Model class A and class L peptides increase the production of apoA-I-containing lipoproteins in HepG2 cells

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Abstract **Class A peptides inhibit atherosclerosis and protect cells from class L peptide-mediated lysis. Because the cytolytic process is concentration dependent, we hypothesized that at certain concentrations both classes of peptides exert similar effect(s) on cells. To test this hypothesis, we studied the effects of a class L peptide (18L = GIKKFLGSIWKFIKAFVG) and a class A peptide, 18A-Pro-18A (18A = DWLKAFYDKVAEKLKEAF) (37pA), on apolipoprotein and lipoprotein production in HepG2 cells. Secretion of ^{35}S-labeled apolipoprotein A-I (apoA-I) was stimulated by both 18L (110%) and 37pA (135%) at 10 and 20 nM of peptides, respectively. Both peptides enhanced the secretion of ^{3}H-labeled phospholipids by 140% and ^{14}C-labeled HDL-cholesterol (HDL-C) by 35% but had no significant effect on the total cholesterol mass or secretion. These results indicate that class L and class A peptides cause redistribution of cholesterol among lipoproteins in favor of HDL-C. Both peptides remodeled apoA-I-containing particles forming preβ- as well as α-HDL. This study suggests that increased secretion of phospholipids and apoA-I and the formation of preβ-HDL particles might contribute to the antiatherogenic properties of these peptides.**—Dashti, N., G. Datta, M. Manchekar, M. Chaddha, and G. M. Anantharamaiah. **Model class A and class L peptides increase the production of apoA-I-containing lipoproteins in HepG2 cells.** ***J. Lipid Res.*** **2004.** 45: **1919–1928.**

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Recent attention has focused on HDL as a target for the therapeutic intervention of atherosclerosis. Thus, the infusion of recombinant HDL consisting of apolipoprotein A-I Milano (apoA-I_{Milano}) and lipid complexes in humans has been shown to dramatically reduce atherosclerosis burden (1). Therefore, new and more effective approaches to increase HDL by increasing apoA-I production are of great importance.

The amphipathic helix is a structural motif present not only in apoA-I but also commonly found in biologically active peptides and proteins. We have grouped amphipathic helical motifs into several classes (2). Among them, class A (apolipoprotein-like) and class L (lytic peptide-like) peptides have been shown to exert opposite effects on membranes (3). Although class L peptides are cytolytic, class A peptides stabilize cell membranes and also inhibit class L-mediated cell lysis (4). Analysis of class L peptides and peptide hormones (class H) reveals a close similarity in structure (2). That is, both classes possess a wide hydrophobic face with a high hydrophobic moment value and both consist of a polar face of only cationic amino acids, with class L peptides containing exclusively Lys residues. Peptide hormones exert their physiological effects at very low (nanomolar to picomolar) concentrations (5).

Class A amphipathic helical peptides have been shown to interact with cell membranes to exert several beneficial effects, including the inhibition of neutrophil activation, human immunodeficiency virus-induced fusion, and antiatherogenic properties such as cellular cholesterol efflux and scavenging of "seeding molecules" from the surface of LDL (6–11). Administration of class A peptides into atherosclerosis-sensitive mice has shown that the peptides inhibit atherosclerosis but without changes in plasma cholesterol levels (12, 13). However, administration of a class A peptide into mice infected with influenza A virus increased the levels of apoA-I and HDL and decreased the

Abbreviations: apoA-I, apolipoprotein A-I; HDL-C, high density lipoprotein-cholesterol; LDH, lactic dehydrogenase; PL/C, phospholipid-to-cholesterol ratio; R_f, relative mobility.

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levels of LDL compared with those in control mice exposed to influenza virus (14). Furthermore, a Pro-linked dimer of the model class A amphipathic helical peptide 18A, 18A-Pro-18A, also referred to as 37pA (with a primary amino acid sequence of 18A = DWLKAFYDKVAEKLKEAF), has been shown to be more effective than human apoA-I in activating the plasma enzyme LCAT (15) and in interacting with trophoblasts to stimulate human placental lactogen (9). The peptide 37pA is also the most effective peptide to compete out HDL bound to scavenger receptor class B type I receptor in murine adrenal cells (16).

To explain the release of human placental lactogen by 37pA, Jorgenson et al. (17) reasoned that the association of the class A peptide analogs with the phospholipids of the cell membrane may lead to focal changes in the properties of the membrane such as membrane fluidity and/or permeability, causing, for example, an influx of calcium ions, which in turn could stimulate second messenger production. Importantly, peptide 37pA has been shown to be involved in ABCA1-mediated cholesterol efflux and in the assembly of discoidal HDL (18). In these studies, and in the studies involving human placental lactogen stimulation, it is not clear whether the peptide by itself has any effect on the levels of apoA-I, which may explain the phenomenon described above. It has been shown, by the synthesis of a Pro-punctuated dimer of 18L (18L = GIKKFLGSIWKFIKAFVG), that this peptide does not stimulate ABCA1-mediated cholesterol efflux despite the presence of a wide hydrophobic face and high lipid-associating ability (18).

Lytic peptides, because of their large hydrophobic face, interact with membranes and self-associate to form pores (3). This involves a concentration-dependent reorientation of the α helical peptide from perpendicular to the lipid acyl chain to parallel to the lipid acyl chain to form self-associated barrel-like assemblies, which are responsible for the cytotoxic effects of this class of peptides (3). On the other hand, class A peptides have been shown to be nontoxic even at high micromolar concentrations (13). Therefore, we hypothesized that at certain concentration ranges both classes of peptides exert similar effect(s). We undertook to study the effects of the two well-characterized, highly membrane-active peptide analogs from these two classes (18A-Pro-18A, a member of class A, and 18L, a member of class L) on cell viability and the hepatic synthesis and secretion of apoA-I- and apoB-containing lipoproteins using the human hepatoblastoma HepG2 cell line. Helical wheel diagrams of 18A and 18L are shown in **Fig. 1A** and **1B**, respectively.

