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Possible Involvement of Protein Kinase C in the Induction of Adipose Differentiation-Related Protein by Sterol Ester in RAW 264.7 Macrophages

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Abstract The accumulation of lipid droplets in macrophages contributes to the formation of foam cells, an early event in atherosclerosis. It is, therefore, important to elucidate the mechanisms by which lipid droplets accumulate and are utilized. Sterol ester (SE)-laden RAW 264.7 macrophages accumulated lipid droplets in a time-dependent manner up to 16 h, which was enhanced by cotreatment with 0.1 μM phorbol 12-myristate 13-acetate (PMA). Inhibition of protein kinase C (PKC) activity by cotreatment with 0.3 μM calphostin CCAL for 16 h resulted in coalescence of small lipid droplets into large ones and increased accumulation of lipid droplets, although to a lesser extent than after PMA cotreatment. Immunostaining for adipose differentiation-related protein (ADRP) revealed a fluorescent rim at the surface of each medium to large lipid droplet. ADRP appearance correlated with lipid droplet accumulation and was regulated by PMA in a time-dependent manner. Induction of ADRP expression by PMA or CAL required SE, since ADRP levels in PMA- or CAL-treated non-SE-laden macrophages were comparable to those in untreated cells. Removal of SE from the incubation medium resulted in the concomitant dissolution of lipid droplets and down-regulation of ADRP. In conclusion, the above results suggest that ADRP may be an important protein in the regulation of lipid droplet metabolism in lipid-laden macrophages and that this regulation may be mediated by PKC activity. J. Cell. Biochem. 83: 187–199, 2001.

Key words: lipid droplet accumulation; protein kinase C; adipose differentiation-related protein; macrophage

Uptake of chemically modified low density lipoprotein (LDL) by macrophages results in the accumulation of lipid droplets and the formation of foam cells, an early event in atherosclerosis [Ross, 1993]. Macrophages bind and internalize acetyl and oxidized LDLs by means of a specific scavenger receptor [Sparrow et al., 1989; Han and Nicholson, 1998; Han et al., 1999; Lougheed et al., 1999]. These modified forms of LDL are taken up more rapidly by macrophages than native LDL in a manner that leads to the formation of foam cells [Wiklund et al., 1991; Juul et al., 1996; Kritharides et al., 1998]. Expression of macrophage scavenger receptor mRNA can be induced by exposure to cholesterol [Han et al., 1999] or lipoproteins [Han and Nicholson, 1998], and the subsequent removal of these agents decreases scavenger receptor mRNA levels, indicating reversible regulation of this receptor. In addition, in the

Abbreviations used: ADRP, adipose differentiation-related protein; DIC, differential interference contrast; DMEM, Dulbecco's modified Eagle's medium; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SE, sterol ester; CAL, calphostin C; LDL, low density lipoprotein; PBS, phosphate-buffered saline.

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human monocyte cell line THP-1, induction of acetyl LDL (scavenger) receptor has been shown to be dependent on activation of PKC [Via et al., 1989]. Similar regulation of scavenger receptor by PKC has been observed in rabbit smooth muscle cells, which also accumulate lipid droplets and develop into foam cells in atherosclerotic lesions [Pitas et al., 1992; Mietus-Snyder et al., 1997]. When cholesterol acceptors, such as high density lipoprotein, are present in the culture medium, PKC activation is coupled to cholesterol efflux in adipose cells and human skin fibroblasts [Theret et al., 1990; Mendez et al., 1991] and accelerates the secretion of apolipoprotein E, a surface component of lipoproteins, in rat ovarian granulosa cells [Wyne et al., 1989]. Moreover, PKC inhibition down-regulates apolipoprotein-mediated cellular cholesterol efflux in macrophages [Li et al., 1997]. Thus, depending on whether cholesterol acceptors are present, PKC may play a dual role in scavenger receptor expression or in cholesterol efflux.

Adipose differentiation-related protein (ADRP) was first identified as a protein whose expression increased during adipocyte differentiation [Jiang and Serrero, 1992]. Further investigations revealed that this protein is expressed ubiquitously in diverse cell types, such as MA-10 Leydig cells, Chinese hamster ovary fibroblasts, human HepG2 hepatoma cells, and lactating mammary epithelial cells [Heid et al., 1996, 1998; Brasaemle et al., 1997]. In 3T3-L1 preadipocytes and early differentiated adipocytes, ADRP can be detected on small lipid droplets, but is undetectable in mature adipocytes. Interestingly, although not expressed during early differentiation, perilipin, another lipid droplet-associated protein in adipocytes and steroidogenic cells [Greenberg et al., 1991; Servetnick et al., 1995], colocalizes with ADRP on small lipid droplets; but as adipocytes further differentiate, only perilipin is present on the surface of lipid droplets. Distinct proteins are therefore expressed and associated with lipid droplets at different differentiation stages, presumably stabilizing the droplets. Induction of expression of ADRP and its mRNA can be achieved by stimulation of the undifferentiated mouse 1246 adipogenic cell line with cyclooxygenase inhibitors, such as ibuprofen and indomethacin, which have been shown to stimulate the differentiation of a variety of cell lines [Ye and Serrero, 1998] and to activate peroxisome proliferator-activated receptors α and γ [Lehmann et al., 1997]. ADRP while normally not detected in rat hepatocytes, its expression can also be induced by treatment with a carnitine palmitoyltransferase I inhibitor, etomoxir, which causes neutral lipid accumulation [Steiner et al., 1996]. The findings that stable expression of ADRP in COS-7 cells transfected with ADRP cDNA stimulates long chain fatty acid uptake [Gao and Serrero, 1999] and that ADRP expression is activated by long chain fatty acids in 1246 cells [Gao et al., 2000] may imply an intimate relationship between ADRP expression and fatty acid metabolism.

