Selective Down-Regulation by Protein Kinase C Inhibitors of Apolipoprotein-Mediated Cellular Cholesterol Efflux in Macrophages[†]

Qianqian Li,[‡] Maki Tsujita,[‡,§,||] and Shinji Yokoyama*[,‡,§]

Biochemistry I, Nagoya City University Medical School, Kawasumi 1, Mizuho-ku, Nagoya 467, Japan, and Lipid and Lipoprotein Research Group, Department of Medicine, The University of Alberta, Edmonton, Alberta, Canada T6G 2S2

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ABSTRACT: Extracellular apolipoprotein A-I removed cholesterol and phospholipid from cholesterol-loaded mouse peritoneal macrophage and thereby generated a preβ high-density lipoprotein (HDL) particle having a weight ratio of cholesterol to phosphatidylcholine of approximately 1:1. Treatment of the cells with phorbol myristate slightly increased cholesterol efflux by this mechanism without influencing the nonspecific cholesterol efflux to the lipid microemulsion. When the cells were treated by protein kinase C (PKC) inhibitors, H7 and staurosporine, apolipoprotein-mediated cellular cholesterol efflux was substantially reduced without a significant change in phosphatidylcholine efflux, resulting in generation of cholesterol-poor preβ-HDL particles having a weight ratio of cholesterol to phosphatidylcholine as low as 1:10. In spite of this change, specific binding of apoA-I to the cellular surface was unaffected. Cellular cholesterol available for acylCoA:cholesterol acyltransferase (ACAT) was rapidly depleted by adding apoA-I to the medium, and the PKC inhibitor treatment reversed this effect. In contrast, nonspecific cellular cholesterol efflux to the lipid microemulsion did not influence the ACAT-available cellular cholesterol pool, and it was not influenced by the PKC inhibitors. Thus, we concluded that apolipoprotein-mediated cellular cholesterol efflux is linked to mobilization of cholesterol from an intracellular pool used by ACAT to a specific pool for apolipoprotein-mediated preβ-HDL generation, in response to apolipoprotein−cell interaction and subsequent intracellular signaling. Binding of apolipoprotein to the cell surface is required for assembly of the preβ-HDL particle with cellular phospholipid, and the intracellular cholesterol mobilization is needed for enrichment with cholesterol of the preβ-HDL. These reactions are largely independent of diffusion-mediated nonspecific cell cholesterol efflux.

Cholesterol efflux is one of the essential events in cellular cholesterol homeostasis and metabolism because animal body cells are unable to catabolize cholesterol except for hepatocytes that synthesize bile acids and steroidogenic cells that use it as a precursor. It is interesting, however, that this topic has been studied by many researchers not directly from such a point of view, but rather has been approached from the viewpoint that excessive intracellular accumulation of cholesterol occurs at the initial stage of atherosclerotic vascular lesion and such accumulation may be reversed by stimulation of the cholesterol efflux mechanism.

Much of the communication between the cells and extracellular lipoproteins is carried out by receptor-mediated mechanisms, especially for the delivery of cholesterol to the cells. The low-density lipoprotein (LDL)[1] receptor is responsible for the uptake of LDL that is linked to subsequent down-regulation of cholesterol biosynthesis, stimulation of intracellular cholesterol esterification, and down-regulation of receptor expression (Brown & Goldstein, 1986). In addition, many membrane proteins with scavenger function for denatured protein seem to play roles in the pathological uptake of naturally degenerated LDL without regulation (Krieger et al., 1993). One such molecule may also function for physiological selective uptake of cholesteryl ester from high-density lipoprotein (HDL) in certain organs (Acton et al., 1996). On the other hand, HDL is believed to play a central role in the efflux of cholesterol for a number of reasons. HDL has been shown to induce the decrease of cellular cholesterol when incubated with cholesterol-loaded cells in vitro (Ho et al., 1980). This is consistent with the finding in clinical epidemiology that HDL is a negative risk factor for coronary heart disease (Gordon & Rifkind, 1989). In such a context, it has been questioned for years if HDL can reverse or prevent intracellular cholesterol accumulation in the vascular walls.

In contrast to various cellular cholesterol uptake pathways, the mechanism of HDL-mediated cellular cholesterol removal has been poorly understood. This is perhaps due to controversial views created by conflicting experimental data generated in various different conditions. This has also been related to the dynamic equilibrium essentially involved in

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* To whom correspondence should be addressed at Biochemistry I, Nagoya City University Medical School, Kawasumi 1, Mizuho-cho, Mizuho-ku, Nagoya 467, Japan. Telephone: +81-51-853-8139. FAX: +81-52-841-3480. E-mail: syokoyam@med.nagoya-cu.ac.jp.

[‡] The University of Alberta.

[§] Nagoya City University Medical School.

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[1] Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo, apolipoprotein; PMA, phorbol 12-myristate 13-acetate; PBS, 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.5 mM ethylenediaminetetraacetic acid; PDGF, platelet-derived growth factor; MCSF, macrophage colony stimulating factor; ACAT, acylCoA:cholesterol acyltransferase.

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the structure of the HDL particle to provide its lipid and protein components with various different physical status. However, the findings in the past few years in this field have gradually unveiled the mechanism of cellular cholesterol efflux (Oram & Yokoyama, 1996).

