Decreased lipid efflux and increased susceptibility to cholesterol-induced apoptosis in macrophages lacking phosphatidylcholine transfer protein

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Macrophages are the predominant cellular component of atherosclerotic lesions, where they scavenge oxidatively modified lipoproteins while defending themselves against cholesterol-induced cytotoxicity by adaptive mechanisms that depend in part on the synthesis, distribution and efflux of phosphatidylcholines. PC-TP (phosphatidylcholine transfer protein) is a START (steroidogenic acute regulatory protein-related lipid transfer) domain protein that catalyses the intermembrane transfer of phosphatidylcholines and promotes apolipoprotein AI-mediated lipid efflux when overexpressed in the cytosol of Chinese-hamster ovary cells. To explore a role for PC-TP in the adaptive responses of macrophages to cholesterol loading, we utilized peritoneal macrophages from mice with homozygous disruption of the gene encoding PC-TP ($Pctp^{-/-}$) and wild-type littermate controls. PC-TP was abundantly expressed in macrophages from wild-type but not $Pctp^{-/-}$ mice. In cholesteryl ester-loaded macrophages from $Pctp^{-/-}$ mice, the apolipoprotein AI-mediated efflux of phospholipids and cholesterol was decreased. This could be attributed to proportional decreases in the expression levels of ATP-binding cassette A1. Also, in response to free cholesterol loading, the absence of PC-TP from macrophages was associated with marked increases in apoptotic cell death. These findings suggest that PC-TP in macrophages may serve an atheroprotective role by defending against cholesterol-induced cytotoxicity.

Key words: apolipoprotein AI, atherosclerosis, cholesterol, macrophage, mice, phosphatidylcholine transfer protein.

INTRODUCTION

Atherosclerotic lesions are populated by macrophages, which ingest modified lipoproteins through scavenger receptor-dependent pathways [1]. Since scavenger receptors are not down-regulated in response to cholesterol accumulation [1], macrophages utilize alternative mechanisms to defend against the cytotoxicity induced by excess FC [free (unesterified) cholesterol] [2,3]. Much of the cholesterol is esterified due to the activity of ACAT (acyl-CoA:cholesterol acyltransferase) and sequestered into cytoplasmic lipid droplets. Macrophages also eliminate cholesterol by efflux through ABCA1 (ATP-binding cassette protein A1) and ABCG1 [3–5].

In the face of ongoing lipoprotein internalization, the capacity of macrophages to defend against cytotoxicity by cholesteryl esterification and efflux is eventually overwhelmed, as reflected by increases in FC content and cellular degradation of ABCA1 [3,6]. Under these circumstances, macrophages adapt by up-regulating the *de novo* synthesis of phosphatidylcholines, which are assembled into intracellular lamellar structures that sequester FC and delay the onset of cell death [3,7].

PC-TP (phosphatidylcholine transfer protein) is a 25 kDa soluble lipid transfer protein that catalyses the intermembrane transfer of phosphatidylcholines between model membranes *in vitro* [8]. PC-TP is a member of the START (steroidogenic acute regulatory protein-related lipid transfer) domain protein superfamily [9] that binds phosphatidylcholine molecules exclusively [10]. Overexpression of PC-TP in CHO cells (Chinese-hamster ovary cells) promotes ABCA1-mediated pre-β HDL (high-density lipoprotein) formation [11].

On the basis of our observation that PC-TP is expressed in cultured mouse peritoneal macrophages, we utilized mice with homozygous disruption of the gene encoding PC-TP ($Pctp^{-/-}$) to explore its role(s) in lipid efflux from cultured macrophages after loading with CE (cholesteryl ester) and FC, as well as in the cellular adaptation to FC-induced cytotoxicity. Our results demonstrate that the absence of PC-TP from CE-loaded macrophages is associated with decreased apoAI (apolipoprotein AI)-mediated lipid efflux due to decreased expression of ABCA1, as well as increased susceptibility to FC-induced apoptotic cell death. These findings suggest an important function of PC-TP in the defence of macrophages against cholesterol-induced cytotoxicity.

EXPERIMENTAL

Reagents

DMEM (Dulbecco's modified Eagle's medium), Phenol Red-free DMEM, L-glutamine, penicillin/streptomycin solutions, fetal bovine serum and PBS were purchased from Invitrogen (Carlsbad, CA, U.S.A.). ConA (concanavalin A), 9-*cis* retinoic acid, 22(*R*)-hydroxycholesterol, Brefeldin A, a rat anti-mouse β-actin monoclonal antibody and fatty acid-free BSA were purchased from Sigma (St. Louis, MO, U.S.A.). Compound CP-113,818, an inhibitor of ACAT, was a gift from Pfizer (Groton, CT, U.S.A.). Purified human apoAI and polyclonal antibodies specific to mouse

Abbreviations used: ABCA1, ATP-binding cassette protein A1; ACAT, acyl-CoA:cholesterol acyltransferase; LDL, low-density lipoprotein; AcLDL, acetylated LDL; apoAI, apolipoprotein AI; CE, cholesteryl ester; CHOP, C/EBP-homologous protein; ConA, concanavalin A; DMEM, Dulbecco's modified Eagle's medium; FC, free (unesterified) cholesterol; HDL, high-density lipoprotein; HDL_3, subfraction 3 of HDL; LCM, L-cell conditioned medium; LDH, lactate dehydrogenase; LXR, liver X receptor; PC-TP, phosphatidylcholine transfer protein; *Pctp*, gene encoding PC-TP; RXR, retinoid X receptor.

