Redistribution of macrophage cholesteryl ester hydrolase from cytoplasm to lipid droplets upon lipid loading[1]

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Abstract Hydrolysis of intracellular cholesteryl esters (CEs) represents the first step in the removal of cholesterol from lipid-laden foam cells associated with atherosclerotic lesions. Neutral cholesteryl ester hydrolase (CEH) catalyzes this reaction, and we recently cloned the cDNA for the human macrophage CEH and demonstrated increased mobilization of intracellular CE droplets by CEH overexpression. The present study was undertaken to test the hypothesis that for CE hydrolysis, CEH must become associated with the surface of the cytoplasmic lipid droplets. Our data show the redistribution of CEH from cytosol to lipid droplets upon lipid loading of human THP-1 macrophages. Depletion of triacylglycerol (TG) by incubation with the acyl-CoA synthetase inhibitor Triacsin D had no effect on CEH association with the lipid droplets, suggesting that CEH associates with mixed (CE + TG) as well as TG-depleted CE droplets. However, CEH had 2.5-fold higher activity when mixed droplets were used as substrate in an in vitro assay, consistent with the reported higher cholesterol efflux from cells containing mixed isotropic droplets. Perilipin as well as adipophilin, two lipid droplet-associated proteins, were also present on the lipid droplets in THP-1 macrophages. In conclusion, CEH associates with its intracellular substrate (lipid droplets) and hydrolyzes CE more efficiently from mixed droplets.—Zhao, B., B. J. Fisher, R. W. St. Clair, L. L. Rudel, and S. Ghosh. **Redistribution of macrophage cholesteryl ester hydrolase from cytoplasm to lipid droplets upon lipid loading. *J. Lipid Res.* 2005.** 46: **2114–2121.**

Supplementary key words perilipin • adipophilin • THP-1 macrophages

Unregulated uptake of modified LDL by blood monocyte-derived macrophages results in the cytoplasmic accumulation of cholesteryl ester (CE)-rich lipid droplets and the formation of foam cells. Continued generation of foam cells within the intimal wall of the artery results in fatty streak formation, one of the earliest morphological changes associated with atherosclerosis (1). Efficient cholesterol efflux from macrophages is critical for the prevention of foam cell formation and subsequent protection against atherosclerosis. Because there is no significant release of CE from cells, including macrophages, for efflux to occur, CE must first be hydrolyzed to free cholesterol. The obligatory first step in reverse cholesterol transport, therefore, is the hydrolysis of CE to release free cholesterol, a reaction catalyzed by a neutral cholesteryl ester hydrolase (CEH). Because the rate of CE hydrolysis is often slower than free cholesterol movement to the extracellular acceptor, CE hydrolysis is increasingly being recognized as the rate-limiting step in cellular cholesterol efflux (2, 3). Thus, macrophages with high neutral CEH activity do not accumulate CE in the presence of atherogenic lipoproteins such as β-VLDL, compared with macrophages with low CEH activity (4). In addition, animal models of atherosclerosis, such as the hypercholesterolemic rabbit and the White Carneau pigeon, appear to possess macrophages in which stored CE is resistant to hydrolysis and subsequent mobilization (5, 6).

Ghosh (7) recently reported the cloning and characterization of the human macrophage CEH cDNA and demonstrated the expression of CEH mRNA in the human monocyte/macrophage cell line THP-1 as well as in human peripheral blood monocyte-derived macrophages. The rate-limiting role of this enzyme in intracellular CE mobilization was confirmed by the observed concentration-dependent decrease in intracellular lipid droplets and cellular CE mass after overexpression (8). Although this enzyme hydrolyzes CEs presented as lipid droplets in vitro and effectively mobilizes intracellular CE droplets, a direct association of CEH with cytoplasmic lipid droplets has not been demonstrated.

Lipid droplets are spherical organelles found in many types of eukaryotic cells; they are composed of a core of neutral lipids covered by a monolayer of phospholipids,

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free cholesterol, and proteins. The lipid droplets present in adipocytes are well characterized as containing a core of triglycerides and surface proteins. In preadipocytes and early differentiated adipocytes, adipose differentiation-related protein (ADRP) is found associated with small lipid droplets. With maturation, perilipin A becomes the most abundant protein on the surface of large lipid droplets; ADRP is undetectable in mature adipocytes (9). Perilipin A functions to increase cellular triglyceride storage by decreasing the rate of triglyceride hydrolysis (10, 11). Furthermore, perilipin A is phosphorylated by cAMP-dependent protein kinase and serves an additional role in controlling the hormone-stimulated lipolysis in adipose tissue (12–14) by regulating the activity of hormone-sensitive lipase. Although perilipin A is most abundant, the other isoforms, perilipins B and C, occur primarily in adipose and steroidogenic cells, respectively (9).

