Stimulation of CTP:Phosphocholine Cytidylyltransferase by Free Cholesterol Loading of Macrophages Involves Signaling through Protein Dephosphorylation*

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Free cholesterol-loaded macrophages in atheroma synthesize excess phosphatidylcholine (PC), which may be an important adaptive response to the excess free cholesterol (FC) load. We have recently shown that FC loading of macrophages leads to 2–4-fold increases in PC mass and biosynthesis and to the post-translational activation of the membrane-bound form of CTP:phosphocholine cytidylyltransferase (CT), a key enzyme in PC biosynthesis. Herein, we explore further the mechanism of CT activation in FC-loaded macrophages. First, enrichment of membranes from control macrophages with FC in vitro did not increase CT activity, and PC biosynthesis in vivo is up-regulated by FC loading even when CT and FC appear to be mostly in different intracellular sites. These data imply that FC activates membrane-bound CT by a signaling mechanism. That the proposed signaling mechanism involves structural changes in the CT protein was suggested by data showing that two different antibodies against synthetic CT peptides showed increased recognition of membrane-bound CT from FC-loaded cells despite no increase in CT protein. Since CT is phosphorylated, two-dimensional maps of peptides from 32P-labeled control and FC-loaded macrophages were compared: six peptide spots from membrane-bound CT, but none from soluble CT, were dephosphorylated in the FC-loaded cells. Furthermore, incubation of FC-loaded macrophages with the phosphatase inhibitor, calyculin A, blocked increases in both PC biosynthesis and antipeptide-antibody recognition of CT. Last, treatment of membranes from control macrophages with χ phage protein phosphatase in vitro increased both CT activity (2-fold) and antipeptide-antibody recognition of CT; soluble CT activity and antibody recognition were not substantially affected by phosphatase treatment. In summary, FC loading of macrophages leads to the partial dephosphorylation of membrane-bound CT, and possibly other cellular proteins, which appears to be important in CT activation. This novel regulatory action of FC may allow macrophages to adapt to FC loading in atheroma.

Macrophages are a prominent cell type in both early and advanced atherosclerotic lesions (1–3) and undoubtedly play important roles in the clinical progression of these lesions (4, 5). Most atheroma macrophages are loaded with both cholesterol esters (6) and, particularly in advanced lesions, free cholesterol (FC)1 (7–10). In addition, lesion macrophages appear to have increased rates of phospholipid biosynthesis (11–13) and to have increased phospholipid mass (14, 15), in the form of intracellular membrane whorls (10). This excess phospholipid may serve to prevent the decreased fluidity of FC-rich membranes (16) and/or to inhibit cholesterol crystal formation (15). Thus, the increased phospholipid biosynthesis seen in atheroma macrophages may be part of an adaptive response to prevent FC-mediated cellular toxicity.

To gain insight into the biochemistry of this in vivo phenomenon, we recently explored phospholipid metabolism in FC-loaded macrophages in cell culture (17). Our work revealed that FC loading of macrophages leads to the accumulation of excess PC mass, in the form of intracellular membrane whorls, via activation of the enzyme CTP:phosphocholine cytidylyltransferase, which is a rate-limiting enzyme in PC biosynthesis (17). Initial mechanistic studies in that report disclosed two important points. First, FC loading of macrophages led to the activation of membrane-bound CT without a substantial change in the activity of soluble CT (17). Thus, the activation cannot be explained by soluble-to-membrane CT translocation, which is a process thought to be involved in the activation of CT in certain other systems (18–20). Second, there was no increase in CT mRNA in FC-loaded macrophages, and stimulation of PC biosynthesis in the FC-loaded cells did not depend upon new protein synthesis (17). Therefore, the activation occurs via a post-translational mechanism.

The goal of the present study was to explore further the mechanism of CT activation in FC-loaded macrophages. Our new data indicate that FC loading activates CT in macrophages via a signaling mechanism that appears to involve the dephosphorylation of membrane-bound CT and possibly other cellular proteins. This novel intracellular signaling effect of FC may be part of an important adaptive response of macrophages to the potential toxicity of excess FC accumulation.

