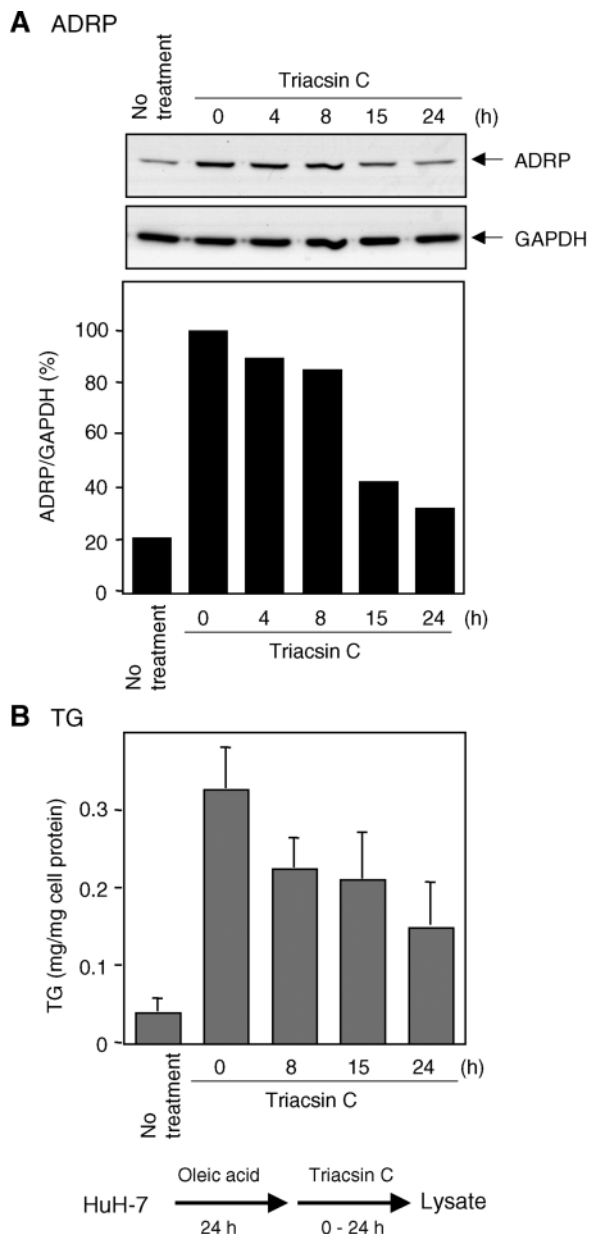
Fig. 6. Correlated changes of the amounts of ADRP and TG in oleic acid-treated HuH-7 human hepatic cells. HuH-7 cells (1 × 10^6 cells/dish) were incubated for 24 h in medium containing 600 μM oleic acid. Then the cells were further incubated for up to 24 h in a new medium with 5% LPDS, 25 mg/ml BSA, and 10 μg/ml triacsin C. ADRP and GADPH in the cell lysates were detected by immunoblotting. The intensity of the bands was quantified with NIH Image, and the amount of ADRP relative to GAPDH was calculated (A). Under the same experimental conditions, the amounts of TG in the cell lysates were measured using the enzymatic method (B). "No treatment" represents the cells incubated without oleic acid for 24 h. Values represent mean ± standard deviation of triplicate measurements. Shown are typical data from three independent experiments.