Little is known about the lipid droplet-associated proteins in macrophage foam cells and whether the hydrolysis of stored CEs is regulated by these lipid droplet-associated proteins. In murine macrophages (RAW264.7), Chen et al. (15) demonstrated the association of ADRP with the cytoplasmic lipid droplets (both small and large) and the concomitant increased expression of ADRP with lipid droplet accumulation. However, the presence of neither perilipin nor adipophilin, the human ortholog of ADRP, is described in human blood monocyte-derived macrophages or in THP-1 cells. Furthermore, it is not known whether CEH associates with cytoplasmic lipid droplets in a manner similar to hormone-sensitive lipase association with lipid droplets in adipocytes, a process regulated by perilipin A (16).

The present study was undertaken to determine the subcellular localization of CEH in human macrophages and to gain insight into the association of CEH with its physiological substrate (CE stored as cytoplasmic lipid droplets). We show here the redistribution of CEH from cytoplasm to lipid droplets upon lipid loading, demonstrating the association of CEH with its intracellular substrate, an obligatory step for the subsequent hydrolysis of CE present in the lipid droplets. Furthermore, CEH activity is affected by the presence or absence of triacylglycerol (TG) associated with CE in the intracellular lipid droplets. In addition, these data also show, for the first time, the association of perilipin and adipophilin with lipid droplets in lipid-laden THP-1 cells.

MATERIALS AND METHODS

Materials

THP-1 and HEK293T cells were obtained from ATCC (Manassas, VA). Acetylated LDL (AcLDL) was purchased from Intracel, Inc. (Frederick, MD). 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-23,24-bisnor-5-cholen-3-ol (NBD-cholesterol), Alexa® 546 conjugated goat anti-rabbit IgG, and Alexa® 488 conjugated goat anti-guinea pig IgG were from Molecular Probes (Eugene, OR). Peroxidase-labeled goat anti-guinea pig IgG was obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD), and HRP-conjugated goat anti-rabbit IgG was from Bio-Rad (Hercules, CA). Guinea pig polyclonal antibodies to adipophilin and perilipin that cross-react with human, mouse, and rat were purchased from Research Diagnostics, Inc. (Flanders, NJ), and mouse monoclonal antibodies to Na^+K^+ATPase were from Santa Cruz Biotechnology (Santa Cruz, CA). The TNT™ Quick Coupled transcription/translation system and canine microsomes were from Promega (Madison, WI). [^{35}S]methionine, cholesteryl [1-^{14}C]oleate, and Western Lightning Chemiluminescence Reagent Plus were from New England Nuclear (Boston, MA). Phorbol 13-myristate, 12-acetate (PMA) and BSA were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). All other reagents used were of analytical grade and obtained from Fisher Scientific (Pittsburgh, PA).

Cell culture

THP-1 cells were maintained in RPMI-1640 medium containing 10% FBS according to the instructions supplied. For induction of macrophages, PMA (100 nM) was added to the medium and the cells were seeded at a density of 0.1×10^6 cells/cm^2 into tissue culture dishes and maintained in a humidified atmosphere of 95% air and 5% CO_2. Media containing PMA were replaced every 2 days, and experiments were started after 5–7 days in culture, when the cells were phenotypically macrophage (7). THP-1 macrophage cells were lipid loaded by incubation with AcLDL (50 μg/ml) for 48 h. In some studies, the loading medium was also supplemented with NBD-cholesterol (5 μg/ml) to fluorescently label the intracellular lipid droplets. Nonloaded cells were maintained in lipoprotein-deficient serum for the same period. HEK293T cells were maintained and propagated in DMEM supplemented with 10% FBS according to the guidelines provided.

In vitro translation

The human macrophage CEH expression vector, pCMV-CEH, described previously (8), was used for in vitro transcription/translation using the TNT™ Quick Coupled system with or without added canine microsomes according to the manufacturer's protocol. [^{35}S]methionine was included to label the newly synthesized proteins. At the end of the incubation, half of the reaction was subjected to proteinase K (200 μg/ml) digestion (30 min on ice) to assess the susceptibility of newly synthesized protein to proteolytic degradation (17). Cytosolic proteins are susceptible to degradation, and proteins localized in the endoplasmic lumen are protected under these conditions. Core glycosylation control RNA (provided in the kit) was used as a positive control to assess the functionality of the added microsomes. The reaction products were separated on a 12.5% SDS-PAGE gel. The gel was dried and subjected to autoradiography.