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apoAI and apoE were from Biodesign (Saco, ME, U.S.A.). [1,2-^{3}H(N)]Cholesterol (40–60 Ci/mmol) and [methyl-^{3}H]choline chloride (60–90 mCi/mmol) were from PerkinElmer (Boston, MA, U.S.A.). Polyclonal antibodies directed against mouse ABCA1 were obtained from Novus Biologicals (Littleton, CO, U.S.A.). A polyclonal antibody to PC-TP was prepared as described previously [11]. Multiwell Becton Dickinson Primaria™ plates were from BD Biosciences (San Jose, CA, U.S.A.). Cell-culture dishes and general chemical reagents were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Freshly collected human plasma from healthy volunteers was obtained from the New York Blood Center (New York, NY, U.S.A.).

Mouse peritoneal macrophages

Pctp$^{-/-}$ mice [12] backcrossed for eight generations to a C57BL/6J genetic strain and wild-type littermate controls were kindly provided by Dr M. K. Wu (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) [13]. Peritoneal macrophages were elicited by intraperitoneal injection of ConA (40 μg in 0.5 ml of sterile PBS) [6,14]. Three days after injection, peritoneal exudates were harvested by peritoneal lavage into 5 ml/mouse DMEM supplemented with 10 % (v/v) fetal bovine serum, 4 mmol/l L-glutamine, penicillin/streptomycin solution (100 units/ml and 100 μg/ml respectively) and LCM (20 %, v/v, L-cell conditioned medium) (DMEM–LCM) [15]. Cells were plated in Primaria dishes, non-adherent cells were removed after 2 h by three washes with PBS, and fresh DMEM–LCM was added to the peritoneal macrophages. The medium was replenished every 24 h, and all experiments were initiated within 36–48 h of macrophage isolation.

Preparation of apolipoproteins and lipoproteins

ApoAI, supplied as 2 mg in approx. 1.5 ml of 8 M guanidinium chloride, was dialysed four times against 1.5 litres of PBS before use. The concentration of protein was determined by measuring the absorption at 280 nm, using a molar absorption coefficient of 1.13 $M^{-1} \cdot cm^{-1}$. LDL (low-density lipoprotein; $\rho = 1.019$–1.063 g/ml) and subfraction 3 of HDL (HDL_3; $\rho = 1.125$–1.21 g/ml) were isolated from fresh human plasma by sequential density ultracentrifugation [16]. Lipoproteins were dialysed four times, each against 3 litres of PBS using 10 000 molecular-mass cut-off Slide-A-Lyzer dialysis cartridges (Pierce Biotechnology, Rockford, IL, U.S.A.). The dialysed lipoproteins were sterilized by filtration through a 0.45 μm filtration unit (Corning, Corning, NY, U.S.A.) and stored in the dark at 4 °C. Protein concentrations of lipoprotein preparations were determined using a Bio-Rad Bradford reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with BSA as a standard.

LDL was acetylated by the method of Basu et al. [17]. Briefly, the desired mass of LDL was diluted 1:1 (v/v) into a saturated solution of sodium acetate. A mass of acetic anhydride equivalent to 1.5× that of the LDL protein was divided evenly into seven aliquots, and these were added every 10 min during the course of 1 h with slow stirring at 4 °C. After the addition of the final aliquot of acetic anhydride, the acetylation reaction was allowed to proceed for an additional 30 min. AcLDL (acetylated LDL) was then dialysed and sterilized as described for lipoproteins. Acetylation was confirmed by a shift in electrophoretic mobility from pre-α to α on agarose gels (Lipogel; Beckman Coulter, Fullerton, CA, U.S.A.) [17].

Lipid efflux assays

For the measurement of phospholipid efflux, cellular phospholipids of confluent macrophages were radiolabelled with 2 μCi/ml [^{3}H]choline in DMEM–LCM for 24 h. For cholesterol loading, radiolabelled monolayers were washed twice with warm PBS and incubated for 24 h in DMEM/BSA (DMEM containing 2 mg/ml BSA) plus 100 μg/ml AcLDL (CE-loaded macrophages) or for 6 h with 100 μg/ml AcLDL plus 2 μg/ml CP-113,818 (FC-loaded macrophages). After labelling/loading, monolayers were washed twice with warm PBS and equilibrated with DMEM/BSA for 2 h. At the end of the equilibration period, the efflux was initiated by the addition of either apoAI (0–50 μg/ml) or HDL_3 (50 μg/ml) in fresh DMEM/BSA. After a 4 h period, media and cells were collected for quantification of efflux by liquid-scintillation counting [11].

