Differential effects of an acyl-coenzyme A:cholesterol acyltransferase inhibitor on HDL-induced cholesterol efflux from rat macrophage foam cells

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Abstract When rat macrophages were converted to foam cells with acetylated low density lipoprotein (acetyl-LDL) and then reacted with high density lipoprotein (HDL) and an inhibitor of acyl-coenzyme A:cholesterol acyltransferase (58-035) (sequential incubation system), 58-035 did not enhance HDL-induced cholesterol efflux. In contrast, when macrophages were exposed to acetyl-LDL in the presence of both HDL and 58-035 (simultaneous incubation system), HDL-induced cholesterol efflux was enhanced 1.6-fold by 58-035. Cholesterol efflux with HDL alone was 2-fold greater in simultaneous incubation than in sequential incubation. These results suggest the presence of an efficient cholesterol efflux pathway in simultaneous incubation which is not available in sequential incubation. This pathway, which we refer to as the neutral cholesterol ester hydrolase-independent pathway, is characterized by the efflux of lysosome-derived cholesterol without re-esterification.

Key words: Macrophage foam cell; Cholesteryl ester cycle; ACAT inhibitor; High density lipoprotein; Cholesterol efflux; Intracellular cholesterol transport

1. Introduction

Macrophage foam cells (MFC) are a prominent feature of the early stage of atherosclerotic lesions [1]. Macrophages are known to take up chemically modified low density lipoproteins (modified LDLs) such as acetyl-LDL and oxidized LDL through the scavenger receptor pathway, which leads to the accumulation of intracellular cholesteryl esters (CE) or the formation of foam cells [2]. Among these modified LDLs, oxidized LDL is regarded as a major atherogenic lipoprotein in vivo [3]. Cellular CE in MFC undergoes continual hydrolysis to free cholesterol by neutral cholesterol ester hydrolase (NCEH) and re-esterification to CE by acyl-coenzyme A:cholesterol acyltransferase (ACAT). This metabolic cycle is known as the cholesteryl ester cycle [4].

In contrast to LDL, high density lipoprotein (HDL) is widely believed to play a protective role in atherogenesis because of its capacity to promote cholesterol efflux from peripheral cells, the first step in 'reverse cholesterol transport' [5–8]. Most of the previous studies on HDL-mediated cholesterol efflux from MFC have been performed with established foam cells in which macrophages were first converted to foam cells with atherogenic lipoproteins, such as beta-VLDL and acetyl-LDL, followed by incubation with HDL (sequential incubation, Fig. 1A) [4–7]. However, in some experiments, cells were incubated with atherogenic lipoproteins simultaneously with HDL, and the inhibitory effect on CE accumulation in macrophages was examined (simultaneous incubation, Fig. 1B) [8–10]. The simultaneous incubation system was first introduced to this field by Innerarity et al. [10]. Using the simultaneous incubation system, we recently demonstrated that discoidal complexes of apoA-I and dimyristoylphosphatidylcholine, a model of nascent HDL in vivo, interacted with acetyl-LDL and reduced its ligand activity for the macrophage scavenger receptor, indicating that this incubation system could be used to demonstrate another aspect of HDL anti-atherogenicity [9]. We also observed that HDL did not affect the cellular uptake of acetyl-LDL, but significantly inhibited CE accumulation by promoting cholesterol efflux [8,9]. However, the mechanisms and the pathophysiological implications of the anti-atherogenic properties of HDL in simultaneous incubation is unclear [8–10]. In the present study, we directly compared the efficiencies of HDL and an ACAT inhibitor (58-035) in enhancing cholesterol efflux in sequential incubation with those in simultaneous incubation and correlated them with the in vivo effects of an ACAT inhibitor in the progression and the regression of experimental atherosclerosis [11]. The results showed that HDL and 58-035 reduced cellular cholesterol much more efficiently in the simultaneous incubation system than in the sequential incubation system.

2. Materials and methods

2.1. Materials

[1α, 2α(n)-^{3}H]Cholesteryl oleoyl ether (1.7 TBq/mmol) and Na^{125}I (3.7 GBq/ml) were purchased from Amersham. The ACAT inhibitor 58-035 was a generous gift from Dr. J.G. Heider of Sandoz, Inc. (East Hanover, NJ). Tissue culture media and reagents were obtained from Life Technologies, Inc. Other chemicals were of the best grade available from commercial sources.