Immunocytochemistry

Cells were plated on fibronectin-coated Lab-Tek Cover Glass chambers. Three to 5 days after plating and specified treatments, the culture media were aspirated and cells were fixed in 3.7% paraformaldehyde in PBS for 10 min at 4°C. This is the method of choice for fixation because the cells retain their lipid content and the lipid droplet structure is unaffected (18). Cells were permeabilized by exposure to 0.15% Triton X-100 in PBS for 3 min at 4°C, washed with PBS, and blocked with 0.5% BSA in PBS for at least 1 h at room temperature. Cells were then incubated with primary antibody overnight at 4°C. Cells incubated in the absence of primary antibody were used as negative controls. For detection, cells were subsequently incubated with either Alexa Fluor® 546 or Alexa Fluor® 488 conjugated secondary antibody in 0.5% BSA for 1 h at room temperature. Cells were washed three times with PBS and once with distilled water. Cells were imaged at 90× magnification using an Olympus model IX70 inverted phase microscope fitted with a MagnaFire™ digital camera

using either a tetramethylrhodamine-5-(and-6)-isothiocyanate [5(6)] (TRITC) filter cube (530–560 nm excitation, 590–650 nm emission; Chroma Technology Corp.) or a FITC filter cube (460–500 nm excitation, 510–560 nm emission). Although highly cross-adsorbed secondary antibodies were used, additional negative controls included the use of cross-species secondary antibodies (e.g., the use of Alexa Fluor® 488 conjugated anti-guinea pig secondary antibody with rabbit polyclonal antibody to CEH and the use of Alexa Fluor® 546 conjugated anti-rabbit antibody with guinea pig polyclonal antibody to perilipin or adipophilin). No cross-species reactivity was observed.

Isolation of lipid droplet-associated proteins

THP-1 cells (8×10^6 cells) were plated in 100 mm tissue culture dishes in the presence of PMA. After 3 days of differentiation, one set was lipid loaded using AcLDL (50 μg/ml) for 48 h. After a wash with cold PBS, cells were harvested and lysed by sonication in 200 mM phosphate buffer containing 0.25 M sucrose, 80 mM KCl, 5 mM 2-mercaptoethanol, and protease inhibitors, as described previously (7). Cell lysates were centrifuged at 100,000 *g* for 1 h to separate the cytosolic and total membrane fractions. The intracellular lipids floating on top of the cytosolic fraction were carefully collected and washed twice with the homogenizing buffer by centrifuging at 100,000 *g* for 1 h. Total proteins associated with equal volumes of lipid fraction from nonloaded and AcLDL-loaded cells were precipitated with TCA (final concentration, 10%) at 4°C for 1 h instead of the 24 h acetone precipitation described earlier (19). The protein precipitates were dissolved in the harvesting buffer and immediately neutralized to pH 7.4. Protein concentration was determined using the Bio-Rad protein assay kit.

Western blot analyses

An aliquot containing 10–20 μg of total protein was separated on a 4–20% SDS-PAGE gel, and proteins were transferred to a polyvinylidene difluoride membrane. After a brief wash in TBS and blocking in 5% nonfat dry milk, the blots were incubated with primary antibody appropriately diluted in 5% nonfat dry milk overnight at 4°C. After three to five washes in TBS, the blots were subsequently incubated with secondary antibody appropriately diluted in 5% nonfat dry milk for 1 h at room temperature. After three to five washes in TBS, the blots were developed using Lightning Chemiluminescence Reagent Plus and exposed to X-ray film. Densitometric analyses were performed using Kodak 1D Image Analysis Software.

Triglyceride depletion

THP-1 macrophages were loaded with lipid using AcLDL as described above. Incubation with the acyl-CoA synthetase inhibitor Triacsin D and albumin was used to modulate the cellular triglyceride concentration, as described previously (20). In brief, after 48 h of loading, the AcLDL-containing medium was removed, and cells were washed with medium and then incubated with medium containing 12.5 μM Triacsin D and 400 μM BSA for 24 h. Under these conditions, >90% of cellular triglyceride was removed without any significant decrease in the levels of cellular CE content, and CE was present as anisotropic intracellular droplets (20). Lipid droplet-associated proteins were isolated as described above.

CEH activity measurements

HEK 293T cells were plated in six-well tissue culture dishes (6×10^5 cells/well) and transfected with either the empty vector pCMV or the CEH expression vector pCMV-CEH, as described previously (8). Cell lysates were prepared, and CEH activity was determined using a radiometric assay (21). Cholesteryl [1-^{14}C] oleate was used as the radioactive substrate at a final concentration of 75 μM and presented in the reaction tube as droplets in acetone, determined to be the most suitable mode of substrate presentation (22). To measure CEH activity toward a mixed droplet substrate, triolein (60%) and cholesteryl oleate (40%), based on the composition determined by Lada, Willingham, and St. Clair (20) for isotropic droplets, were mixed, dried under nitrogen, dissolved in acetone, and used at the same final concentration in the assay (75 μM) as pure cholesteryl oleate.

