# Apolipoprotein A-I stimulates the transport of intracellular cholesterol to cell-surface cholesterol-rich domains (caveolae)

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We have studied the effect of lipid-free human plasma apolipoprotein A-I (apoA-I) on the transport of newly synthesized cholesterol to cell-surface cholesterol-rich domains, which in human skin fibroblasts are mainly represented by caveolae. Changes in transport of newly synthesized cholesterol were assessed after labelling cells with [$^{14}$C]acetate at 15 °C and warming cells to permit the transfer of cholesterol, followed by the selective oxidation of cholesterol in cholesterol-rich domains (caveolae) in the plasma membrane before their partial purification. ApoA-I, but not BSA added in an equimolar concentration, enhanced the transport of cholesterol to the caveolae up to 5-fold in a dose- and time-dependent manner. The effect of apoA-I on cholesterol transport exceeded its effect on cholesterol efflux, resulting in an accumulation of intracellular cholesterol in caveolae. Methyl-$\beta$-cyclodextrin, added at a concentration promoting cholesterol efflux to the same extent as apoA-I, also stimulated cholesterol trafficking, but was 3-fold less effective than apoA-I. Progesterone inhibited the transport of newly synthesized cholesterol to the caveolae. Treatment of cells with apoA-I stimulated the expression of caveolin, increasing the amount of caveolin protein and mRNA by approx. 2-fold. We conclude that apoA-I induces the transport of intracellular cholesterol to cell-surface caveolae, possibly in part through the stimulation of caveolin expression.

Key words: caveolin, cholesterol trafficking, high-density lipoprotein, lipoproteins, reverse cholesterol transport.

## INTRODUCTION

Caveolae are components of the plasma membrane (PM) seen as non-coated vesicles 50–100 nm in diameter and containing caveolins [1–3]. The function of caveolae and caveolin seems to involve the targeting and co-ordination of the intracellular trafficking of several proteins and lipids, including cholesterol, from the intracellular compartments to the PM [1,2,4–7]. The suggestion that caveolin is involved in intracellular cholesterol trafficking is based on several lines of evidence: (1) caveolin binds free cholesterol [4]; (2) cholesterol transport to the cell surface in cells expressing caveolin is much faster than in corresponding cells lacking caveolin [4,8]; (3) progesterone blocks the movement of both caveolin and cholesterol from the endoplasmic reticulum (ER) to the PM [4]; and (4) expression of the gene encoding caveolin responds to cell cholesterol content [9], a regulation that is mediated by sequences similar to sterol regulatory elements [10]. In addition, most cholesterol released from cells to extracellular acceptors originates from caveolae [5,11]; SR-B1, a receptor for high-density lipoprotein (HDL), is also located in this region of the PM [12]. Most peripheral cells contain caveolae. Cells lacking caveolae still possess regions of PM ('rafts'), which share some properties of caveolae, such as high concentration of cholesterol, sphingolipids and lipid-modified signalling molecules [13]. The role, if any, of rafts in cholesterol homoeostasis has not yet been defined. It is unclear whether any rafts distinct from caveolae exist in caveolae-containing cells; throughout this paper we refer to the free-cholesterol-rich domains of the PM as caveolae.

HDL is the principal extracellular acceptor of cholesterol released from extrahepatic cells and is a key element of the reverse cholesterol transport pathway, a protective pathway that prevents the excessive accumulation of cholesterol in the vessel wall and the development of atherosclerosis (reviewed in [14]). Apolipoprotein A-I (apoA-I) is the main protein of HDL that determines this activity [14]. Although HDL and apoA-I are efficient acceptors of PM cholesterol [15], other evidence is accumulating to suggest that apoA-I is also involved in the regulation of intracellular events leading to cholesterol efflux, including the ability of apoA-I to stimulate the translocation of intracellular cholesterol to the PM [16,17] and to promote the efflux of intracellular cholesterol [18–21]. ApoA-I also triggers signalling pathways, which could be related to cholesterol efflux [22–24].

In the present study we have investigated a possible link between the capacity of apoA-I to stimulate the efflux of intracellular cholesterol and the caveolin-dependent transport of cholesterol. We found that apoA-I stimulates the transfer of newly synthesized cholesterol from the intracellular compartments to the caveolae region of the PM of human skin fibroblasts. Our findings also show that part of the effect of apoA-I could be due to up-regulation of caveolin expression.

## MATERIALS AND METHODS

### Cells

Human skin fibroblasts were grown in a $CO_2$ incubator [air/$CO_2$ (19:1)] in 75 cm² flasks (Falcon; Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cultures were maintained in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum, 20 mM Hepes, 1 % (w/v) non-essential amino acids, 2 mM L-glutamine, 100 i.u./ml penicillin, 100 $\mu$g/ml streptomycin and

Abbreviations used: apoA-I, apolipoprotein A-I; CyD, methyl-$\beta$-cyclodextrin; ER, endoplasmic reticulum; HDL, high-density lipoprotein; PM, plasma membrane.

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3.7 mg/ml sodium bicarbonate (all from ICN, Seven Hills, NSW, Australia). Cells were confluent at the beginning of the experiments.