2.2. Lipoproteins and their modification

LDL (d = 1.019–1.063 g/ml) and HDL (d = 1.063–1.21 g/ml) were isolated by sequential ultracentrifugation. Traces of apolipoprotein B and E were removed from HDL by a heparin agarose column [12]. Acetyl-LDL was prepared as previously described [13]. Iodination of acetyl-LDL with ^{125}I was performed according to the method of McFarlane [14]. Acetyl-LDL was also labeled with the non-hydrolyzable cholesteryl ester analog [^{3}H]cholesteryl oleoyl ether, as previously described [9]. Protein concentrations were determined by BCA protein assay reagents [5] and expressed as mg protein/ml.

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Abbreviations: ACAT; acyl-coenzyme A:cholesterol acyltransferase, HDL; high density lipoprotein, LDL; low density lipoprotein, acetyl-LDL; acetylated LDL, CE; cholesteryl esters, MFC; macrophage foam cells, BSA; bovine serum albumin, NCEH; neutral cholesterol ester hydrolase.

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2.3. Cell culture

Rat peritoneal macrophages were collected (1×10^7/rat) from non-stimulated male Wistar rats (170–200 g) [15], and suspended at 2×10^6/ml with Dulbecco's modified Eagle's medium (DMEM) containing 3% bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (10 mM, pH 7.4), streptomycin (0.1 mg/ml) and penicillin (100 U/ml) (DMEM 3% BSA). One ml of the cell suspension was added to each 35-mm dish, and the cells were incubated at 37°C in 5% CO_2 for 2 h. Cell monolayers which formed were washed 3 times with 1 ml of PBS and used for the following experiments.

Macrophage monolayers were incubated in two incubation systems (Fig. 1). In sequential incubation (Fig. 1A), cells were initially converted to foam cells by incubating them for 18 h with 50 μg/ml of acetyl-LDL. After washing three times with 1 ml of PBS, cells were incubated for an additional 18 h with 0.25 mg/ml of HDL and/or 5 μg/ml of 58-035. In simultaneous incubation (Fig. 1B), cells were incubated with 50 μg/ml of acetyl-LDL simultaneously with HDL and/or 58-035. After washing three times with 1 ml of PBS, cells were incubated for an additional 18 h with medium alone so that the total incubation time in simultaneous incubation was equal to that in sequential incubation. In a preliminary experiment, cholesterol esterification was completely inhibited by 58-035 at 5 μg/ml when macrophages were incubated with 50 μg/ml of acetyl-LDL and 0.1 mM of [^{3}H]oleate.

2.4. Mass determination of cellular cholesterol contents

Cellular lipids were extracted and both the free cholesterol mass and CE mass were quantified by a modification [8] of the enzymatic fluorometric methods of Heider and Boyett [16]. Cells were dissolved in 0.1 M of NaOH to determine cell proteins using a BCA protein assay reagent [5].

2.5. Endocytic degradation of ^{125}I-acetyl-LDL by rat macrophages

Macrophage monolayers (2×10^6) prepared as above were incubated with 50 μg/ml of ^{125}I-acetyl-LDL (79 cpm/ng protein) in the absence or presence of 0.25 mg/ml of HDL and 5 μg/ml of 58-035. Endocytic degradation was determined by trichloroacetic acid-soluble radioactivity in the medium as follows. Ice-cold 40% trichloroacetic acid (0.25 ml) was added to 0.75 ml of the medium to a final concentration of 10%. $AgNO_3$ (0.7 M, 0.2 ml) was then added to the mixture and incubated at room temperature for 30 min to precipitate free iodine [17]. After centrifugation at $700 \times g$ for 10 min, supernatants were counted by a gamma-counter.

2.6. Statistical analysis

Data were evaluated by Student's *t*-test. When the *P*-value was less than 0.05, the difference was judged to be significant.

3. Results

To confirm that HDL and 58-035 did not affect the endocytic pathway of acetyl-LDL, rat macrophages were incubated with ^{125}I-acetyl-LDL in the absence or presence of HDL and 58-035. Endocytic degradation of ^{125}I-acetyl-LDL was not altered by the addition of HDL and/or 58-035 (data not shown). Moreover, the accumulation of cellular [^{3}H]cholesteryl oleoyl ether was also not affected by these agents when incubated with [^{3}H]cholesteryl oleoyl ether-labeled acetyl-LDL (data not shown). Thus, neither HDL nor 58-035 has any effects on the endocytic pathway of acetyl-LDL or its cholesterol supply to cells under the present conditions.