RESULTS

Reconfirmation of the cytosolic localization of CEH

Intracellular CEs are stored as cytoplasmic lipid droplets and hydrolyzed by the neutral cytosolic CEH. To determine whether CEH encoded by the human macrophage cDNA is associated with the cytoplasm or lumen of the endoplasmic reticulum, as predicted by its high degree of homology with members of the carboxylesterase family, it was transcribed and translated in vitro in the absence or presence of microsomes. Resistance to proteinase K digestion was used as a measure of the movement of newly synthesized protein into the lumen of microsomes (17). As seen in **Fig. 1**, newly synthesized CEH remained susceptible to proteinase K in the presence of microsomes (lane 4), demonstrating the lack of CEH movement into the lumen of the microsomes and reconfirming the cytosolic localization of neutral CEH encoded by cDNA isolated from human macrophages. Control plasmid expressing a protein that is glycosylated by the microsomes was used as a positive control for assessing the functionality of the microsomes. In the absence of canine microsomes, a single protein was synthesized (Fig. 1, lane 5). This precursor was processed into higher molecular weight glycosylated pro-

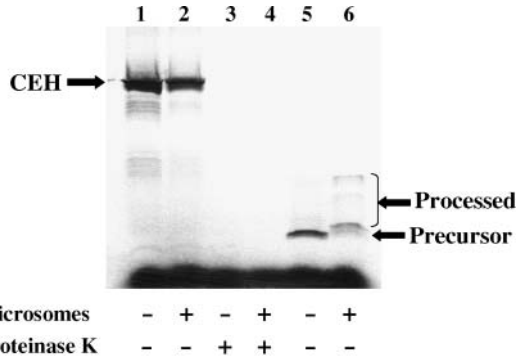

Fig. 1. Reconfirmation of the cytosolic localization of cholesteryl ester hydrolase (CEH). pCMV-CEH plasmid DNA (500 ng/reaction) was translated using the TNT™ Quick Coupled system with (lanes 2, 4) or without (lanes 1, 3) added canine microsomes, as described in Materials and Methods. At the end of the 90 min incubation, half of the reaction was subjected to proteinase K (200 μg/ml) digestion for 30 min on ice (lanes 3, 4). The reaction was stopped by the addition of PMSF (final concentration, 10 mM), and proteins were separated by SDS-PAGE. The gel was dried and subjected to autoradiography. Lane 5 shows the translated product using a control plasmid expressing a core glycosylated protein. This precursor is processed into higher molecular mass glycosylated proteins in the presence of microsomes (lane 6).

teins in the presence of microsomes (Fig. 1, lane 6), confirming the functionality of the canine microsomes used.

Redistribution of CEH upon lipid loading of THP-1 macrophages with AcLDL

CEH was immunolocalized within THP-1 macrophages that were either nonloaded or lipid loaded with AcLDL. In both sets, NBD-cholesterol was included in the culture medium to fluorescently label the intracellular CE droplets. Indirect immunofluorescence imaging revealed that in nonloaded cells, CEH was uniformly distributed within the cytoplasm of the cell [**Fig. 2**, diffuse red fluorescence associated with the two large cells (marked with arrows) and two small cells (marked with S→)], with no apparent lipid droplets in the cytoplasm (diffuse green fluorescence attributable to NBD-cholesterol). In contrast, in the presence of AcLDL, THP-1 macrophage cells accumulated large amounts of cholesterol visible as fluorescent (green) intracellular CE droplets (marked with D→) in the cytoplasm (marked with C→). In these cells, CEH was not uniformly distributed in the cytoplasm but, instead, localized encircling the NBD-cholesterol-labeled cytoplasmic lipid droplets, indicating redistribution of CEH upon lipid loading in these cells. The movement of CEH from the cytosol to lipid droplets is clearly evident from the CEH overlay on the phase-contrast image, in which the peripheral cytoplasm (marked with C→) of the cell shows negligible staining for CEH. However, the association of CEH with very small cytoplasmic droplets in cells not loaded with AcLDL cannot be completely ruled out, and Western blot analyses were used to determine the distribution of CEH between the cytoplasm and lipid droplets.

Confirmation of intracellular localization of CEH by Western blot analyses

To further confirm the localization of CEH in nonloaded and lipid-loaded cells, cytoplasmic and lipid droplet fractions were isolated and examined for CEH protein content by Western blot analyses using rabbit polyclonal antibody to CEH, as described previously (8). As seen from the representative Western blots in **Fig. 3B**, CEH protein is primarily associated with the cytoplasmic fraction in nonloaded cells (No AcLDL), with only a minor amount associated with the lipid-droplet fraction (Fat). Upon lipid loading, this distribution is reversed, with CEH being predominantly associated with the lipid droplet fraction. The relative distribution of immunoreactive CEH protein in these fractions was determined by densitometric analyses of the Western blots and is shown in Fig. 3A. In nonloaded cells (No AcLDL), ~72 ± 1.75% of CEH protein was associated with the cytosolic fraction and 28 ± 1.75% was associated with the lipid droplets. Upon lipid loading with AcLDL, this distribution was essentially reversed, and 24 ± 8.58% of CEH protein was associated with the cytosolic fraction and 76 ± 8.58% was associated with the lipid droplets. Together with the immunocytochemical detection of CEH in THP-1 cells described above, these data clearly demonstrate the redistribution of CEH from the cytoplasm to the lipid droplets upon lipid loading. Possible contamination of the lipid droplet fraction with plasma membrane vesicles was ruled out by analyzing for the presence of the plasma membrane marker Na^+K^+ATPase. As seen from the representative blots in Fig. 3C, no immunoreactivity was observed in the lipid droplet fraction (Fat), although a strong immunoreactive band was