### Transfer of newly synthesized cholesterol to caveolae

To label the entire cellular cholesterol pool, human skin fibroblasts were incubated in a serum-containing medium with [1$\alpha$,2$\alpha$(n)-$^3$H]cholesterol (Amersham, Castle Hill, NSW, Australia; specific radioactivity 1.81 TBq/mmol; final radioactivity 75 kBq/ml) for 48 h at 37 °C in a $CO_2$ incubator. After labelling, cells were washed six times with Hanks balanced salt solution and incubated for a further 18 h in serum-free medium at 37 °C in a $CO_2$ incubator to down-regulate from the possible chronic effects of exposure to serum apoA-I. The viability of the cells did not change as a result of incubation in a serum-free medium. The cells were then incubated with various concentrations of apoA-I in Leibovitz L-15 serum-free medium (ICN) for the indicated periods at 37 °C. Flasks were cooled on ice, [1-$^{14}$C]acetic acid sodium salt (ICN, specific radioactivity 2.2 GBq/mmol, final radioactivity 18 MBq/ml) was added and the flasks were incubated for a further 3 h at 15 °C. Under these conditions intracellular cholesterol trafficking is blocked while cholesterol biosynthesis proceeds [25,26]. It was shown in preliminary experiments that the amount of newly synthesized [$^{14}$C]cholesterol in cell homogenates and the post-nuclear supernatant was not affected by preincubation with apoA-I. At the end of the incubation, synthesis of labelled cholesterol was arrested by the addition of a 1000-fold excess of unlabelled sodium acetate; the flasks were quickly warmed and incubated for 20 min at 37 °C to allow a portion of the newly synthesized cholesterol to be transferred to the PM. The cells were cooled on ice, medium was removed for analysis of cholesterol efflux and the cells were washed three times with ice-cold PBS. Cholesterol oxidase (Boehringer Mannheim, Nunawading, Vic., Australia) was added to the cells at a final concentration of 1 unit/ml and flasks were incubated for 3 h at 4 °C. Under these conditions only cholesterol in caveolae (and possibly any caveolin-free rafts if they exist in these cells) is oxidized [5,27], forming cholestenone (referred to as oxysterol throughout this paper); the reaction is performed to completion [5]. The proportion of [$^3$H]cholesterol oxidized under these conditions was 0.02–0.05, reflecting the amount of cholesterol in caveolae (similar to that found previously [5,27]). After the end of the incubation, cells were washed three times with ice-cold PBS and the PM was isolated by the method of Smart et al. [4,28] with modifications. Each flask was washed twice with buffer A [0.25 M sucrose/1 mM EDTA/20 mM Tricine (pH 7.8)] and cells were collected by scraping into 3 ml of buffer A. The cells were pelleted by centrifugation for 5 min at 1500 ***g***, resuspended in 1 ml of buffer A and homogenized with 30 strokes in a 2 ml tissue grinder (Wheaton). The suspension was centrifuged at 1000 ***g*** for 10 min in an Eppendorf centrifuge in 1.5 ml centrifuge tubes. The post-nuclear supernatant was mixed with 3 ml of 65% (v/v) Percoll (Pharmacia Biotech, Uppsala, Sweden) in buffer A, and centrifuged for 30 min at 50000 ***g*** in a 50.3 Ti rotor. The PM fraction (a visible band) was collected and subjected to further analysis. Analysis of 5′-nucleotidase and NADH:cytochrome *c* reductase content in the gradient fractions showed that most of the PM was recovered in this band without major contamination with the ER (less than 11% of the ER was recovered in the PM fraction). All of these isolation procedures were performed at 4 °C.

Four controls were included in each experiment in addition to the experimental samples: (1) no preincubation with apoA-I; (2) no preincubation with apoA-I and no oxidation; (3) no preincubation with apoA-I and no 'warm up' stage; and (4) no preincubation with apoA-I, no warming and no oxidation. The amount of cholesterol oxidized by cholesterol oxidase under controlled conditions reflects the amount of cholesterol in caveolae [5,27]. The amount of oxidizable cholesterol was calculated in two different ways. First, we determined the total amount of [$^{14}$C]oxysterol per flask ('A' panels). Because a fraction of cholesterol that transferred from intracellular compartments to caveolae was released to the medium within the duration of the experiment, the amount of released cholesterol was added, when indicated, to the amount of oxidized cholesterol to quantify the total amount of cholesterol transferred to or through caveolae. To quantify cholesterol efflux, the amount of specifically released [$^{14}$C]cholesterol (i.e. the difference between the amount of cholesterol released in the presence and in the absence of an acceptor) was corrected for total losses of membrane cholesterol pool that occurred during isolation. Secondly, we determined the amount of [$^{14}$C]oxysterol as a fraction of total non-oxidized [$^3$H]cholesterol in the sample (entire cholesterol pool), thus correcting for losses during membrane purification and lipid analysis ('B' panels). In both cases the 'no oxidase' control, which was 10–25% of 'oxidized' samples, was subtracted from the experimental values.

### Lipid analysis

Lipids from the medium, cells and membrane preparation were extracted as described previously [29] and samples together with lipid standards (free cholesterol and cholest-4-en-3-one; Sigma, Castle Hill, NSW, Australia) were fractionated by TLC on silica-gel layers developed in light petroleum (boiling point range 40–60 °C)/diethyl ether/acetic acid (80:20:1, by vol.). $^3$H and $^{14}$C label in each fraction was determined by liquid-scintillation spectrometry.

### Expression of caveolin

Human skin fibroblasts grown in 75 cm$^2$ flasks were incubated in a serum-free medium for 18 h at 37 °C in a $CO_2$ incubator. Medium was then replaced with Leibovitz L-15 serum-free medium with or without apoA-I at a final concentration of 10 or 100 $\mu$g/ml and flasks were incubated for the indicated periods at 37 °C. Total protein and RNA were isolated from the cells by using a TriPure isolation kit (Boehringer Mannheim) in accordance with the manufacturer's instructions.