Efficiencies of HDL and 58-035 in promoting cholesterol efflux from rat macrophages were examined in two experimental systems (Fig. 1). When macrophages were converted to foam cells with acetyl-LDL and then reacted with HDL (sequential incubation), the CE level was reduced by only 12% (Fig. 2A). The addition of 58-035 to HDL did not significantly enhance the HDL-induced reduction in the cellular CE level, or that of total cholesterol (Fig. 2A), as we previously demonstrated [7]. When cells were incubated with 58-035 alone, the

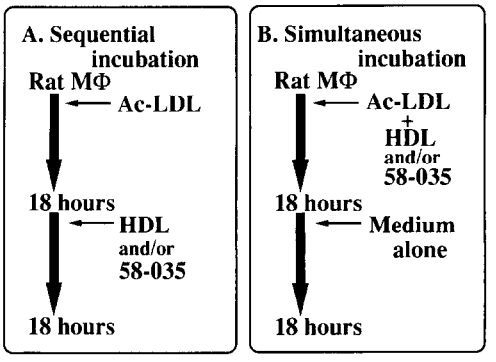


Fig. 1. Two different experimental systems for examining the effects of HDL and the ACAT inhibitor 58-035 on cholesterol efflux from rat macrophages. In sequential incubation (panel A), macrophages were incubated for 18 h with acetyl-LDL to be converted to foam cells. After washing, cells were incubated for an additional 18 h with HDL and/or 58-035. In simultaneous incubation (panel B), macrophages were incubated for 18 h with acetyl-LDL in the presence of HDL and/or 58-035. After washing, cells were incubated for an additional 18 h with medium alone before harvesting cells.

CE level was significantly reduced while total cholesterol was not significantly altered (Fig. 2A).

When macrophages were incubated with acetyl-LDL together with HDL from the onset of the experiment (simultaneous incubation), HDL reduced the cellular CE level by 51% (Fig. 2B), which was significantly greater than the CE reduction by HDL (12%) ($P < 0.01$) in sequential incubation (Fig. 2A). When 58-035 was added to HDL, the cellular CE level was

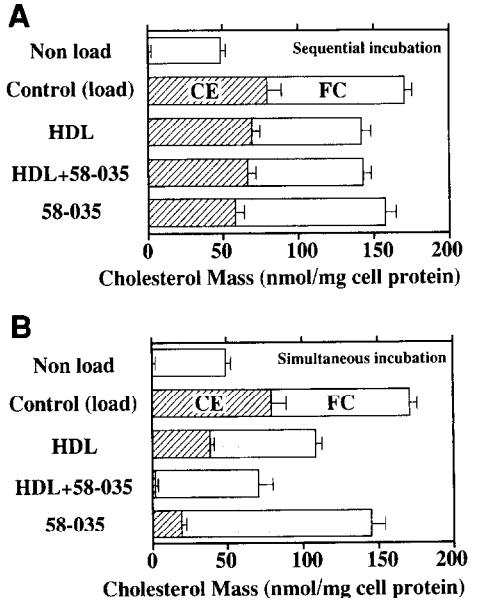
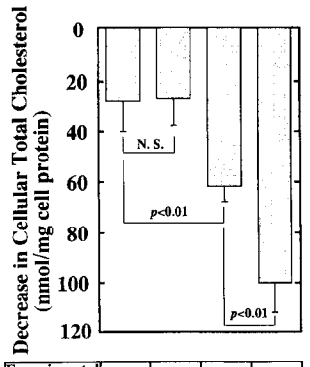


Fig. 2. Effects of HDL and the ACAT inhibitor 58-035 on cholesterol efflux from rat macrophages in sequential (A) and simultaneous (B) incubation. A. Rat macrophages (2×10^6) were converted to foam cells by 18 h of incubation with 50 μg/ml of acetyl-LDL. After washing, cells were incubated for an additional 18 h with 0.25 mg/ml of HDL and/or 5 μg/ml of 58-035. B. Rat macrophages (2×10^6) were incubated for 18 h with 50 μg/ml of acetyl-LDL together with 0.25 mg/ml of HDL and/or 5 μg/ml of 58-035. After washing, cells were incubated for an additional 18 h with medium alone. Cellular lipids were extracted and cholesterol mass was determined as described in the 'Materials and Methods'. Each value is the mean of quadruplicate experiments with a bar showing SD.