According to the results from several laboratories, there are at least two independent mechanisms for cellular lipid efflux. One is nonspecific exchange of cholesterol and phospholipid between the cell membrane and the lipoprotein surface (Karlin et al., 1987; Johnson et al., 1991; Czarnecka & Yokoyama, 1996). This is bidirectional transfer of lipids by diffusion through an aqueous phase so that the rate of cholesterol transfer is much faster than phospholipid (Li et al., 1993), and the cholesterol content in the membrane limits the rate (Czarnecka & Yokoyama, 1996). The net efflux may only be generated by a cholesterol gradient created between the cell and lipoprotein, and cholesterol esterification in lipoprotein in HDL generates such a gradient (Czarnecka & Yokoyama, 1996). The other mechanism based on the newer findings is a pathway directly mediated by apolipoprotein. Apolipoproteins of HDL interact with the cell surface in their lipid-free form and generate new HDL particles with the cellular phospholipid and cholesterol (Hara & Yokoyama, 1991; Hara et al., 1992; Bielicki et al., 1992; Forte et al., 1993; Mendez et al., 1994). Since this is a one-way removal of both lipids, it causes a net lipid efflux by itself.

Apolipoprotein-mediated efflux has been characterized in detail particularly in the past few years. Lipid-free helical apolipoprotein directly interacts with the cellular surface and generates "preβ-HDL"-like particles with the apolipoprotein and cellular phospholipid and cholesterol (Li et al., 1993; Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Bielicki et al., 1992; Forte et al., 1993, 1995, 1996; Mendez et al., 1994; Komaba et al., 1992; Yancy et al., 1995). The reaction is carried out by many apolipoproteins of broad spectrum including most of the helical mammalian apolipoproteins (apo), A-I, A-II, A-IV, and E, and insect apolipophorin III (Hara & Yokoyama, 1991; Hara et al., 1991; Bielicki et al., 1992; Yancy et al., 1995). The reaction is weak with small helical apolipoproteins such as apoC-III and carboxymethylated human apoA-II that have only one (or two) helical segment(s) (Hara et al., 1992; Bielicki et al., 1992). A synthetic helical peptide with two helical segments is active for this reaction but that with one helix is inactive (Mendez et al., 1994; Yancy et al., 1995). Thus, the structural requirement for the reaction seems to be related to the number of amphiphilic helical segments in a protein molecule. The K_m value of the reaction is less than the common dissociation constants of those apolipoproteins with the lipid surface (10^{-8} to $\sim 10^{-7}$ versus 10^{-7} to $\sim 10^{-6}$ M) (Hara & Yokoyama, 1991; Komaba et al., 1992; Tajima et al., 1983; Yokoyama et al., 1985; Okabe et al., 1988) and as low as 1/1000 of the plasma apoA-I concentration. Cholesterol esterification on the generated preβ-HDL particle does not cause further enhancement of cholesterol efflux (Czarnecka & Yokoyama, 1995). Physical and reversible binding of apolipoprotein is required for the reaction (Li et al., 1995; Tsujita & Yokoyama, 1996; Smith et al., 1996). Cellular cholesterol available for efflux by this pathway is likely to be supplied from a certain cellular pool by a specific transport mechanism (Li & Yokoyama, 1995).

Accordingly, this pathway is biologically regulated by several cellular factors. Among them are a putative specific interaction site of the cell surface (Kawano et al., 1993; Francis et al., 1995; Li & Yokoyama, 1995; Czarnecka & Yokoyama, 1996; Li et al., 1995; Tsujita & Yokoyama, 1996; Smith et al., 1996) and a specific intracellular cholesterol transport system linked to the efflux that is potentially initiated by a signaling pathway in the cells (Mendez et al., 1991; Li & Yokoyama, 1995; Francis et al., 1996). Activation of protein kinase C subsequent to the interaction of HDL with cells has been reported by several research groups, in relation to cholesterol mobilization or in more general manners (Aviram et al., 1989; Mendez et al., 1991; Wu & Handwerger, 1992; Nazih et al., 1994; Walter et al., 1995). We have recently proposed that protein kinase C activation is required for mobilization of intracellular cholesterol to the pool selectively available for the apolipoprotein-mediated efflux (Li & Yokoyama, 1995). This was found in rat vascular smooth muscle cells with which interaction of apolipoprotein generates cholesterol-poor preβ-HDL. The cells were transformed to the macrophage-like stage by treatment with growth factors, and yet cholesterol was poorly available for the reaction. Preβ-HDL generated by the apolipoprotein−cell interaction was enriched in cholesterol only when the cells were further treated with a phorbol ester, and this effect was reversed by protein kinase C inhibitors. This result suggested that, even when the cells were transformed into the macrophage-like stage defined by several parameters, rat vascular smooth muscle cells failed to link between cellular interaction with apolipoprotein and mobilization of intracellular cholesterol for this particular efflux pathway. Therefore, direct pharmacological stimulation of the cells by phorbol ester was required for such mobilization.

To study further the mobilization of intracellular cholesterol for apolipoprotein-mediated efflux, we attempted to extrapolate our observation above to a more generalized condition using the cells in which cholesterol is readily available for apolipoprotein-mediated efflux. In this paper, by using mouse peritoneal macrophages we demonstrate selective inhibition by protein kinase C inhibitors of cholesterol incorporation from the intracellular pool into preβ-HDL generated by the cellular interaction with apolipoprotein. This was independent of nonspecific cholesterol efflux by a physicochemical mechanism and of the interaction of apolipoprotein with the cells to generate preβ-HDL particles from cellular phospholipid.