lets (1–4). ADRP is a major lipid droplet-associated protein that is expressed in various types of cells, including macrophage-derived foam cells in human atherosclerotic lesions (14) and liver cells (3). In this study, we investigated
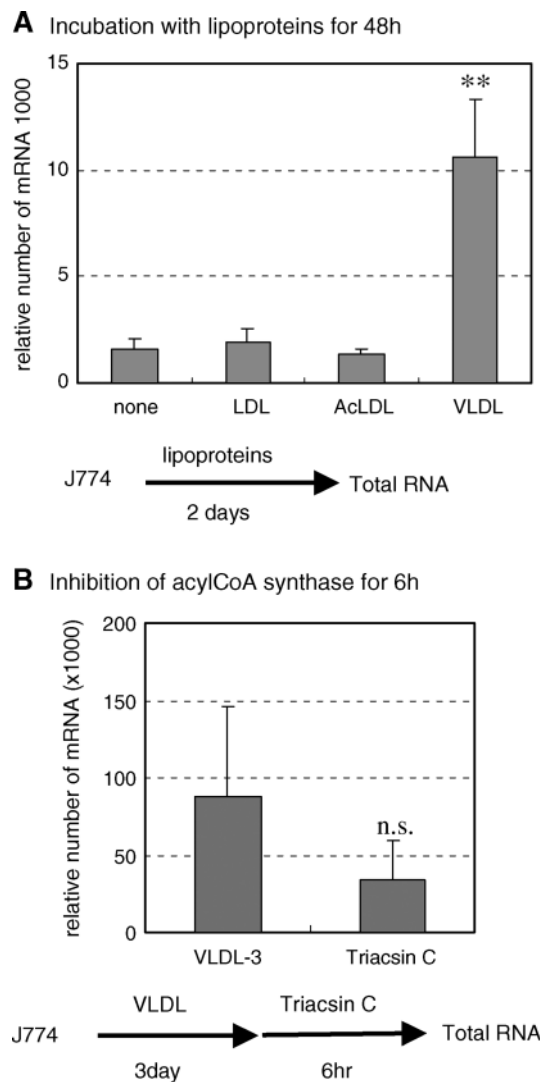
Fig. 7. Changes in ADRP mRNA level during formation and regression of foam cells. J774 macrophages (1 × 10^6 cells/dish) pretreated overnight with LPS (10 μg/ml) were then incubated for 2 days with lipoproteins (50 μg protein/ml) (A). J774 macrophages pretreated with LPS (1 × 10^6 cells/dish) were incubated for 3 days with VLDL (50 μg protein/ml). Then the foam cells were incubated for a further 6 h after changing the medium to a new one containing 5% LPDS, 10 μg/ml triacsin C, and 25 mg/ml BSA (B). Total RNA was extracted from the cells, and single-strand cDNA was prepared using oligo-dT as primer. Real-time PCR experiments were carried out to evaluate the amount of mRNAs for ADRP and GAPDH. Values are reported as amount of ADRP mRNA relative to GAPDH mRNA. Values represent mean ± standard deviation (n = 3). **$P < 0.005$.