Since ADRP is widely distributed in various cell types containing lipid droplets, we were interested to determine whether it is associated with lipid droplets in SE-laden RAW 264.7 macrophages and its possible regulation. Since PKC activation regulates scavenger receptor expression and therefore lipoprotein uptake, the effects of PKC activity on lipid droplet accumulation and ADRP expression were also investigated in order to elucidate the relationship between PKC activity and foam cell formation in vitro. We here show that SE loading of RAW 264.7 macrophages induces expression of ADRP protein, which, unlike that in 3T3-L1 preadipocytes, is associated with both small and large lipid droplets. Treatment with PMA up-regulates both lipid droplet accumulation and ADRP expression in a time-dependent manner. Moreover, hydrolysis of lipid droplets occurs concomitantly with ADRP down-regulation. Our results suggest that PKC-regulated ADRP expression is involved in the formation of macrophage foam cells.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

RAW 264.7 macrophages (ATCC TIB-71) were purchased from the American Type Culture Collection (Rockville, MD) and cultured on coverglasses in DMEM (Gibco BRL, Long Island, NY) supplemented with 10% CPSR-1 (a low-lipid serum replacement) (Sigma), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. To study lipid droplet accumulation, macrophages were loaded with SE [Jepson et al., 1996] by incubation for 1–24 h in SE medium, i.e., DMEM containing 80 μg/ml of 25-hydroxycholesterol (Sigma), 100 μg/ml of oleic acid (Sigma), and 2 mg/ml of BSA in the presence or absence of

0.1 μM PMA (Sigma), a PKC activator, or 0.3 μM CAL (Sigma), a PKC inhibitor.

Isolation of Lipid Droplets

Lipid droplet fraction was isolated from SE-laden macrophages as described by Mrotek et al. [1981]. Briefly, macrophages were collected and homogenized in 1 ml of 0.25 M sucrose solution and layered on top of 3 ml of 0.5 M sucrose solution in a 10 ml centrifuge tube. After addition of a top layer of 0.125 M sucrose solution (1.2 ml), the sample was centrifuged at 13,000*g* (4°C) for 3 h and the lipid droplets collected from the top layer. Isolated lipid droplets were also subjected to electrophoresis and immunoblotting using aliquots of less than 200 μl of the top sucrose layer from the isolation procedure.

Immunofluorescence Microscopy

RAW 264.7 macrophages cultured on coverslips were fixed in cold methanol (−20°C) for 1 min, washed, and incubated with mouse monoclonal A2 antibody, which recognizes a 160 kDa capsular protein on the surface of lipid droplets in steroidogenic cells [Wang and Fong, 1995], rabbit anti-perilipin antiserum [Souza et al., 1998], or rabbit anti-ADRP antibody, a kind gift from Dr. Thomas Keenan [Heid et al., 1996, 1998]. Incubation with cells in the absence of these antibodies and antiserum or with unrelated antibodies were used as negative controls. After PBS washes, the cells were then reacted with FITC-conjugated anti-rabbit or anti-mouse IgG (Sigma), washed, mounted, and photographed using a Zeiss epifluorescence microscope (Zeiss, Inc., Thornwood, NY).

Immunoblotting

RAW 264.7 macrophages were scraped off the culture dishes and homogenized by sonication and the protein concentration of the homogenate determined (protein assay kit, BioRad). The cell lysates (30 μg/lane) were electrophoresed on a 10% SDS–polyacrylamide gel [Fritz et al., 1989] and the proteins transferred to a nitrocellulose membrane. After blocking with 5% non-fat milk for 30 min, the membrane was reacted with rabbit anti-ADRP antibody, followed by alkaline phosphatase-conjugated anti-rabbit IgG (Promega, Madison, WI). Negative control was performed by omitting anti-ADRP antibody. Reactive bands were visualized using a combination of nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) in alkaline phosphatase assay buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris–Base, pH 9.5). The blots were photographed and the relative density of each positive band on the nitrocellulose membrane quantified by densitometric scanning using an Image Master (Pharmacia Biotech, Hong Kong).

Nile Red Staining

To identify lipid droplets, macrophages were first fixed in 10% formalin in PBS for 10 min, then stained with Nile red (Sigma) at a final concentration of 10 μg/ml [Greenspan et al., 1985]. Following brief washes with PBS, the cells were mounted and observed.

Data Analysis

To calculate the surface areas and diameters of lipid droplets, SE-laden macrophages were stained with Nile red and photographed. The mean surface area and diameter range of the stained lipid droplets in each cell were measured using the BRS-2 version of the Microsoft Computer Image Device System (Brock University, St. Catharines, Ontario, Canada). On the basis of their diameter, lipid droplets were classified as small (less than 0.5 μm), medium (0.5–1.5 μm), or large (greater than 1.5 μm).

RESULTS

PMA Potentiated the Effect of SE on Lipid Droplet Accumulation

Since scavenger receptors mediate the uptake of lipoproteins and are up-regulated by PMA [Via et al., 1989; Pitas et al., 1992; Mietus-Snyder et al., 1997], our initial aim was to investigate the effect of PMA in regulating SE-induced lipid droplet formation. As shown in Figure 1, when RAW 264.7 macrophages were loaded with SE in the absence of PMA, small lipid droplets started to appear in the cytoplasm and accumulated in a time-dependent manner, as shown by Nile red staining (Figs. 1A, C and E; the diffuse background staining seen is possibly due to an extensive reticulum which may represent intracellular membranes and organelles [Greenspan et al., 1985] and cannot be eliminated). This process was enhanced by co–treatment with 0.1 μM PMA (Figs. 1B, D and F). This effect was more marked after long-term treatment, since PMA cotreatment of SE-laden macrophages for 16 h significantly

