Exchange and mass efflux of cholesterol in macrophages. Evidence for a common mechanism and a role for plasma membrane proteins

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Abstract Exchange and net mass efflux of cholesterol were investigated in [^{3}H]cholesterol-labeled or cholesteryl ester-loaded murine peritoneal macrophages, respectively. Macrophages were subjected to mild proteolysis prior to measurements of mass efflux or exchange to assess whether plasma membrane proteins participated in either process. Cholesterol exchange and mass efflux were inhibited up to 70% following trypsinization. The inhibitory effect was reversible as cells regained normal efflux and exchange 6–8 hr following treatment. Incubation of trypsinized cells with cycloheximide prevented recovery, indicating that protein synthesis was necessary for restoration of normal cholesterol efflux. Studies with peptide and nonpeptide inhibitors of proteolysis suggested that active catalytic activity of trypsin was necessary for the inhibitory effect to be expressed. The degree of inhibition for both cholesterol exchange and mass efflux was dependent in a quantitatively similar manner on the time of incubation and the concentration of trypsin, suggesting that the mechanism of cholesterol exchange and mass efflux were similar at the level of the plasma membrane. Two other serine-proteases, thrombin and elastase, were also capable of inhibiting cholesterol removal in a similar manner. No cell death was observed by altered morphology, detachment, changes in DNA or protein content, or trypan blue exclusion even under the most severe proteolytic conditions. These studies suggest that protease-sensitive plasma membrane proteins play a role in cholesterol efflux in macrophages. —**Randolph, R. K., and H. F. Hoff.** Exchange and mass efflux of cholesterol in macrophages. Evidence for a common mechanism and a role for plasma membrane proteins. *J. Lipid Res.* 1986. **27:** 307–315.

Supplementary key words trypsin • thrombin • elastase • cycloheximide

The maintenance of cellular cholesterol homeostasis is undoubtedly the result of a balance between opposing mechanisms which contribute cholesterol to or remove cholesterol from the cell. Understanding of the cellular events that contribute to cholesterol removal or efflux is vital to a complete description of the mechanism of cholesterol accumulation in foam cells of developing atherosclerotic lesions. Previous studies have shown that the rate of cholesterol removal can be a function of extracellular acceptor concentrations (1), cell type (1), the size of intracellular storage pools of cholesterol or cholesteryl ester (2), the site of cholesteryl ester deposition (2, 3), or the physical state of stored cholesteryl esters (4). Independent of which factor is rate-limiting in any of the above circumstances, all cholesterol that exits the cell presumably passes through the plasma membrane. This is the terminal cellular step in cholesterol efflux and is thought to occur by the desorption of cholesterol out of the plasma membrane into the extracellular space (5). According to the "aqueous diffusion" model, which has been advanced to explain this aspect of cholesterol efflux (6), two general situations must be considered. First, when extracellular acceptor concentrations are sufficiently high such that efflux is zero order with respect to acceptor concentration, the rate of cholesterol desorption from the membrane is a function of the degree and type of nearest neighbor interactions between desorbing cholesterol molecules and other membrane components in the bilayer (1, 7). In this context, most of the evidence to date points to the importance of membrane lipid components in contributing to interactions of this kind and, thus, in determining removal rates. Second, when extracellular acceptor concentrations are low, the rate-limiting step in cholesterol removal is no longer the desorption step but the diffusion time of desorbed cholesterol in the unstirred water layer (1, 5). Any interaction or binding of acceptor particles with the plasma membrane that would increase the local concentration of acceptors conceivably could enhance the cholesterol removal rate by increasing the frequency of collisions between desorbed cholesterol molecules and acceptor particles. For either of these cases, relatively little is known about the possible requirements for or contribu-

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; PBS, phosphate-buffered saline; LDL, low density lipoproteins; HDL_3, high density lipoproteins$_3$.

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tion of membrane proteins to the mechanism of cholesterol efflux.

In 1971, Werb and Cohn (2) reported that cholesterol exchange was reversibly inhibited in murine macrophages that had been subjected to mild trypsin treatment. This result suggested a role for plasma membrane proteins in cholesterol exchange. In order to pursue this interesting observation further, studies utilizing trypsin treatment as a tool were undertaken to explore the participation of plasma membrane proteins in cholesterol efflux.

MATERIALS

Trypsin (bovine pancreas), elastase (porcine pancreas, type 1), thrombin (human plasma), phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), and cycloheximide were obtained from Sigma Chemical Company, St. Louis, MO. Sodium ^{51}chromate (200–900 Ci/g) and [1,2,-^{3}H(N)]-cholesterol (55 Ci/mmol) were obtained from New England Nuclear, Boston, MA, and [1-^{14}C]oleic acid (48 mCi/mmol) was obtained from ICN Biochemicals, Inc., Irvine, CA.