EXPERIMENTAL PROCEDURES

Materials—The Falcon tissue culture plasticware used in these studies was purchased from Fisher. Tissue culture media and reagents were provided by the Alberta Heritage Foundation for Medical Research (to M.H.). The cost of publication of this article was defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: FC, free cholesterol; ACAT, acyl-CoA: cholesterol O-acyltransferase; CHO, Chinese hamster ovary; CT, CTP: phosphocholine cytidylyltransferase; DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline; PC, phosphatidylcholine; TLC, thin-layer chromatography; PVDF, polyvinylidene difluoride.
obtained from Life Technologies, Inc. Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT). Lipoprotein-deficient serum (LPSD) was prepared from fetal calf serum by preparative ultracentrifugation (density, 1.21 g/ml) (21). [methyl-\(^{3}H\)] choline, carrier-free 2\(^{32}\)PO\(_4\), and the ECL (enhanced chemiluminescence) immunoblotting detection reagent were purchased from DuPont NEN. Compound \(\text{SBO}_{35}\) (3-decyl-\(\text{O}\)-palmitoyl-\(\text{L}\)-lysine (4-methyleneglycine fragment, \(\text{L}\)-lysylglycine) (22)) was generously provided by Dr. J. ohn Heider of Sandoz, Inc. (East Hanover, NJ). Stock solutions (10 mg/ml) were prepared in dimethyl sulfoxide; the final dimethyl sulfoxide concentration in both treated and control cells was 0.05%. Compound \(\text{U18666A}\) (3β-2-(diethylamino)ethoxy)androst-5-en-17-one) was generously provided by Dr. Laura Liscum, Tufts University (Medford, MA). Stock solutions (10 mg/ml) were prepared in ethanol. Recombinant \(\lambda\) phage protein phosphatase (cf. Ref. 23) was purchased from New England Biolabs (Beverly, MA). All organic solvents were purchased from Fisher. Goat anti-rabbit IgG and calyculin A were from Life Technologies, Inc., modified trypsin (sequencing grade) was from Boehringer Mannheim, and cholera (≥99% pure) was from Nu-Chek-Prep, Inc. (Elysian, MN). All other chemicals and reagents were purchased from Sigma.

Anti-CT Antiserum—Rabbit antiserum against recombinant CT was provided by Dr. Suzanne ackowski (St. J.ude Children’s Research Hospital); the antigen was made by baculovirus-mediated expression of rat liver CT DNA in Sf9 cells and then purified by SDS-polyacrylamide gel electrophoresis (24). Rabbit antiserum against a synthetic N-terminal CT peptide corresponding to amino acids 1–17 of rat liver CT (25) was provided by Dr. Claudia Kent (University of Michigan). Rabbit anti-serum against a synthetic mid-molecule CT peptide (corresponding to amino acids 164–176 of rat liver CT) (26) was provided by Dr. Nyc-ance (University of Alberta).

Cells—Monolayer cultures of J774A1 cells (from the American Type Culture Collection) were grown and maintained in spinner culture with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 \(\mu\)g/ml), and glucose (252 \(\mu\)g/ml) as described previously (27, 28). CHO-mSRA1I cells, which were transfected with a murine macrophage scavenger receptor (class AI) cDNA (29), were generously provided by Dr. Mrtty Kriss (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The cells were grown in monolayer culture in Ham’s F-12 medium containing 10% fetal bovine serum. For each experiment, the J774 or CHO-mSRA1I cells were plated in 16 × 22-mm dishes at a density of 10\(^6\) cells/dish in medium, 10% FCS and then incubated at 37 \(^\circ\)C in an atmosphere containing 5% CO\(_2\). After 2 h of incubation, the monolayers were washed with warm PBS and then incubated with medium, 10% LPSD alone (control) or containing 100 units/ml of bacterial \(\text{L}\)-alanine (1:40 dilution) (30). All LPSD solutions were adjusted to given in terms of their protein content with bovine serum albumin as a standard. All LPSD preparations were stored under argon at 4 \(^\circ\)C and used within 4 weeks.

\[^{1}H\]Choline Labeling Studies—Incorporation of \([^{1}H]\) choline into phosphatidylcholine in intact cells was determined as described previously (17). For the phosphatidylcholine studies, macrophages incubated under various conditions were pulsed for 1 h with 2 \(\mu\)Ci of \([^{1}H]\) choline/ ml (80.0 Ci/mmol). Lipid extracts of the cells were then separated by TLC in a solvent system of chloroform methanol/acetic acid/water (50: 25:84, \(v/v\)), and the radioactivity in phosphatidylcholine was quantified.

Scintillation Fractionation—After preincubation under control or lipid loading conditions, monolayers of J774 cells were washed three times with ice-cold PBS, and the cells were scraped with a rubber policeman into each assay buffer on ice and sonicated twice for 30 s (homogeneous fraction). A portion of the cell homogenate was then centrifuged at 100,000 \(\times\) \(g\) for 1 h at 4 \(^\circ\)C. The supernatant was collected (soluble fraction) and the pellet was resuspended in assay buffer (membrane fraction). Note that the sonication procedure disrupted the nuclei of the macrophages, as verified by DNA measurements, and so the soluble fraction included nuclear soluble contents, and the membrane fraction included nuclear membranes (cf. Refs. 25 and 31).

For immunoblotting, two-dimensional phosphoprotein mapping, and in vitro protease treatment, the macrophages were fractionated by the digitonin treatment method of Wright et al. (32). In brief, the cell monolayers were washed three times with ice-cold PBS and then incubated for 8 min at 4 \(^\circ\)C with digitonin-release buffer, which contained 10 mM Tris-HCl, pH 7.4, 0.25 mM sucrose, 0.8 mg/ml digitonin, 33 mM sodium fluoride, 33 mM sodium vanadate, 3.3 mM EDTA, 3.3 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonl fluoride, 1 mM leupeptin, and 1 \(\mu\)g/ml pepstatin. The release buffer was then collected (soluble fraction), and the cell ghosts, still attached to the culture dish, were carefully washed once with PBS and scraped into digitonin-release buffer (membrane fraction). The ghosts were then permeabilized by scraping the monolayer in digitonin-release buffer and homogenizing the suspension by repeated aspiration and expulsion from a Pasteur pipette.