Cholesterol efflux experiments were performed essentially as described for phospholipids with the following modifications: cells were labelled with [^{3}H]cholesterol in DMEM–LCM for 24 h. To simultaneously label and enrich cells with cholesterol, [^{3}H]cholesterol-labelled AcLDL was prepared by incubating 1 μCi of [^{3}H]cholesterol with 100 μg of AcLDL protein in 1 ml of DMEM/BSA for 30 min at 37 °C. CE-loaded macrophages were prepared by labelling/loading in the presence of 100 μg/ml [^{3}H]cholesterol-labelled AcLDL in DMEM/BSA for 24 h. FC-loaded macrophages were prepared by labelling/loading with 100 μg/ml [^{3}H]cholesterol-labelled AcLDL in the presence of 2 μg/ml CP-113,818 in DMEM/BSA for 6 h. After the labelling/loading phase, cells were washed twice with PBS and then equilibrated for 2 h in DMEM/BSA. Efflux was then initiated by the addition of either apoAI or HDL_3 in fresh DMEM/BSA. After a period of 4 h, media and cells were collected and the efflux quantified by liquid-scintillation counting [11]. In preliminary experiments, saturation curves were obtained which revealed that apoAI and HDL_3 concentrations of 20 and 50 μg/ml respectively yielded maximal lipid efflux for both wild-type and *Pctp*$^{-/-}$ macrophages. In some experiments, cultures were also incubated with hydroxycholesterol (10 μM) and 9-*cis* retinoic acid (10 μM) with or without Brefeldin A (0.5–1.0 μg/ml).

Assay of apoptosis

Susceptibility of peritoneal macrophages to FC-induced apoptosis was assessed using a Vybrant apoptosis assay kit (Molecular Probes, Eugene, OR, U.S.A.), which utilizes a fluorescence-based method to distinguish non-staining live cells from green Alexa-488 annexin V-staining apoptotic and red propidium iodide-staining necrotic cells [18]. Peritoneal macrophages were incubated in DMEM/BSA in the absence or presence of CP-113,818 (2 μg/ml) or treated with 100 μg/ml AcLDL in DMEM/BSA in the absence or presence of CP-113,818 (2 μg/ml) for 10 h. Cells were washed twice with cold PBS and incubated for 15 min at 25 °C in annexin-binding buffer containing Alexa-488 annexin V (5 μl/100 μl) and propidium iodide (1 μg/100 μl). Cells were then washed twice to remove unbound annexin V and propidium iodide. Monolayers were immediately visualized using a Nikon Diaphot-TMD fluorescence microscope to quantify cell death.

Cytotoxicity assay

Cytotoxicity in response to cellular accumulation of FC was assessed by the measurement of LDH (lactate dehydrogenase) release, which occurs when the integrity of cellular plasma membranes is compromised. Briefly, peritoneal macrophages were harvested and cultured in 12-well culture dishes. Cells were then enriched with CE by incubation with DMEM/BSA containing AcLDL (100 μg/ml). Accumulation of FC was initiated by the addition of CP-113,818 (2 μg/ml) in Phenol Red-free DMEM/BSA. Appearance of LDH in the media was quantified

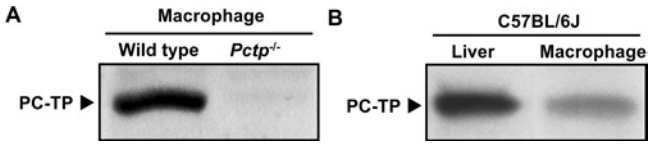

Figure 1 Expression of PC-TP in mouse peritoneal macrophages

Western-blot analysis of PC-TP expression (**A**) in peritoneal macrophages derived from wild-type and *Pctp*-deficient mice (*Pctp*$^{-/-}$) and (**B**) in liver and peritoneal macrophages obtained from wild-type C57BL/6J mice. Equal amounts of cellular homogenate (**A**, 50 μg of protein; **B**, 30 μg of protein) were electrophoresed through 12.5 % (w/v) polyacrylamide gels, transferred on to nitrocellulose membranes and probed with an anti-PC-TP antibody [11].

colorimetrically using a cytotoxicity kit (Roche Applied Science, Indianapolis, IN, U.S.A.). Values for the release of LDH from CE-enriched cells treated with DMEM/BSA, containing an equivalent volume of vehicle, were used as controls and subtracted from each measurement. At the completion of each experiment, control cells were solubilized in Phenol Red-free DMEM/BSA containing 2 % (v/v) Triton X-100, using a volume equivalent to that of medium initially present in each well. Cytotoxicity was quantified by determining the percentage of total cellular LDH released [19].

Western-blot analysis

Cellular homogenates were prepared by adding chilled (4 °C) lysis buffer [20 mM sodium phosphate, 150 mM NaCl, 0.1 % SDS, 1 % Nonidet P40 and Complete protease inhibitor (Roche)] for 15 min. Cellular membranes were further disrupted by brief sonication, and cellular debris was pelleted by centrifugation at 24 000 ***g*** for 20 min at 4 °C. Equal amounts of protein were subjected to SDS/PAGE, transferred on to nitrocellulose membranes and probed using specified antibodies. Detection was by enhanced chemiluminescence, followed by autoradiography. Blots were quantified by densitometry using a FluorChem 8900 Imaging system (Alpha Innotech Corp., San Leandro, CA, U.S.A.). Protein expression was normalized to the expression of β-actin.

Statistical analysis

Results are presented as means $\pm$ S.D. and were compared using independent samples of Student's *t* tests. Statistical significance was defined as $P < 0.05$.

RESULTS

Whereas PC-TP mRNA has been detected in leucocytes from both mice and humans [20,21], protein expression in macrophages has not been investigated. Figure 1(A) demonstrates that PC-TP was readily detectable by Western-blot analysis in ConA-elicited peritoneal macrophages derived from wild-type C57BL/6J mice, but was not present in macrophages derived from *Pctp*$^{-/-}$ mice. Comparable expression of PC-TP was also observed in immortalized monocyte/macrophage-like mouse (RAW264.7 and J774A.1) and human THP-1 cell lines (results not shown). Figure 1(B) compares PC-TP expression in peritoneal macrophages with hepatic expression. PC-TP is highly expressed in livers of C57BL/6J mice [13], indicating that macrophage expression, while being lower, was nevertheless substantial.