The human hepatoblastoma cell line HepG2 was selected as the experimental model representing human liver because it is the most suitable and accessible human-derived cell line. These cells retain many of the biochemical functions of human liver parenchymal cells, including the nutritional and hormonal regulation of the synthesis and secretion of plasma lipoproteins (19, 20). We report here that in HepG2 cells both class L and class A peptides increased the secretion of apoA-I-containing lipoproteins and modified the subpopulation of secreted HDL.

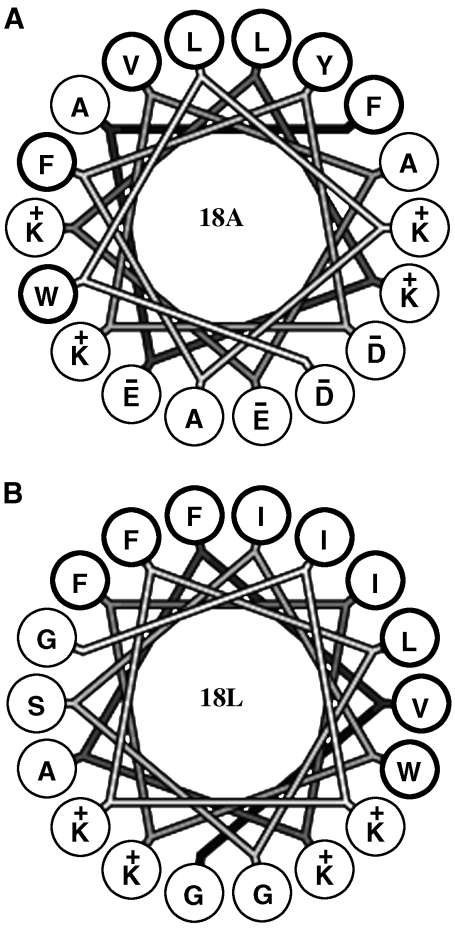

Fig. 1. Helical wheel representation of 18A (A), the parent form of 37pA, and 18L (B). The hydrophobic amino acids are shown in boldface, and the charged residues have the charge indicated on them. 18A has a net zero charge, whereas 18L is cationic.

EXPERIMENTAL PROCEDURES

Materials

MEM, trypsin, sodium pyruvate, L-glutamine, minimum essential vitamin solution, and FBS were purchased from Grand Island Biological Co. (Grand Island, NY). Sodium deoxycholate, TCA, Triton X-100, benzamidine, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin A, and reduced diphosphopyridine nucleotide were from Sigma Chemical Co. (St. Louis, MO). Protein G-Sepharose CL-4B, L-[^{35}S]methionine, [^{3}H]glycerol, [^{14}C]acetate, ^{125}I-labeled iodide, and Amplify were from Amersham Pharmacia Biotech (Piscataway, NJ). All reagents used for gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA). Iodo-Beads were obtained from Pierce Biotechnology (Rockford, IL). Monospecific antibodies to apoA-I, apoB, and apoE were generated in our laboratory.

Peptide synthesis and iodination

Peptides 18L and 37pA were synthesized by solid-phase peptide synthesis (21). The purity of the synthetic peptides was established by analytical HPLC and ion spray mass spectrometry. The peptides were dialyzed against distilled water and lyophilized before use. Peptide was iodinated with Na^{125}I using Iodo-Beads (22), as previously described (23).

Cell culture

The human hepatoblastoma HepG2 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were seeded onto tissue culture dishes in MEM containing 10% (v/v) FBS and were incubated at 37°C in a 95% air/5% CO_2 atmosphere as previously described (19). Medium was changed 48 h later and daily thereafter. At the start of the experiments, the maintenance medium was removed and monolayers were washed twice with PBS. After the addition of serum-free MEM, cells were incubated for the indicated time period in the presence or absence of peptides. In studies designed to determine the effects of peptides on the net accumulation of apolipoproteins and HDL-cholesterol in the medium, a longer incubation time (i.e., 18–24 h) was necessary to obtain a sufficient quantity of samples for accurate measurements of the mass of HDL-cholesterol and apoA-I, apoB, and apoE because of the sensitivity limits of the assays used. In contrast, a short-term incubation time (i.e., 2–6 h) is routinely used to assess potential changes in the early stages of the synthesis and secretion of lipids and apolipoproteins. Therefore, the incorporation of [^{3}H]glycerol into lipids and [^{35}S]methionine into proteins was determined after 3–5 h of incubation. After incubation, conditioned medium was collected and preservative cocktail consisting of 500 U/ml penicillin G, 50 μg/ml streptomycin sulfate, 20 μg/ml chloramphenicol, 1.3 mg/ml ε-amino caproic acid, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml EDTA was added to prevent oxidative and proteolytic damage (19, 20). The medium was centrifuged at 2,000 rpm for 30 min at 4°C to remove small amounts of broken cells and debris. The monolayers were washed three times with PBS, scraped off the plate in PBS, and sonicated. Cell protein was measured by the method of Lowry et al. (24). Cell viability was assessed by cellular Trypan blue uptake and the determination of lactic dehydrogenase (LDH) activity in the conditioned medium after an overnight incubation as previously described (25).