EXPERIMENTAL PROCEDURES

Materials. RPMI medium 1640, penicillin−streptomycin, Tris, and fetal calf serum were purchased from Life Technologies Inc. Fatty acid-free bovine serum albumin (cell culture grade), chloramine T, and triolein (>99%) were from Sigma Chemical, and sodium bromide and ethylenediaminetetraacetic acid were from BDH Inc. Egg phosphatidylcholine was purchased from Avanti. Phorbol 12-myristate 13-acetate (PMA), staurosporine, and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) were all obtained from Sigma. [1,2-^{3}H]Cholesterol oleate (45 Ci/mmol), [*methyl*-^{3}H]choline chloride (15 Ci/mmol), and [4-^{14}C]cholesterol (53 mCi/mmol) were obtained from Amersham. ^{125}I carrier-free (17 Ci/mg in 10^{-5}M NaOH) was purchased from Du Pont Canada Inc.

Lipoproteins and Apolipoproteins. Lipoproteins were isolated from fresh human plasma by sequential ultracentrifugation in sodium bromide at a density of 1.006−1.063 g/mL for LDL and 1.063−1.21 g/mL for HDL. The lipoprotein fractions were thoroughly dialyzed against 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.5 mM ethylenediaminetetraacetic acid (PBS). ApoA-I was isolated from the HDL fraction using delipidation followed by anion exchange column chromatography in 6 M urea as previously described (Yokoyama et al., 1982). ApoA-I was dissolved in buffer before use in the experiments according to the method previously described (Yokoyama et al., 1982). LDL was labeled with [1,2-^{3}H]cholesteryl oleate (45.4 Ci/mmol, purchased from Amersham, Canada) according to the previous method (Nishikawa et al., 1986). The labeled LDL was acetylated by the method previously described for the purpose of loading radiolabeled cholesterol to macrophages (Hara & Yokoyama, 1991). Lipid microemulsions having a homogeneous diameter of 26 nm were prepared from egg phosphatidylcholine and triolein as described in our previous work (Tajima et al., 1983) for the experiment to induce nonspecific cellular lipid efflux (Hara & Yokoyama, 1992), by sonicating both lipids at a weight ratio of 1:1 and isolating the emulsions by ultracentrifugation and gel permeation chromatography.

Loading Mouse Peritoneal Macrophages with the Acetylated LDLs. Peritoneal macrophages were obtained from BALB mice (20−35 g) by peritoneal lavage with cold RPMI medium 1640 containing 5 units/mL penicillin and 5 μg/mL streptomycin (medium A) as described previously (Hara & Yokoyama, 1991). The radiolabeled and acetylated LDL (50 μg of protein) in 1 mL of medium A containing bovine serum albumin (0.2% w/v) was incubated with the cells in the presence of [*methyl*-^{3}H]choline (2 μCi/mL) for 24 h in order to load the cells with cholesterol and to label cellular cholesterol and choline−phospholipid in a 3.5 cm culture dish as described in our previous paper (Li et al., 1993; Hara & Yokoyama, 1991). The cells were washed and maintained in the lipoprotein-free medium for another 24 h before any further experiment.

Measurement of Cholesterol and Phospholipid Flux. The cholesterol-loaded cells were incubated with various amounts of lipid-free apoA-I in 1 mL of medium A for 2−24 h. The culture medium was collected and centrifuged at 12400*g* for 15 min to remove cell debris, and lipid was extracted as described elsewhere (Hara & Yokoyama, 1991). Lipid from the cellular fraction was extracted directly (Hara & Yokoyama, 1991). The extracted lipid was analyzed by thin-layer chromatography to measure the radioactivity in various lipid fractions. The mass amount of lipid efflux was calculated by using the specific radioactivity of each lipid in the cellular pool immediately before the flux experiments, assuming cholesterol biosynthesis is negligible and showing a change of the specific radioactivity of the phospholipid is insignificant (<10%) in the cholesterol-loaded cells during the efflux (Li et al., 1993; Hara & Yokoyama, 1991). Lipid efflux to the lipid microemulsion was also observed by measuring the efflux of radiolabeled cellular lipid. The mass amount of the efflux was expressed as the transfer of the originally cell-associated lipid to the microemulsion calculated from the efflux of radiolabeled lipid and the specific radioactivity of each cellular lipid before the efflux event (Li et al., 1993; Hara & Yokoyama, 1992). Background without apoA-I or microemulsion was subtracted to show apoA-I/microemulsion-specific efflux in the figures.

Modulation of Cellular Lipid Efflux. The labeled cells were pretreated for stimulation with recombinant platelet-derived growth factor BB chain (PDGF) (Sigma), 10 ng/mL, and/or recombinant macrophage colony stimulating factor (MCSF) (a generous gift from Dr. Nobuhiro Yamada, University of Tokyo), 100 ng/mL, for 24 h (Inaba et al., 1992). In order to observe the effect of further activation of protein kinase C, the cells were incubated with PMA, 160 nM, for the last 1 h of this incubation period. To examine the effect of protein kinase C inhibition, lipid efflux was observed in the presence of staurosporine or H-7 (Tamaoki et al., 1986; Hidaka et al., 1984). Staurosporine was dissolved in dimethyl sulfoxide as 4 mM and added to the medium to make a final concentration of 0.5−50 nM (Tamaoki et al., 1986). H-7 was dissolved in water as 5 mM and added to the medium to make a final concentration of 12.5−100 μM (Hidaka et al., 1984).