In vitro Enzyme Assays—CT:phosphocholine cytidylyltransferase (EC 2.7.7.15) was assayed by measuring the incorporation of \([^{1}C]\)cholesterol into cholesteryl esters, as described previously (17). Acetyl-CoA:cholesterol O-acetytransferase (ACAT) activity in microsomes isolated from macrophages was determined by measuring the incorporation of \([^{3}H]\)oleate from \([^{3}H]\)Jae-CoA into cholesteryl esters on a 514 line from a 25-milliwatt argon laser. Western blot Analysis—Immunoblot analysis of CT was performed as described by Wang et al. (34). Cells were scraped on ice in digitonin-release buffer, and this mixture was immediately mixed with 4-fold concentrated Laemmli sample buffer (\(\text{L}\)-alanyl-\(\text{L}\)-leucyl-\(\text{L}\)-phenylalanine, 1:200 rhodamine-labeled goat anti-rabbit IgG in buffer B). The proteins were then separated by 10% reducing SDS-polyacrylamide gel electrophoresis (35) using \(100 \mu\)g protein/lane; protein masses were determined by the Bio-Rad protein assay, based upon the method of Bradford (36), using bovine serum albumin as standard. After transfer to nitrocellulose or PVDF membrane, the blots were incubated with 5% nonfat dry milk in buffer C (24 ml) and then incubated with, and then incubated with 1:200 rhodamine-labeled goat anti-rabbit IgG in buffer B for 30 min. After removing the antibody solution, the cells were washed and then incubated with buffer B for 15 min and then incubated with 1:200 rhodamine-labeled goat anti-rabbit IgG in buffer B for 30 min. After removing the secondary antibody solution, the cells were washed with and incubated for 45 min with buffer B. Finally, the cells were rinsed three times with 1 ml of buffer B and examined by fluorescence microscopy as described in the previous section. Rhodamine was visualized with a 514-nm bandpass excitation filter, a 540-nm dichroic mirror, and a 550-nm long pass emission filter. Confocal fluorescence images were obtained with a laser confocal microscope (MRC 600, 200\(\times\) zoom, Bio-Rad Laboratories, Hercules, CA) on an inverted microscope (Axiovert; Zeiss, Oberkochen, Germany) using a \(\times\) 63, NA 1.4 Zeiss Plan-Apo infinity corrected objective. The illumination source for rhodamine was a 514 line from a 25-milliwatt argon laser.

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800 μl of lysis buffer and incubated on ice for 30 min. Soluble and membrane lysate samples were preclarified with 40 μl of preimmune serum and shaken for 1 h at 4°C. This was followed by the addition of 100 μl of protein A-Sepharose CL-4B, which was prepared as follows: incubation with wash buffer (50 mM Tris, pH 8.0, 0.15M NaCl, 1% sucrose, 0.73 mg/digitonin/ml, 2 mM dithiothreitol, 0.9 mM phenylmethysulfon fluoride, 0.9 μg leupeptin/ml, and 0.9 μg pepstatin/ml) containing 400 units of α phage protein phosphatase, according to the procedure of Wang et al. (34). Control incubations were with buffer F alone.

Cellular Free Cholesterol Analyses—Cellular free cholesterol mass measurements and filipin staining of intracellular free cholesterol were carried out exactly as described previously (39).

RESULTS

The Mechanisms Responsible for CT Activation by Cellular FC Loading in Macrophages Are Not Present in CHO Cells—We first sought to determine if the mechanisms used by macrophages to activate CT in response to FC loading were present in CHO cells, since much is known about CT activation in this cell type (19). We utilized the CHO-mSRAII line, which is stably transfected with the murine macrophage scavenger receptor (29), so that we could load the cells with FC to a similar degree as macrophages. In fact, when these cells were incubated with acetyl-LDL or with the ACAT inhibitor, compound 58035, their FC content increased approximately 2-fold (see legend to Fig. 1), and their absolute level of FC was similar to that of macrophages under similar conditions (Fig. 1C). Nonetheless, incorporation of labeled choline into PC, as a measure of phosphatidylcholine biosynthesis,2 in FC-loaded CHO cells was not increased compared with that in unloaded CHO cells (Fig. 1A). The –2-fold increment in FC in the CHO cells (see legend to Fig. 1) should have led to a –2-fold increase in PC biosynthesis (cf. Ref. 17). In contrast, the macrophages showed a 3-fold increase in PC biosynthesis (Fig. 1B), as predicted by their –3-fold increase in FC (cf. Ref. 17). In experiments not displayed here, we were able to reproduce the up-regulation of PC biosynthesis in CHO-mSRAII cells by phospholipase C treatment and show that this was associated with soluble-to-membrane CT translocation (cf. Ref. 40); note that soluble-to-membrane CT translocation has been shown not to be the mechanism of CT activation in FC-loaded macrophages (17). Thus, the cellular machinery responsible for the up-regulation of PC biosynthesis by cellular FC loading in macrophages, mediated by the activation of CT (17), is not