Determination of the net accumulation of apolipoproteins in the medium

Cells were grown as described above and incubated in serum-free MEM in the presence and absence of peptides for 22 h. Conditioned medium was processed as above and was concentrated ~10- to 15-fold as previously described (19, 20, 26). The masses of apoA-I, apoB, and apoE were determined by electroimmunoassays using monospecific polyclonal antibodies as described in detail elsewhere (27–29).

Determination of the net accumulation of total cholesterol, HDL-cholesterol, and LDL-cholesterol in the medium

HepG2 cells were grown for 4–5 days in phenol red-free MEM (to prevent interference with the enzymatic colorimetric method used to measure the concentration of cholesterol) containing 10% FBS. Cells were incubated for 22 h in phenol red- and serum-free MEM in the presence and absence of peptides. The HDL accumulated in the conditioned medium was isolated by heparin-manganese precipitation as described (30). Cholesterol concentrations in total medium and heparin-manganese supernatant (non-apoB-containing lipoproteins) were determined by enzymatic determination using the Data Medical Association kit (Arlington, TX). The cholesterol content of apoB-containing lipoproteins (heparin-manganese precipitate) was calculated by subtracting the cholesterol concentration in heparin-manganese supernatant from total cholesterol.

De novo synthesis and secretion of apoA-I and apoB

HepG2 cells were grown for 4 days as described above. The maintenance medium was removed, cells were washed twice with PBS, and serum-free MEM was added. The incorporation of L-[^{35}S]methionine into newly synthesized apolipoproteins in the presence and absence of peptides was determined after 3.5 h of incubation. The [^{35}S]apoA-I and [^{35}S]apoB were isolated by immunoprecipitation as described below and in the figure legends. The incorporation of L-[^{35}S]methionine into total protein was determined by precipitation with TCA at a final concentration of 10%. The mixture was kept on ice for 1 h, and the precipitated proteins were collected on filters, washed extensively with 5% TCA, dried, and counted in a scintillation counter.

Immunoprecipitation

After metabolic labeling with [^{35}S]methionine, the conditioned medium was collected and cells were washed with cold PBS and analyzed for protein. Preservative cocktail described above plus leupeptin (50 μg/ml), pepstatin A (50 μg/ml), and aprotinin (100 kallikrein-inactivating units/ml) were added to the medium to prevent oxidative and proteolytic damage (26, 31). The [^{35}S]apoA-I and [^{35}S]apoB secreted into the medium were immunoprecipitated using monospecific polyclonal antibodies to human apoA-I or apoB-100, respectively, coupled to protein G-Sepharose CL-4B as previously described (20, 31). The [^{35}S]apoA-I or [^{35}S]apoB was extracted from protein G by boiling for 4 min in sample buffer [0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.02% (w/v) bromophenol blue] and run on 4–12% SDS-PAGE (32). After electrophoresis, the gels were dried and analyzed by autoradiography or PhosphorImager.

Determination of [^{3}H]glycerol incorporation into various lipid fractions

HepG2 cells were plated and grown in MEM containing 10% FBS as described above. Serum-free MEM was added and the incorporation of [^{3}H]glycerol (5 μCi/ml of medium) into cellular and secreted lipids in the presence and absence of peptides was determined after 5 h of incubation. ^{3}H-labeled conditioned medium was removed and processed as above. Cell monolayers were washed three times with PBS, scraped off the plates, and sonicated. Total lipids were extracted from conditioned medium and cell suspension by the method of Folch et al. (33). The final extracts were applied to TLC plates, and various lipids (i.e., phospholipids, monoglycerides plus diglycerides, and triglycerides) were separated using a hexane-diethyl ether-acetic acid (80:20:1) solvent system, visualized with iodine and counted as previously described in detail (34).

Determination of [^{14}C]acetate incorporation into total cholesterol and HDL-cholesterol

Cells were incubated with serum-free MEM and [^{14}C]acetate (2 μCi/ml of medium) in the presence and absence of peptides. Lipoproteins with d < 1.063 g/ml (VLDL plus LDL) and d = 1.063–1.21 g/ml (HDL) were isolated from conditioned medium by sequential ultracentrifugation and dialyzed against PBS. The incorporation of ^{14}C-labeled acetate into digitonin-precipitable sterols in cell suspension, secreted into the medium, and lipoprotein fractions was determined as previously described (34).

Determination of the apoA-I-containing lipoprotein subpopulation

Preβ and α-HDL subpopulations in concentrated conditioned medium of cells incubated in the presence and absence of 18L and 37pA were determined by crossed immunoelectrophoretic technique, a two-dimensional method combining electrophore-

sis and electroimmunoassay as previously described (35). Samples were electrophoresed in the first dimension in 2% agarose and in the second dimension into gels containing polyclonal antibody to human apoA-I.

Determination of ^{125}I-labeled 37pA association with the apoA-I-containing subpopulation

To assess the potential association of peptides with apoA-I-containing lipoproteins, cells were incubated with serum-free MEM and ^{125}I-labeled 37pA; 18L was not tested because it does not have a tyrosine and hence cannot be iodinated. The ^{125}I-labeled conditioned medium was immunoprecipitated with polyclonal antibody to human apoA-I coupled to protein G-Sepharose CL-4B under nondenaturing conditions as previously described (31). As controls, conditioned medium was also immunoprecipitated with antibody to human apoB and nonimmune rabbit IgG. The immunoprecipitated complexes were washed with PBS four to six times until background counts were detected in the wash. The complexes were counted in a Beckman γ counter. In a separate experiment, the ^{125}I-labeled conditioned medium was concentrated and subjected to two-dimensional electroimmunoassay as described above. The gel was stained, dried, and subjected to autoradiography.