Density Gradient Ultracentrifugation. The medium was analyzed to detect the lipid efflux product by using density gradient ultracentrifugation in sucrose as described earlier (Hara & Yokoyama, 1991). Sucrose solution of density 1.30 g/mL, 0.7 mL, was overlayered with the solution of density 1.10 g/mL, 1.2 mL, and then the culture medium, 1.2 mL, was overlayered on the top in a 3-mL quick-seal centrifuge tube for a Beckman TL100.3 rotor. After centrifugation at 99 000 rpm in a TL100 ultracentrifuge for 16 h at 4 °C, 200-μL fractions were collected from the bottom. For each fraction, the density was measured, and the radioactivity of free cholesterol and phosphatidylcholine was counted after lipid extraction and thin-layer chromatography. The mass of each lipid in every fraction was calculated by using the specific radioactivity of the respective lipid in the cellular pool (Li et al., 1993; Hara & Yokoyama, 1991).

^{125}I-Apolipoprotein A-I Binding to the Cell Surface. Binding of apoA-I to the cell surface was measured by the method previously described (Tsujita & Yokoyama, 1996). ApoA-I was dissolved as 50 μg/mL in 10 mL of the 0.4 M glycine−0.4 N NaOH, pH 8.5, buffer (McFarlane, 1958). ^{125}I, 1 mCi, was added to the solution, and the labeling reaction was carried out and stopped by adding 50 μL of chloramine T solution (4 mg/mL) and stirring for 15 min at room temperature, followed by adding 100 μL of 16 mg/mL sodium metabisulfite and further stirring for 10 min (Greenwood & Hunter, 1963). The mixture was added to 40 mL of acetone/diethyl ether (3:1, v/v), left overnight at 4 °C, and centrifuged at 2500 rpm for 25 min to recover the pellet. The pellet was washed once more with the same organic solvent, dried by evaporation, dissolved in the PBS, and finally dialyzed against PBS. The final solution was concentrated by Amicon Centriflo 25 (Amicon, Danvers, MA). The analysis of the product by electrophoresis in SDS−polyacrylamide showed that more than 80% of the total radioactivity was found with the band of apoA-I and the specific radioactivity was 2 270 000 cpm/μg. The cholesterol-loaded macrophages with and without H-7 treatment for 5 h were washed and incubated in the 3.5 cm dish with various concentrations of ^{125}I-apoA-I at 0 °C for 2 h in RPMI 1640 culture medium (1 mL), pH 7.4, containing 25 mM HEPES, 0.2% (w/v) bovine serum albumin, and PCSM (medium B). The cells were then washed twice by incubating at 0 °C for 2 h with 1 mL of culture medium B, or with

medium B containing 50 μg of nonradiolabeled apoA-I for displacement of reversible binding of the labeled apoA-I. The cells were further washed with 1 mL of the chilled PBS without EDTA 3 times, and cell protein was solubilized with 1 N NaOH by 1 h incubation at room temperature. The radioactivity and the protein in the NaOH solution were determined. The counts after displacement were subtracted from the counts without displacement in order to calculate the specific reversible binding of apoA-I to the cellular surface. As each measurement was duplicated, the specific binding was calculated as an average of quadruple values of the difference.

Cellular Cholesterol Esterification. AcylCoA:cholesterol acyltransferase (ACAT) activity in the cells after 24 h incubation with various amounts of apoA-I was measured according to Francis et al. (1993). After incubation with apoA-I for a certain period of time, culture medium containing [^{14}C]oleate was applied to the cell for 1 h at 37 °C, and incorporation to the cellular cholesteryl ester was determined. The assay was triplicated.

Analytical Methods. Quantitative measurement of protein was performed by the method of Lowry et al. (1951) for lipoproteins, apolipoproteins, and cells. Cholesterol, cholesteryl ester, and choline phospholipid were measured by using the enzymatic fluorescent method (Hara & Yokoyama, 1991).

All the experiments were repeated at least 3 times, and the data demonstrated as the figures and tables represent a typical set. Over the ranges of cell protein from 20 to 200 μg/dish and cellular free cholesterol from 40 to 100 μg/mg of protein, cholesterol removal by apoA-I varied by 20% with respect to the percentage of the cellular free cholesterol pool (Komaba et al., 1992; Li et al., 1993), and by 25% with respect to the relative inhibitory effect of the protein kinase C inhibitors on apoA-I-mediated cellular cholesterol efflux (Li & Yokoyama, 1995).

RESULTS

Mouse peritoneal macrophages were treated with PMA for 1 h immediately before apoA-I was added to the culture medium. When rat vascular smooth muscle cells were treated in the same manner in our previous study, those cells required pretreatment with PDGF or PDGF and MCSF to demonstrate the effect of PMA on the specific increase of cholesterol incorporation into apolipoprotein-mediated cell lipid efflux (Li & Yokoyama, 1995). Therefore, the effect of these growth factors was also tested in the experiment with the macrophages. As shown in Figure 1, a significant increase of cholesterol efflux was observed in the apoA-I-mediated reaction regardless of the pretreatment of the cells with growth factors. On the other hand, there was no effect of PMA on nonspecific cholesterol efflux to the lipid microemulsion. The data were not inconsistent with the similar experiment with the macrophage presented in our previous work though it did not reach a significant difference (Li & Yokoyama, 1995). Thus, short period treatment of macrophages with PMA results in the specific increase of apolipoprotein-mediated cholesterol efflux.