In the absence of CE loading, efflux of phospholipids and cholesterol to apoAI (0–50 μg/ml) was not detected (results not shown). Figure 2 shows phospholipid and cholesterol efflux in experiments performed using *Pctp*$^{-/-}$ and wild-type peritoneal macrophages that were loaded with CE. Wild-type macrophages

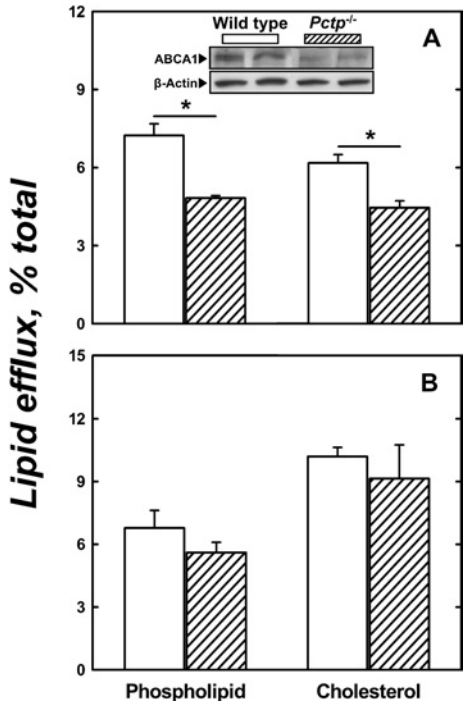

Figure 2 Impaired apoAI-mediated lipid efflux and decreased ABCA1 expression in CE-loaded *Pctp*$^{-/-}$ macrophages

Peritoneal macrophages from wild-type (open bars) and *Pctp*$^{-/-}$ (hatched bars) mice were CE-loaded and radiolabelled as described in the Experimental section. Lipid efflux was measured after 4 h incubations with DMEM/BSA containing either (**A**) apoAI (20 μg/ml) or (**B**) HDL_3 (50 μg/ml). Results are the means $\pm$ S.D. for triplicate determinations from one of (**A**) three or (**B**) two independent experiments. The inset displays representative Western blots in which equal amounts of macrophage homogenate (50 μg of protein) were subjected to SDS/PAGE on 4–20 % gradient polyacrylamide gels, transferred on to nitrocellulose membranes and probed with polyclonal antibodies specific for ABCA1. (Blots are representative of two independent experiments.) *$P < 0.01$ wild-type versus *Pctp*$^{-/-}$ mice.

displayed substantial efflux of cellular phospholipid and cholesterol to apoAI (Figure 2A). The absence of PC-TP was associated with a 33 % decrease in apoAI-mediated phospholipid efflux and a 27 % decrease in the efflux of cholesterol. In duplicate determinations in two independent experiments, before initiating lipid efflux, we did not observe significant differences in the mean contents of CE (wild-type, 52 μg/mg cell protein; *Pctp*$^{-/-}$, 64 μg/mg cell protein), FC (wild-type, 33 μg/mg cell protein; *Pctp*$^{-/-}$, 39 μg/mg cell protein) and phospholipids (wild-type, 84 μg/mg cell protein; *Pctp*$^{-/-}$, 94 μg/mg cell protein). This suggested that efflux of radiolabelled lipids was an indicator of mass efflux. To determine whether the decrease in apoAI-mediated lipid efflux in *Pctp*$^{-/-}$ cells was due to altered cellular expression of ABCA1, Western-blot analysis was performed using homogenates of macrophages. Before treatment with AcLDL, ABCA1 levels were the same in *Pctp*$^{-/-}$ and wild-type macrophages (results not shown). However, as shown in the inset to Figure 2(A), ABCA1 expression was decreased by 39 % in CE-loaded macrophages from *Pctp*$^{-/-}$ mice relative to controls. Figure 2(B) shows cellular efflux of phospholipids or cholesterol when purified human HDL_3 was used as the acceptor. Whereas values for phospholipid efflux mediated by HDL_3 in CE-loaded wild-type and *Pctp*$^{-/-}$ macrophages were similar in magnitude to those observed for apoAI-mediated efflux (Figure 2A), HDL_3-mediated cholesterol efflux was nearly 2-fold higher in both wild-type and *Pctp*$^{-/-}$ macrophages compared with the efflux of cholesterol to apoAI in wild-type cells. Neither phospholipid nor cholesterol efflux to HDL_3

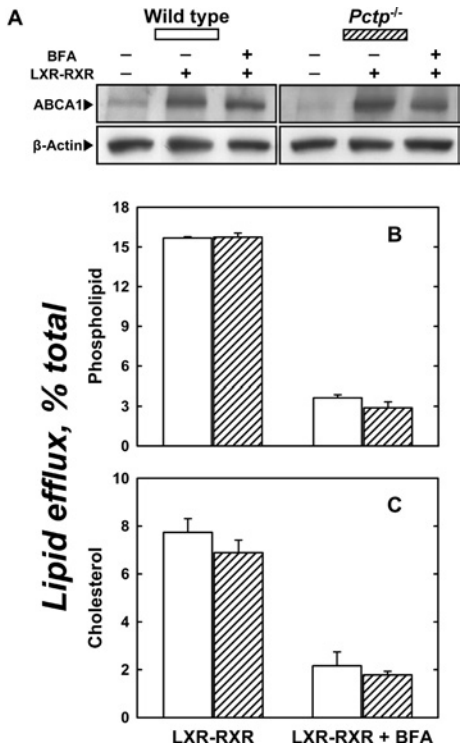

Figure 3 PC-TP expression does not influence the responses of apoAI-mediated lipid efflux to LXR/RXR ligands and Brefeldin A in CE-loaded macrophages