2 Although the incorporation of labeled choline into PC can be affected by pool sizes of precursors, we have previously shown that cellular FC loading does not affect these pool sizes (17). Thus, changes in the incorporation of labeled choline into PC should accurately reflect changes in PC biosynthesis.
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FIG. 2. Enrichment of membranes from J774 macrophages with FC in vitro effect on CT activity. Monolayers of J774 macrophages were incubated for 36 h in DMEM, 10% LPDS alone (Con Mø) or containing 50 μg acetyl-LDL/ml plus 5 μg 58035/ml (FC-loaded Mø). Membrane fractions from these cells were preincubated in the absence or presence of 300 μM cholesterol (FC), 30 μM 25-hydroxycholesterol (250HC), or 300 μM cholesterol in cholesterol/phosphatidylcholine liposomes (FC-lip). The cholesterol was added from a 50 × stock (15 mM) in acetonitrile, and 25-hydroxycholesterol was added from a 1000 × stock (3 mM) in ethanol; separate controls contained solvent alone (2% acetone or 0.1% ethanol, respectively) which had no effect on CT or ACAT activities. The membranes were then assayed, also in the absence or presence of the above cholesterol preparations, for CT activity (A) or, as a control, for ACAT activity (B). The CT assay was done in the absence of PC-deacyl liposomes.

Shared by all cell types.

FC Loading of Membranes in Vitro Does Not Lead to CT Activation—The up-regulation of CT activity in FC-loaded macrophages does not depend upon new protein synthesis, is not associated with an increased level of CT mRNA, and, as mentioned above, does not involve soluble-to-membrane translocation (17). Thus, we considered two general mechanisms of CT activation in these cells: direct activation of CT by FC, for example by an allosteric mechanism or by affecting the lipid microenvironment of the enzyme (cf. Refs. 41 and 42), and alteration in the CT molecule by a FC-mediated signaling event, such as a change in the phosphorylation state of CT (cf. Refs. 43 and 44). To address the former possibility, CT-containing membranes from unloaded macrophages were incubated in vitro in the absence or presence of one of three sources of sterol, namely, cholesterol, 25-hydroxycholesterol, or FC-rich liposomes, and then assayed for CT activity in the absence of PC-oleate liposomes. As shown in Fig. 2A, none of the sterol treatments substantially increased CT activity in these membranes. In contrast, CT activity in membranes isolated from FC-loaded macrophages (last bar of Fig. 2A) was ~3-fold greater than that in membranes from unloaded cells (cf. Ref. 17). This finding indicates that, under the conditions of our CT assay, an increase in CT activity can be detected. To determine if the steroids were added in a manner which would likely allow interaction with a membrane enzyme, membranes from unloaded macrophages were incubated with the same three sources of sterol and assayed for acyl-CoA:cholesterol O-acyltransferase (ACAT) activity, which is known to be stimulated by cholesterol by both substrate delivery and an allosteric mechanism (42, 45) (Fig. 2B). The data show that all three sources of sterol, especially FC-rich liposomes, caused substantial increases in membrane-bound ACAT activity. Thus, under conditions of efficient sterol delivery to membranes, CT activity was not activated in vitro by incubation with steroids.

In Vivo Support for a Signaling Mechanism in the Activation of CT by FC Loading of Macrophages—To further support the idea that activation of CT in FC-loaded macrophages involves a signaling mechanism rather than direct interaction of the enzyme with cholesterol, we sought to show that FC loading could activate PC biosynthesis even when most of the cholesterol and CT were in different intracellular sites. First, the intracellular location of CT in control and FC-loaded macrophages was determined by indirect immunofluorescence microscopy using an anti-N-terminal CT peptide antibody (cf. Ref. 25). Using in vitro CT enzyme activity measurements, we demonstrated that this antibody was able to immunoprecipitate ~95% of CT from control and FC-loaded macrophages (data not shown). The images in Fig. 3 show that the pattern of fluorescence in both control (A) and FC-loaded macrophages (B) was predominantly nuclear, similar to that found in several other cell types (31), although there was some cytoplasmic staining as well (cf. Ref. 46). In an experiment with FC-loaded macrophages in which the antibody was absorbed with purified recombinant CT, this pattern of fluorescence was almost entirely absent (Fig. 3C).