Figure 2 shows the effect of a protein kinase C inhibitor, H-7, on apoA-I-mediated efflux of cholesterol and phospholipid from the macrophage. During the first 24 h of incubation, apoA-I induced the efflux of cholesterol (left)

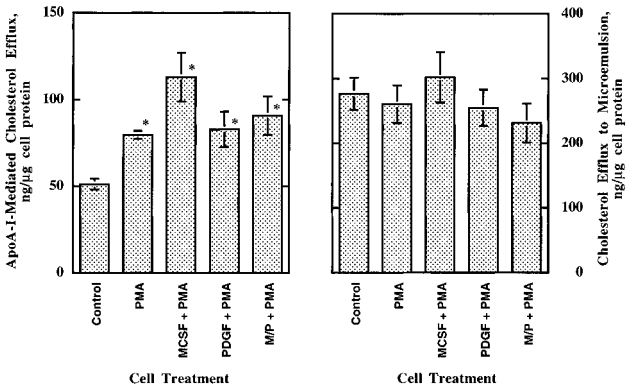

FIGURE 1: Effect of growth factors and PMA on cholesterol efflux from mouse peritoneal macrophage induced by apoA-I and lipid microemulsion. Cholesterol-loaded macrophages (119 ± 19 μg of protein/dish, free cholesterol was 39.5 ± 2.1% of total cellular cholesterol of 7.0 ± 2.0 μg/dish) were pretreated with PDGF (10 ng) and/or MCSF (100 ng) and then stimulated by PMA (160 nM) as described in the text. The efflux of cholesterol was observed for 24 h, in the presence of apoA-I (10 μg) or lipid microemulsion (125 μg of phospholipid). Control, no growth factor without PMA; PMA, no growth factor with PMA; PDGF + PMA or MCSF + PMA, PDGF or MCSF with PMA, respectively; P/M + PMA, PDGF and MCSF with PMA. The values are the mean ± standard error of triplicate assays. An asterisk indicates a significant difference from the control with $p < 0.01$.

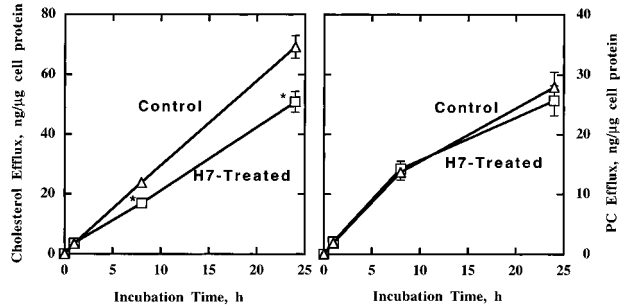

FIGURE 2: Effect of H-7 on apoA-I-mediated efflux of cholesterol and phosphatidylcholine from macrophages. Cholesterol-loaded macrophages (28.7 ± 3.8 μg of protein/dish, free cholesterol was 25.8 ± 3.3% of total cellular cholesterol of 11.7 ± 2.4 μg/dish) were incubated with 10 μg of apoA-I in the presence (□) and absence (△) of H-7 (5 μM). Efflux of cholesterol and phosphatidylcholine into the medium was measured in a time course. The values are the average and SE of triplicated data points. The asterisk indicates a significant difference from the control with $p < 0.01$.

and phosphatidylcholine (right). With a moderate concentration of 10 μM, H-7 suppressed the efflux of cholesterol but not phosphatidylcholine. The efflux time course of both lipids was largely linear regardless of the presence of H-7. Figure 3 further demonstrates the effect of H-7 on cellular lipid efflux from the macrophages. H-7 did not show a significant influence on the base line lipid efflux in the absence of an additional "acceptor" in the medium (data not shown in the figure). In apoA-I-mediated cell lipid efflux, H-7 inhibited cholesterol efflux in a dose-dependent manner, reaching more than 90% inhibition of the apoA-I-induced part at a concentration of 20 μM. In the same incubation mixture, phospholipid efflux was not significantly suppressed by H-7. In contrast, H-7 showed only an insignificant decreasing effect on cholesterol efflux from the macrophage to the lipid microemulsion, and had no influence on phosphatidylcholine efflux to the emulsion. Thus, the inhibitory effect of H-7 on cellular lipid efflux was limited to cholesterol efflux by the apolipoprotein-mediated pathway,

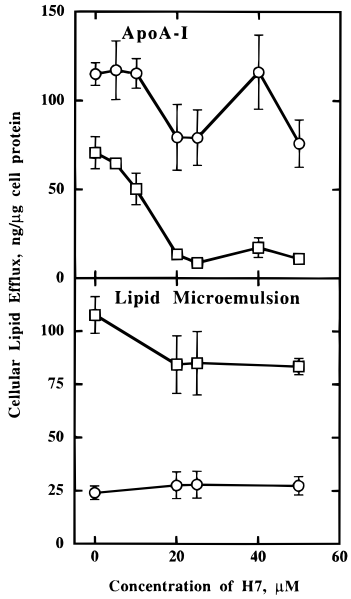

FIGURE 3: Effect of H-7 on cellular lipid efflux mediated by apoA-I (upper panel) and lipid microemulsion (lower panel). Cholesterol-loaded macrophages (28.6 ± 4.5 μg of protein/dish, free cholesterol was 32.5 ± 5.3% of total cellular cholesterol of 8.7 ± 0.9 μg/dish) were incubated with apoA-I, 10 μg, or lipid microemulsion, 196 μg of phospholipid, in 1 mL of the medium for 24 h in the presence and absence of various amounts of H-7. The efflux of cholesterol (□) and phosphatidylcholine (○) into the medium was measured. The values are the average and SE of triplicated data points.