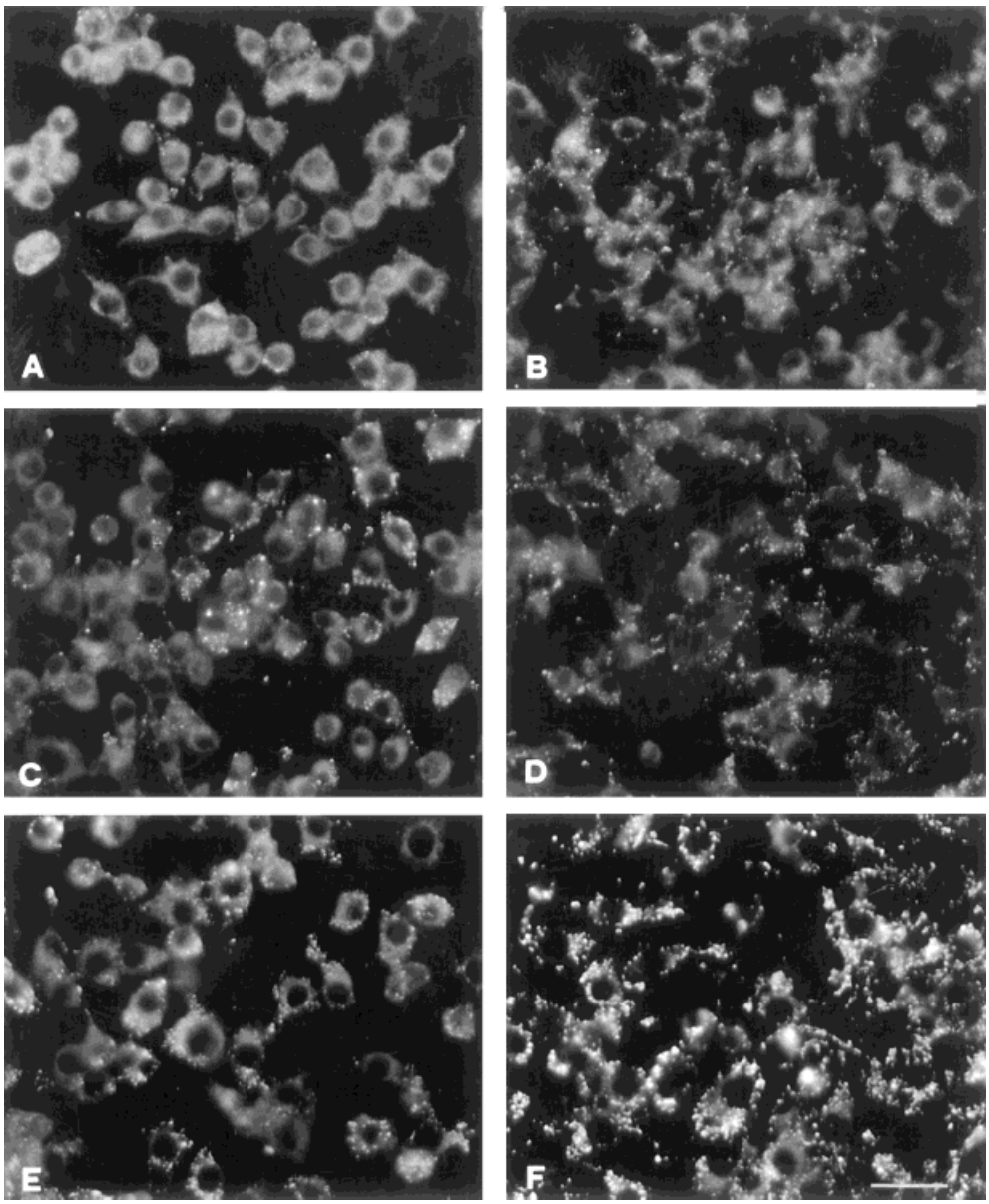

Fig. 1. Time-dependent accumulation of SE-induced lipid droplets. RAW 264.7 macrophages were incubated with 80 μg/ml of 25-hydroxycholesterol, 100 μg/ml of oleic acid, and 2 mg/ml of BSA in the absence (**A**, **C**, and **E**) or presence (**B**, **D**, and **F**) of 0.1 μM PMA for 1 h (A and B), 3 h (C and D), and 6 h (E and F). Lipid droplets are visualized by Nile red staining. Bar = 30 μm.

increased the accumulation of lipid droplets (Fig. 2B) compared to that seen in cells not treated with PMA (Fig. 2A), which showed no further accumulation of lipid droplets after 6 h of treatment. Since activation of PKC by PMA resulted in marked accumulation of lipid droplets, we next examined the effect of inhibiting PKC activity on lipid droplet accumulation. Short-term treatment of SE-laden RAW 264.7 macrophages with 0.3 μM CAL did not affect the degree of lipid droplet accumulation. Surprisingly, long-term application (16 h) resulted in the formation of a few extremely large lipid droplets (Fig. 2C), possibly as the result of the coalescence of small droplets. Statistical analysis confirmed that the mean surface area of lipid droplets per cell increased from $\sim 6.1 \pm 0.3\ \mu m^2$ in SE-laden cells to about $26.2 \pm 4.5\ \mu m^2$ or $16.0 \pm 3.2\ \mu m^2$ in cells cotreated with 0.1 μM PMA or 0.3 μM CAL, respectively (Fig. 3A).

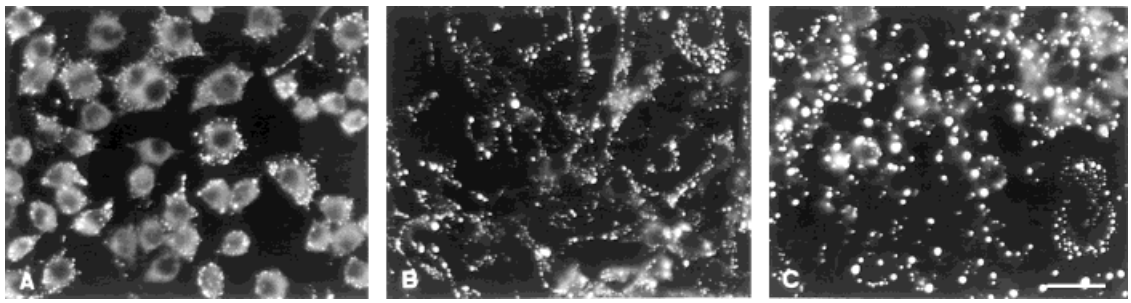

Fig. 2. Effect of PKC activity on the formation of lipid droplets. Macrophages incubated with SE for 16 h show moderate accumulation of lipid droplets (**A**). Cotreatment with 0.1 μM PMA results in marked accumulation of lipid droplets (**B**). Prominent lipid droplet enlargement is induced by 0.3 μM CAL (C). Bar = 30 μm.

These results indicate that both activation and inhibition of PKC result in increased accumulation of lipid droplets, although PKC inhibition has less of an effect.

Measurement of lipid droplet diameters (Fig. 3B) showed that about 64% of the lipid droplets in SE-laden macrophages were small.