the changes in expression of ADRP protein in J774 murine macrophages and human hepatic cell line HuH-7 cells during formation and regression of lipid-storing cells. In the current study, we found: first, that expression of ADRP was strongly induced in association with accumulation of cellular TG; second, that levels of ADRP are decreased when cellular TG is consumed and that the proteasome pathway is involved in this decrease in ADRP; and third, that ADRP prevents a decrease in TG in the lipid-storing cells.
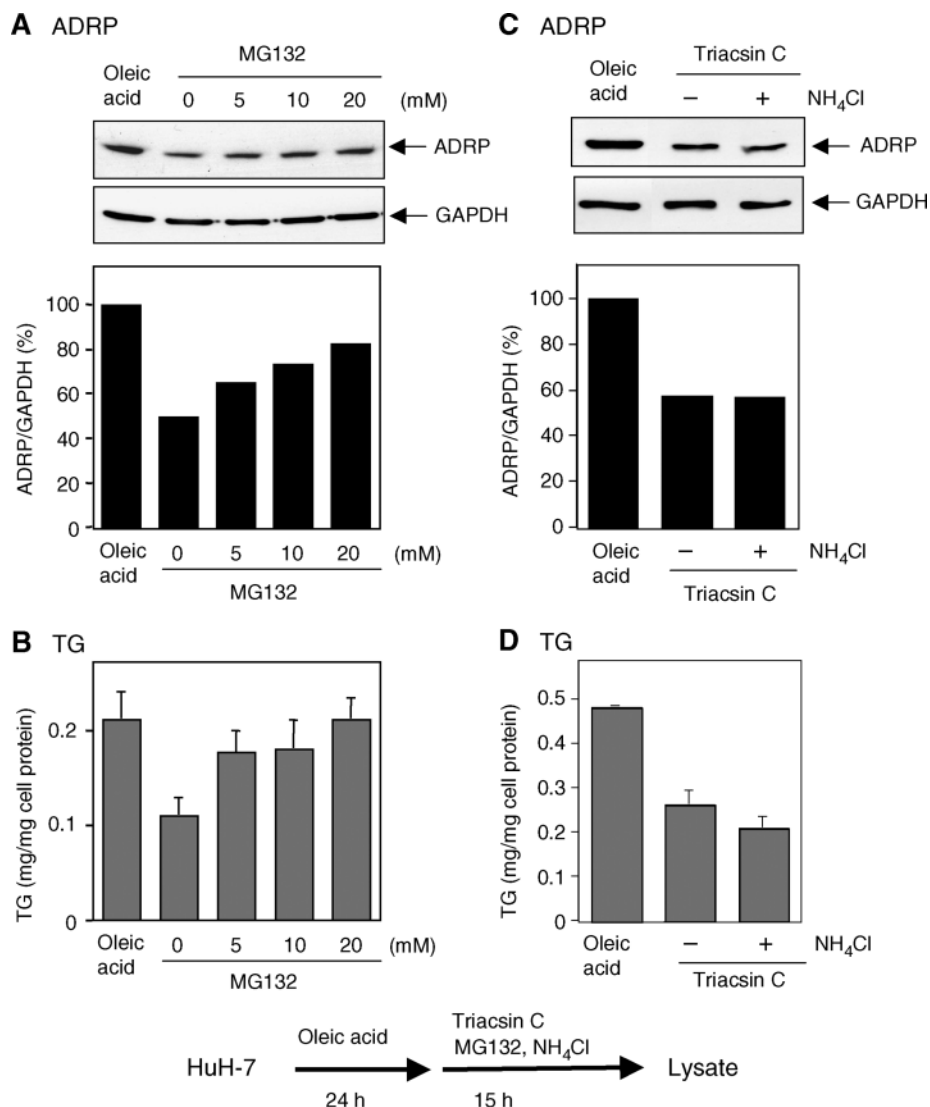
Fig. 8. The proteasome inhibitor abolishes the decrease in ADRP protein during regression of oleic acid-treated HuH-7cells. HuH-7 cells (1×10^6 cells/dish) were incubated for 24 h in medium containing 600 μM oleic acid. Then the cells were further incubated for 15 h in new medium with 5% LPDS, 25 mg/ml BSA, 10 μg/ml triacsin C, and various concentrations of MG132. ADRP and GADPH in the cell lysates were detected by immunoblotting. The intensity of the bands was quantified with NIH Image, and the amount of ADRP relative to GAPDH was calculated (A). Under the same experimental conditions, the amounts of TG in the cell lysates were measured by the enzymatic method (B). Similar sets of experiments were carried out using ammonium chloride, which neutralizes lysosomal pH. HuH-7 cells preincubated with 600 μM oleic acid were further incubated for 15 h in new medium with 5% LPDS, 25 mg/ml BSA, 10 μg/ml triacsin C, and 10 mM ammonium chloride (C, D). Values represent mean ± standard deviation of triplicate measurements. Shown are typical data in three independent experiments.

Foam cell formation from macrophages is induced by various lipoproteins, particularly by modified lipoproteins. It is well established that several types of scavenger receptors expressed on macrophages take up modified LDLs such as AcLDL or OxLDL, resulting in the accumulation of massive amounts of lipids in the cells. However, under our experimental conditions, we found that VLDL is the most effective lipid source for foam cell formation. VLDL is recognized and taken up by the VLDL receptor, a member of the LDL receptor family, in a process that has been shown to induce foam cell formation (16). However, it