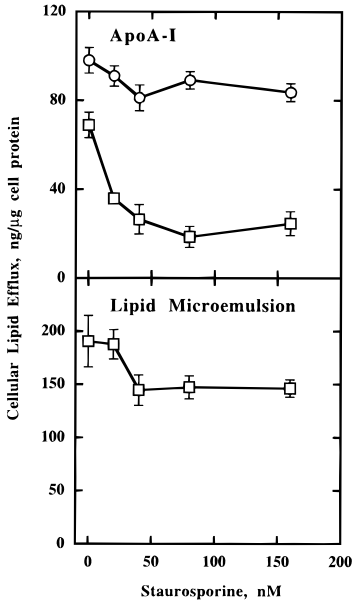

FIGURE 4: Effect of staurosporine on cellular lipid efflux mediated by apoA-I (upper panel) and lipid microemulsion (lower panel). Cholesterol-loaded macrophages (37.5 ± 8.2 μg of protein/dish, free cholesterol was 25.8 ± 3.0% of total cell cholesterol of 12.8 ± 3.2 μg/dish) were incubated with apoA-I, 10 μg, and lipid microemulsion, 196 μg of phospholipid, in 1 mL of the medium for 24 h in the presence and absence of various amounts of staurosporine. The efflux of cholesterol (□) and phosphatidylcholine (○) into the medium was measured. The values are the average and SE of triplicated data points.

and H-7 inhibited neither phosphatidylcholine removal by apolipoprotein nor nonspecific cellular lipid efflux. Figure 4 shows a similar effect of another protein kinase C inhibitor, staurosporine. The base line was uninfluenced by staurosporine (data not shown in the figure). Dose-dependent inhibition of cholesterol efflux by staurosporine was almost identical to that of H-7. It selectively inhibited apoA-I-mediated cholesterol efflux from the macrophage by a maximum of 80% at a concentration of 75 nM, without having much influence on phosphatidylcholine removal by apoA-I and showing only a 20% decrease of nonspecific cholesterol efflux to the lipid microemulsion.

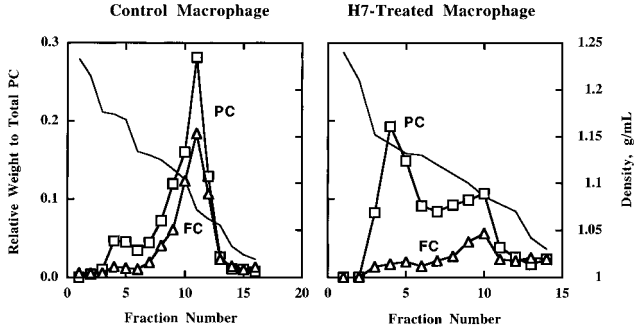

FIGURE 5: Density gradient analysis of the preβ-HDL particle generated by exogenous apoA-I and the cholesterol-loaded macrophages with and without treatment by H-7. The cellular condition was the same as that described for Figure 3. After 24 h incubation of the cell with 10 μg of apoA-I in the presence and absence of 50 μM H-7, 1 mL of medium was analyzed by density gradient ultracentrifugation with sucrose as described in the text. The values of free cholesterol (△) and phospholipid (□) are expressed as weight percentage to the total phospholipid in the analyzed sample.

It is known that apolipoprotein induces cellular lipid efflux and generates a new preβ-HDL-like particle from cellular lipid (Hara & Yokoyama, 1991). This preβ-HDL-like particle was analyzed when the apoA-I-mediated cholesterol efflux was selectively decreased by H-7. Figure 5 shows the density gradient profiles of the medium after apoA-I generated lipid efflux from the macrophage. In the control experiment shown in the left panel, cholesterol and phospholipid formed the coincidental major peak at a density of 1.08 g/mL, where the weight ratio of cholesterol to phosphatidylcholine was 0.8. This major peak was accompanied by a minor peak of density 1.16 g/mL, where the weight ratio of cholesterol to phosphatidylcholine was much lower. When the cells were treated with H-7 (the right panel of Figure 5), there were still two peaks of the lipids in density gradient analysis of the medium. The major peak is formed at a density of 1.14 g/mL mostly with phosphatidylcholine, and the minor peak was at a density of 1.1 g/mL and contains cholesterol and less than half of phosphatidylcholine. Thus, suppression of cholesterol efflux by H-7 resulted in generation of preβ-HDL-like particles containing much less amount of cholesterol. The density of the particle is shifted to a higher range by losing cholesterol.

In order to examine the mechanism of the effect of protein kinase C inhibitors, direct binding of apoA-I to the cell was measured. Figure 6 represents reversible binding of apoA-I, measured as displacement of ^{125}I-apoA-I binding by an excess amount of cold apoA-I (Tsujita & Yokoyama, 1996). No significant difference was observed between the control cells and the H-7-treated cells. Thus, we have concluded that protein kinase C inhibitors do not interfere with direct interaction of apolipoprotein with the cells to generate preβ-HDL, but selectively decrease enrichment of the preβ-HDL with cholesterol.