Proteins were separated by SDS/PAGE [12% (w/v) gel], transferred to nitrocellulose membrane and probed with polyclonal anti-(human caveolin-1) antibody (Transduction Laboratories, Lexington, KY, U.S.A.). The Western blots were developed with an ECL® kit (Amersham) and the resulting film was scanned by trans-illumination and analysed with Optimas software (BioScan, Edmonds, WA, U.S.A.).

RNA was separated on a 1.2% (w/v) agarose gel, transferred to a nylon membrane and probed with $^{32}$P-labelled caveolin cDNA [9] and $^{32}$P-labelled actin cDNA as an internal standard. The membrane was exposed to a PhosphorImager plate and analysed on the Bioimager BAS-1000 (Fuji).

### Miscellaneous

Human plasma apoA-I was isolated and purified as described previously [30]. The protein contents of cells, membrane fractions and protein solutions were determined by the method of Bradford [31].

## RESULTS

### Effect of apoA-I on the transport of newly synthesized cholesterol

To assess the effect of apoA-I on the trafficking of newly synthesized cholesterol, cells were preincubated with apoA-I, cholesterol in the ER was labelled by incubation with [$^{14}$C]acetate at 15 °C and then the cells were warmed to allow newly synthesized cholesterol to move to caveolae (see the Materials and methods section for details). The dependence of the transport of newly synthesized cholesterol to caveolae on the apoA-I concentration is presented in Figure 1. In the absence of apoA-I, warming cells to 37 °C resulted in only a small increase in the amount of [$^{14}$C]oxysterol in caveolae compared with cells incubated at 4 °C (Figure 1A). In the presence of apoA-I the amount of [$^{14}$C]oxysterol in caveolae increased in a dose-dependent manner up to 5-fold (Figure 1A). A similar effect was evident when the amount of newly synthesized cholesterol in caveolae ([$^{14}$C]oxysterol) was normalized to the total amount of cholesterol in PM ([$^{3}$H]cholesterol) (Figure 1B) accounting for the possible losses during isolation of PM and lipid analysis. The amount of [$^{3}$H]oxysterol, reflecting the total amount of cholesterol in caveolae, was minimally affected by apoA-I (2090 d.p.m. with

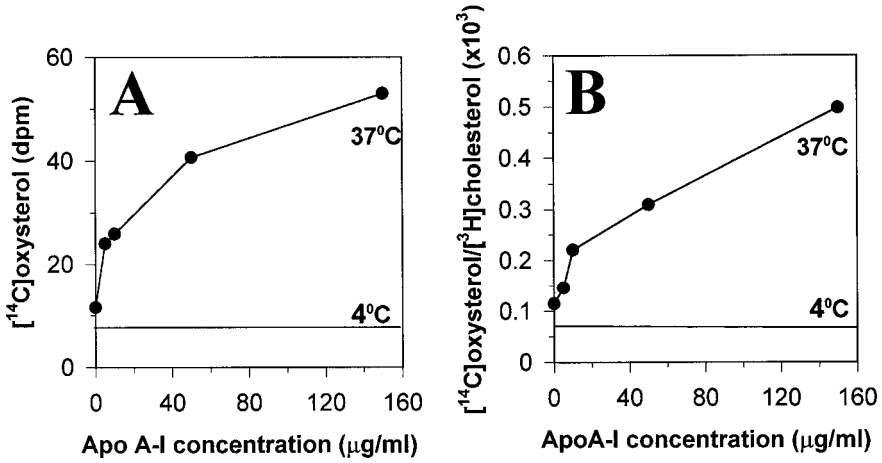


**Figure 1 Dose dependence of the effect of apoA-I on the transport of newly synthesized cholesterol to the caveolae**

Human skin fibroblasts in 75 cm$^2$ flasks were labelled with [$^{3}$H]cholesterol by incubation for 48 h at 37 °C in serum-containing medium containing labelled cholesterol (final concentration 75 kBq/ml). The label was removed and cells were washed, incubated in serum-free medium for 18 h at 37 °C and then for a further 2 h in the presence of the indicated concentrations of apoA-I. Intracellular cholesterol was labelled by incubation for 3 h at 15 °C with [$^{14}$C]acetate (final concentration 18 MBq/ml); the reaction was arrested by the addition of a 1000-fold excess of unlabelled sodium acetate. Cells were warmed to 37 °C for 20 min to allow the newly synthesized cholesterol to be transported to the caveolae; cholesterol in caveolae was oxidized by incubation for 3 h at 4 °C with cholesterol oxidase (final concentration 1 unit/ml). PM was then isolated as described in the Materials and methods section, lipids were extracted and cholesterol and oxysterol were separated by TLC. (**A**) Total amount of [$^{14}$C]oxysterol found in the PM; (**B**) amount of [$^{14}$C]oxysterol in the PM normalized to the amount of [$^{3}$H]cholesterol in the PM preparation.