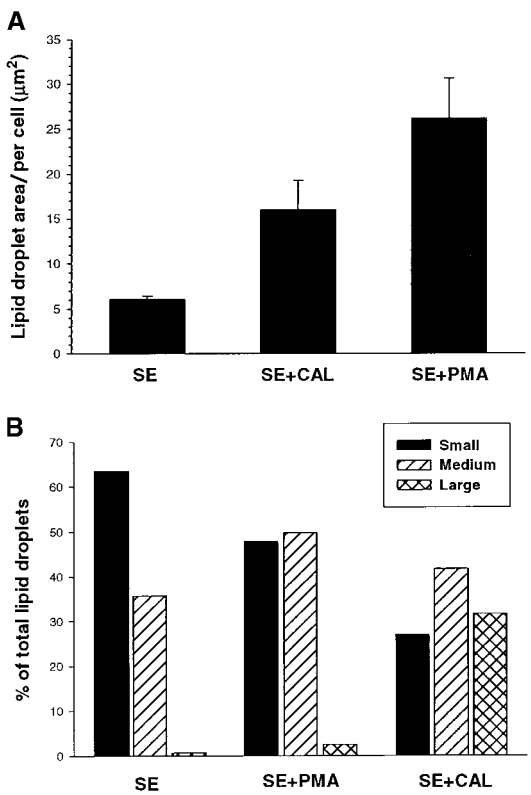

Fig. 3. Effects of PMA and CAL on the surface area and diameter of lipid droplets. **A:** The lipid droplet surface area per cell increases significantly in PMA- or CAL-treated cells. **B:** Enlargement of lipid droplets as a result of CAL stimulation, shown by the shift to large lipid droplets. Thirty-five cells were counted per treatment condition.

In contrast, in CAL-cotreated cells, only about 27% of droplets were small, while 31% were large droplets, rarely seen in cells loaded with SE alone. The effect of PMA cotreatment on lipid droplet enlargement was not apparent, as it only slightly increased the percentage of medium-sized lipid droplets from 36% in SE-laden cells to 50%. These results indicate that inhibition of PKC by CAL not only stimulates the storage of lipid droplets, but also promotes the formation of large lipid droplets.

Presence of ADRP on SE-Induced Lipid Droplets

To evaluate the properties of these nascent lipid droplets, we screened for the presence of three lipid droplet-associated proteins found on the surface of endogenous lipid droplets in 3T3-L1 adipocyte and rat adrenocortical cells [Greenberg et al., 1991; Brasaemle et al., 1997; Fong and Wang, 1997; Londos et al., 1999]. Rabbit anti-perilipin antiserum or mouse monoclonal A2 antibody did not reveal any specific staining pattern of the lipid droplet surface in immunofluorescence studies or detect any specific protein band on immunoblots (not shown). However, immunostaining of SE-laden cells with anti-ADRP antibody demonstrate numerous small immunostained droplets (Figs. 4C and D), not normally seen in untreated control macrophages (Figs. 4A and B). The short fixation time in cold methanol did not extract out lipid droplets, since the presence of which can be identified by DIC image. Incubation with cells in the absence of anti-ADRP antibody or an unrelated primary antibody did not reveal any specific staining around lipid droplets (data not shown). Treatment of SE-laden macrophages with 0.1 μM PMA for 16 h resulted in the