is unlikely that this receptor is directly coupled to ADRP induction, because we observed strong induction of ADRP in macrophages by oleic acid. It should be noted that we used lipoproteins on the basis of their protein content, meaning that the amounts of lipids added to the cells varied. On average, 1 mg protein of VLDL contains approximately 7.5 mg of TG and 2.5 mg of cholesterol, whereas 1 mg protein of LDL contains 1 mg of TG and 2 mg of cholesterol. Thus, it is possible that the effective induction of ADRP by the VLDL fraction is a result of the high content of neutral lipids in this lipoprotein. It was
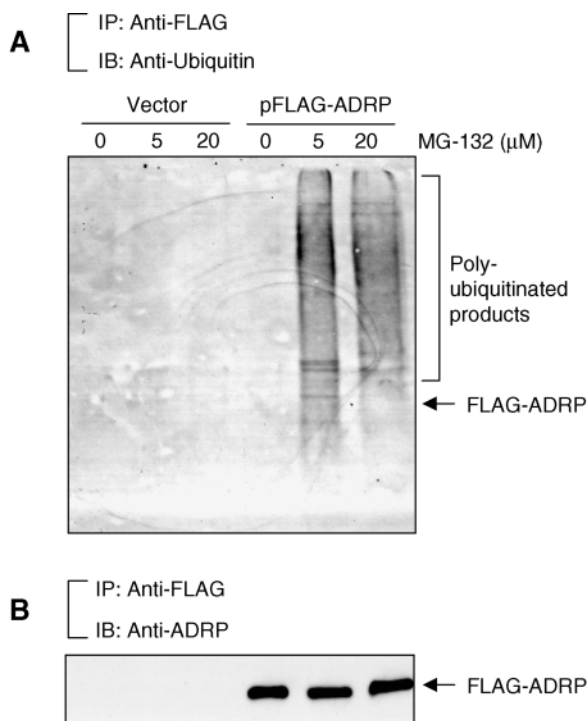
Fig. 9. The ubiquitinated form of ADRP increases in the presence of proteasome inhibitor in HuH-7 cells. HuH-7 cells (1×10^6 cells/dish) were transfected either with empty vector (pFLAG-CMV-2) or with expression plasmid for human ADRP (pFLAG-ADRP) by using TransIT transfection reagent for 48 h. Under these conditions, FLAG-tagged ADRP is overexpressed without accumulation of neutral lipids in the cells. The cells were then treated for 3 h with 20 μM of MG132, and the cell lysates were incubated for 3 h with anti-FLAG M2 affinity gel to immunoprecipitate FLAG-tagged proteins. The gels were collected by centrifugation and washed three times with the lysis buffer. The proteins eluted with 0.1 M glycine-HCl (pH 3.5) from the gels were analyzed by immunoblotting with an anti-polyubiquitin pAb and the anti-human ADRP antibodies.

shown that ADRP was induced by AcLDL in macrophages (37). The reason(s) for this discrepancy between our present data is not known, but one possible factor is that we added a relatively low concentration of AcLDL (50 μg/ml) to the macrophages compared to other studies, in which more than 100 μg/ml were used.

We found that the changes in the amount of ADRP were better correlated with cellular TG content than with cholesterol content. Chawla et al. (17) used macrophages obtained from PPARδ-null mice to show that expression of ADRP depends on activation of PPARδ, and demonstrated by reporter-gene assay a close relationship between TG accumulation and PPARδ activation. A highly selective agonist for PPARδ promotes lipid accumulation in human primary macrophages (38). The physiological ligand(s) for PPARδ has yet to be identified, but fatty acids, rather than TG, are probable candidates. Our observations, based on measurement of ADRP protein and lipids, accord well with those of Chawla et al.