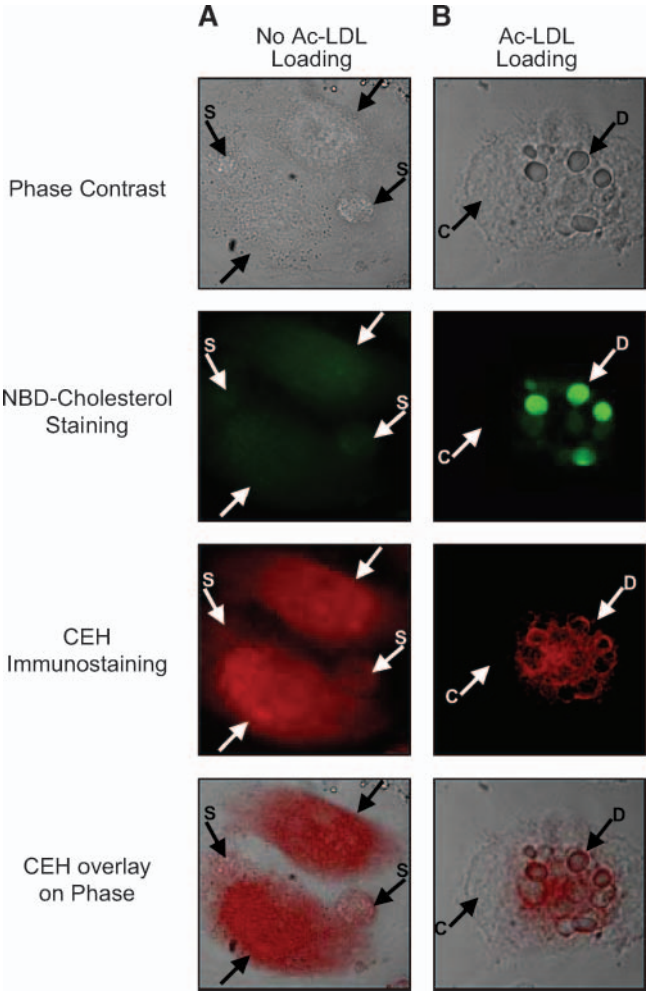

Fig. 2. Redistribution of CEH upon lipid loading. THP-1 macrophages, with or without lipid loading using AcLDL, were fixed and processed for immunocytochemistry, as described in Materials and Methods. Representative images taken at 90× magnification are shown. The left panels show the cells without AcLDL, and the right panels show cells incubated with AcLDL. Two large cells (marked with arrows) and two small, rounded cells (marked with S→) are visible in the left panels (No Ac-LDL). A single cell is shown in the right panels (Ac-LDL) showing distinct large lipid droplets (marked with D→) in the cytoplasm (marked with C→). Intracellular lipid droplets were visualized by monitoring the NBD-cholesterol fluorescence (green). Intracellular CEH was stained using anti-CEH rabbit polyclonal antibodies and Alexa Fluor® 546 conjugated goat anti-rabbit IgG (red). The phase-contrast images were overlaid with CEH immunostaining (red) to show the redistribution of CEH upon lipid loading. Note the uniform distribution of the red stain in the cells in the left panel (No Ac-LDL) and the absence of CEH (red) staining from the peripheral cytoplasm with concomitant increased red staining surrounding the lipid droplets in the right panel (Ac-LDL).