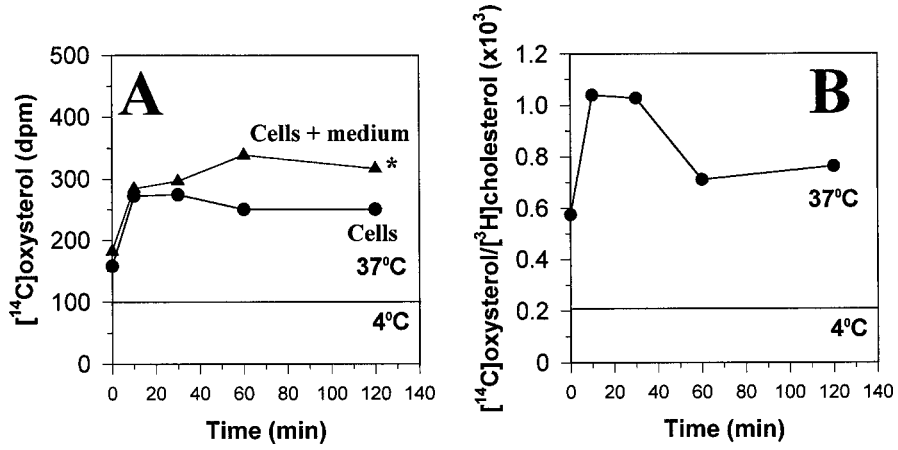


**Figure 2 Time course of the effect of apoA-I on the transport of newly synthesized cholesterol to the caveolae**

Human skin fibroblasts in 75 cm$^2$ flasks were labelled with [$^{3}$H]cholesterol by incubation for 48 h at 37 °C in serum-containing medium containing labelled cholesterol (final concentration 75 kBq/ml). The label was removed and cells were washed, incubated in serum-free medium for 18 h at 37 °C and then for the indicated periods in the presence of 100 $\mu$g/ml apoA-I. Intracellular cholesterol was labelled by incubation for 3 h at 15 °C with [$^{14}$C]acetate (final concentration 18 MBq/ml); the reaction was arrested by the addition of a 1000-fold excess of unlabelled sodium acetate. Cells were warmed to 37 °C for 20 min to allow the newly synthesized cholesterol to be transported to the caveolae, then cholesterol in caveolae was oxidized by incubation for 3 h at 4 °C with cholesterol oxidase (final concentration 1 unit/ml). PM was then isolated as described in the Materials and methods section, lipids were extracted and cholesterol and oxysterol were separated by TLC. (**A**) Total amount of [$^{14}$C]oxysterol found in the PM; (**B**) amount of [$^{14}$C]oxysterol in the PM normalized to the amount of [$^{3}$H]cholesterol in the PM preparation. *$P < 0.05$ (compared with cells).

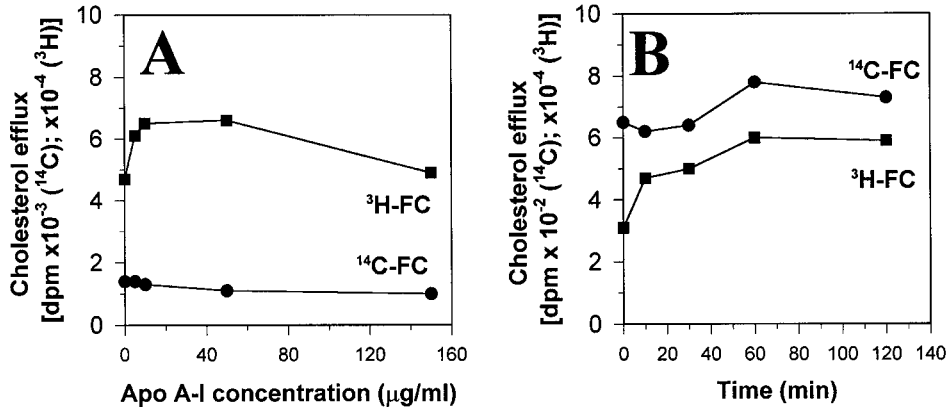


**Figure 3 Dose dependence (A) and time course (B) of the effect of apoA-I on cholesterol efflux**

Experiments were performed as described in the legends to Figures 1 and 2. After the end of the 'warming up' incubation the medium was taken and cholesterol was isolated by TLC. Note that the scales for [$^{3}$H]cholesterol and [$^{14}$C]cholesterol are different.

100 $\mu$g/ml of apoA-I, compared with 1643 d.p.m. without apoA-I).

The effect of the duration of preincubation of cells with apoA-I on the transport of newly synthesized cholesterol to caveolae is presented in Figure 2. Preincubation of cells with apoA-I for as little as 10 min resulted in an approx. 2-fold increase in the transport of newly synthesized cholesterol to caveolae; further incubation had little additional effect. A portion of newly synthesized cholesterol was also found in the medium. The time dependence of the effect of apoA-I on the transport of newly synthesized cholesterol to caveolae combined with its efflux, shown in Figure 2(A), also indicates that most of the effect occurred in the first 10 min of incubation (Figure 2A). When the amount of newly synthesized cholesterol in caveolae ([$^{14}$C]-oxysterol) was normalized to the total amount of cholesterol in PM ([$^{3}$H]cholesterol), the ratio increased within the first 10 min, but decreased when preincubation with apoA-I was extended to 60 min (Figure 2B). The decrease in the [$^{14}$C]oxysterol-to-[$^{3}$H]-cholesterol ratio at longer incubations with apoA-I is most likely to be attributable to the faster efflux of [$^{3}$H]cholesterol, which is already on the cell surface, than that of newly synthesized cholesterol, which needs to be transported there.

When the amount of cholesterol released from cells to apoA-I was analysed, the efflux of [$^{3}$H]cholesterol clearly depended on both the apoA-I concentration (Figure 3A) and the duration of incubation with apoA-I (Figure 3B). The efflux of [$^{14}$C]cholesterol also depended on the duration of incubation (Figure 3B) but not on apoA-I concentration in the medium (Figure 3A), indicating that the transfer of newly synthesized cholesterol to caveolae could be rate-limiting in its efflux. The amount of newly synthesized [$^{14}$C]cholesterol specifically released to the medium was minimal; however, if related to the amount of [$^{14}$C]oxysterol found in the caveolae it accounted for up to 50 % of newly synthesized cholesterol transported from the ER to caveolae.