The mechanism of incorporation of cholesterol into the preβ-HDL-like lipoprotein was further studied by measuring intracellular acylesterification of cholesterol by radiolabeled

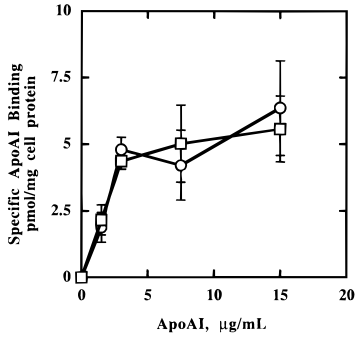

FIGURE 6: Reversible binding of apoA-I to cholesterol-loaded macrophages with (○) and without (□) treatment by H-7. Cell protein was 89.7 ± 13.8 μg/dish, and total cell cholesterol was 28 ± 4.5 μg/dish. The cells were pretreated with and without 50 μM H-7 for 24 h. Reversible binding was measured after displacement of the bound ^{125}I-labeled apoA-I by an excess amount of cold apoA-I. Details of the experimental procedure are described in the text. The values are the average and SE of the quadruple-calculated data as described in the text.

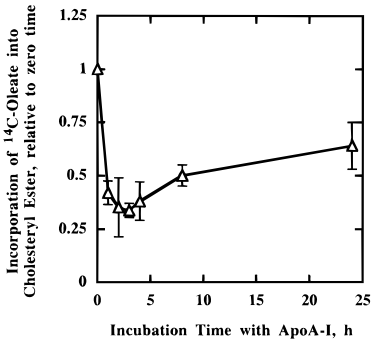

FIGURE 7: Decrease of intracellular cholesterol esterification by incubating the macrophages with apoA-I. Cell protein was 72.0 ± 5.5 μg/dish, and total cell cholesterol was 19.5 ± 3.5 μg/dish. The cells were incubated with apoA-I, 10 μg, for various times, and then the cells were incubated with [^{14}C]oleic acid for 1 h to observe its incorporation into cholesteryl ester. The values are the average and SE of triplicated data points.

fatty acid. This parameter is considered to be parallel to the cellular cholesterol pool size available for its esterification by ACAT (Francis et al., 1993). As demonstrated in Figure 7, incorporation of [^{14}C]oleate into the cholesteryl ester fraction was rapidly decreased by incubating the cells with apoA-I, reaching near the maximum effect within 1 h or so. Figure 8 shows the effect of H-7 on this reaction within a short time of incubation (2 h). Adding apoA-I to the medium reduced the ACAT-available cholesterol pool size, and the presence of H-7 during the incubation of the cells with apoA-I canceled this effect. On the other hand, lipid microemulsion that causes nonspecific cholesterol efflux had no influence on the ACAT-available cellular cholesterol pool size, and additional H-7 did not cause a change of this parameter either. Interestingly, adding H-7 alone to the medium caused a significant increase of the ACAT-available cell cholesterol pool size, perhaps indicating the presence of autocrine stimulation of the macrophage by apoE secreted by the macrophage itself (Zhang et al., 1996; Smith et al., 1996).

DISCUSSION

Cholesterol-loaded mouse peritoneal macrophages were shown to respond to short-time treatment with PMA by moderately increasing cholesterol efflux mediated by apo-

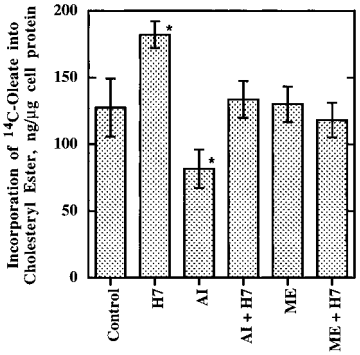

FIGURE 8: Effect of apoA-I and H-7 on intracellular cholesterol esterification in the cholesterol-loaded macrophages. Cell protein was 105.1 ± 9.1 μg/dish, and total cell cholesterol was 29 ± 5.5 μg/dish. The cells were incubated for 2 h with apoA-I, 10 μg, or lipid microemulsion, 196 μg, as phospholipid, with and without 50 μM H-7. After the cells were washed, incorporation of [^{14}C]-oleic acid into cholesteryl ester was measured by 1-h incubation as described in the text. Control, incubation of the cells in the blank medium; H7, incubation of the cells in the medium containing H-7 alone; AI, in the medium containing apoA-I alone; AI + H7, apoA-I and H-7; ME, lipid microemulsion alone; ME + H7, lipid microemulsion and H-7. The values are the average and SE of triplicated data points. The asterisk indicates a significant difference from the control with $p < 0.01$.

lipoprotein. The same treatment, however, did not influence nonspecific cellular lipid efflux to the lipid microemulsion. This finding was consistent with our previous observation with rat vascular smooth muscle cells (Li & Yokoyama, 1995), where cholesterol was poorly available for apolipoprotein-mediated efflux and treatment with growth factors plus PMA resulted in the significant increase of this poor cholesterol availability. The macrophages whose cholesterol is readily available for apolipoprotein-mediated efflux, however, did not require pretreatment with the growth factors for their response to PMA, and the extent of the relative increase of apolipoprotein-mediated cholesterol efflux by PMA was less than in the pretreated smooth muscle cells.

In contrast, apolipoprotein-mediated cholesterol efflux from macrophages was markedly suppressed by protein kinase C inhibitors, H-7 and staurosporine. However, this treatment had no or little effect on phospholipid efflux by apolipoprotein and only a small effect on nonspecific cholesterol efflux to the lipid microemulsion. In addition, reversible binding of apoA-I to the cell was unaffected by the inhibitors. Density gradient ultracentrifugation analysis demonstrated that the selective decrease of apoA-I-mediated cholesterol efflux by such cellular treatment resulted in generation of cholesterol-poor preβ-HDL with apoA-I.