Statistical analysis

Statistical analysis was performed by a Student's *t*-test.

RESULTS

Effects of class L and class A on cell viability

To ensure that the amounts of peptides used in this study were not cytotoxic, HepG2 cells were incubated for 24 h in serum-free medium and in the presence of increasing concentrations of either 18L or 37pA. This pilot study showed that incubation of cells with either 18L ranging from 5 to 25 nM or 37pA ranging from 10 to 100 nM final concentration had no cytotoxic effect on the cells as determined by light microscopy, Trypan blue exclusion, red blood cell lysis (data not shown), and cell protein, which was 5.08 ± 0.15, 5.28 ± 0.18, and 5.20 ± 0.1 mg/dish in control, 10 nM 18L-treated, and 20 nM 37pA-treated cells, respectively (mean ± SEM of 17 samples from 7 experiments). The activity of LDH in the conditioned medium, a measure of the extent of cell damage by peptides, was 10.40 ± 1.3, 9.95 ± 1.35, and 9.55 ± 0.15 U/ml of medium in control, 18L-treated (10 nM), and 37pA-treated (20 nM) cells, respectively, indicating no cytotoxic effect of peptides at these concentrations. The activity of LDH in sonicated cell suspension was 10,980 ± 578 U/ml. Addition of 18L at 25 nM caused minor cell damage that was reflected in slightly higher cellular Trypan blue uptake and LDH activity in the conditioned medium.

Class L and class A peptides stimulate the secretion of newly synthesized apoA-I without significantly affecting apoB secretion

To establish the effects of the peptides on the secretion of newly synthesized apoA-I and apoB, the incorporation of L-[^{35}S]methionine into secreted apolipoproteins after 3.5 h of incubation was determined. As shown in **Fig. 2A**,

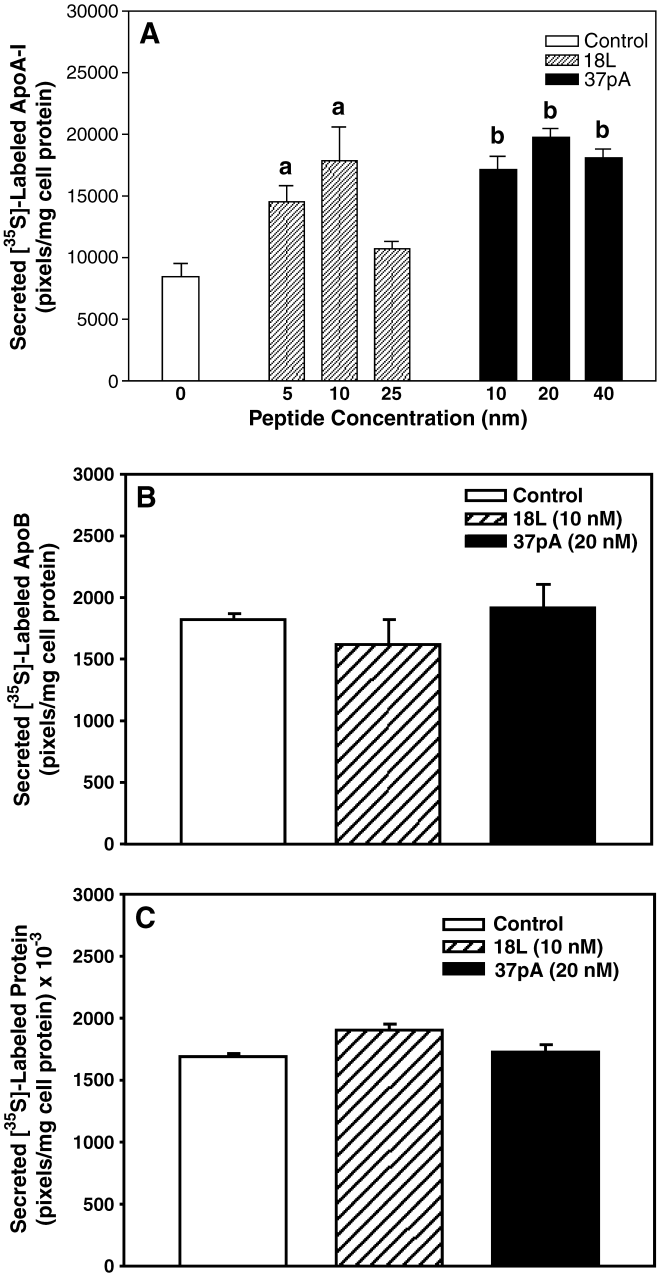

Fig. 2. Addition of 18L and 37pA peptides enhances the secretion of newly synthesized apolipoprotein A-I (apoA-I) but has no significant effect on the secretion of newly synthesized apoB and total protein. HepG2 cells were grown in MEM containing 10% FBS for 4 days. The maintenance medium was removed, and monolayers were washed twice with PBS and incubated for 3.5 h in serum- and methionine-free MEM containing [^{35}S]methionine (50 μCi/ml of medium) in the presence or absence of the indicated concentrations of 18L or 37pA. The [^{35}S]apoA-I (A) and [^{35}S]apoB (B) secreted into the medium were immunoprecipitated with monospecific polyclonal antibody to human apoA-I and apoB-100, applied to 4–12% SDS-PAGE, and autoradiographed as described in Experimental Procedures. The intensity of the bands was determined by densitometry of the autoradiograms and is expressed as pixels per milliliter of medium. The secretion of ^{35}S-labeled total proteins (C) was determined by TCA precipitation of conditioned medium. Values shown are means ± SEM of triplicate samples. The difference between control and peptide-treated cells was significant at $^{a}P = 0.03$ and $^{b}P = 0.004$.