Because the effect of apoA-I on cholesterol trafficking to caveolae could have resulted from changes in the cholesterol content of caveolae, which might have occurred after incubation of cells with the apoA-I, we compared the effect of apoA-I with another, non-specific cholesterol acceptor, methyl-$\beta$-cyclodextrin (CyD) [32]. Cholesterol efflux to both CyD and apoA-I was dose-dependent, with CyD removing up to 90 % of cellular cholesterol at high CyD concentration (Figure 4, upper panel). Analysing the dose-dependence curve (Figure 4, lower panel) we then selected two concentrations of CyD and apoA-I that promoted an equivalent efflux of cholesterol. These concentrations were (1) apoA-I 20 $\mu$g/ml (0.71 nM), CyD 60 $\mu$g/ml (54 $\mu$M), and (2) apoA-I 100 $\mu$g/ml (3.6 nM), CyD 100 $\mu$g/ml (91 $\mu$M).

Preincubation of cells with both apoA-I and CyD increased the amount of newly synthesized cholesterol found in caveolae to a similar extent (1.7-fold) (Figure 5A). However, whereas apoA-I promoted the efflux of a fraction of the newly synthesized cholesterol, the efflux of [$^{14}$C]cholesterol to CyD was negligible. When the amount of [$^{14}$C]cholesterol released to the medium was taken into account, apoA-I caused a 3-fold increase in the amount of newly synthesized cholesterol transported to caveolae (Figure 5A). When the amount of newly synthesized cholesterol in caveolae ([$^{14}$C]oxysterol) was normalized to the total amount of cholesterol in PM ([$^{3}$H]cholesterol), apoA-I was clearly more effective then CyD in stimulating the transfer of cholesterol to caveolae (Figure 5B).

We also compared the effect of apoA-I with that of BSA. As expected, apoA-I, when added at 1 and 4 $\mu$M, caused respectively 30 % and 250 % increases in both the amount of newly synthesized cholesterol in caveolae and the [$^{14}$C]oxysterol-to-[$^{3}$H]cholesterol ratio. BSA, added at the same molar concentration as apoA-I, did not stimulate the transport of newly synthesized cholesterol to caveolae. BSA did not promote the efflux of [$^{14}$C]cholesterol; neither did it affect cholesterol biosynthesis (results not shown).

The effect of apoA-I was also compared with that of progesterone, an inhibitor of intracellular cholesterol trafficking [3,33] and especially of the transport of cholesterol to caveolae [4]. Two modifications were made to the experimental design when the effect of progesterone was studied. First, because progesterone had to be added in a complex with BSA, we included the vehicle in all control incubations. Secondly, to account for any effect of progesterone on cholesterol metabolism, such as delayed biosynthesis, an additional time point was included when progesterone was added to the incubation mixture after cholesterol biosynthesis was concluded. The time course of the effect of progesterone on cholesterol trafficking is presented in Figure 6. (The point at $-20$ min refers to the control incubation without progesterone.) Progesterone effectively inhibited both the amount of newly synthesized cholesterol in caveolae (Figure 6A) and the [$^{14}$C]oxysterol-to-[$^{3}$H]cholesterol ratio (Figure 6B); it had no effect on cholesterol biosynthesis.

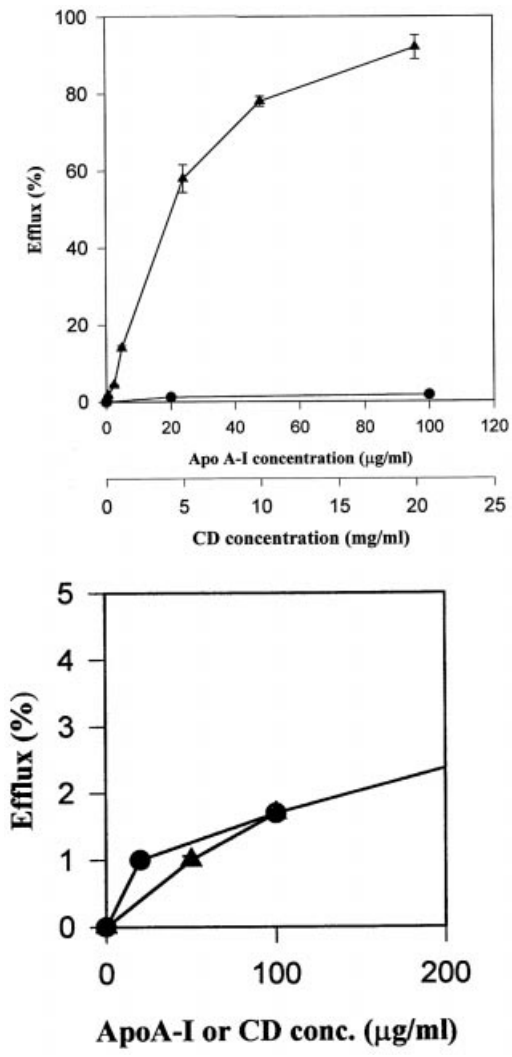


**Figure 4 Dose dependence of the effect of apoA-I (●) and CyD (▲) on cholesterol efflux**

Upper panel: human skin fibroblasts in 12-well plates were labelled with [$^3$H]cholesterol by incubation for 48 h at 37 °C in serum-containing medium containing labelled cholesterol (final concentration 75 kBq/ml). The label was removed and cells were washed, incubated in serum-free medium for 18 h at 37 °C and then for a further 1 h in the presence of the indicated concentrations of apoA-I or CyD (CD). Medium was removed, cells were washed and the amounts of [$^3$H]cholesterol in the cells and medium were determined by $\beta$-counting. Lower panel: expansion of the low-concentration portion of the curves. Results are means ± S.E.M. for quadruplicate determinations.