METHODS

Resident peritoneal macrophages were obtained by lavage from young male Swiss Webster mice, Charles River Animal Breeding Labs, Wilmington, MA. Peritoneal cells were plated at a density of 2–3 $\times$ 10^6 cells/well into 24-well tissue culture plates (Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium (DMEM) (Whitaker M.A. Bioproducts, Walkersville, MD) containing 10% calf serum (Hyclone Laboratories, Logan, UT). Following an overnight incubation (37°C, 95% air, 5% CO_2), non-adherent cells were washed away by multiple rinses of Dulbecco's phosphate-buffered saline (PBS). The resulting cultures contained approximately 1 $\times$ 10^6 macrophages (40 μg of cell protein or 4 μg of DNA per well).

Low density lipoproteins (LDL) and high density lipoproteins$_3$ (HDL_3) were obtained from normocholesterolemic human plasma by sequential ultracentrifugation at d 1.019–1.063 g/ml and d 1.125–1.210 g/ml, respectively. Low density lipoproteins were acetylated as described by Basu et al. (8).

Activity of scavenger receptors was measured by the increase in the incorporation of [^{14}C]oleic acid into cholesteryl [^{14}C]oleate in macrophages incubated with acetylated-LDL (50 μg of protein/ml) and [^{14}C]oleate/albumin (0.27 mM and 1 μCi/ml) (9).

Macrophage Fc receptors were measured by the number of IgG-opsonized erythrocytes bound during a 1-hr incubation at 4°C (10). A minimum of 100 cells were scored for erythrocyte rosettes. Sheep erythrocytes and goat anti-sheep erythrocyte IgG preparations were obtained from the Department of Microbiology, Cleveland Clinic Foundation, Cleveland, OH.

Cytotoxicity was assessed by monitoring the release of cellular lactate dehydrogenase (LDH) activity (Sigma Chemical Co., LDH Kit No. 340) and by measuring the release of cellular ^{51}chromium radioactivity to medium containing 20% calf serum. Labeling of cells with ^{51}chromium was achieved during a 3-hr incubation with sodium ^{51}chromate (100 μCi/ml) at 37°C in the presence of 10% calf serum.

Cholesterol exchange experiments were initiated by labeling macrophages with [^{3}H]cholesterol. Heat-inactivated serum was labeled with thin-layer chromatography-purified [^{3}H]cholesterol (>99% purity) as described by Werb and Cohn (11). A 24-hr incubation with the [^{3}H]cholesterol-labeled serum (10% and 1 μCi/ml) was sufficient for cellular cholesterol to achieve isotopic steady state (average [^{3}H]cholesterol specific activities for 10% serum and cells were 1.0 $\times$ 10^4 cpm/μg and 8.5 $\times$ 10^3 cpm/μg, respectively). Less than 10% of total cell cholesterol radioactivity was found in the cholesteryl ester fraction of the labeled cells. For mass efflux experiments, macrophages were loaded with cholesteryl ester by incubation with medium containing acetylated-LDL (50 μg protein/ml). Cellular cholesteryl ester mass was increased approximately 40-fold following a 24-hr incubation. Cholesteryl ester comprised greater than 70% of total cholesterol in loaded cells. Following labeling or loading, medium containing labeled serum or acetylated-LDL was removed and cells were incubated for an additional 24 hr with medium containing human serum albumin (2 mg/ml) in order to allow surface bound and undegraded lipoproteins to undergo complete processing. On the day of experiment, cells were treated with trypsin or other proteolytic enzymes and the appropriate controls. Following treatment, enzyme preparations were aspirated and residual proteolytic activity was quenched by washing the cells with PBS containing SBTI (1 mg/ml) or serum (10%). The exchange or efflux phase of the experiment was initiated by the addition of medium containing serum or isolated HDL_3. After various periods of time, cells were washed with PBS and harvested by scraping. Cells were further washed by centrifugation and were resuspended in 0.1 M phosphate buffer. Following disruption of the cells by sonication, aliquots were taken for determination of [^{3}H]cholesterol radioactivity or cholesterol mass and DNA. Cellular cholesterol mass and DNA were determined as described by Gamble et al. (12) and Labarca and Paigen (13), respectively. Protein was assayed on lipoproteins and disrupted cells by a modification of the Lowry method (14). Tritiated cholesterol radioactivity was measured by liquid scintillation spectrophotometry to a 2 sigma error of <1%. Exchange and mass efflux data are expressed as the fractional amount of initial radioactivity

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or cholesterol mass remaining in cells (Figs. 1 and 3) or as the fractional amount of initial radioactivity or cholesterol mass exchanged or excreted (Figs. 4 and 5 and Tables 2 and 3), respectively. We chose to use the term "exchange" in experiments where the loss of cellular [^{3}H]cholesterol radioactivity was monitored since no change in cholesterol mass was detected in the labeled cells under any experimental conditions. From this result we concluded that unlabeled cholesterol from the acceptor pool was exchanging with the labeled cholesterol.