(**A**) Western-blot analysis demonstrating ABCA1 expression in CE-loaded wild-type and *Pctp*$^{-/-}$ macrophages before and after activation of LXR using 22(*R*)-hydroxycholesterol (10 μM) and RXR using 9-*cis* retinoic acid (10 μM) in the absence or presence of Brefeldin A (BFA; 0.5 μg/ml) during CE loading. (Blots are representative of two independent experiments). (**B**) Phospholipid and (**C**) cholesterol efflux from CE-loaded peritoneal macrophages from wild-type (open bars) and *Pctp*$^{-/-}$ (hatched bars) mice that were incubated for 24 h with 22(*R*)-hydroxycholesterol (10 μM) and 9-*cis* retinoic acid (10 μM) in DMEM/BSA in the absence (LXR/RXR) or presence of 0.5 μg/ml BFA (LXR/RXR + BFA) during CE loading. Lipid efflux was measured after 4 h incubation with 20 μg/ml apoAI in DMEM/BSA plus 10 μM each of 22(*R*)-hydroxycholesterol and 9-*cis* retinoic acid in the absence (LXR/RXR) or presence of 0.5 μg/ml BFA (LXR/RXR + BFA). Results are the means $\pm$ S.D. for triplicate determinations obtained from one of two independent experiments.

was affected by PC-TP expression. Considering recent evidence that ABCG1 expression in macrophages largely controls lipid efflux to HDL_3 [5], our results suggest that PC-TP does not influence the functional expression of ABCG1 in CE-loaded macrophages.

Macrophages synthesize and secrete apoE, which promotes lipid efflux by ABCA1-dependent and -independent mechanisms [22–24]. Therefore we tested whether PC-TP expression may have influenced intracellular concentrations or secretion rates of apoE in macrophages. Before or after CE loading, which up-regulates the synthesis and secretion of apoE [25–27], Western-blot analysis demonstrated that levels of apoE within cells or appearing in the medium were the same in wild-type and *Pctp*$^{-/-}$ macrophages (results not shown).

We next sought to determine whether PC-TP might influence the transcriptional up-regulation of ABCA1 expression that occurs in CE-loaded macrophages in response to LXR (liver X receptor) activation [28]. The LXR and RXR (retinoid X receptor) ligands 22(*R*)-hydroxycholesterol and 9-*cis* retinoic acid respectively were included during CE loading of macrophages. As shown in Figure 3(A), Western-blot analysis revealed that expression of ABCA1 in both wild-type and *Pctp*$^{-/-}$ macrophages was of comparable magnitude after LXR/RXR activation. These expression levels represent 4.5- and 9-fold increases in ABCA1 expression compared with CE-loaded wild-type and *Pctp*$^{-/-}$ macrophages respectively in the absence of LXR/RXR activation. Compared with the absence of LXR/RXR ligands (Figure 2A), LXR-mediated induction of ABCA1 was accompanied by increases in apoAI-mediated phospholipid (Figure 3B) and cholesterol efflux (Figure 3C). Consistent with equivalent expression of ABCA1 (Figure 3A), efflux of phospholipids (Figure 3B) and cholesterol (Figure 3C) was the same in CE-loaded wild-type and *Pctp*$^{-/-}$ macrophages after stimulation with LXR/RXR ligands. Under these experimental conditions of LXR/RXR activation, there was only a modest up-regulation of PC-TP by 42 % (results not shown), which did not reach statistical significance by densitometric analysis ($P = 0.14$). Therefore the up-regulation of lipid efflux in CE-loaded wild-type macrophages due to LXR/RXR can be attributed to the marked up-regulation of ABCA1, without a contribution from PC-TP up-regulation.

The integrity of the ABCA1-dependent lipid efflux pathway depends on a functional vesicular transport mechanism and an intact Golgi apparatus [29], which serve to deliver ABCA1 from intracellular sites of synthesis and storage to the cell surface [30,31]. To explore whether an ABCA1-independent requirement of PC-TP for apoAI-mediated lipid efflux might be unmasked under conditions in which plasma-membrane expression of ABCA1 was decreased, we inhibited vesicular transport using Brefeldin A [32–34]. As shown in Figure 3(A), Brefeldin A treatment did not appreciably alter the total expression of ABCA1 in either wild-type or *Pctp*$^{-/-}$ macrophages. Consistent with decreased delivery of ABCA1 to the plasma membrane, treatment with Brefeldin A of CE-loaded macrophages after incubation with LXR/RXR ligands led to marked inhibition of apoAI-mediated phospholipid efflux by 80 % (Figure 3B) and cholesterol efflux by 73 % (Figure 3C). These decreases in lipid efflux notwithstanding, Brefeldin A treatment failed to elicit differences between wild-type and *Pctp*$^{-/-}$macrophages. The same was true when longer Brefeldin A pretreatment periods (6 and 12 h) or a higher dose (1 μg/ml) was used (results not shown).