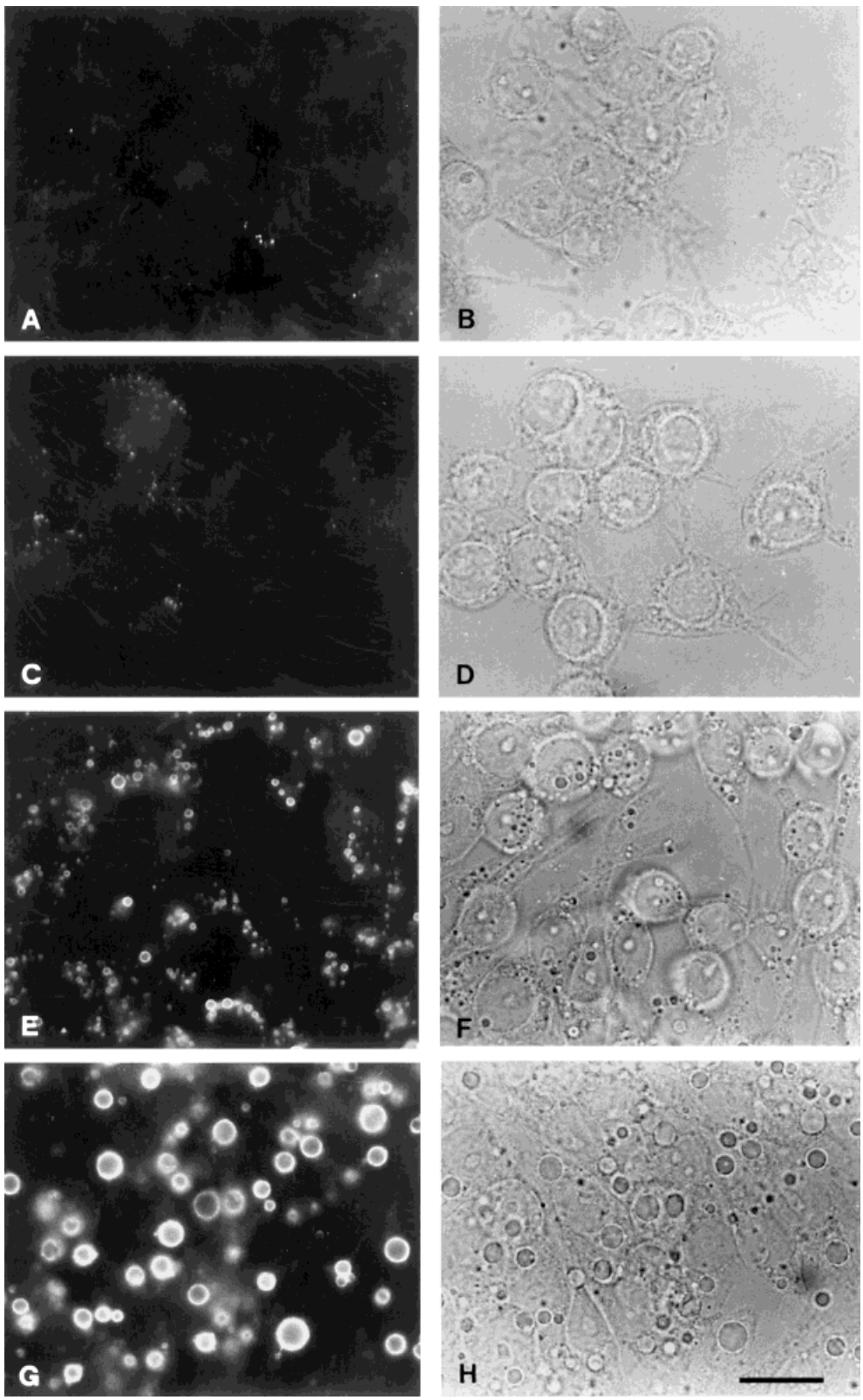

Fig. 4. Distribution of ADRP in normal and lipid-laden macrophages. **B**, **D**, **F**, and **H** are corresponding DIC images of **A**, **C**, **E**, and **G** (immunostained with anti-ADRP antibody). Untreated control macrophages display almost no ADRP staining (A and B). In macrophages loaded with SE, an ADRP staining pattern of small spots is seen in the cytoplasm (C) and the lipid droplets are too small to be seen in the corresponding DIC image (D). PMA (0.1 μM) stimulates the accumulation of lipid droplets, the surface of which are labeled with anti-ADRP antibody (E and F). ADRP staining is most prominent at the periphery of enlarged lipid droplets in CAL-treated cells, seen as bright circles around each lipid droplet (G and H). Bar = 15 μm.

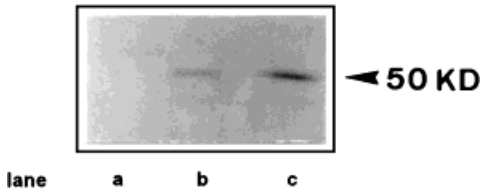

Fig. 5. Association of ADRP with lipid droplets as evaluated by subcellular fractionation. Isolated lipid droplets were analyzed for ADRP by immunoblotting. **Lane a:** Untreated macrophages. **Lane b:** SE-laden macrophages. **Lane c:** SE-laden macrophages co-incubated with 0.1 μM PMA. The data shown here is one typical result from three independent experiments.

accumulation of abundant small- to medium-sized ADRP-positive lipid droplets (Figs. 4E and F). Similarly, the large CAL-induced lipid droplets (Figs. 4G and H) were also strongly stained with anti-ADRP antibody; in this case, ADRP staining was more intense and clearly encircled the lipid droplets. Isolation and immunoblotting of lipid droplets confirmed the association of ADRP with lipid droplets (Fig. 5) and showed that more ADRP protein was expressed in cells containing plentiful lipid droplets induced by PMA cotreatment.

Time-Dependent Induction of ADRP by PMA in SE-Laden Macrophages

Since lipid droplets in RAW 264.7 macrophages were surrounded by ADRP, we next determined whether lipid droplet accumulation was accompanied by induction of ADRP expression. Figure 6 shows the time-dependent induction and up-regulation of ADRP protein in SE-laden macrophages, which was more marked in PMA-cotreated cells. In normal cells without accumulated lipid droplets, only trace amounts of ADRP were detected (lane a), in good agreement with the weak ADRP immunostaining seen in Figure 4A. Negative control omitting anti-ADRP antibody did not label any specific band on the blot. Densitometric analysis showed that, although the amount of ADRP protein in SE-laden macrophages did not increase continuously from 6 to 16 h, PMA-induced ADRP expression continued to increase throughout the 16 h of incubation. Taken together with

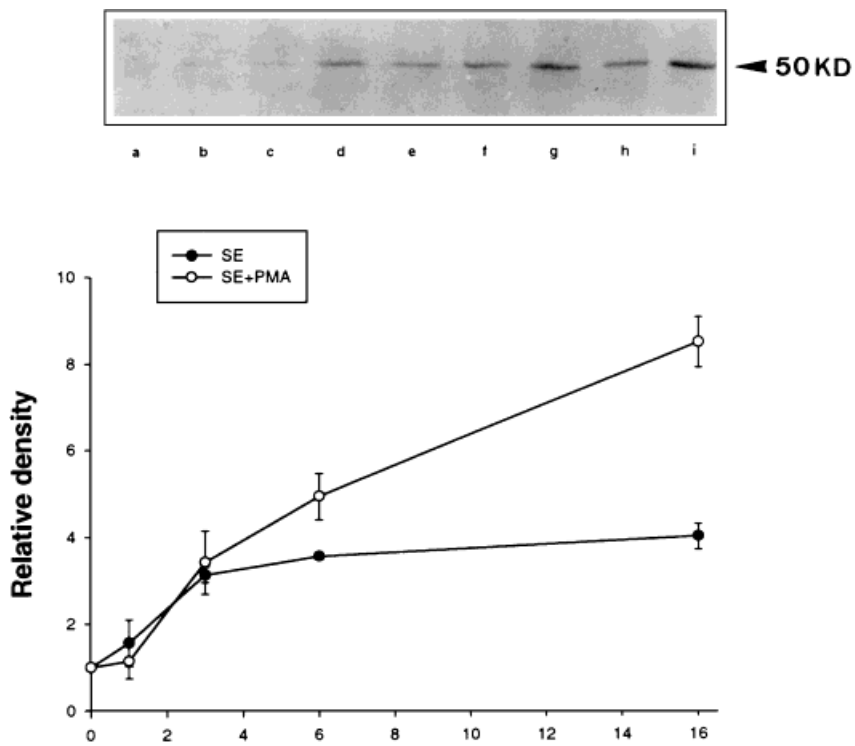

Fig. 6. Time-course study of ADRP expression by immunoblot analysis. Upper trace: Immunoblots. **Lane a:** Untreated macrophages, showing no ADRP expression. **Lanes b–i:** SE-laden macrophages incubated in the absence (lane b, d, f, and h) or presence (lane c, e, g, and i) of 0.1 μM PMA for 1 h (lanes b and c), 3 h (lanes d and e), 6 h (lanes f and g), and 16 h (lanes h and i). ADRP expression increases with incubation time, and is increased in the presence of PMA. Lower trace: Densitometric scans showing that PMA significantly up-regulates ADRP protein level in a time-dependent manner. The densitometric results are shown as the mean ± SD from three independent experiments.