RESULTS

Inhibition of the rate of cholesterol exchange in macrophages by trypsin treatment was reported initially by Werb and Cohn (2). In confirmation of this earlier report, the data in **Fig. 1** and **Fig. 2** show that trypsin treatment of macrophages resulted in a marked reduction in the rate of cholesterol exchange as measured by both the rate of label removal from cells (Fig. 1) and its appearance in the medium (Fig. 2). This inhibition persisted 6 to 8 hr following treatment, at which time the cells recovered to the control rate. Incubation of the trypsin-treated cells with cycloheximide prevented this recovery, suggesting that active protein synthesis was necessary. The effect of trypsin was likely due to its proteolytic activity on the cell surface as indicated by the normal rate of exchange in cells incubated with inactivated trypsin preparations (SBTI- and PMSF-treated trypsin in Fig. 1).

In order to determine whether the data shown in Figs. 1 and 2 were, in fact, a reflection of an exchange reaction between cells and the serum, we tested the effect of trypsin treatment on the uptake of labeled cholesterol from serum. The results in **Table 1** indicate that trypsin treatment inhibited the rate of label uptake by approximately 60–70% compared to control cells. This magnitude of inhibition was similar to that observed for cholesterol removal (Fig. 1). Moreover, trypsin-treated cells demonstrated a normal rate of label uptake 10 hr after treatment, suggesting recovery from the trypsin treatment.

In other studies we sought to determine the requirement of cholesterol exchange for protein synthesis. For these experiments, control cells, i.e., no trypsin treatment, were incubated with medium containing 20% serum alone or the same medium plus cycloheximide (1.6 μg/ml). The rate was similar in these two groups (0.060/hr) through the first 8 hr of incubation, at which time a 55% reduction in [^{3}H]cholesterol exchange was apparent in the cells incubated with cycloheximide compared to cells incubated in the absence of cycloheximide (0.033/hr vs. 0.060/hr, respectively). This result was also consistent with the observation of Werb and Cohn (2) in control cells treated with puromycin.

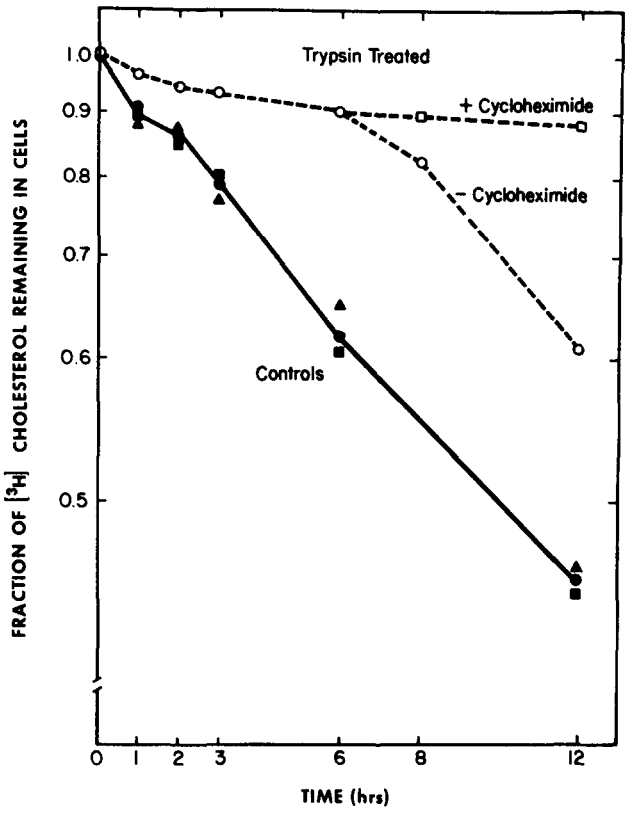

Fig. 1. Effect of trypsin treatment on exchange of [^{3}H]cholesterol in mouse peritoneal macrophages. Following labeling and equilibration as described under Methods, cells were incubated for 1 hr at 37°C with medium alone (●), medium containing 250 μg of trypsin/ml (○), medium containing 250 μg PMSF-inactivated trypsin/ml (▲), or medium containing 250 μg trypsin/ml plus 1 mg SBTI/ml (■). All treatment media were then aspirated and cells were washed with medium containing 10% calf serum. The exchange assay was initiated by incubating the cells with medium containing 20% calf serum. To one-half of the dishes containing trypsin-treated cells was added cycloheximide (1.6 μg/ml) (□). Cells were harvested at the indicated times and washed by centrifugation, and [^{3}H]cholesterol radioactivity was determined. The average zero-time (post-treatment) specific activity (± SD) for all treatments was 8,055 cpm/μg of total cholesterol ± 243. The average total cellular cholesterol mass (± SD) was 0.31 ng/ng of DNA ± 0.05 and did not change with incubation time. The data from this experiment represent one of three separate experiments in which similar results were obtained. All values are the average of duplicate cultures.