### Effect of apoA-I on caveolin expression

To investigate the effect of apoA-I on caveolin expression, cells were incubated in the presence or absence of apoA-I (10 or 100 $\mu$g/ml); protein and RNA were extracted and analysed by Western and Northern blots. Two bands with a molecular mass of approx. 23 kDa and similar to the bands produced with control human endothelium caveolin were identified on the Western blot by polyclonal anti-caveolin antibody (Figure 7A). When analysed by quantitative densitometry, an up to 2-fold increase in the amount of caveolin was observed in a time- and dose-dependent manner when the cells were treated with apoA-I (Figure 7C). Other proteins were not affected by this treatment.

Probing of cellular RNA with a human caveolin cDNA probe by Northern blot hybridization detected a single transcript approx. 3.0 kb in size (Figure 7B), which is in agreement with the results reported by others [34]. Actin mRNA was used as a control and its concentration was not affected by incubation of cells with apoA-I (results not shown). An up to 2-fold increase in the amount of caveolin mRNA was observed when the cells were treated with apoA-I (Figure 7D). Thus, treatment of cells with apoA-I led to an increased expression of caveolin in human skin fibroblasts.

## DISCUSSION

One of the key features of atherosclerosis is the accumulation of cholesterol in the artery wall, which is a result of an imbalance between cholesterol influx and efflux. The incoming cholesterol originates from two sources: (1) endogenous cholesterol biosynthesis and (2) uptake of exogenous cholesterol, mainly from low-density lipoprotein. Both pathways for cholesterol delivery are subject to tight metabolic regulation [35]. Removal of excess cholesterol from cells occurs by the reverse cholesterol transport pathway, the key element of which is HDL [14]. Our present understanding of the regulation of this pathway is very limited. Because the concentration of HDL in plasma is inversely correlated with the risk of developing atherosclerosis [36], it was believed that the availability of HDL was the rate-limiting factor in the activity of the reverse cholesterol transport pathway [37]. However, evidence is accumulating to suggest that HDL is not restricted to being a passive acceptor of cellular cholesterol but also participates in regulating intracellular pathways involved in the release of cholesterol. [14,17,21]. Most reports on cellular cholesterol efflux agree that the concentration of apoA-I required for maximum recruitment of cholesterol is below the concentration of apoA-I in plasma [38,39]. Recently Dietschy et al. [40,41] suggested that the rate-limiting step in the reverse cholesterol transport pathway is not the HDL concentration in plasma but intracellular events resulting in cholesterol efflux. To reconcile both suggestions we have investigated the hypothesis that one regulates the other.

The hypothesis that extracellular events regulate cholesterol efflux is supported by the suggestion that different HDL subfractions have different roles in the efflux. The finding that lipid-poor pre-$\beta_1$-HDL is the initial acceptor of cellular cholesterol [42] led to the development of the 'shuttle and sink' model (reviewed in [15]). According to this model only a small portion of apoA-I, which is lipid-free or lipid-poor, participates in the initial release of cholesterol from the cells with the subsequent delivery of this cholesterol to the lipid-rich fractions of HDL. Cholesterol efflux occurs from specific regions of the PM; however, it is not clear whether the availability of cholesterol for the efflux is determined by packing of cholesterol in some regions of the PM [43], specific morphological features such as caveolae [5,11], or cellular HDL receptors such as SR-B1 [44], ABCA1 [45–47] or cubilin [48]. All three mechanisms can co-exist; however, the involvement of caveolae and/or HDL receptors in cholesterol efflux indicates that intracellular events might be implicated in the regulation of cholesterol efflux.

One of the factors that might regulate cholesterol efflux is intracellular cholesterol trafficking, especially transport to the regions of the PM where it becomes available for efflux. The transport of intracellular cholesterol to the PM is regulated by apoA-I [16,19–21]; it has been demonstrated that the region of the PM responsible for cholesterol efflux contains caveolae [5,11]. Caveolae are also characterized by the presence of a large number of proteins involved in intracellular signalling [2] and SR-B1 [12].