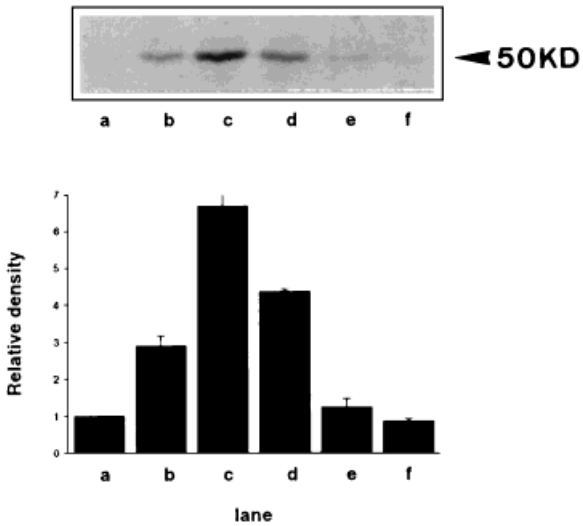

Fig. 7. Immunoblot study of effect of PMA and CAL on ADRP expression. Upper trace: immunoblots; lower trace: densitometric scans. **Lane a:** Normal untreated cells; **lane b:** SE-laden cells; **lane c:** 0.1 μM PMA-cotreated cells; **lane d:** 0.3 μM CAL-cotreated cells; **lane e:** PMA-treated non-SE-laden cells; **lane f:** CAL-treated non-SE-laden cells. The densitometric results are shown as the mean±SD from three independent experiments.

the data shown in Figures 1 and 2, these results demonstrate that ADRP induction is closely correlated with the accumulation of lipid droplets.

SE is Required for the Effects of PMA and CAL

Stimulation of non-SE-laden macrophages for 16 h with either 0.1 μM PMA (Fig. 7, lane e) or 0.3 μM CAL (Fig. 7, lane f) did not result in ADRP protein expression than that observed in untreated cells, whereas in the presence of SE, PMA, and CAL stimulated ADRP expression (Fig. 7, lanes c and d). Moreover, densitometric scanning showed that the amount of ADRP protein in CAL-cotreated macrophages was about 160% of that in SE-laden cells, but only 68% of that in PMA-cotreated cells (Fig. 7), consistent with the previous result (Fig. 3A) that the extent of lipid droplet accumulation, i.e., mean lipid droplet area per cell, induced by CAL cotreatment is greater than that in SE-laden macrophages, but less than that induced by PMA cotreatment.

Lipolysis is Accompanied by Down-Regulation of ADRP Protein

When SE-laden macrophages (Fig. 8B) or PMA-cotreated cells (Fig. 8D) were placed in sterol-free medium for 16 h, almost no lipid droplets were detected by Nile red staining. In contrast, cells constantly cultured in SE medium contained numerous lipid droplets (Fig. 8A) which became more crowded in PMA-cotreated cells (Fig. 8C). As before, immunofluorescence studies showed that the lipid-laden macrophages were immunostained with anti-ADRP antibody (Figs. 8E and G), while SE-deprived cells displayed sparse, weak ADRP staining, irrespective of the absence (Fig. 8F) or presence (Fig. 8H) of PMA. The down-regulation of ADRP was confirmed by immunoblot analysis (Fig. 9), which also demonstrated ~2.3-fold increase in ADRP protein levels in PMA-cotreated cells. These results clearly indicate that ADRP up-regulation occurs concomitantly with increased lipid droplet accumulation and its down-regulation is associated with lipolysis.

DISCUSSION

Our results demonstrated that both the activation and inhibition of PKC activity result in increased accumulation of lipid droplets in lipid-laden macrophages, although to different extents. In addition, inhibition of PKC resulted in enlargement of lipid droplets. We also demonstrated, for the first time, that these lipid droplets in lipid-laden macrophages are surrounded by ADRP, and that ADRP expression is regulated by SE loading and enhanced by cotreatment with a PKC activator or inhibitor. The involvement of PKC in lipid droplet accumulation and in ADRP induction required the presence of SE. Dissolution of lipid droplets in macrophages occurred when SE was removed from the culture medium and was accompanied by down-regulation of ADRP. Taken together, these results indicate that ADRP expression is closely linked to lipid droplet metabolism, which is regulated by PKC activity.

The expression of ADRP in lipid-laden macrophages provides further evidence in support of the fact that ADRP is ubiquitously expressed in cells containing lipid droplets [Brasaemle et al., 1997]. Our results also indicate that ADRP expression is induced by SE, since almost no ADRP was detected in normal macrophages. ADRP induction has been seen in etomoxir-treated rats which accumulate lipid in their livers [Steiner et al., 1996] and in the mouse 1246 adipogenic cell line treated with cyclooxygenase inhibitors to stimulate differentiation