Treatment of cholesteryl ester-loaded macrophages with trypsin also resulted in a reduction in the rate of net cholesterol efflux (**Fig. 3**). Impaired cholesterol efflux was evident through the first 8 hr of incubation following treatment. By 12 hr the cells had regained efflux approaching that of the control cells. The cholesterol excreted from these cells was predominantly from the cholesteryl ester storage pool but was recovered in the medium as unesterified cholesterol as reported by others (3, 15).

Inhibition of cholesterol exchange or mass efflux, as a result of trypsin treatment, was not dependent on type or concentration of acceptor, as results similar to these shown in Figs. 1 and 2 (50–60% inhibition relative to

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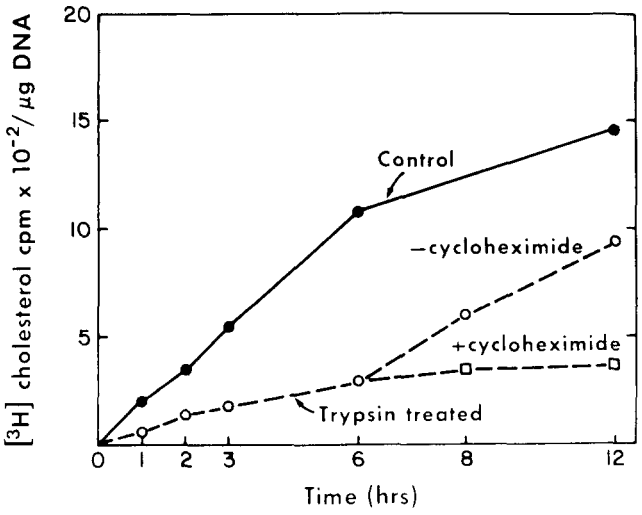

Fig. 2. Effect of trypsin treatment on the efflux of [^{3}H]cholesterol to the medium from labeled mouse peritoneal macrophages. Duplicate aliquots of medium (from the experiment shown in Fig. 1) were taken from the cultures at the time indicated and [^{3}H]cholesterol radioactivity was determined. Values are the average of duplicate cultures.

controls) have been achieved utilizing serum (5–30%) and isolated human HDL_3 (5–400 μg HDL_3 cholesterol/ml) as acceptor (data not shown).

From these data (Figs. 1 and 3), it was apparent that trypsin was perturbing both cholesterol exchange and mass efflux in a similar manner. In order to explore this possibility further, the effect of trypsin concentration and time of treatment on cholesterol exchange and mass efflux were compared. For these studies, rate measurements were made 6 hr following treatment, as exchange and efflux were linear and the effect of trypsin persisted over this time period. The data in **Fig. 4** demonstrate that the inhibitory effect of trypsin on cholesterol exchange and mass efflux was dependent on the enzyme concentration. Both processes were similarly sensitive to trypsin treatment with maximal inhibition observed in cells that had been treated with trypsin concentrations of 125 μg/ml. The inhibitory effect of trypsin as a function of time of treatment was similar for exchange and mass efflux (**Fig. 5**). No additional inhibition was observed in treatments longer than 40 min. In no instance was cholesterol exchange or mass efflux completely inhibited. Approximately 30–40% of total fractional efflux was conserved even under the most extreme proteolytic conditions.

The optimal conditions for the present studies included treatment of cells with trypsin concentrations of ≅250 μg/ml for up to 40 min duration. Typical use of trypsin in other tissue culture systems for the release of cells for passage or analysis involves roughly equivalent concentrations, but shorter durations (5–10 min). For this reason, a series of control studies was conducted in order to determine whether the trypsin treatment was inhibiting efflux as a result of overt cell damage or cell death (**Table 2**). Under optimal conditions for the inhibition of cholesterol exchange, activity of the scavenger receptor was almost totally abolished. This result was expected as the scavenger receptor has been reported to be protease-sensitive (16). Another macrophage membrane protein, the Fc receptor, which has been reported to be resistant to all but the most severe proteolysis (17), was unaffected by the trypsin treatment. Two indicators of cytotoxicity, ^{51}chromium and lactate dehydrogenase activity release, were also unaffected by optimal trypsin treatment. Trypsin treatment did not result in detachment or loss of cells or DNA from the tissue culture dishes. In addition, staining by the vital dye, trypan blue, was not different between control and trypsin-treated cells (<1% cells staining positively). There was some alteration in morphology of treated cells, however, which was consistent with a previous report by Rabinovitch and DeStefano (18), in which they described how trypsinized macrophages quickly spread on the tissue culture dish and took on a more flattened appearance. This change occurred at very low trypsin concentrations and could not be correlated with the dose-dependent inhibition of cholesterol efflux.