secretion of [^{35}S]apoA-I was markedly stimulated by 18L at 5 nM (+72%; $P = 0.026$) and 10 nM (+111%; $P = 0.034$) and to a lesser extent at 25 nM (+27%; $P = 0.15$). Similarly, there was a significant stimulation in the secretion of [^{35}S]apoA-I with 37pA added at 10 nM (+104%; $P = 0.004$), 20 nM (+135%; $P < 0.001$), and 40 nM (+114%; $P = 0.002$) (Fig. 2A). These results showed that the maximum effect of 18L and 37pA on apoA-I secretion was achieved at 10 and 20 nM, respectively, and that the stimulatory effect of 18L was diminished at concentrations $\geq$ 25 nM (Fig. 2A). Based on these results, we used 18L and 37pA at 10 and 20 nM, respectively, in all subsequent studies.

In contrast to apoA-I secretion, addition of 18L and 37pA had no significant effect on the secretion of [^{35}S]apoB (Fig. 2B), resulting in significantly higher apoA-I/apoB ratios with 18L (+115%; $P = 0.04$) and 37pA (+126%; $P = 0.005$) compared with the control. The secretion of total ^{35}S-labeled proteins, measured as TCA-precipitable radioactivity in the medium, was not altered by the peptides (Fig. 2C). Thus, peptides 18L and 37pA exerted their effect on the secretion of newly synthesized apoA-I and not of apoB or total protein.

Class L and class A peptides enhance the net accumulation of apoA-I in the medium but have no major effect on apoB and apoE levels

To test the effects of peptide on the mass of apolipoproteins, the net accumulation of apoA-I, apoB, and apoE in the conditioned medium after 22 h of incubation was determined. As shown in **Table 1**, the stimulatory effect of peptides on the secretion of newly synthesized apoA-I was also observed, albeit to a lesser extent, on the net accumulation of apoA-I in the conditioned medium, which was significantly increased with both 18L (+26%; $P = 0.004$) and 37pA (+30%; $P = 0.006$). Consistent with the unchanged secretion of newly synthesized apoB (Fig. 2B), the net accumulation of apoB in the medium was not altered by either peptide. Similarly, 18L and 37pA had no effect on the net accumulation of apoE in the medium (Table 1).

Class L and class A peptides do not significantly alter the net accumulation of total cholesterol mass in the medium but cause redistribution of cholesterol among lipoproteins in favor of HDL-cholesterol

The observed increase in the net accumulation of apoA-I in the conditioned medium suggested that the HDL-cholesterol (HDL-C) levels may also increase by these peptides. As shown in Table 1, the net accumulation of total cholesterol in the conditioned medium was not altered by 37pA, and a moderate 18% decrease by 18L was not statistically significant. Determination of cholesterol concentration in the heparin-manganese supernatant fraction (non-apoB lipoproteins) of conditioned medium demonstrated that this fraction contained 39% of total cholesterol in control cells and was increased to 50% with 18L and 70% ($P = 0.003$) with 37pA (Table 1). These results indicate that both peptides caused redistribution of cholesterol between secreted lipoproteins in favor of increased cholesterol content of apoA-I-containing lipoproteins.

Class L and class A peptides increase the secretion of de novo-synthesized ^{3}H-labeled phospholipids

To evaluate the potential correlation between changes in the secretion of apolipoproteins and lipids, the effects of 18L and 37pA on the incorporation of ^{3}H-labeled glycerol into the secreted and cellular phospholipids and triglycerides were determined. The significant 110–135% increase in the secretion of apoA-I by peptides was paralleled by an equally significant enhancement in the secretion of newly synthesized ^{3}H-labeled phospholipids with both 18L (+139%; $P = 0.017$) and 37pA (+144%; $P < 0.001$) (**Table 2**). Cellular ^{3}H-labeled phospholipids were not affected by 37pA and were decreased by 10% ($P = 0.013$) with 18L. There was a modest, although not significant, decrease in the secretion of newly synthesized triglycerides with both 18L (−18%; $P = 0.065$) and 37pA (−22%; $P = 0.056$) (Table 2). Cellular ^{3}H-labeled triglycerides were not affected by 18L or 37pA (Table 2).

As shown in Table 2, in the absence of peptides, 0.6% of total ^{3}H-labeled phospholipids (cellular plus medium)

TABLE 1. Effects of 18L and 37pA peptides on the net accumulation of apolipoproteins, total cholesterol, and HDL-C in conditioned medium of HepG2 cells

Peptide	apoA-I	apoB	apoE	TC	HDL-C
		μg/g cell protein/h			*% of TC*
None	84.22 ± 4.16	68.67 ± 6.24	35.95 ± 2.20	830.13 ± 64.89	38.94 ± 4.50
18L (10 nM)	106.00 ± 4.65[a]	75.59 ± 3.64	37.50 ± 1.05	681.87 ± 66.77	50.25 ± 6.7
37pA (20 nM)	108.78 ± 6.17[b]	67.35 ± 3.24	35.07 ± 4.74	877.04 ± 51.08	69.24 ± 1.32[c]

apoA-I, apolipoprotein A-I; HDL-C, high density lipoprotein-cholesterol; TC, total cholesterol. HepG2 cells were grown in MEM containing 10% FBS for 4 days. The maintenance medium was removed and cells were washed twice with PBS. Serum-free MEM and the indicated concentration of peptides were added, and cells were incubated for 22 h. Conditioned medium was concentrated and analyzed for the mass of apoA-I, apoB, and apoE and total cholesterol and heparin-manganese supernatant (HDL plus very high density lipoprotein) as described in Experimental Procedures. Values shown are means ± SEM of eight dishes from four separate experiments for apoA-I and apoB and triplicate dishes for apoE and cholesterol. The difference between control and peptide-treated values was significant as described in the footnotes.