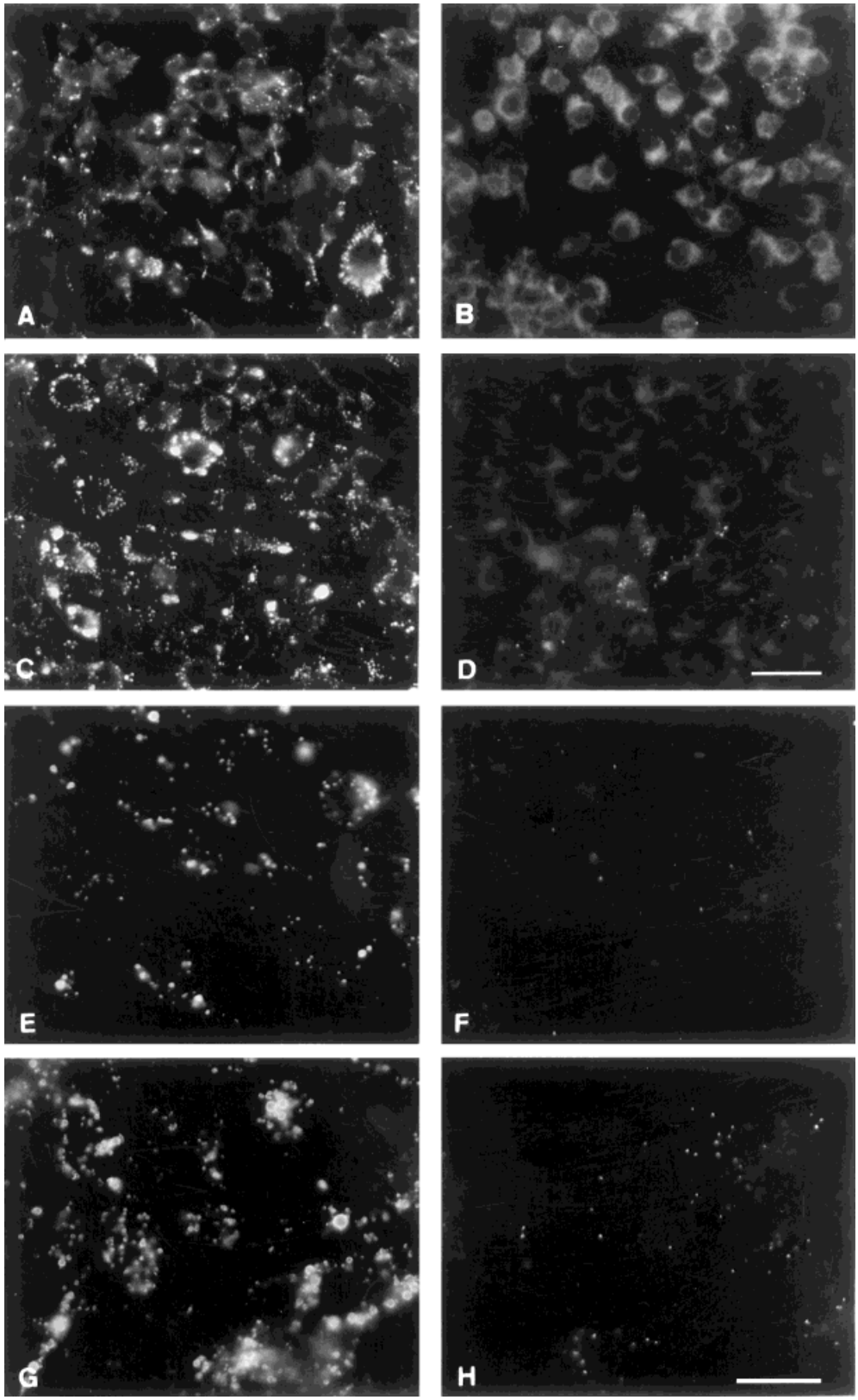

Fig. 8. Immunofluorescence study of down-regulation of ADRP during lipolysis. Macrophages were either loaded with SE in the absence (**A** and **E**) or presence (**C** and **G**) of 0.1 μM PMA for 22 h or first loaded with SE (**B** and **F**) or SE plus 0.1 μM PMA (**D** and **H**) for 6 h, then incubated in fresh DMEM for another 16 h. A–D: Stained with Nile red. E–H: Immunostained for ADRP. SE-laden macrophages (A) accumulate lipid droplets that display ADRP staining (E). PMA-cotreated cells have more lipid droplets in the cytoplasm (C) and show extensive ADRP labeling (G). Removal of SE (B and F) or SE and PMA (D and H) results in the almost complete hydrolysis of lipid droplets (B and D) and abolishment of ADRP staining (F and H). Bars: A–D = 30 μm; E–H = 15 μm.

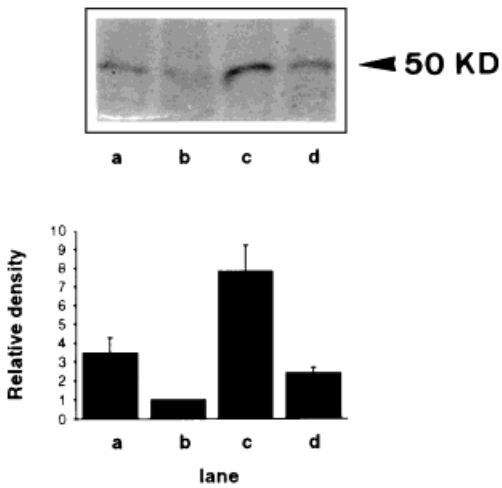

Fig. 9. Immunoblot study of the effect of SE removal on PMA-induced ADRP expression. Upper trace: blots; lower trace: densitometric scan. Macrophages were either loaded with SE alone (**lane a**) or cotreated with 0.1 μM PMA (**lane c**) for 22 h, or first loaded with SE in the absence (**lane b**) or presence (**lane d**) of PMA for 6 h, then changed to fresh DMEM for another 16 h. PMA increases 2–3 folds of ADRP protein level as compared with that of SE-laden group (**lane a**). Withdrawal of SE (lane b) results in down-regulation of ADRP in PMA-treated (lane d) and -untreated group (lane b). The densitometric results are shown as the mean±SD from three independent experiments.

[Ye and Serrero, 1998]. The fact that ADRP, but not perilipin or the 160 kDa capsular protein, was associated with lipid droplets in SE-laden macrophages supports the findings that ADRP is widely distributed in several lipid droplet-containing cell types, whereas perilipin and the capsular protein are only found associated with lipid droplets in adipocytes and steroidogenic cells [Greenberg et al., 1991; Servetnick et al., 1995; Blanchette-Mackie et al., 1995; Wang and Fong, 1995].