Experimental system	A	A	B	B
HDL	+	+	+	+
58-035	–	+	–	+

A. Sequential incubation

B. Simultaneous incubation

Fig. 3. Effects of HDL and 58-035 on cellular total cholesterol in rat macrophages. The effects of HDL and 58-035 on the levels of total cellular cholesterol in rat macrophages were calculated and re-plotted from the data shown in Fig. 3. N. S.; Not significant.

markedly reduced to the basal level (Fig. 2B). The ability of HDL to reduce total cholesterol was also markedly enhanced by 58-035, from 36% to 59% in simultaneous incubation ($P < 0.01$) (Fig. 2B), which was in sharp contrast to the results in sequential incubation (Fig. 2A). When cells were incubated with acetyl-LDL and 58-035, free cholesterol accumulated in cells while a significant amount of CE was detected (Fig. 2B). However, when the cells were harvested just after 18-h incubation with acetyl-LDL and 58-035, cellular CE was virtually at the basal level (data not shown). This indicates that a significant portion of free cholesterol was converted to CE in the absence of 58-035 during an additional 18 h incubation with medium alone (see Fig. 1), probably because the free cholesterol level far exceeded the threshold level for re-esterification to CE by ACAT [18].

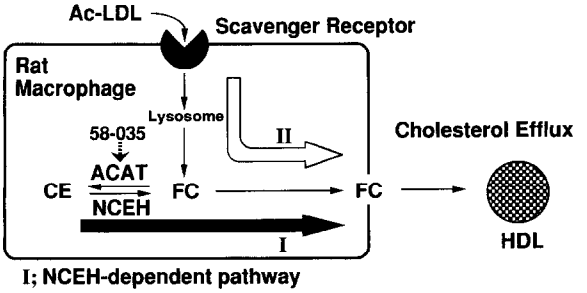
Since the endocytic degradation of acetyl-LDL and the subsequent cholesterol supply were not affected by HDL or 58-035, the reducing effects of HDL and 58-035 on cellular cholesterol levels in simultaneous incubation are most likely due to their effects on cholesterol efflux. From the data shown in Fig. 2, we determined the absolute amounts of cholesterol efflux from rat macrophages, and the results were re-plotted in Fig. 3. It became clear that the amount of cholesterol efflux with HDL alone was 2-fold greater in simultaneous incubation than in sequential incubation ($P < 0.01$). Moreover, addition of 58-035 to HDL in the simultaneous incubation system enhanced HDL-induced cholesterol efflux by 1.6-fold ($P < 0.01$), but had no effect in the sequential incubation system.

4. Discussion

In the present study, we used two experimental protocols to determine the effect of HDL and the ACAT inhibitor 58-035 on cholesterol efflux from rat macrophages (Fig. 1). One was a conventional protocol in which macrophages were first converted to foam cells with acetyl-LDL and then incubated with HDL (sequential incubation) (Fig. 1A). In the other protocol, macrophages were incubated with both acetyl-LDL and HDL from the onset of the experiment (simultaneous incubation) (Fig. 1B).

ACAT inhibitors are well known to enhance HDL-induced cholesterol efflux from mouse MFC in sequential incubation because they increase cellular free cholesterol derived from cytoplasmic CE [7,19,20]. However, in contrast to mouse MFC, 58-035 did not enhance HDL-induced cholesterol efflux from rat MFC (Figs. 2A and 3), as we recently reported [7]. This species difference may be due to the slower turnover rate of the CE cycle in rat MFC [7]. In sequential incubation, the enhancement of HDL-induced cholesterol efflux by 58-035 is closely related to the turnover rate of the CE cycle in each species.

In contrast to sequential incubation, 58-035 strongly enhanced cholesterol efflux from rat macrophages in simultaneous incubation (Figs. 2B and 3). This suggests that the mechanism for cholesterol efflux in simultaneous incubation may be different from that in sequential incubation. Fig. 4 shows possible intracellular pathways for cholesterol transport in each incubation system. In sequential incubation, cholesterol efflux greatly depends on the hydrolytic conversion of cytoplasmic CE to free cholesterol. We tentatively referred to this efflux pathway the 'NCEH-dependent pathway'. The simultaneous incubation system may involve an intracellular pathway for cholesterol efflux in addition to the NCEH-dependent pathway. In this system, some of the free cholesterol released into the cytoplasm from lysosomes after degradation of acetyl-LDL is esterified to CE, which could enter the NCEH-dependent pathway (Fig. 4). However, some of the free cholesterol released into the cytoplasm from lysosomes is directly transported to plasma membranes to be effluxed by HDL without undergoing esterification by ACAT (NCEH-independent path-