[a] $P = 0.004$.
[b] $P = 0.006$.
[c] $P = 0.003$.

TABLE 2. Effects of 18L and 37pA peptides on the incorporation of [^{3}H]glycerol into the cellular and secreted phospholipids and triglycerides in HepG2 cells

Peptide	^{3}H-Labeled Phospholipids			^{3}H-Labeled Triglycerides		
	Cellular	Secreted	Secreted	Cellular	Secreted	Secreted
	dpm/mg cell protein		*% of total*	*dpm/mg cell protein*		*% of total*
None	1,235,765 ± 20,530	7,360 ± 430	0.58 ± 0.04	436,885 ± 35,995	27,485 ± 1,210	6.01 ± 0.62
18L (10 nM)	1,111,750 ± 20,470[a]	17,580 ± 1,625[b]	1.56 ± 0.17[b]	464,490 ± 7,420	22,615 ± 1,500	4.64 ± 0.22
37pA (20 nM)	1,252,820 ± 14,830	17,955 ± 315[c]	1.41 ± 0.03[c]	473,745 ± 8,790	21,455 ± 1,915	4.32 ± 0.31

Cells were incubated in serum-free MEM and the incorporation of [^{3}H]glycerol (5 μCi/ml of medium) into the cellular and secreted phospholipids and triglycerides after 5 h of incubation was determined as described in Experimental Procedures. Values shown are means ± SEM of triplicate dishes.

[a] $P = 0.013$.
[b] $P = 0.017$.
[c] $P < 0.001$.

was secreted into the medium, and this was increased to 1.56% ($P = 0.021$) with 18L and 1.4% ($P < 0.001$) with 37pA. In contrast, the percentage of total triglycerides that was secreted into the medium was decreased, albeit not significantly, from 6% in control cells to 4.6% and 4.3% with 18L and 37pA, respectively.

Class L and class A peptides do not significantly alter the secretion of de novo-synthesized cholesterol but cause the redistribution of cholesterol among lipoproteins in favor of HDL-C

Results of peptide-mediated changes on HDL-C (Table 1) were based on isolation of HDL by the heparin-manganese method. Because the heparin-manganese supernatant contains all non-apoB-containing particles, the measured HDL-C might also contain peptide-cholesterol complexes. Therefore, we assessed the effect of peptides on the de novo synthesis and secretion of total cholesterol and measured HDL-C in lipoproteins isolated by ultracentrifugation. Although the secretion of ^{14}C-labeled total cholesterol was not significantly affected by 18L and 37pA, the HDL-C was significantly increased with both peptides (**Table 3**). These results indicate that both peptides caused the redistribution of newly synthesized cholesterol between secreted lipoproteins in favor of increased cholesterol content of apoA-I-containing lipoproteins.

The above results suggest that 18L at low concentration and 37pA at a relatively higher level markedly enhanced the secretion of phospholipid-rich apoA-I-containing particles. As shown in **Table 4**, the apoA-I/apoB ratio was increased significantly with 18L (115%; $P = 0.04$) and 37pA (126%; $P = 0.005$), the phospholipid-to-cholesterol ratio (PL/C) was also increased with both 18L (109%; $P = 0.04$) and 37pA (106%; $P = 0.012$), and the HDL-C/non-HDL-C ratio was increased, albeit not significantly, with both 18L (14%) and 37pA (28%) (Table 4). These results suggest that in HepG2 cells, both 18L and 37pA improve the antiatherogenic indices.

Class L and class A peptides modify apoA-I-containing lipoproteins

To determine if the peptide-mediated increase in apoA-I-containing particles resulted in a change in particle composition, two-dimensional immunoelectrophoresis was carried out. Concentrated conditioned media from control and peptide-treated cells were analyzed. Human plasma was included in the analysis for comparison. The apoA-I-containing lipoproteins in plasma showed two peaks with preβ and α mobilities (**Fig. 3A**). The apoA-I-containing particles in the control conditioned medium showed one broad peak [relative mobility (Rf) ∼ 0.33], indicating a heterogeneous population of apoA-I-containing particles (Fig. 3B). In the presence of 18L (Fig. 3C) and 37pA (Fig. 3D), two apoA-I-containing peaks corresponding to preβ and α mobilities, similar but not identical to those in plasma, were observed. The preβ particles in plasma and in the presence of peptides had a similar mobility (R_f ∼ 0.27). On the other hand, the peptide-induced α particles

TABLE 3. Effects of 18L and 37pA peptides on the incorporation of [^{14}C]acetate into cellular cholesterol, secreted cholesterol, and HDL-C in HepG2 cells

Peptide	Cellular Cholesterol	Secreted Cholesterol	Secreted Cholesterol	HDL-C
	dpm/mg cell protein		*% of total*	*dpm/mg cell protein*
None	118,932 ± 2,153	3,607 ± 206	2.94 ± 0.11	735 ± 24
18L (10 nM)	120,914 ± 2,085	3,911 ± 126	3.13 ± 0.05	967 ± 31[a]
37pA (20 nM)	115,563 ± 2,491	4,066 ± 332	3.67 ± 0.01[b]	1005 ± 31[c]

Cells were incubated in serum-free MEM and the incorporation of [^{14}C]acetate (2 μCi/ml of medium) into the cellular cholesterol, secreted cholesterol, and HDL-C (d = 1.063–1.21 g/ml) was determined as described in Experimental Procedures. Values shown are means ± SEM of triplicate dishes.