Despite the wide distribution of ADRP in various cell types, little is known about its function in lipid droplet storage. The association of ADRP with tiny or nascent lipid droplets and the association of perilipin, but not ADRP, with large droplets in adipocytes 3 days after initiation of differentiation led Brasaemle et al. [1997] to propose that ADRP may be responsible for the nucleation of minute lipid droplets. Our results, however, reveal that ADRP is consistently found on the surface of small and large lipid droplets in lipid-laden macrophages. The discrepancy may be due to the fact that the diameter of large lipid droplets in differentiated adipocytes usually exceeds 20 μm, while "large" lipid droplets in RAW 264.7 macrophages rarely have diameters exceeding 4 μm. In the present study, the enlargement of lipid droplets caused by CAL cotreatment in RAW 264.7 macrophages is probably dependent on ADRP expression, as evidenced by an increased protein level of ADRP. More recently, ADRP expression was shown to accelerate the initial uptake rate of long-chain, but not short-chain, fatty acids in COS-7 cells transfected with ADRP cDNA [Gao and Serrero, 1999] and this fact, together with the observation that ADRP is localized near the cell membrane of transfected COS-7 cells, led Gao and Serrero [1999] to propose that ADRP is involved in carrier-mediated fatty acid uptake. Evidence in support of the postulate that ADRP may act as a fatty acid binding protein was that purified recombinant mouse ADRP binds fatty acid in a stoichiometric manner. Interestingly, in mouse steroidogenic 1246 cells, ADRP expression is stimulated by long-chain fatty acid [Gao et al., 2000] possibly through by activating a PPAR receptor [Spiegelman, 1998] or stabilizing its expression by post-translational mechanisms. These results may suggest that ADRP expression can be positively regulated by fatty acid internalized by the cells.

The close association of ADRP with both small and large lipid droplets suggests that ADRP expression is essential to lipid droplet formation. This suggestion was further supported by the finding that the induction or down-regulation of ADRP in RAW 264.7 macrophages was reversibly regulated, respectively, by lipid accumulation or lipid hydrolysis. In fact, ADRP was hardly detectable in either lipid droplet-deprived cells induced by SE removal or in normal untreated cells. Thus, stimulation of lipid droplet formation is accompanied by newly expressed ADRP. Recently, ADRP has also been demonstrated to bind cholesterol and may be important in moving cholesterol to the droplet for esterification [Atshaves et al., 2000; Frolov et al., 2000]. However, the exact temporal relationship between the first appearance of lipid droplets and ADRP expression requires further investigation. Whether the effects of PKC on stimulating ADRP expression and lipid droplet accumulation is mediated by regulating the interaction between ADRP and cholesterol or fatty acid remains to be determined.

Unlike ADRP, the 160 kDa capsular protein and perilipin are translocated from the surface of lipid droplets to the cytosol during CAL-

induced steroidogenesis, as shown by the loss of immunoreactivity around lipid droplets and increased cytoplasmic staining intensity [Fong and Wang, 1997]. This detachment of lipid droplet-associated proteins is accompanied by a reduction in the total area of cytoplasmic lipid droplets, implying a possible correlation between capsular protein dissociation and steroidogenesis [Fong and Wang, 1997]. Surprisingly, ADRP is never found to dissociate from the lipid droplet surface in adrenal cells actively involved in steroidogenesis (unpublished data). Thus, the functions of individual lipid droplet-associated proteins in steroidogenesis require further exploration. RAW 264.7 macrophages seem to be a simple model to study the regulation of ADRP in lipid metabolism.

The modulation of scavenger receptors by lipoproteins and cellular cholesterol [Han and Nicholson, 1998; Han et al., 1999] and the regulation of these receptors by PKC in smooth muscle cells and the THP-1 human monocyte cell line indicate the involvement of PKC in scavenger receptor-mediated uptake of lipoproteins [Via et al., 1989; Pitas et al., 1992; Mietus-Snyder et al., 1997]. Enhanced expression and transport of macrophage scavenger receptors can be modulated by okadaic acid through phosphorylation/dephosphorylation of cellular proteins [Fong, 1996]. These results demonstrate that PKC may mediate lipoprotein internalization by activation of scavenger receptor mRNA and phosphorylation of cellular proteins or even direct phosphorylation of receptors themselves. However, whether PKC is involved in the subsequent formation of lipid droplets has not been studied. Our results demonstrate that PKC simultaneously up-regulated both ADRP expression and lipid droplet accumulation, suggesting a role of PKC in the increased ADRP expression as well as in the expression of surface scavenger receptor. Similar effect of PKC activation on lipid body formation induced by *cis*-unsaturated fatty acids has been demonstrated in neutrophils [Weller et al., 1991]. In their study, however, PMA treatment of neutrophils induced lipid body formation, although in a lower degree than those induced by PMA and fatty acids. Moreover, inhibition of PKC inhibited fatty acid-induced lipid body formation. In our study, the ADRP level of macrophages treated with PMA alone was nearly identical to that of normal untreated cells which accumulated very few lipid droplets. It is plausible that PKC activity is regulated differentially in different cell types. Nevertheless, these results indicate that PKC is involved in regulating lipid metabolism in different cell types.

In contrast to extensive data showing the important involvement of the cAMP/PKA system in steroidogenesis and in lipid droplet accumulation in macrophages, little is known about the involvement of PKC in these processes. Activation of PKC has been shown to inhibit production of progesterone and testosterone in cultured rat granulosa and Leydig cells [Welsh et al., 1984; Trzeciak et al., 1987], and inhibition of PKC in Y-1 cells results in increased steroidogenesis [Reyland, 1993]. However, Hartigan et al. [1995] found that the production of glucocorticoid by cells in the zona fasciculata of the adrenal glands was increased following PKC activation. In the present study, we demonstrated that alteration of PKC activity by activation or inhibition resulted in the accelerated accumulation, but not hydrolysis, of lipid droplets in macrophages. Whether the conflicting data on the effects of PKC on steroidogenic cells and macrophages are due to the different cell types used requires further study. However, the data presented in this study are not the sole evidence that inhibition and activation of PKC give rise to similar results, since treatment of adrenocortical cells with either PMA or CAL stimulates steroidogenesis, although to differing degrees (unpublished data). It is possible that different PKC isoforms are activated by PMA and CAL, respectively.

Although much effort has been expended on studies of the mechanism by which macrophages take up and metabolize lipoproteins, little is known about the structural characteristics of lipoprotein-derived lipid droplets in macrophage foam cells. Our study therefore focused on the expression and regulation of the lipid droplet-associated protein, ADRP, in the hope that this would provide some insights into the structural properties of lipid droplets and the mechanisms that regulate lipid droplet formation. The findings that ADRP is associated with lipid droplets and that its expression is regulated by PKC in SE-laden macrophages, an in vitro model for foam cells, provide a new insight into the possible role of ADRP in lipid metabolism.

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