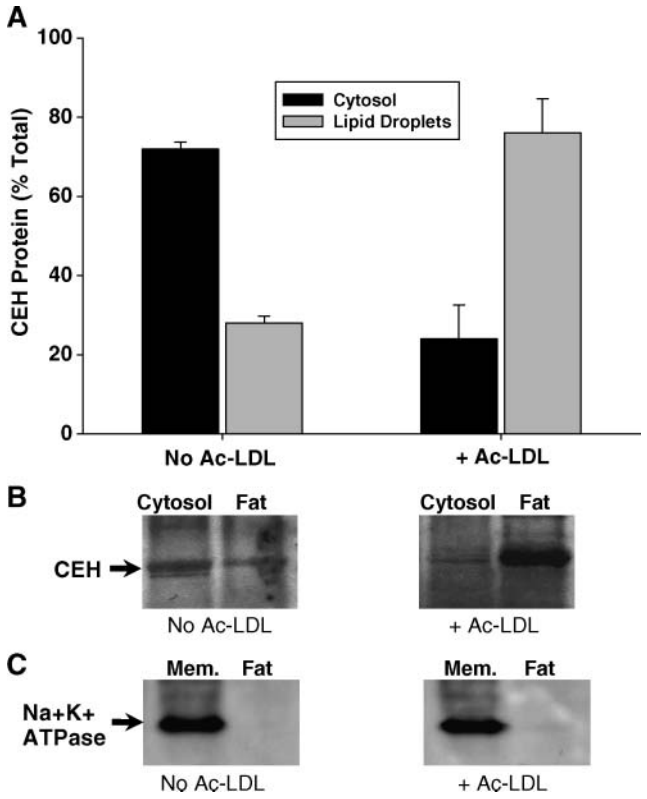

Fig. 3. Association of CEH with isolated lipid droplets. For Western blot analyses, lipid droplet-associated proteins were isolated from the same volume of floating lipid layer from THP-1 macrophages (8×10^6 cells) cultured in the absence or presence of AcLDL and resuspended in 50 μl. Equivalent amounts of cytosol and lipid droplet-associated proteins (Fat) from cells not loaded with AcLDL (No Ac-LDL) and those loaded with AcLDL (+Ac-LDL) were separated by SDS-PAGE and processed for Western blot analysis using anti-CEH rabbit polyclonal antibodies. The immunoreactive band at ~66 kDa was quantified densitometrically. A: Distribution of CEH in cells loaded with AcLDL and nonloaded cells (No Ac-LDL). Data represent means ± SD of three independent experiments. B: Representative Western blot. Lipid droplet-associated proteins were also analyzed for the presence of the plasma membrane marker Na^+K^+ATPase using mouse monoclonal antibodies. C: Representative Western blot. Total membrane fraction (Mem.) from the same cells was used as a positive control.

present in the membrane fraction from the same cells (Mem.).

Effect of TG depletion on CEH association with intracellular lipid droplets

Incubation of THP-1 cells with AcLDL results in the accumulation of both CEs and TG, and TG can greatly influence the physical state of CE. To determine whether the association of CEH to intracellular lipid droplets is affected by the TG content of the droplets, cellular TGs were selectively depleted by incubation of loaded cells with Triacsin D, as described previously (20). Lipid droplet fractions were examined by Western blot analyses for the associated CEH protein. As shown in **Fig. 4**, depletion of cellular TG did not affect the association of CEH with lipid droplets, suggesting that CEH associates with mixed (TG + CE) as well as TG-depleted CE droplets within the cell.

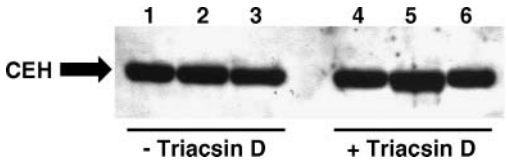

Fig. 4. Association of CEH with lipid droplets after cellular triacylglycerol (TG) depletion. Lipid droplet-associated proteins were isolated from AcLDL-loaded THP-1 macrophages without (lanes 1–3) or with TG depletion (lanes 4–6) by incubation with Triacsin D for 24 h. Ten micrograms of total protein from each of the samples was separated by SDS-PAGE and processed for Western blot analysis using anti-CEH rabbit polyclonal antibodies.

CEH activity toward CE droplets versus mixed (CE + TG) droplets as substrates

CEs present as mixed droplets along with TG are in a liquid or isotropic state and are effluxed more rapidly from cells than CEs present as liquid crystalline or anisotropic droplets without much TG (20). To determine the activity of CEH toward pure CE droplets versus mixed CE + TG droplets, HEK293T cells were transfected with an expression vector for human macrophage CEH (pCMV-CEH), and cell lysates were assayed for CEH activity using either cholesteryl oleate alone (representing pure CE droplets) or cholesteryl oleate + triolein (representing CE + TG mixed droplets) as substrate. CEH-mediated hydrolysis of cholesteryl [1-^{14}C]oleate presented as mixed cholesteryl oleate + triolein droplets was 2.5-fold higher than with pure cholesteryl oleate droplets (**Table 1**), suggesting that the higher efflux from cells containing mixed droplets is probably the result of the increased hydrolysis of these droplets by intracellular CEH.