[a] $P = 0.013$.
[b] $P = 0.015$.
[c] $P = 0.02$.

TABLE 4. Effect of 18L and 37pA peptides on the atherogenic variables in HepG2 cells

Addition	[^{35}S]apoA-I/[^{35}S]apoB Ratio	Phospholipid/Cholesterol Ratio	HDL-C/Non-HDL-C Ratio
None	0.92 ± 0.07	2.17 ± 0.18	0.29 ± 0.01
18L (10 nM)	1.98 ± 0.34[a]	4.53 ± 0.55[a]	0.33 ± 0.02
37pA (20 nM)	2.08 ± 0.19[b]	4.47 ± 0.31[c]	0.37 ± 0.07

Values shown are means ± SEM of triplicate dishes and were obtained from results described in Fig. 2 and Tables 2 and 3. The difference between control and peptide-treated cells was significant as described in the footnotes.
[a] $P = 0.04$.
[b] $P = 0.005$.
[c] $P = 0.012$.

had a R_f value (~0.66) greater than that seen in plasma (~0.50).

To test a potential association of peptides with apoA-I-containing particles, in a separate experiment cells were incubated for 18 h with ^{125}I-labeled 37pA and the concentrated conditioned medium was subjected to two-dimensional immunoelectrophoresis. The gel was stained, dried, and subjected to autoradiography. Results showed radioactivity predominantly in the α-migrating peak (**Fig. 4A**). These results suggest that 37pA associates with α-HDL and to a lesser extent with preβ-HDL. To further confirm this observation, aliquots of ^{125}I-labeled conditioned medium were immunoprecipitated with antibodies to human apoA-I and apoB under nondenaturing conditions as described previously (31). An aliquot of ^{125}I-labeled conditioned medium was subjected to the same procedure using rabbit nonimmune IgG as a control for background noise. As shown in Fig. 4B, ^{125}I-labeled 37pA coimmunoprecipitated with apoA-I, suggesting the presence of complexes containing both the peptide and apoA-I. The peptide association with apoA-I-containing particles was 5-fold higher than that with apoB-containing particles (Fig. 4B). To rule out the possibility that radioactivity in the anti-apoA-I immunoprecipitate might be attributable to the immunoprecipitation of free ^{125}I-labeled 37pA, an aliquot of nonconditioned medium containing ^{125}I-labeled 37pA (before its addition to the cells) was also immunoprecipitated using anti-apoA-I. As shown in Fig. 4B, only a small amount of radioactivity was detected in the immunoprecipitate, indicating only a minor contribution of free ^{125}I-labeled 37pA under these conditions. We did not include 18L in this series of experiments because it does not possess a tyrosine residue for iodination. However, because the two-dimensional gel showed a similar profile to that observed with 37pA (Fig. 3), we assume that 18L might also associate with apoA-I-containing particles in a similar manner.

DISCUSSION

With the report on the apoA-I_{Milano}-lipid complex infusion studies (1, 36, 37), interest in HDL therapy has increased. Although the study in humans by Nissen et al. (1) yielded significant results, the large amount of protein-lipid complex needed to be infused limits its use as a therapeutic approach. In this context, studies using small class A amphipathic helical peptides have exponentially increased as a result of their remarkable effect on atherosclerosis in dyslipidemic mouse models of atherosclerosis (12–14, 38). The mechanism by which class A peptides exert their effects is not clear. The consequence of the incorporation of amphipathic helices into the membranes on membrane properties is dependent, at least in part, on the size of the hydrophobic face and the charge distribution on the hydrophilic face. For example, class A peptides (a motif present in exchangeable apolipoproteins) with positively charged residues at the polar-nonpolar interface and negatively charged residues at the center of

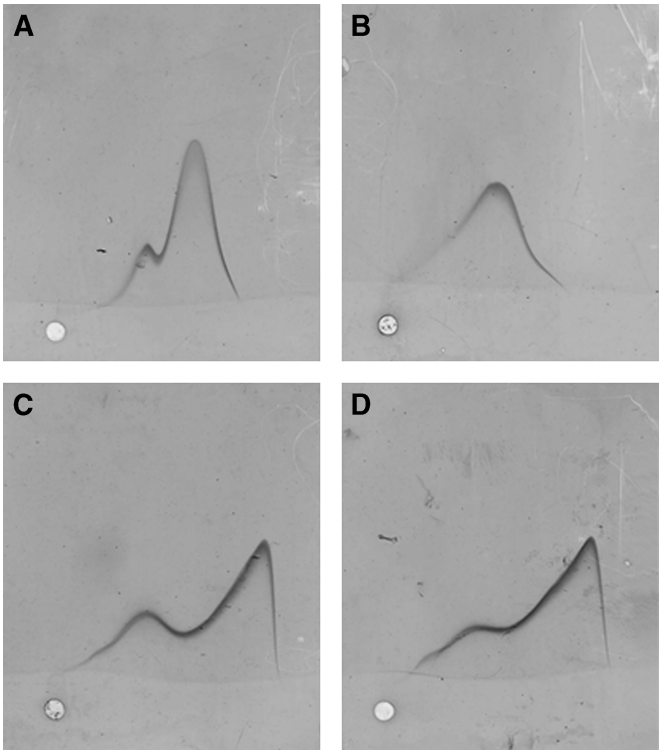

Fig. 3. Class L and class A peptides modify apoA-I-containing lipoproteins. HepG2 cells were incubated overnight with serum-free MEM in the presence or absence of 18L and 37pA. The conditioned medium was concentrated and subjected to two-dimensional immunoelectrophoresis. Samples were electrophoresed in the first dimension in 2% agarose and in the second dimension on the gel containing polyclonal antibody to human apoA-I. A shows human plasma for comparison with the conditioned medium from control cells (B), 18L-treated cells (C), and 37pA-treatead cells (D).