One of the major issues in the cell biological aspects of lipid droplets is how the droplets are formed, in particular

whether there exist nascent lipid droplets composed of lipid-poor cores containing ADRP. A related question is the fate of ADRP when lipids are lost from lipid droplets. We found that the cellular content of ADRP decreased during TG loss from foam cells. Triacsin C is a potent inhibitor of acyl-CoA synthase (33), and is able to deplete macrophages of cellular TG (34). The level of ADRP mRNA decreased after 6 h treatment of lipid-storing cells with triacsin C, suggesting that ADRP expression could be regulated transcriptionally by cellular TG content. However, the rapid decrease in ADRP protein in foam cells cannot be explained by the decrease in mRNA level alone, implying that ADRP content is also decreased by proteolytic degradation. When a proteasome inhibitor was added to HuH-7 human hepatic cells pretreated with oleic acid, it abolished the decrease in ADRP. Smear bands of ubiquitinated ADRP that correspond to its polyubiquitinated forms were detected when overexpressed FLAG-tagged ADRP was immunoprecipitated with anti-FLAG beads from the cells coincubated with MG132. These results indicate that proteasome-dependent proteolysis is involved in the reduction of ADRP content. This is the first study, to our knowledge, to show that the amount of lipid droplet-associated protein is regulated in part by proteasome-dependent proteolysis.

Proteasome forms a proteolytic machinery in the cytosol that is crucial for quality control of cellular proteins (39). Because proteasome efficiently degrades ubiquitinated proteins, it has been suggested that ubiquitin ligases, the enzymes responsible for the ubiquitination reaction, are intracellular sensors for redundant proteins. This raises the next question of what triggers the ubiquitination of ADRP during regression of lipid-storing cells. A large lipid-associated protein related to plasma lipoproteins, apolipoprotein B, is known to be degraded by proteasome during lipoprotein assembly in cells when addition of lipids to the nascent protein is not properly processed (36, 40). It is possible that ADRP is stabilized by its association with lipids, and thus that certain conformational changes may occur after dissociation from lipids.

The physiological role of ADRP has yet to be fully established. Perilipin A, another lipid droplet-associated protein, is phosphorylated by protein kinase A and is directly involved in hormone-dependent regulation of lipolysis (21). ADRP contains a sequence, called the PAT domain, homologous to that in perilipin A (2), and both proteins localize on the surface of lipid droplets (7). However, there are some important differences between them: perilipin A is expressed only in hormone-sensitive tissues such as adipocytes or adrenal gland, and ADRP lacks phosphorylation sites. ADRP may not be involved in hormone-dependent regulation of lipid metabolism. Overexpression of ADRP in fibroblastic cells induces lipid accumulation and enhances incorporation of fatty acids (18, 19), suggesting that ADRP stabilizes lipid droplet structures or even facilitates lipid droplet formation. Very recently, Larigauderie et al. (37) reported that reduction of ADRP expression by 80–90% following treatment with siRNA caused an approximately 50% reduction in the lipid con-

tent of THP-1 macrophages in the presence of AcLDL. This observation supports the possibility that ADRP contributes to lipid droplet formation, but the moderate reduction in cellular neutral lipids after siRNA treatment suggests that other proteins on the lipid droplets might compensate for the loss of ADRP function. In our study, inhibition of the proteasome-dependent decrease in ADRP significantly blocked the depletion of TG in HuH-7 cells in the presence of triacsin C. Our observation suggests the possibility that ADRP prevents the hydrolysis of TG in lipid droplets that contributes to lipid storage in the lipid-storing cells.

While this manuscript was being revised, Xu et al. (41) reported on the Web that ADRP transiently overexpressed in Chinese ovary cells was degraded by the proteasome-dependent pathway upon removal of the lipid source in the medium. Although our study has tried to examine the regulatory behavior of endogenous ADRP in macrophages and liver cells, the good correspondence between their study and ours would strongly suggest that the proteasomal degradation of ADRP is a common regulatory mechanism of ADRP expression and lipid storage.

It has been shown that the lipid droplet is a rather ubiquitous constituent of cells (7, 8) and a site of active lipid metabolism (3, 4). Massive accumulation of intracellular lipids is seen in several diseases, including atherosclerosis, fatty liver, and xanthoma. Understanding the role of ADRP in these disorders may suggest a direction for the development of therapies.

In conclusion, we found that a proteolytic process is involved in the regulation of cellular ADRP content during regression of lipid-storing cells. This may represent one of the mechanisms by which the hormone-independent components of lipid droplet-associated proteins are regulated.▯

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