In order to determine whether the effect of trypsin on cholesterol efflux was enzyme specific, [^{3}H]cholesterol exchange was measured in macrophages treated with trypsin or with two other serine proteases, thrombin or elastase (**Table 3**). Both thrombin and elastase were capable of inhibiting [^{3}H]cholesterol exchange to a degree similar to that observed for trypsin. Recovery of normal rates of [^{3}H]cholesterol exchange in thrombin- and elastase-treated cells was observed in the 6–8 hr "window" following treatment (data not shown).

DISCUSSION

Utilizing extracellular proteolysis, we have demonstrated inhibition and recovery of cholesterol movement from macrophages which appears similar for both ex-

TABLE 1. Effect of trypsin treatment on the uptake of [^{3}H]cholesterol from labeled serum by mouse peritoneal macrophages

Time following Treatment	[^{3}H]Cholesterol Uptake	
	Control Cells	Trypsin-Treated Cells
hr	*cpm/μg DNA per hr*	
1	263	61
2	212	90
3	202	72
6	244	89
10	237	265

Uptake of [^{3}H]cholesterol was measured during 1-hr incubations with labeled serum (20% and 1 μCi/ml) at the times indicated following a 1-hr treatment with medium containing no additions (control) or 250 μg trypsin/ml (trypsin). Values are the average of duplicate cultures.

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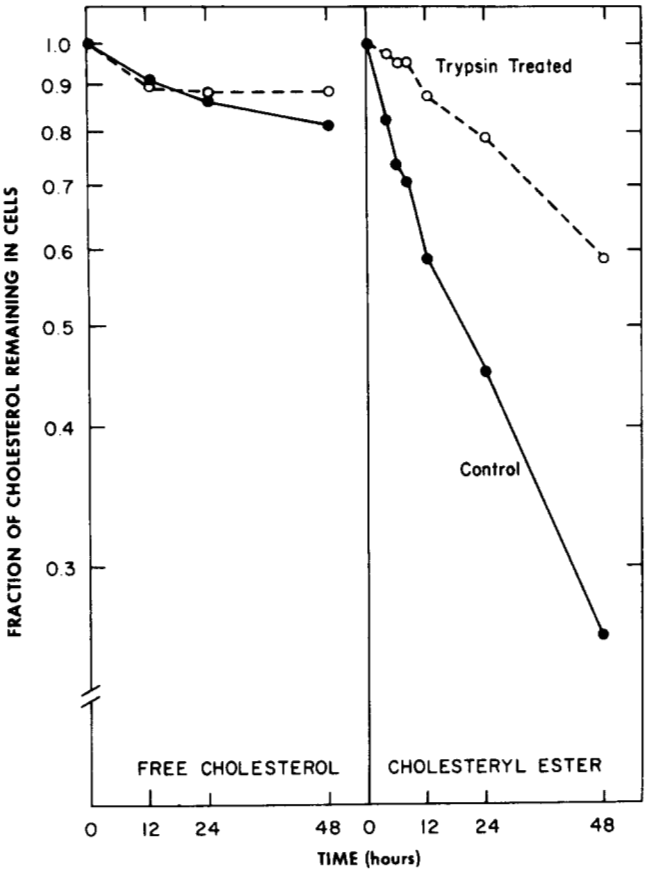

Fig. 3. Effect of trypsin treatment on mass efflux of cholesterol in mouse peritoneal macrophages. Following cholesteryl ester loading and equilibration as described under Methods, cells were incubated for 1 hr at 37°C with medium containing 250 μg of trypsin/ml (○) or medium containing 250 μg of trypsin/ml plus 1 mg SBTI/ml (●). Treatment media were aspirated and cells were washed with medium containing 10% calf serum. Cholesterol efflux was initiated by the addition of fresh medium containing 20% calf serum. At the indicated times, cells were harvested by scraping and washed by centrifugation, and free cholesterol and cholesteryl ester mass were determined. Free cholesterol and cholesteryl ester mass at zero-time (post treatment) were 0.56 ng/ng DNA and 2.61 ng/ng DNA, respectively, for control cells and 0.59 ng/ng DNA and 2.70 ng/ng DNA, respectively, for trypsin-treated cells. Results similar to those shown were obtained in one other experiment. Values are the average of duplicate cultures.

change and mass efflux. As expressed by trypsin-sensitivity, the mechanism of cholesterol removal does not shift or change under conditions in which there is increased cellular "need" (40-fold increase in cellular cholesteryl esters) to excrete cholesterol or when there are large differences in the absolute rates of cholesterol removal (0.12 ng/ng DNA per 6 hr for exchange vs. 0.63 ng/ng DNA per 6 hr for mass efflux). We conclude that a common mechanism of cholesterol removal is operative at the plasma membrane for net mass efflux and exchange reactions in macrophages. By extension, this suggests that the mechanism for cholesterol removal (at the plasma membrane) is similar in cholesteryl ester-loaded and non-loaded macrophages. This conclusion is perhaps intuitive from other studies of cholesterol exchange and net transfer in human erythrocytes (19), viral membranes (20, 21), and rabbit brush border membrane vesicles (22) but has not, to our knowledge, previously been demonstrated in a model system in which the source of excreted cholesterol was an intracellular storage pool of cholesteryl ester. Since cholesterol exchange in macrophages presumably occurs in a manner similar to that suggested for other mammalian cells, i.e., as described by the aqueous diffusion model (5), our data imply that net mass efflux also occurs by this mechanism.