Interestingly, despite no increase in CT protein in FC-loaded macrophages (see above and below), the fluorescence signal was brighter in the FC-loaded cells (compare A and B). This important point will be explored in detail in the following sections. We next performed confocal microscopy on these cells to assess two parameters that, given the thickness of the nucleus, are difficult to evaluate by conventional fluorescence microscopy: the degree to which the nuclear envelope was stained with the anti-CT antibody and the relative proportion of nuclear versus cytoplasmic staining. The data in Fig. 3D clearly show that a portion of the cells have a nuclear envelope pattern of fluorescence, as well as a nuclear matrix pattern. In addition, even in these confocal images, the overall pattern of fluorescence appears to be predominantly nuclear.

To examine where cholesterol accumulates in FC-loaded macrophages, control and FC-loaded macrophages were fixed, stained with filipin to visualize intracellular accumulations of free cholesterol (47), and viewed by fluorescence microscopy (Fig. 4, A and B). The filipin signal was much brighter in the FC-loaded cells, as expected, and was located predominantly in perinuclear vesicles. Although this localization, rather than a predominantly plasma membrane localization, was somewhat surprising (cf. Ref. 48), the data clearly show that the filipin signal was almost entirely absent from the nucleus or nuclear envelope (Fig. 4B). This finding, together with the CT immunofluorescence data in Fig. 3, show that most of the cholesterol in FC-loaded macrophages is in a different intracellular location from most of the CT, suggesting that the presence of the
bulk of cholesterol and CT in the same intracellular compartment is not necessary for CT activation in intact FC-loaded macrophages.

To further support this conclusion, we took advantage of a steroid, called U18666A, which is known to block the exit of lipoprotein-derived cholesterol from lysosomes (49). Note that the pattern seen in Fig. 4B, although not yet defined in terms of the identity of the fluorescent vesicles, is reminiscent of that seen in cells loaded with cholesterol in the presence of inhibitors of lysosomal cholesterol export, including U18666A (49, 50). In fact, when macrophages were incubated with acetyl-LDL plus U18666A, the pattern of fluorescence was very similar to that seen with cells incubated with acetyl-LDL plus the ACAT inhibitor 58035 (compare C and B in Fig. 4). The data in Fig. 5 clearly show that, under conditions of similar cellular FC loading (inset), the activation of PC biosynthesis in macrophages incubated with acetyl-LDL plus U18666A was very similar to that in cells incubated with acetyl-LDL plus 58035.

In this system, we cannot rule out a small amount of contact between FC and CT in the FC-loaded cells, and the FC-containing vesicles in either the U18666A- or 58035-treated cells have not yet been definitively identified (cf. Ref. 50). Nonetheless, these data, together with those in Figs. 2-4, strongly suggest that activation of PC biosynthesis in FC-loaded macrophages involves a signaling mechanism rather than direct interaction of CT with cholesterol.

Differential Recognition of Control and Activated CT on Immunoblots by Anti-CT Peptide Antibodies—In view of the possibility that the signaling mechanism proposed above could involve an alteration in the structure of the CT molecule, we were intrigued by the finding in Fig. 2 that the immunofluorescence signal with an antisynthetic peptide antibody was brighter in FC-loaded macrophages than in control cells, despite no increase in CT protein (Ref. 17 and below). This finding might imply that the antibody can detect an alteration of the CT molecule that is related to its activation. To examine this point in more detail, we used immunoblot analysis to compare the relative ability of this N-terminal antibody, which was made against amino acids 1–17 of CT (25), to interact with CT from unloaded and FC-loaded macrophages. The data in Fig. 6A show that at three different durations of macrophage FC loading, the antipeptide antibody interacted much more strongly with CT from FC-loaded macrophages (lane 4) compared with CT from the three non-FC-loaded cells (lanes 1–3). That this 42-kDa band is, in fact, CT was demonstrated by showing an absence of signal when the antibody was preabsorbed with purified recombinant CT (data not shown, cf. Fig. 3C). As a control, recognition of CT by this antibody was not increased by loading CHO-mSRAII cells with FC (data not shown), which does not lead to an increase in PC biosynthesis (Fig. 1).

The increased recognition of CT by the antipeptide antibody was seen as early as 4 h after FC loading and continued to increase modestly up to 12 h of FC loading. As demonstrated...
against holorecombinant rat liver CT (24) reacted equally well with CT from control and FC-loaded macrophages, which is consistent with our previous conclusion (17) that synthesis of CT is not increased in activated cells. Furthermore, this finding indicates that the increases in CT activity and antipeptide antibody immunoreactivity seen with FC loading cannot be explained by a decrease in degradation of the CT protein or a masking of CT by another 42-kDa protein on the blot. Note that neither the N-terminal nor mid-molecule antibody showed increased recognition of soluble CT from FC-loaded macrophages (not shown), which is important since only membrane-bound CT is activated by FC loading (17). Although the molecular basis of these findings has not yet been definitively determined (see “Discussion”), the data suggest that activation of CT in FC-loaded macrophages is associated with an alteration of the structure of the membrane-bound CT molecule.