Distribution of lipid droplet surface proteins, perilipin, adipophilin, and CEH in THP-1 macrophages

Intracellular lipid droplets found in adipocytes are associated with proteins such as perilipin and adipophilin (the human ortholog of the murine ADRP), and these proteins are thought to play an important role in regulating lipolysis. However, the expression of these or similar lipid droplet-associated proteins in human macrophages remains unclear. Immunofluorescence microscopy was used to determine the expression and association of these two proteins with lipid droplets in lipid-loaded THP-1 macrophages. As shown in **Fig. 5**, adipophilin (top panels) as well as perilipin (bottom panels) were expressed in THP-1 macrophage and were associated with cytoplasmic drop-

TABLE 1. CEH activity toward CE versus CE + TG mixed droplets

Cholesteryl Oleate (CE Droplets)	Cholesteryl Oleate + Triolein (CE + TG Droplets)	Fold Increase
49.7 ± 11.5	133.3 ± 12.1	2.52 ± 0.77

CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; TG, triacylglycerol. HEK 293T cells were transfected with pCMV or pCMV-CEH vector. CEH activity toward cholesteryl oleate or cholesteryl oleate + triolein as substrate was assayed in the cell lysates 48 h after transfection as described in Materials and Methods. Net CEH activity was calculated by subtracting the background activity associated with cells transfected with the empty vector. Values shown are in pmol/h/mg protein (mean ± SD; n = 3).

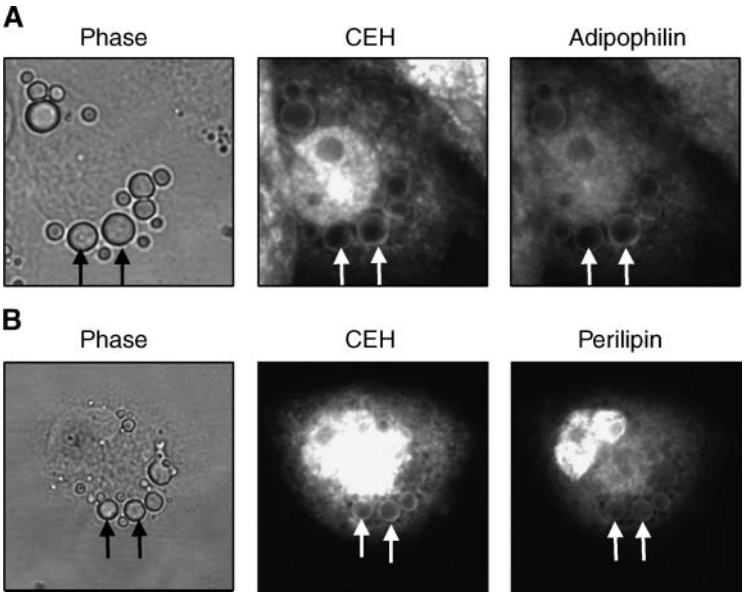

Fig. 5. Presence of CEH, perilipin, and adipophilin on lipid droplets in THP-1 macrophages. THP-1 macrophages grown in coverglass chambers were fixed and processed for immunocytochemistry as described in Materials and Methods. Cells were immunostained for CEH and adipophilin (top panels) or CEH and perilipin (bottom panels) using the corresponding two primary antibodies. For fluorescence detection, Alexa Fluor® 546 conjugated goat anti-rabbit IgG (for CEH detection) and Alexa Fluor® 488 conjugated goat anti-guinea pig IgG (for perilipin or adipophilin detection) were used. Representative single cell images taken at 90× magnification are shown. Lipid droplets and immunostaining of CEH, perilipin, or adipophilin on the surface of the lipid droplets are marked by arrows.

lets along with CEH (see the fluorescent staining visible as white rings around the droplets, marked with arrows). In these double-stained cells, a very bright perinuclear staining was also observed with almost all cells that was not associated with any particular subcellular structure. Negative controls in which cross-species fluorescent secondary antibodies were used did not show any cross-reactivity (absence of any fluorescence; data not shown). These data were confirmed by Western blot analyses of lipid droplet-associated proteins. A single immunoreactive band was obtained using polyclonal antibody to either adipophilin (~50 kDa) or perilipin (~42 kDa) (data not shown). Based on the molecular mass, it appears that perilipin C is expressed in THP-1 macrophages.

DISCUSSION

The data presented here demonstrate, for the first time, the redistribution of neutral CEH from the cytoplasm to lipid droplets in human monocyte/macrophage THP-1 cells (Figs. 2, 3), thereby confirming the association of CEH with its physiological substrate, an obligatory step toward enzymatic hydrolysis of intracellular CE stored as lipid droplets. Consistent with these data, overexpression of human macrophage CEH indeed leads to the mobilization of intracellular lipid droplets and the depletion of cellular CEs reported previously (8). Unlike hormone-sensitive lipase, which associates with the lipid droplets in adipocytes after hormonal stimulation (10), the movement of CEH from the cytoplasm to lipid droplets upon lipid loading does not require any additional stimulation and is probably a spontaneous rather than a regulated step.