Three lines of evidence from the present studies suggest that the plasma membrane proteins play a role in the movement of cholesterol out of the plasma membrane. First, cholesterol exchange and efflux are protease-sensitive. Extracellular proteolysis has long been utilized as a tool to explore the participation of plasma membrane

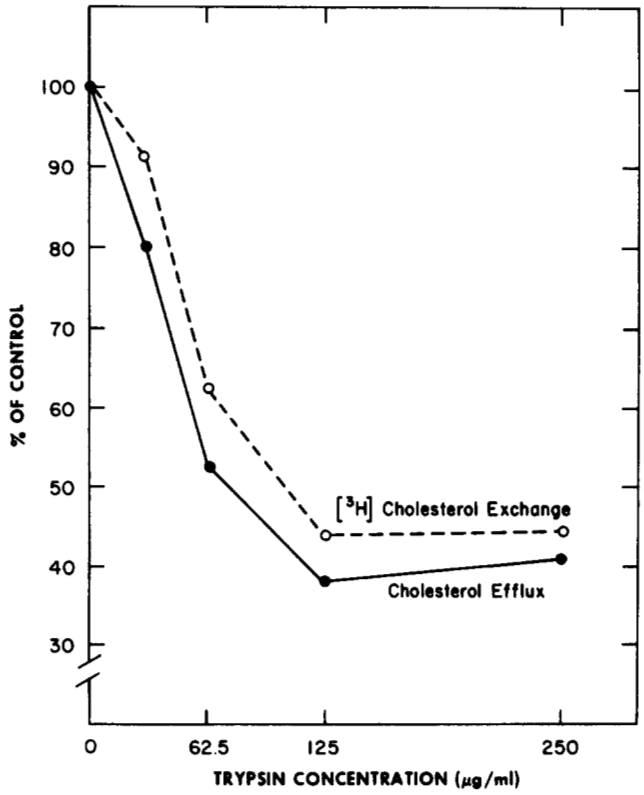

Fig. 4. Effect of trypsin concentration on [^{3}H]cholesterol exchange and cholesterol mass efflux in mouse peritoneal macrophages. Cells were labeled with [^{3}H]cholesterol or loaded with cholesteryl ester and allowed to equilibrate as described under Methods. Medium containing the indicated concentrations of trypsin or PMSF-inactivated trypsin (control) was added and incubations were continued for 1 hr at 37°C. Following treatments, cells were washed and harvested (zero-time controls) or incubated in medium containing 20% calf serum. Following 6 hr of incubation, the remaining cells were harvested and [^{3}H]cholesterol radioactivity (exchange) or total cholesterol mass (efflux) was determined. The fractional rate for [^{3}H]cholesterol exchange (100% control) was 0.38/6 hr and 0.27/6 hr for cholesterol mass efflux (100% control). This experiment was repeated twice with similar results. Values are the average of duplicate cultures.

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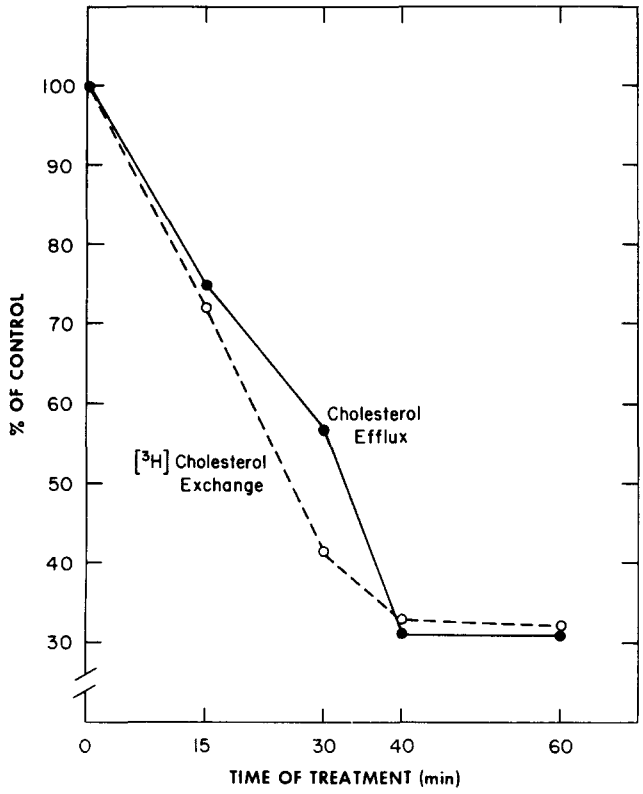