The Effect of Cellular FC Loading on the Phosphorylation State of CT in Macrophages—Since CT is a phosphorylated protein (18–20), one possible alteration of the enzyme induced by cellular FC loading could be increased or decreased phosphorylation (cf. Refs. 43 and 44). To begin to examine this idea, 32P-labeled control and FC-loaded macrophages were fractionated into soluble and membrane fractions, and CT was isolated by immunoprecipitation. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, and in all cases, a distinct 32P-labeled band at ~42 kDa was detected by autoradiography. The intensities of the soluble CT bands from control and FC-loaded macrophages appeared identical. The intensity of the membrane-bound CT band from FC-loaded cells appeared somewhat less than that from control cells, but it was clear that two-dimensional peptide analysis was necessary for a thorough analysis of this point (cf. Ref. 51). Thus, the CT bands were eluted from the gels and treated with trypsin, and equal protein masses of the resultant peptides were subjected to two-dimensional analysis (Fig. 7). The autoradiograms in Fig. 7, A and B, show that the pattern of 32P-labeled peptides derived from soluble CT from control (A) and FC-loaded (B) macrophages appeared very similar. In contrast, several, but not all, peptides derived from membrane-bound CT from FC-loaded macrophages (D) were clearly labeled less intensely than those from control macrophages (C; see arrows). Thus, FC loading of macrophages is associated with dephosphorylation of certain regions of membrane-bound, but not soluble, CT.

The Role of Protein Dephosphorylation in the Activation of CT in FC-Loaded Macrophages—To begin to address the issue of whether the dephosphorylation of CT and/or other cellular proteins may be important in enzyme activation, control, and FC-loaded macrophages were treated in the absence or presence of calyculin A, a potent, cell-permeable inhibitor of protein phosphatase 1 and, to a lesser extent, phosphatase 2a (52). The data in Fig. 8A show that treatment with 5 nM calyculin A inhibited substantially the induction of PC biosynthesis seen with FC loading of the cells (compare the second and fourth bars in Fig. 8A). In contrast, the phosphatase inhibitor had no effect on PC biosynthesis in unloaded macrophages (compare the first and third bars in Fig. 8A). Thus, calyculin A is not simply an inhibitor of CT or any other enzyme in the PC biosynthetic pathway. Furthermore, the inhibitory effect of

Fig. 5. Effect of inhibition of FC export from lysosomes on PC biosynthesis in macrophages incubated with acetyl-LDL. Monolayers of J774 macrophages were preincubated for 30 min in the absence or presence of 1 μM U18666A and then incubated for 12 h in DMEM, 10% LPDS containing 50 μg of acetyl-LDL/ml alone, acetyl-LDL plus 1 μM U18666A, or acetyl-LDL plus 5 μg of 58035/ml. The cells were then incubated for 1 h in the same medium containing 2 μCi of [3H]choline/ml, and the radioactivity in cellular [3H]phosphatidylcholine was determined. The cells were also assayed for their content of FC (inst).

Fig. 6. Anti-CT immunoblots of CT from control and FC-loaded macrophages. A, monolayers of J774 macrophages were incubated for the indicated timepoints as follows: lane 1, DMEM, 10% LPDS alone; lane 2, DMEM, 10% LPDS containing 50 μg of acetyl-LDL/ml; lane 3, DMEM, 10% LPDS containing 5 μg of 58035/ml; lane 4, DMEM, 10% LPDS containing acetyl-LDL plus 58035 (i.e. FC loading conditions). Homogenates of the cells were then subjected to reducing SDS-10% polyacrylamide electrophoresis, blotted to nitrocellulose, and immunoblotted with an anti-N-terminal-CT synthetic antibody. The bands shown migrated in a region of the gel corresponding to ~42 kDa. B, monolayers of J774 macrophages (Me) were incubated for 12 h in DMEM, 10% LPDS alone (Con) or DMEM, 10% LPDS containing 50 μg of acetyl-LDL/ml plus 5 μg of 58035/ml (FC). Membrane fractions were prepared by the digitonin method and subjected to immunoblot analysis using the anti-N-terminal-CT synthetic peptide antibody employed above (N-term Ab), an anti-mid-molecule-CT synthetic peptide antibody (Mid-mol Ab), and an anti-holo-CT antibody (Holo Ab).

previously (17), the induction of PC biosynthesis in macrophages first becomes apparent at 4 h of FC loading and continues to peak up to 12 h of FC loading. Furthermore, as shown in Fig. 6B, an antibody made against another synthetic CT peptide (mid-molecule, amino acids 164–176) (26) also reacted more intensely with CT in membranes from FC-loaded macrophages, although the effect was not as marked as that seen with the N-terminal antibody. In contrast, an antibody made

3In contrast to what one may have predicted from the immunoblot data, soluble and membrane-bound CT from both control and FC-loaded macrophages were quantitatively immunoprecipitated by the N-terminal antibody. This was documented by showing that the supernatants from these precipitations had no CT signal as detected by immunoblot analysis with the mid-molecule antibody, which recognizes all forms of CT in macrophages.
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...calyculin A was not due to blockage of FC loading of the cells, as documented by the cellular cholesterol mass data in the inset to Fig. 8A.