Figure 4 shows that PC-TP does not influence lipid efflux in FC-loaded macrophages. As was the case in CE-loaded macrophages, we did not observe significant differences in mean contents of CE (wild-type, 55 μg/mg cell protein; *Pctp*$^{-/-}$, 63 μg/mg cell protein), FC (wild-type, 53 μg/mg cell protein; *Pctp*$^{-/-}$, 47 μg/mg cell protein) and phospholipids (wild-type, 107 μg/mg cell protein; *Pctp*$^{-/-}$, 100 μg/mg cell protein) before initiating lipid efflux from FC-loaded macrophages. As shown in Figure 4(A), apoAI-mediated phospholipid and cholesterol effluxes were the same in FC-loaded macrophages derived from wild-type and *Pctp*$^{-/-}$ mice. Consistent with these findings, cellular levels of ABCA1 protein were not affected by PC-TP expression (inset to Figure 4A). Figure 4(B) demonstrates that HDL_3-mediated lipid efflux was not influenced by PC-TP expression.

We next determined whether PC-TP influences the susceptibility of macrophages to FC-induced apoptosis. After FC loading for 10 h, macrophages in relatively early stages of apoptosis were detected as green-coloured cells due to the binding of Alexa-488 annexin V to phosphatidylserine, which is exposed on the outer leaflet of the plasma membrane [35]. Later stages of post-apoptotic necrosis [7,36,37] were detected as reddish orange colour by the membrane-impermeable nucleic acid stain propidium iodide. Infrequent cell death was observed in wild-type and *Pctp*$^{-/-}$ macrophages incubated in the presence of CP-113,818 alone (Figures 5A and 5B) or in CE-loaded macrophages in the absence of the ACAT inhibitor (results not shown). In contrast, much more abundant green and red fluorescence was detected in CE-loaded cells exposed to CP-113,818 (Figures 5C and 5D). As

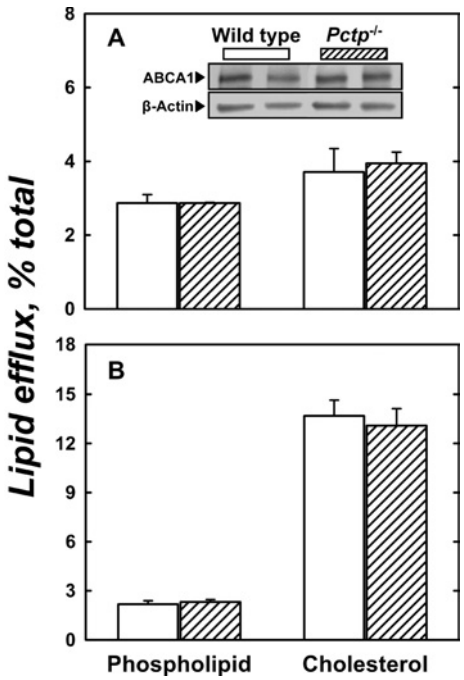

Figure 4 PC-TP expression does not influence lipid efflux in FC-loaded macrophages

Peritoneal macrophages from wild-type (open bars) and *Pctp*$^{-/-}$ (hatched bars) mice were FC-loaded and radiolabelled as described in the Experimental section. Lipid efflux was measured from radiolabelled FC-loaded macrophages after 4 h incubations with DMEM/BSA containing either (**A**) apoAI (20 μg/ml) or (**B**) HDL_3 (50 μg/ml). Results are means $\pm$ S.D. for triplicate determinations obtained from one of two independent experiments. The inset displays representative Western blots in which equal amounts of macrophage homogenate (50 μg of protein) were subjected to SDS/PAGE on 4–20 % gradient polyacrylamide gels, transferred on to nitrocellulose membranes and probed with polyclonal antibodies specific for ABCA1. (Blots are representative of two independent experiments).

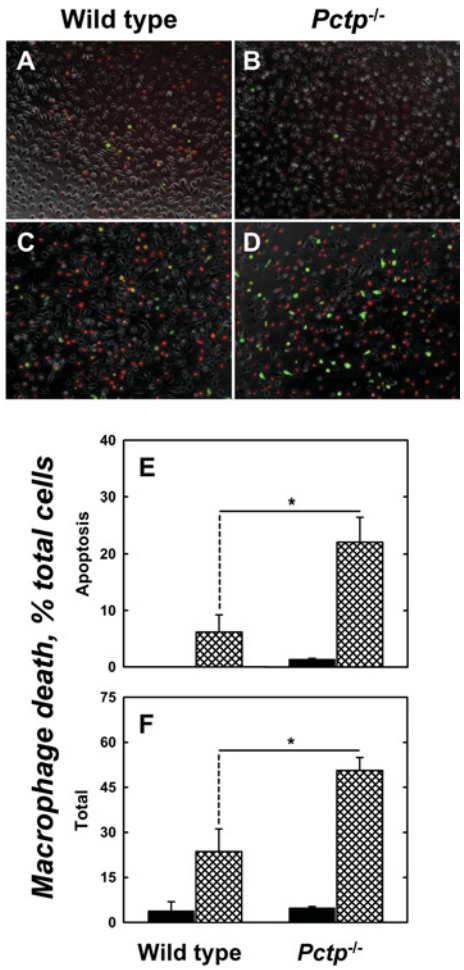

Figure 5 FC-induced cell death is enhanced in *Pctp*$^{-/-}$ peritoneal macrophages