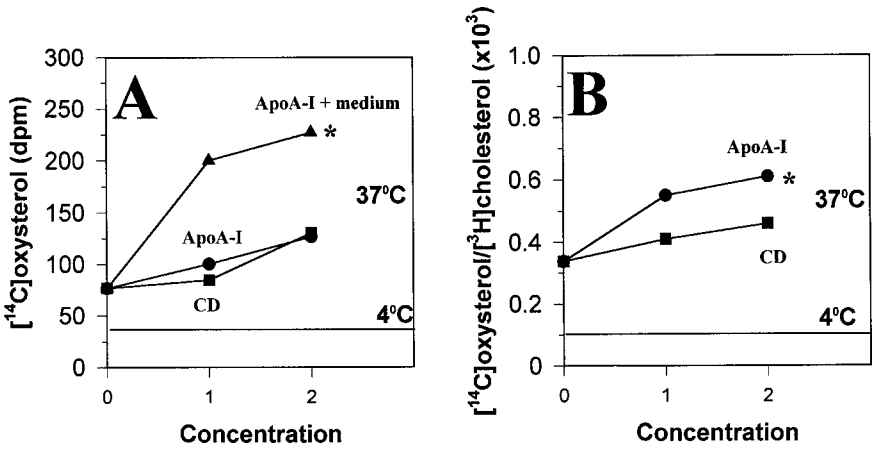


**Figure 5 Effect of apoA-I and CyD on the transport of newly synthesized cholesterol to the caveolae**

Human skin fibroblasts in 75 $cm^2$ flasks were labelled for 48 h at 37 °C with [$^3$H]cholesterol by incubation in serum-containing medium containing labelled cholesterol (final concentration 75 kBq/ml). The label was removed and cells were washed, incubated in serum-free medium for 18 h at 37 °C and then for a further 30 min in the presence of apoA-I or CyD (CD). The concentrations were as follows: 1, apoA-I 20 $\mu$g/ml (0.71 nM)/CyD 60 $\mu$g/ml (54 $\mu$M); 2, apoA-I 100 $\mu$g/ml (3.6 nM)/CyD 100 $\mu$g/ml (91 $\mu$M). Intracellular cholesterol was labelled by incubation for 3 h at 15 °C with [$^{14}$C]acetate (final concentration 18 MBq/ml); the reaction was arrested by the addition of a 1000-fold excess of unlabelled sodium acetate. Cells were warmed to 37 °C for 20 min to allow the newly synthesized cholesterol to be transported to the caveolae; cholesterol in caveolae was oxidized by incubation for 3 h at 4 °C with cholesterol oxidase (final concentration 1 unit/ml). PM was then isolated as described in the Materials and methods section, lipids were extracted and cholesterol and oxysterol were separated by TLC. (**A**) Total amount of [$^{14}$C]oxysterol found in the PM; (**B**) amount of [$^{14}$C]oxysterol in the PM normalized to the amount of [$^3$H]cholesterol in the PM preparation. $^*P < 0.02$ (compared with CyD).

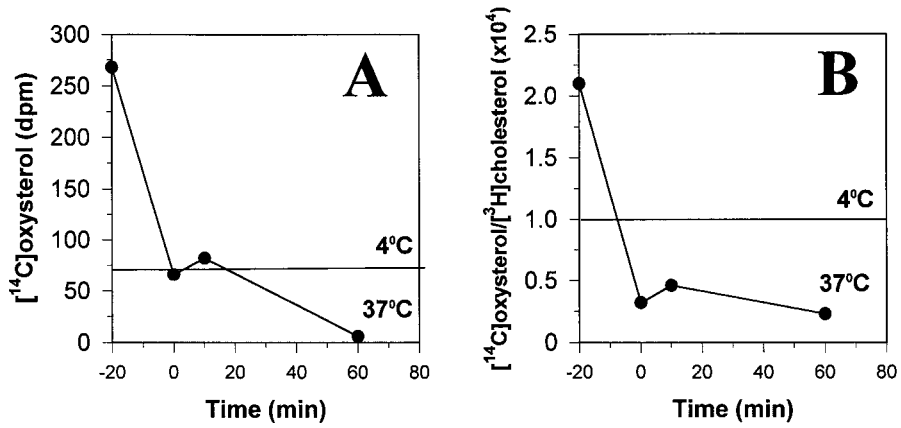


**Figure 6 Effect of progesterone on the transport of newly synthesized cholesterol to the caveolae**

Human skin fibroblasts in 75 $cm^2$ flasks were labelled with [$^3$H]cholesterol by incubation for 48 h at 37 °C in serum-containing medium containing labelled cholesterol (final concentration 75 kBq/ml). The label was removed and cells were washed, incubated in serum-free medium for 18 h at 37 °C and then for the indicated periods in the presence of BSA (final concentration 0.5 mg/ml) or progesterone (final concentration 10 $\mu$g/ml) added as a complex with BSA (see the text for an explanation of the *x*-axis). Intracellular cholesterol was labelled by incubation for 3 h at 15 °C with [$^{14}$C]acetate (final concentration 18 MBq/ml); the reaction was arrested by the addition of a 1000-fold excess of unlabelled sodium acetate. Cells were warmed to 37 °C for 20 min to allow the newly synthesized cholesterol to be transported to the caveolae; cholesterol in caveolae was oxidized by incubation for 3 h at 4 °C with cholesterol oxidase (final concentration 1 unit/ml). PM was then isolated as described in the Materials and methods section, lipids were extracted and cholesterol and oxysterol were separated by TLC. (**A**) Total amount of [$^{14}$C]oxysterol found in the PM; (**B**) amount of [$^{14}$C]oxysterol in the PM normalized to the amount of [$^3$H]cholesterol in the PM preparation.

In the present paper we investigated the regulation of the transfer of intracellular cholesterol to cell-surface caveolae. The cells used were a quiescent culture of human skin fibroblasts. The cholesterol trafficking in these cells is well characterized. They have a large number of caveolae and it seems likely that in these cells caveolae, rather than caveolin-free rafts, are the major participants. Their cholesterol homoeostasis is regulated towards the efflux of free cholesterol [1]. Human skin fibroblasts might be an appropriate model for components of the normal artery wall because quiescent endothelial and smooth-muscle cells have similar parameters of cholesterol trafficking and efflux [1]. However, fibroblasts might not correctly represent events in cells prevalent in the developed atherosclerotic plaque, phenotypically modified smooth-muscle cells or macrophages, where cholesterol trafficking might be very different.