The data in Fig. 8B show the effect of calyculin A treatment on the immunoblot pattern of CT from control and FC-loaded macrophages. As above (Fig. 6), both the N-terminal and mid-molecule antibodies showed an increased signal with CT from FC-loaded cells. This increased signal was blunted in FC-loaded cells treated with calyculin A. Note that the CT signal from unloaded macrophages is not affected by calyculin A treatment. In experiments not displayed here, we also showed that calyculin A treatment of FC-loaded macrophages partially prevented the decreased phosphorylation of peptides derived from membrane-bound CT. Thus, treatment of macrophages with a very low concentration of a potent phosphatase inhibitor blunts the induction of PC biosynthesis, the increased recognition of CT on immunoblots by anti-CT peptide antibodies, and the decreased phosphorylation of membrane-CT peptides.

To further support the idea that dephosphorylation can lead to macrophage CT activation, soluble and membrane fractions from unloaded macrophages were treated in vitro with x phage protein phosphatase (cf. Refs. 23 and 34) and then assayed for CT activity and immunoblot reactivity using the anti-N-terminal CT peptide antibody (Fig. 9). Phosphatase treatment led to only very small increases in soluble CT activity and in anti-N-terminal antibody reactivity. In contrast, membrane-bound CT was activated 2-fold by phosphatase treatment, and reactivity with the anti-N-terminal antibody was also markedly increased. These data, together with the calyculin A data in Fig. 8, strongly suggest that up-regulation of CT activity by FC loading of macrophages involves an intracellular protein dephosphorylation signaling mechanism.

**DISCUSSION**

The mechanistic studies presented in this report have potential importance to three areas of research, namely, FC-mediated intracellular signaling pathways, mechanisms of CT regulation, and adaptive responses of FC-loaded macrophages in atherosclerotic lesions.

**FC-mediated Intracellular Signaling—**A truly novel aspect of the data in this report is the finding that FC can trigger intracellular protein dephosphorylation (Fig. 7). Previous studies by others have elucidated other FC-mediated signaling pathways, including inhibition of the proteolytic activation of the transcription factor, sterol regulatory element binding protein (53), stimulation of the degradation of the enzyme, hydroxy-3-methylglutaryl-coenzyme A reductase (54, 55), and allosteric activation of ACAT (42). LDL and 25-hydroxycholesterol have also been implicated in the phosphorylation of hydroxy-3-methylglutaryl-coenzyme A reductase in human fibroblasts (56), but not in CHO cells (57). There are no previous reports, however, of FC-mediated protein dephosphorylation. The findings in this report raise many questions about this FC-mediated signaling event, including: (a) which intracellular site or sites (e.g. lysosomes and/or Golgi) accumulate and respond to the excess FC in this signaling pathway (see Figs. 4 and 5 and Ref. 50); (b) what proteins in addition to CT may be dephosphorylated, and are they important in the stimulation of CT or other FC-induced events in macrophages; (c) is the decrease in protein phosphorylation due to increased dephosphorylation or decreased phosphorylation; and (d) are specific phosphatase(s) or kinase(s) regulated by FC or does FC change the susceptibility of CT and possibly other proteins to constitutively active phosphatases or kinases? The apparent absence of this particular FC-mediated signaling pathway in CHO cells (Fig. 1) may provide a tool for addressing some of these questions in the future.

**Mechanisms of CT Activation—**Our current knowledge about how CT is activated in FC-loaded macrophages can be summarized as follows: (a) the regulation is post-translational (Ref. 17 and Fig. 6B, bottom blot); (b) the form of CT bound to membranes, presumably mostly nuclear membranes (Fig. 3), is the form that is activated in FC-loaded macrophages (Ref. 17 and Fig. 9), and there is no substantial soluble-to-membrane CT translocation (Ref. 17)\(^4\); (c) simple enrichment of CT-containing membranes with FC in vitro does not lead to CT activation (Fig. 2), and PC biosynthesis is up-regulated by FC-loading even when most of the CT and FC are in different intracellular sites (Figs. 3–5); (d) activated, membrane-bound CT shows increased recognition by antisynthetic CT peptide antibodies (Fig. 6; see also Figs. 3, 8, and 9); (e) activated, membrane-bound CT is partially dephosphorylated (Fig. 7); (f) inhibition of intracellular protein phosphatase activity in FC-loaded macrophages inhibits both the stimulation of PC biosynthesis and the increased recognition of CT by the anti-CT antibodies (Fig. 8); and (g) treatment of membrane fractions, but not soluble fractions, from unloaded macrophages in vitro leads to activation of CT and increased reactivity with the anti-N-terminal CT.

\(^4\) Y. Shiratori, M. Houweling, X. Zha, and I. Tabas, unpublished data.