Experimental System		Efflux pathway	Efflux efficiency
Sequential incubation	58-035 (-)	I	+
	58-035 (+)	I	+
Simultaneous incubation	58-035 (-)	I & II	++
	58-035 (+)	II	+++

Fig. 4 Schematic representation of the pathways for intracellular cholesterol transport in rat macrophages. In the sequential incubation system, cholesterol efflux from rat MFC depends on the rate of CE conversion to cholesterol by NCEH. Since the turnover rate of the CE cycle in rat MFC is significantly slower than that in mouse MFC, 58-035 does not significantly enhance HDL-induced cholesterol efflux in rat macrophages [7]. However, in the simultaneous incubation system, free cholesterol generated by the action of lysosomal acid lipase enters the NCEH-independent pathway in the presence of 58-035. In the absence of 58-035, some of the lysosome-derived cholesterol is esterified to CE by ACAT and enters the NCEH-dependent pathway. The data shown in Fig. 3 strongly suggest that the NCEH-independent pathway is more efficient than the NCEH-dependent pathway in rat macrophages.

way) (Fig. 4) Since the simultaneous incubation system contains both pathways, if 58-035 is present in this system, only the NCEH-independent pathway is available for cholesterol efflux because esterification of free cholesterol is blocked by the inhibitor.

In the simultaneous incubation system without 58-035, the absolute amount of cholesterol efflux induced by HDL was 2-fold greater than that in the sequential incubation system (Fig. 3). Moreover, in simultaneous incubation with 58-035, the amount of HDL-induced cholesterol efflux was still 3.5-fold greater than that in the sequential incubation system. Therefore, it is likely that cholesterol efflux by the NCEH-independent pathway may be much more efficient than that by the NCEH-dependent pathway in rat macrophages (Fig. 4).

This notion is supported by the following observations. The half-time for conversion of cytoplasmic CE to free cholesterol in rat MFC is approximately 36 h , which is much longer than that in mouse MFC (12 h) [7]. In contrast, the half-times for conversion of lysosomal LDL cholesteryl esters to free cholesterol by acid lipase (acidic cholesterol ester hydrolase) are reportedly 37 min in Chinese hamster ovary cells [21] and 60 min in rat hepatoma cells (Fu5AH) [22]. Moreover, transport of lysosomal cholesterol to plasma membrane is also reportedly very rapid (within 1 h) [21,22]. Therefore, the most time-consuming process in cholesterol efflux in rat macrophages is the NCEH-catalyzed conversion of cytoplasmic CE to free cholesterol.

Recently, attention has been focused on ACAT inhibitors as a new drug for controlling atherosclerosis. In vivo experiments with cholesterol-fed rabbits clearly demonstrated that an ACAT inhibitor, CI-976 effectively inhibited the progression of atherosclerosis [11]. However, in contrast to its strong inhibitory effect on the progression of atherosclerosis, its effect on the regression of established lesions was relatively weak [11]. These two aspects of in vivo effects of the ACAT inhibitor has not been well explained by the previous in vitro experiments with mouse MFC in which an enhancing effect of 58-035 on HDL-induced cholesterol efflux was demonstrated by the sequential incubation [19,20]. In the present study, we clearly demonstrated in rat MFC that 58-035 significantly enhanced HDL-induced cholesterol efflux in simultaneous incubation but not in sequential incubation, suggesting that ACAT inhibitors are more efficient in the progression phase than in the regression phase of atherosclerosis. The turnover rate of CE cycle in rat MFC is much slower than that in mouse MFC but faster than that in rabbit MFC, indicating that the turnover rate of CE cycle would be slower in higher mammals [7]. Therefore, rat MFC may be a more appropriate model than mouse MFC to reflect cholesterol metabolism in MFC in higher mammals.

Although the two different intracellular cholesterol pathways shown in Fig. 4 have been known, the present study demonstrated for the first time that NCEH-independent pathway is much more efficient for cholesterol efflux than NCEH-dependent pathway. Since HDL is expected to co-localize with atherogenic LDLs such as Ox-LDL in atherosclerotic lesions in situ, the NCEH-independent pathway might play a crucial role in HDL-mediated cholesterol efflux in atherogenesis, and particularly in protecting macrophages from CE accumulation in the initial phase of foam cell formation.

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