Intracellular lipid droplets are heterogenous with respect to their lipid content, and in vitro loading of THP-1 macrophages with AcLDL results in the accumulation of mixed droplets containing triglycerides as well as CEs. Triglycerides greatly influence the physical state of CEs. In the presence of high concentration of triglycerides, CEs become more fluid (isotropic) and are effluxed more rapidly from THP-1 cells (23, 24). Conversely, in the absence of triglycerides, CEs exist in a liquid-crystalline (anisotropic) state thought to be a poor substrate for CE hydrolysis. Lada, Willingham, and St. Clair (20) used Triacsin D to reduce triglyceride levels in AcLDL-loaded THP-1 macrophages and showed a reduction in cholesterol efflux (20) indicative of decreased CE hydrolysis. Under similar conditions in the current study, we found a continued association of CEH with intracellular lipid droplets after TG depletion, demonstrating no apparent difference in the association of CEH with TG-depleted CE droplets and CE + TG droplets. However, CEs of droplets containing cholesteryl oleate and triolein were hydrolyzed by CEH 2.5 times faster than CEs of pure cholesteryl oleate droplets when assayed in vitro, providing direct evidence for reduced CEH-mediated hydrolysis and subsequent mobilization of CEs present in cells with low TG levels. Because very low levels of TGs are present in atherosclerotic lesions, reduced CEH-mediated hydrolysis of the liquid-crystalline TG-poor, CE-rich droplets and consequently attenuated free cholesterol efflux could plausibly result in increased CE accumulation in the lesions.

Our data also identify the presence of two other lipid droplet-associated proteins, perilipin and adipophilin, on the cytoplasmic lipid droplets of THP-1 macrophages. Perilipins are a family of three protein isoforms, encoded by a single gene, that are believed to be localized exclusively to lipid droplets in adipocytes (25) and steroidogenic cells (26). Perilipin A (~61 kDa) is the most abundant isoform in both cell types; perilipin C (~42 kDa) is considered unique to steroidogenic cells; low levels of perilipin B (~46–48 kDa) are present in both cells types. No reports are available regarding the presence of perilipin isoforms in monocytes/macrophages. Using immunofluorescence, we showed the presence of perilipin on the surface of lipid droplets in THP-1 macrophages (Fig. 5). Based on the mo-

lecular mass of the single immunoreactive band detected by Western blot analysis, it appears that only perilipin C is expressed in these macrophages. Although perilipin A regulates lipolysis in adipocytes, based on the detection of perilipin C on the surface of lipid droplets present in steroidogenic tissues, Servetnick et al. (26) suggested the presence of a direct metabolic link between CE hydrolyzing enzyme and perilipin C. Based on these findings and the data presented here, it may be speculated that the levels of perilipin in human THP-1 macrophages might be involved in regulating intracellular CE hydrolytic activity. Future studies measuring CE hydrolysis after the inhibition of perilipin expression in THP-1 cells could provide proof for this hypothesis. It is noteworthy, however, that ruptured plaques characterized by a large lipid core (in other words, decreased CE mobilization) express perilipin that is completely absent from stable plaques (27).

Similar to the expression of perilipin, our data also demonstrate the association of adipophilin protein with the lipid droplets present in human THP-1 macrophages. Wang et al. (28) reported induced expression of adipophilin mRNA by oxidized LDL in human macrophages and atherosclerotic lesions and suggested an association between adipophilin expression and lipid accumulation in foam cells of atherosclerotic lesions. In support of this view, Larigauderie et al. (29) showed increased lipid accumulation and attenuated lipid efflux after ectopic expression of adipophilin in THP-1 cells. Thus, it appears that the level of adipophilin expression may also play a role in regulating intracellular lipid accumulation and free cholesterol efflux. Its association with lipid droplets along with CEH in THP-1 cells raises the possibility of the potential regulation of CEH activity by intracellular levels of adipophilin.

In conclusion, human macrophage CEH, which is required for the mobilization of intracellular lipid droplets and the reduction of cellular CE stores, redistributes upon lipid loading and associates with cytoplasmic lipid droplets. Such an association places this enzyme in close proximity with its physiological substrate. Although the presence or absence of TG does not affect the association of CEH with lipid droplets, CEH activity is significantly higher with mixed CE + TG droplets, indicating the regulation of CEH-mediated hydrolysis by the composition, and thus the physical state, of the intracellular lipid droplet. With hydrolysis of intracellular CE being recognized as the rate-limiting step in cholesterol efflux, this observed effect of TG on CEH-mediated hydrolysis is consistent with previous reports of increased cholesterol efflux from cells containing mixed or isotropic lipid droplets and decreased cholesterol efflux from cells undergoing TG depletion (20). Although the presence of other proteins, such as perilipin and adipophilin, was also demonstrated by these studies, the role of these two droplet-associated proteins in regulating the activity of CEH and the resulting effect of cellular cholesterol efflux remains to be evaluated.

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