The major finding of this study is that apoA-I stimulates the transport of cholesterol from intracellular compartments to caveolae in a time- and dose-dependent manner. The effect was only evident when cells were preincubated without apoA-I before the experiment. Without such preincubation the background transport was much higher but the effect of apoA-I virtually disappeared. This finding indicates that the stimulation of

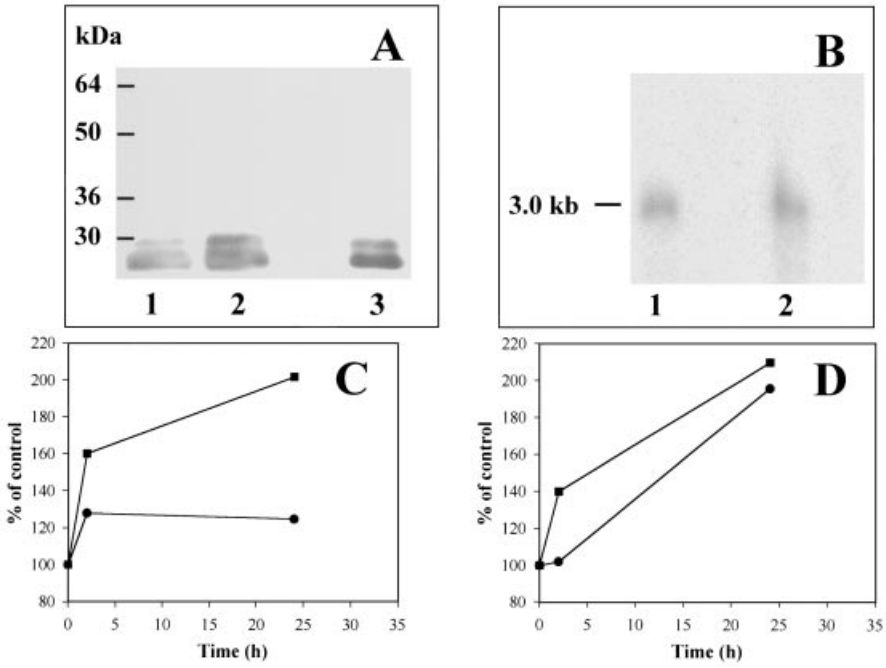


**Figure 7 Effect of apoA-I on the expression of caveolin**

Human skin fibroblasts in 75 $cm^2$ flasks were incubated in serum-free medium for 18 h at 37 °C and then incubated for the indicated periods in the presence or absence of apoA-I (final concentrations 10 and 100 $\mu$g/ml). Protein and RNA were extracted and separated from cells as described in the Materials and methods section. (**A**) Western blot, probed with anti-(human caveolin) polyclonal antibody; lane 1, cells incubated without apoA-I; lane 2, cells incubated with apoA-I (100 $\mu$g/ml for 2 h); lane 3, human endothelial cells (standard). (**B**) Northern blot, probed with caveolin cDNA; lane 1, cells incubated without apoA-I; lane 2, cells incubated with apoA-I (100 $\mu$g/ml for 2 h). (**C**) Time course of the effect of apoA-I on the amount of caveolin in the cells; ●, 10 $\mu$g/ml apo-AI; ■, 100 $\mu$g/ml apo-AI. (**D**) Time course of the effect of apoA-I on the amount of caveolin mRNA in the cells.

cholesterol trafficking by apoA-I might be a constitutive feature under physiological conditions.

It has been demonstrated that the effect of apoA-I on cholesterol trafficking can be partly reproduced by treating cells with CyD. Although CyD was less effective than apoA-I in stimulating cholesterol trafficking, this finding raises the possibility that the effect of apoA-I on cholesterol trafficking might be at least partly explained by changes in the cholesterol content of caveolae. However, it has been shown that the expression of caveolin is up-regulated in response to increased caveolar cholesterol content [9] and down-regulated when cell cholesterol content is decreased by treatment with CyD [49], excluding the possibility that cholesterol efflux in itself causes an increase in caveolae numbers. Rather, selective removal of cholesterol from caveolae might initiate the further transfer of intracellular cholesterol, possibly together with caveolin, to caveolae. Our results also show that under our experimental conditions the effect of apoA-I on cholesterol transport exceeded its effect on efflux, resulting in an accumulation rather than a depletion of intracellular cholesterol in caveolae and/or an increase in the number of caveolae [9]. This makes it possible to speculate that a signalling pathway might be involved in the regulation of intracellular cholesterol transport.

It was also found that apoA-I stimulates the expression of caveolin in human fibroblasts. The expression was increased by approx. 2-fold and was evident at the earliest after 2 h with 100 $\mu$g/ml apoA-I and in 24 h with 10 $\mu$g/ml apoA-I. The effect of apoA-I on the transport of newly synthesized cholesterol was usually larger and was evident in 10 min, making it unlikely that enhancement of caveolin expression was entirely responsible for the effect of apoA-I on cholesterol transport. Moreover, this enhancement could be a response to the accumulation of cholesterol in caveolae if the effect of apoA-I on cholesterol trafficking exceeds its effect on efflux. However, the possibility cannot be excluded that long-term regulation of cholesterol trafficking by apoA-I might depend on the regulation of caveolin expression. More experiments are needed to evaluate the contribution of various mechanisms to the overall process.

In conclusion, we have demonstrated that apoA-I stimulates the transport of intracellular cholesterol to cell-surface caveolae. We hypothesize that this could be a constitutive feature in cells, including the artery wall, and a link between plasma apoA-I concentration and intracellular events leading to the induction of reverse cholesterol transport. A low apoA-I concentration and/or a breakdown in the regulatory effect of apoA-I on intracellular cholesterol trafficking might lead to a decrease in the efficiency of cholesterol efflux, the accumulation of cholesterol in the artery wall and the initiation of development of atherosclerosis.

This work was partly supported by the NIH grant HL 57976, NHF grant G99M 0294 and by the NHLBI-Baker Exchange Program.

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Received 12 February 2001/15 May 2001; accepted 2 June 2001