The intracellular cholesterol available for the ACAT reaction rapidly decreased when apolipoprotein triggered the net cholesterol efflux. The decrease of the ACAT-available pool reaches the maximum within 1 h after adding apoA-I to the medium while the efflux reaction is linear over 24 h (Figure 2). It is therefore reasonable to assume that specific intracellular signaling is involved in triggering rapid depletion of the ACAT-available cholesterol pool. In contrast to the apolipoprotein-mediated efflux, there was no change in the ACAT-available cholesterol even when substantial cholesterol efflux was initiated by the lipid microemulsion. Therefore, the trigger of such mobilization of cholesterol seems more specific than the general plasma membrane cholesterol level, and the mobilization is not simply to fulfill

the loss of cholesterol in the cell surface. A protein kinase C inhibitor, H-7, reversed this change. In the condition that H-7 reduced the apoA-I-mediated cholesterol efflux, it also inhibited the depletion of the ACAT-available intracellular cholesterol by apoA-I.

From these data presented, it is rational to propose the hypothesis that intracellular cholesterol is mobilized selectively for apolipoprotein-mediated efflux in response to apolipoprotein−cell interaction. This process is independent of generation of preβ-HDL-like lipoprotein from the cellular phospholipid that occurs subsequently to the apolipoprotein−cell interaction, and therefore the inhibition of cholesterol mobilization results in generation of the cholesterol-poor preβ-HDL. This is also independent of nonspecific cellular cholesterol efflux mediated by cholesterol diffusion from the plasma membrane through the aqueous phase. It is likely that the apolipoprotein−cell interaction triggers intracellular signal transduction to initiate the cholesterol mobilization.

These results were thus consistent with those obtained from rat vascular smooth muscle cells (Li & Yokoyama, 1995), except for two important differences. First, the smooth muscle cells require pretreatment with growth factors for the increase of the apolipoprotein-derived cholesterol efflux by PMA. PDGF is essential, and MCSF had additional effect (Li & Yokoyama, 1995). In macrophages, cholesterol-rich preβ-HDL is generated without such stimulation of the cells, and the further increase of the cholesterol content by PMA was only moderate. The growth factors seem to induce in the smooth muscle cells a certain condition required for cholesterol mobilization for apolipoprotein-mediated efflux. However, the linkage is still missing between the apolipoprotein−cell interaction and triggering such cholesterol mobilization, and further pharmacological cellular treatment with phorbol ester is required for the final increase of the cholesterol efflux. Second, H-7 increased the cellular ACAT-available pool in macrophages even in the absence of exogenous apolipoprotein in the medium. This finding indicates that mobilization of cholesterol for the efflux is induced in macrophages without stimulation by exogenous apolipoprotein in the culture. We have shown that not only apoA-I but also many other helical apolipoproteins carry out the same reaction, including apoE (Hara & Yokoyama, 1991; Hara et al., 1992). It is therefore likely that apoE synthesized and secreted by cholesterol-loaded macrophages (Basu et al., 1981) causes the autocrine effect to activate the pathway (Hara & Yokoyama, 1991; Zhang et al., 1996; Smith et al., 1996). This view is supported by the finding that such an increase of the ACAT-available cholesterol pool by H-7 disappeared in the presence of lipid microemulsion (Figure 8) that would absorb apoE to its surface (Hara & Yokoyama, 1992). It is also possible that the small decrease by the protein kinase C inhibitors of the diffusion-mediated cholesterol efflux to the microemulsion may account for the contribution of such apoE-mediated intracellular cholesterol mobilization to the nonspecific efflux during 24 h (Figures 3 and 4).

To test such a possibility, monoclonal antibody against mouse apoE, ID7 (a generous gift from Dr. Ross Milne, Ottawa Heart Institute), was used in the attempt to block the potential autocrine effect of apoE presumably secreted by the macrophages into the medium. However, there was no significant effect of this antibody on the intracellular ACAT-available cholesterol pool. Thus, the results were inconclusive since this particular monoclonal antibody may not interfere in the apoE−cell interaction.

Initiation of intracellular signal transduction by HDL has been reported in various conditions. It has been suggested that HDL stimulates protein kinase C and accordingly enhances intracellular cholesterol translocation for the efflux (Aviram et al., 1989; Mendez et al., 1991). Other reports also indicated that HDL seems to trigger multisignal pathways in the cells including protein kinase C, of which the consequence is yet unclear (Wu & Handwerger, 1992; Nazih et al., 1994; Walter et al., 1995). The results of this report as well as the results presented in our previous work with rat vascular smooth muscle cells (Li & Yokoyama, 1995) implicated that protein kinase C is specifically involved in the mechanism to mobilize cellular cholesterol to a specific pool used for generation of preβ-HDL by apolipoprotein. If protein kinase C is indeed involved in the apolipoprotein-mediated cellular cholesterol efflux, it would be either in intracellular signaling to initiate the cholesterol mobilization or in transport of cholesterol from the intracellular pool to the specific pool for the efflux. The evidence is, however, still circumstantial as the pharmacological activation and inhibition are not strictly specific for protein kinase C. More direct evidence is required to prove the involvement of protein phosphorylation in apolipoprotein-mediated cellular cholesterol efflux.

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