Fig. 5. Effect of time of trypsin treatment on [^{3}H]cholesterol exchange and cholesterol mass efflux in mouse peritoneal macrophages. Cells were labeled with [^{3}H]cholesterol or loaded with cholesteryl ester and equilibrated as described under Methods. Medium containing 250 μg trypsin/ml or 250 μg PMSF-inactivated trypsin/ml (controls) was added and incubations were continued for the indicated periods of time at 37°C. Following the respectively timed treatments, cells were washed and harvested (zero-time control) or incubated with medium containing 20% calf serum for an additional 6 hr. Following the exchange or efflux period, cells were havested and [^{3}H]cholesterol radioactivity or total cholesterol mass was determined. Fractional rate for [^{3}H]cholesterol exchange (100% control) was 0.36/6 hr and 0.28/6 hr for cholesterol efflux (100% control). Similar results were obtained in one other separate experiment. Values are the average of duplicate cultures.

proteins in a variety of cellular processes. Second, active cellular protein synthesis is necessary for recovery from the trypsin treatment. That cycloheximide interrupts recovery and potentiates the effect of trypsin suggests that the plasma membrane proteins that have been destroyed or altered by the treatment are being restored by new protein synthesis. Third, cholesterol exchange is impaired in control cells incubated with cycloheximide. This suggests that protein synthesis is necessary for the maintenance of membrane protein(s) that are important in the efflux mechanism. Clearly, the cholesterol efflux mechanism is altered when plasma membrane proteins are disturbed by exogenous (proteolytic digestion) or endogenous (protein synthesis inhibition) means.

Maximal inhibition of cholesterol efflux occurs under conditions of trypsinization that are clearly sufficient to allow destruction of trypsin-sensitive membrane proteins (e.g., scavenger receptor, Table 1). The treatments are not, however, so severe that all membrane proteins are destroyed (e.g., Fc receptor) or that cells are killed or become irreversibly injured or that plasma membranes lose patency. We suggest that the high concentration of trypsin and long incubation time required to maximally reduce cholesterol removal may not necessarily reflect the magnitude of the proteolytic insult to membrane proteins that is required to elicit an effect on cholesterol efflux. Of particular importance in support of this is the finding that significant effects on cholesterol efflux are observed in treatments when the trypsin concentration is low and/or when treatments are of short duration (Fig. 3 and 4). In addition, two other serine-proteases, thrombin and elastase, are also capable of provoking an inhibition of cholesterol efflux in macrophages (Table 2) at concentrations much lower than that for trypsin. However, since the various enzyme preparations cannot be directly compared for activity due to differences in purity and substrate specificity, it is difficult to suggest a relative potency for the various enzymes to inhibit cholesterol exchange. Nevertheless, it does seem that, under the appropriate conditions, relatively mild proteolysis can result in impaired cholesterol efflux.

There are at least two mechanisms whereby perturbation of plasma membrane proteins by protease treatment could affect cholesterol efflux. First, the proteases could be destroying membrane proteins that serve as binding sites or receptors for acceptor particles. This possibility would be particularly important under conditions where the efflux was a function of acceptor concentration. Since the rate-limiting step in this situation is the diffusion of desorbed cholesterol in the unstirred water layer, a local increase in acceptor concentration near the cell surface (e.g., as a result of binding events) could enhance the removal rate (1, 5). A high density lipoprotein binding site has been demonstrated in a number of cell types and has been postulated in certain cases to play such a role in cholesterol efflux (23, 24). Some evidence suggests that this binding site may be a plasma membrane protein (24). This has not been demonstrated directly and there is, in fact, evidence to the contrary (25). In either case, there is agreement that this binding site is protease-resistant (23–25). Preliminary studies in our laboratory comparing the relationship between HDL binding and efflux rate confirm this protease resistance in macrophages and indicate that HDL binding is not altered under conditions which (in the present paper) give maximal inhibition of efflux. As a result, this mechanism seems incompatible as an explanation for the protease sensitivity of cholesterol efflux. Second, protease treatment could be affecting efflux by altering the properties of the membrane in general. This might occur, for example, if proteolytic cleavage of externally accessible peptides produced conformational changes of residual protein within the bilayer.