Peritoneal macrophages from wild-type (**A**, **C**) and *Pctp*$^{-/-}$ (**B**, **D**) macrophages were incubated for 10 h in DMEM/BSA in the presence of 2 μg/ml CP-113,818 (basal; **A**, **B**) or in the presence of 100 μg of AcLDL plus 2 μg/ml CP-113,818 (FC-loaded; **C**, **D**). Cells were then stained with Alexa-488-labelled annexin V (green) and propidium iodide (red) and viewed by fluorescence microscopy. Quantitative analysis for basal (closed bars) and FC-loaded (crosshatched bars) macrophages of (**E**) apoptotic and (**F**) total cell death for which the percentages of total cells that were green-stained (**E**) as well as red- or dual-stained (**F**) were determined in 5 fields of cells (800–1000 total cells for each condition). Results are the means $\pm$ S.D. for triplicate determinations obtained from one of two independent experiments. $^*P < 0.001$ wild-type versus *Pctp*$^{-/-}$ mice.

quantified in Figure 5(E), FC-induced apoptosis was increased 3.6-fold in the absence of PC-TP. Post-apoptotic necrosis also contributed substantially to total cellular death in response to FC, and to similar extents in wild-type and *Pctp*$^{-/-}$ macrophages. Therefore, in the absence of PC-TP, total cell death was increased to a lesser extent (2.1-fold) when compared with apoptosis.

Recent studies have demonstrated that FC accumulation in the endoplasmic reticulum decreases the membrane fluidity [38], resulting in the depletion of calcium stores, activation of the unfolded protein response and increased expression of the cell-death effector CHOP (C/EBP-homologous protein) [39]. To test whether the absence of PC-TP may have accentuated this mechanism of apoptosis, we measured CHOP expression by Western-blot analysis at four time points (3, 4, 5 and 7 h) during the course of FC loading with AcLDL + CP-113,818. When compared with wild-type macrophages, we failed to detect an increase in CHOP expression in macrophages derived from *Pctp*$^{-/-}$ mice (results not shown).

Figure 6 shows LDH release in response to FC accumulation in *Pctp*$^{-/-}$ and wild-type macrophages. No differences were observed in the time courses of cellular LDH release between wild-type and *Pctp*$^{-/-}$ macrophages (Figure 6A). To exclude the possibility that the cytotoxic potential of lysosome-derived FC was insufficient to yield differences in *Pctp*$^{-/-}$ versus wild-type macrophages [2], peritoneal macrophages were CE-loaded before the addition of CP-113,818 (Figure 6B). Under these conditions, values of LDH failed to level off even at 36 h. Notwithstanding this enhancement in cytotoxicity, we failed to observe a difference between wild-type and *Pctp*$^{-/-}$ CE-loaded macrophages. As shown in the inset to Figure 6(B), extension of the CE enrichment period to 36 h did not unmask differences in LDH release between wild-type and *Pctp*$^{-/-}$ macrophages.

DISCUSSION

Phosphatidylcholine synthesis, distribution and efflux are essential components of macrophage defences against cholesterol-induced toxicity [28,40]. The goal of the present study was to elucidate roles for PC-TP, a lipid-binding protein with strict substrate specificity for phosphatidylcholines [8,10], in the responses of cultured mouse peritoneal macrophages to cholesterol loading. The main findings were that PC-TP is abundantly expressed in

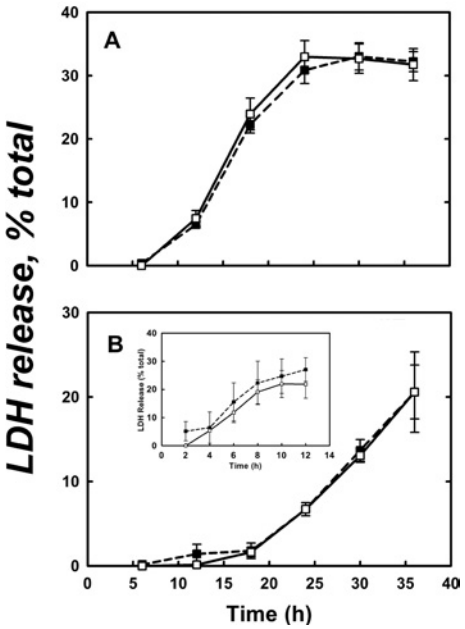

Figure 6 LDH release in response to FC accumulation is not influenced by PC-TP expression

(**A**) LDH release from wild-type (■) and *Pctp*$^{-/-}$ (□) peritoneal macrophages incubated with 100 μg/ml AcLDL + CP-113,818 (2 μg/ml) in DMEM/BSA. (**B**) Wild-type (■) and *Pctp*$^{-/-}$ (□) peritoneal macrophages were CE-loaded by incubation with 100 μg/ml AcLDL in DMEM/BSA for 10 h. Macrophages were then incubated with DMEM/BSA containing CP-113,818 (2 μg/ml) in the absence of AcLDL, and LDH release was measured. The inset presents data from experiments in which macrophages were CE-loaded for 36 h before the addition of 2 μg/ml CP-113,818. Results are the means ± S.D. for one of three independent experiments.

macrophages and that its absence decreased ABCA1-mediated efflux and accelerated apoptotic cell death.