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**Fig. 7. Autoradiography of two-dimensional maps of trypsin-digested CT phosphopeptides from control and FC-loaded macrophages.** Monolayers of J 774 macrophages were labeled for 12 h with \(^{32}P\) (7.5 mCi/well), in the absence (A and C) or presence (B and D) of 50 \(\mu\)g of acetyl-LDL/ml and 5 \(\mu\)g of 58035/ml. Soluble (A and B) and membrane fractions (C and D), made by digitonin method, were immunoprecipitated using an anti-N-terminal-CT synthetic peptide antibody. The immunoprecipitates were subjected to reducing SDS-10% polyacrylamide electrophoresis, blotted to PVDF membrane, and exposed to \(x\)-ray film. CT bands were then cut out and digested with modified trypsin, and the resulting phosphopeptides were resolved by electrophoresis and thin-layer chromatography. Shown are the autoradiograms of the two-dimensional peptide maps. The arrows in C (membranes from control macrophages) point out peptide spots that were diminished in D (membranes from FC-loaded macrophages).

**Fig. 8** The regulation is post-translational (Ref. 17).
antibodies against N-terminal (subjected to immunoblot analysis using the cells from the experiment in cellular [3H]phosphatidylcholine was dephosphorylated, has not been addressed by this study. The protein or proteins other than or in addition to CT need to be involved in enzyme activation, or whether some signal, mediated by intracellular protein dephosphorylation, which in turn leads to the activation of membrane-bound CT.

The apparent differences in the mechanism of CT activation in FC-loaded macrophages versus other situations could be related to the cell type (i.e. macrophages) and/or the inducing agent (i.e. FC loading). In FC-loaded CHO cells, unlike FC-loaded macrophages, PC biosynthesis is not increased (Fig. 1), and there is no increased recognition of CT on immunoblots by the anti-N-terminal CT peptide antibody (data not shown).
Furthermore, in preliminary experiments, we found that incubation of macrophages with oleic acid up-regulates PC biosynthesis and is associated with increased recognition of CT by the anti-N-terminal CT peptide antibody. Thus, pending examination of PC biosynthesis in other cell types loaded with FC and more detailed studies with oleic acid and other possible inducers of PC biosynthesis in macrophages, the mechanistic properties described herein may be part of a macrophage-specific repertoire for the up-regulation of PC biosynthesis.

Throughout this report, we have monitored the immunoreactivity of CT in fixed cells and on immunoblots and found that two antisynthetic peptide antibodies, but not an anti-cho-CT antibody, gave a much stronger signal with CT from FC-loaded cells (Figs. 3 and 6). Furthermore, there was a strong correlation between this increased immunoreactivity of CT and its state of activation (e.g. Figs. 8 and 9). How can these antibody data be interpreted? Since the N-terminal and mid-molecular antibodies were made against unmodified peptides whereas the holo-CT antibody was made against phosphorylated CT (24), and since CT is dephosphorylated when macrophages are FC-loaded (Fig. 7), the simplest explanation is that the antisynthetic peptide antibodies have greater recognition for dephosphorylated, and thus unmodified, CT. The region of CT that is phosphorylated when rat liver CT is expressed in insect cells or CHO cells, however, is the C terminus (amino acids 315–362) (37), not the N terminus or mid-molecular region used to generate the antipeptide antibodies. Thus, it is possible that the N terminus of CT, which contains two potential protein kinase C sites (66), and the mid-molecular region, which contains two serine residues (66), are phosphorylated in macrophages; if so, this may be at least one reason why activation of CT in this cell type is different from that in CHO cells (Fig. 1 and above). Alternatively, the antibody data might be due to the removal of some other modification of CT in the N terminus and mid-molecular region, which might also be unique to macrophages (cf. Ref. 66). Finally, since proteins from SDS gels may reautograph after transfer to nitrocellulose or PVDF membranes (e.g. see Ref. 67), it is theoretically possible that C-terminal dephosphorylation could lead to an N-terminal or mid-molecular conformational change that could be detected by immunoblotting. Interestingly, CT activation in oleic acid-treated HepG2 cells is also associated with increased recognition by an anti-CT antibody (62), but in this case, the antibody used was one made against native rat liver CT, not against a synthetic peptide. In future studies, as we further define the structural changes in macrophage CT that occur with FC loading, the explanation of our antibody data should become apparent.

Adaptive Responses of FC-loaded Macrophages in Atherosclerotic Lesions—The physiological significance of these studies is based upon the observation that macrophages in atherosclerotic lesions are rich in FC and PC and have high rates of PC biosynthesis (10–15, 68–70). We hypothesize that up-regulation of PC biosynthesis in FC-loaded macrophages represents an adaptive response to the excess FC load in these cells. In particular, by maintaining the phospholipid:FC ratio within certain limits, the PC regulatory response would help prevent adverse changes in membrane fluidity as well as intracellular cholesterol crystalization (15, 16). The corollary of this hypothesis is that if this response were diminished, as might occur in the presence of certain cytokines known to be present in atheromatia (cf. Refs. 71 and 72), macrophage necrosis might ensue. In fact, recent experiments in our laboratory have revealed that inhibition of the up-regulation of PC biosynthesis in FC-loaded macrophages leads to accelerated macrophage necrosis.  

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