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TABLE 2. Effect of trypsin treatment on [^{3}H]cholesterol exchange, viability, and function of mouse peritoneal macrophages

Parameter	Cell Treatment	
	Control	Trypsin
[^{3}H]Cholesterol exchange (fraction exchanged/6 hr)	0.32	0.12
Scavenger receptor acetyl-LDL prompted cholesterol esterification (nmol [^{14}C]oleate→cholesteryl [^{14}C]oleate/μg DNA per 6 hr)	0.20	0.07
Fc Receptor (IgG-opsonized erythrocytes bound/macrophage) x ± SD	8 ± 3	10 ± 4
^{51}Chromium-release[a] (fraction of total label released/3 hr)	0.10	0.10
Lactate dehydrogenase release[b] (fraction of total cellular activity released/6 hr)	0.18	0.21

Cultured macrophages were incubated with medium alone or medium containing 260 μg of trypsin/ml for 1 hr. Following treatments, media were aspirated, residual proteolysis was quenched, and dishes were divided into the five groups listed in the table. The respective parameters were measured as described under Methods. All values (except those for Fc receptor assay; n = 100 macrophages) are the average of duplicate dishes.

[a] Total initial cellular ^{51}chromium radioactivity (post-treatment) was 84,075 cpm/μg DNA and 81,365 cpm/μg/DNA for control and trypsin-treated cells, respectively.

[b] Total initial lactate dehydrogenase activity (post-treatment) was 60.5 I.U./μg DNA and 54.2 I.U./μg DNA for control and trypsin-treated cells, respectively.

These changes could result in altered packing or ordering of membrane lipids such that nearest neighbor interactions between desorbing cholesterol molecules and phospholipid acyl chains or hydrophobic regions of proteins are increased. This sequence has been suggested to explain the effects of chemical modification of membrane proteins on cholesterol exchange in a number of cell types. Bittman et al. (26) have demonstrated that cholesterol exchange in human erythrocyte ghosts is increased when membrane proteins are covalently cross-linked by treatment with diamide. Cross-links between plasma membrane proteins and/or phospholipids, on the other hand, may be responsible for decreased cholesterol exchange that is observed in irreversibly sickled erythrocytes or in normal erythrocytes that have been treated with malondialdehyde (27). From the present data and this scant literature base, it is apparent that exogenous chemical modifications or perturbations to plasma membrane proteins (e.g., cross-linking or proteolytic digestion) can result in altered efflux for cholesterol in certain circumstances. Unfortunately, the precise relationship between altered plasma membrane proteins and cholesterol efflux is not yet evident. Nevertheless, we favor this possibility for explaining the effect of protease digestion of membrane proteins on cholesterol efflux in macrophages. Our data and that of Werb and Cohn (2) on the apparent dependence of normal cholesterol exchange on protein synthesis are consistent with the possibility that plasma membrane proteins might also play a role in cholesterol efflux in the "nonperturbed" state. It is conceivable, in this regard, that "native" membrane proteins could influence efflux by some ordering or packing effects on bilayer components as described above. A role for "native" plasma membrane proteins has, in fact, been suggested by Bloj and Zilversmit (22) to account for pools of tightly and loosely bound cholesterol in rabbit intestinal brush border membranes and by Bellini et al. (28) to explain intrinsic differences in efflux from plasma membranes of WIRL-3C rat liver and Fu_5AH rat hepatoma cells.

Our finding that cholesterol efflux in macrophages is protease-sensitive represents a unique observation with respect to cell type. Except for a report by Lange et al. (29) in which vigorous trypsin treatment was reported to have no effect on cholesterol exchange in human erythrocytes, there are no other references in the literature that address the effects of protease treatment on cholesterol efflux in other cell types. As a result, it is not yet clear

TABLE 3. Effect of neutral proteases on [^{3}H]cholesterol exchange in mouse peritoneal macrophages

Enzyme	Concentration	[^{3}H]Cholesterol Exchange
	μg enzyme/ml	*fraction exchanged/6 hr*
None		0.40
Trypsin	125	0.12
Elastase	30	0.18
Thrombin	0.5	0.20

Exchange of [^{3}H]cholesterol was measured as described in Methods following 1-hr treatments with the indicated enzymes. The acceptor for this experiment was 20% calf serum. The sources of the different enzyme preparations are given in Materials. A duplicate experiment yielded similar results. Values are the average of duplicate cultures.

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whether this feature of cholesterol efflux in macrophages is cell specific.

Since thrombin and elastases as well as other neutral proteases could be encountered in vivo by macrophages at sites of inflammation and/or tissue repair (30), a role for this effect in vivo is possible. It is interesting in this regard that macrophages isolated from the lungs of tuberculous mice (31) and inflammatory peritoneal exudates (32) are enriched in cholesterol. We suggest that extracellular proteolytic activity may result in impaired cholesterol efflux from macrophages and, thus, increased cellular cholesterol content. Such a mechanism might contribute to the enrichment of inflammatory macrophages with cholesterol or to the development of cholesteryl ester-laden foam cells in atherosclerotic lesions.

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