On the basis of experiments in CHO cells in which overexpression of PC-TP promoted apoAI-mediated phospholipid and cholesterol efflux, we proposed previously that PC-TP functions to deliver phosphatidylcholines to the plasma membrane for efflux by ABCA1 [11]. In the present study, however, decreases in phospholipid and cholesterol efflux in CE-loaded *Pctp*$^{-/-}$ macrophages were associated with decreased ABCA1 expression. Therefore additional experiments were performed to determine whether the decrease in ABCA1 was principally responsible for decreased lipid efflux. When CE-loaded wild-type and *Pctp*$^{-/-}$ macrophages were stimulated with LXR/RXR ligands, no differences were observed in apoAI-mediated phospholipid or cholesterol efflux, indicating that ABCA1 expression and function were both fully restored in cells lacking PC-TP. We also used Brefeldin A to interrupt vesicular trafficking of ABCA1 from the Golgi apparatus to the plasma membrane [29,31]. Without decreasing the total ABCA1 expression levels, Brefeldin A treatment equally decreased apoAI-mediated lipid efflux in wild-type and *Pctp*$^{-/-}$ macrophages. The absence of an influence of PC-TP on lipid efflux when cell-surface expression of ABCA1 is either high (i.e. after LXR/RXR activation) or low (i.e. after Brefeldin A treatment) argues against a key role in shuttling phosphatidylcholines to the plasma membrane.

One of the early consequences of excess FC accumulation in macrophages is degradation of ABCA1 by a mechanism that involves trafficking of cholesterol out of late endosomes/lysosomes [6]. Excess FC is accommodated by up-regulation of phosphatidylcholine synthesis, which leads to the formation of intracellular membrane whorls that prevent the FC/phospholipid molar ratio from reaching toxic proportions [3]. By altering the intracellular distribution of phosphatidylcholines, the absence of PC-TP may have promoted FC-induced turnover of ABCA1 in CE-loaded *Pctp*$^{-/-}$ macrophages. Although we did not detect an increase in cellular FC content of these cells, it is possible that FC became concentrated in specific subcellular membranes. In this event, only a small increase in FC on a total cell basis may have been sufficient to promote the degradation of ABCA1. Alternatively, a subtle defect in CE hydrolysis could have explained the down-regulation of ABCA1 in the absence of PC-TP. This would have decreased the availability of FC for conversion into LXR ligands. By the same mechanism, a decrease in LXR/RXR activation would also be expected to decrease the lipid efflux to HDL_3 due to decreased ABCG1 expression [5], and this was not observed in our studies. In this connection, the results of Wang et al. [5] also suggest that the magnitude of response of HDL-mediated lipid efflux to LXR/RXR ligands is smaller than that for apoA-1-mediated lipid efflux. Therefore an effect of PC-TP expression on lipid efflux to HDL_3 may have been too small to measure under the current experimental conditions. Whether by altering the cellular distribution of FC or the availability of LXR ligands, PC-TP-related differences in apoAI-mediated phospholipid and cholesterol efflux were overcome by the substantial increases in cellular FC content associated with ACAT inhibition.

Sustained FC accumulation due to ACAT inhibition in macrophages eventually leads to decreased fluidity of the endoplasmic reticulum and apoptotic cell death [38]. This can be attributed in part to activation of the unfolded protein response [39]. Consistent with previous reports [2,7], we readily detected apoptotic cell death in wild-type macrophages after 10 h of FC loading. As indicated by the increase in frequency of annexin V staining, *Pctp*$^{-/-}$ macrophages were much more sensitive to FC-induced apoptosis. In these experiments, the concomitant increase in the frequency of propidium iodide-stained cells was most probably due to post-apoptotic necrosis [2,7]. Whereas the downstream transcription factor CHOP has been shown to effect FC-induced apoptosis [39], the absence of PC-TP expression was not associated with early CHOP induction. This suggests the involvement of other non-CHOP pathways, which have been shown to account for approx. 30 % of apoptosis under the current experimental conditions [39]. It will also be interesting to determine whether the *Pctp*$^{-/-}$ cells are more susceptible to apoptosis by themselves and not only under conditions of FC loading.

A relatively late measure of cytotoxicity is increased permeability of the plasma membrane, as indicated by release of LDH or adenine from macrophages into the medium [19]. Under conditions of ACAT inhibition in CE-loaded macrophages, accumulation of FC within a cytotoxic plasma membrane pool eventually compromises bilayer integrity [41,42]. Consistent with an endoplasmic reticulum-related role for PC-TP in the adaptive response to FC loading, we did not detect any difference in LDH release between wild-type and *Pctp*$^{-/-}$ peritoneal macrophages after challenging with FC.

Considering recent evidence that the proapoptotic effects of FC accumulation are due to increased molecular ordering within the phospholipid-rich bilayer of the endoplasmic reticulum [38], our current findings suggest that PC-TP in macrophages might function to preserve the fluidity of this and possibly other intracellular membranes. This could explain both the early down-regulation of ABCA1 and the accelerated apoptosis observed in *Pctp*$^{-/-}$ macrophages that were challenged with cholesterol. Additional studies, including subcellular membrane composition and fluidity, should help to elucidate the cytoprotective mechanism of PC-TP and determine whether this translates into an atheroprotective role *in vivo*.

This work was supported by National Institutes of Health (grants DK56626 and DK48873), an Established Investigator Award from the American Heart Association and an International HDL Research Awards Program grant to D. E. C. J. M. B. was supported by a MARC Predoctoral Fellowship (GM64043) from the National Institutes of Health.

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Received 15 November 2004/21 December 2004; accepted 4 January 2005
Published as BJ Immediate Publication 4 January 2005, DOI 10.1042/BJ20041899