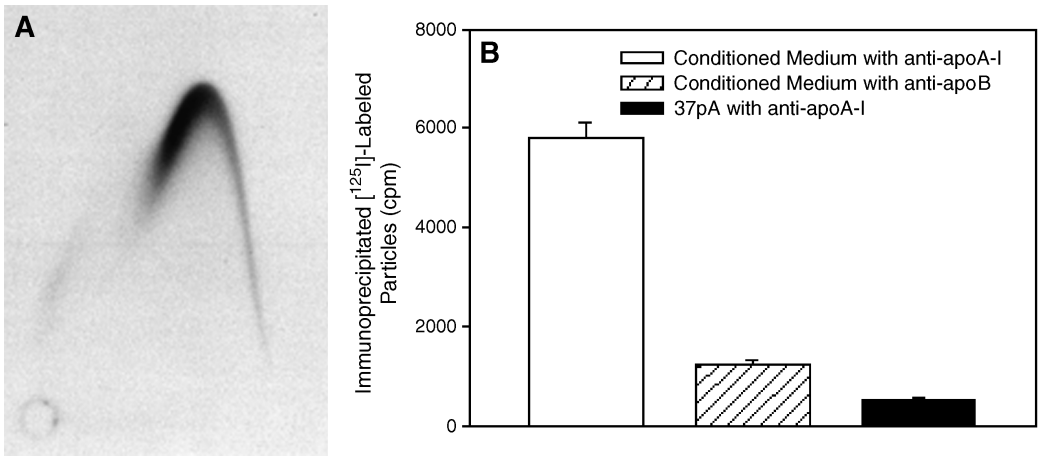

Fig. 4. Peptide 37pA associates with apoA-I-containing lipoprotein particles. HepG2 cells were incubated overnight with serum-free MEM containing ^{125}I-labeled 37pA. Conditioned medium was concentrated and applied to two-dimensional immunoelectrophoresis as described above. The gel was stained, dried, and autoradiographed. A: Autoradiogram of the gel. B: One milliliter aliquots of ^{125}I-labeled conditioned medium were immunoprecipitated with antibodies to apoA-I (open bar) or apoB-100 (hatched bar); the radioactivity in the 1 ml sample immunoprecipitated with nonimmune rabbit IgG (111 cpm) was subtracted from that in all other samples. To assess the potential contribution of free ^{125}I-labeled 37pA to the radioactivity obtained with ^{125}I-labeled conditioned medium immunoprecipitated with anti-apoA-I, 1 ml of nonconditioned medium containing ^{125}I-labeled 37pA was immunoprecipitated with anti-apoA-I (solid bar) and the result is shown. The values are means ± SE of triplicate dishes.

the polar face (Fig. 1A) have been shown to stabilize membranes. It has been shown that stabilization of cell membrane was directly correlated to the ability of the peptide to remove lipid hydroperoxides from plasma (39). The shape of the class A peptides also appears to influence their biological activity. Cylindrical peptides exhibit antiatherogenic properties, whereas wedge-shaped ones do not. Class L peptides with bulky hydrophobic residues and positively charged Lys residues at the center of the polar face (Fig. 1B), on the other hand, destabilize membranes, causing hexagonal phase formation, an intermediate structure in the fusion process (3, 4).

In this study, both 18L and 37pA markedly increased the secretion of newly synthesized apoA-I and phospholipids without significantly altering the secretion of apoB and total cholesterol. This resulted in higher PL/C and apoA-I/apoB ratios in the presence of both peptides compared with control conditioned medium. Several studies have demonstrated a positive correlation between the PL/C of HDL and the capacity of serum to accept cellular cholesterol (40–42). Furthermore, it has been suggested that a low PL/C in HDL is associated with an increased risk for ischemic vascular disease (43) and coronary artery disease (44). Our data, therefore, suggest that the HDL particles secreted in the presence of 18L and 37pA might be more efficient in promoting cholesterol efflux from the cells.

In support of the above observations, 37pA peptide has been shown to be highly efficient in effluxing cellular cholesterol (45). In addition, this peptide has been shown to be involved in ABCA1-mediated cholesterol efflux as well as nonreceptor mediated (i.e., via a mechanism involving membrane microsolubilization) cholesterol efflux (46). This may explain the significantly higher HDL-C in the conditioned medium of 37pA-treated cells. Our observations also support the notion that although 37pA stimulates nonreceptor-mediated cholesterol efflux, increased secretion of apoA-I is responsible for ABCA1-mediated phospholipid efflux. The increased level of HDL-C in the conditioned medium of 18L-treated cells could be attributable to the ABCA1-mediated cholesterol efflux caused by increased secretion of apoA-I.

We have recently shown that the class A amphipathic helical peptide 4F inhibits atherosclerosis in several dyslipidemic mouse models (13, 14). The mechanism appears to be related to the formation of preβ-HDL, which markedly improves HDL-mediated cholesterol efflux and reverse cholesterol transport and possesses anti-inflammatory properties. In these studies, it was shown that the peptide is able to remodel lipoproteins and form apoA-I peptide-containing particles (47). In the present studies, we have shown that when the peptide 37pA is incubated with HepG2 cells, peptide-containing apoA-I particles that possess preβ mobility are formed. Even though the concentration of these particles is not high, they can be effective cholesterol effluxers. The peptide can extract phospholipid from cell membrane, forming peptide-lipid particles. These particles can incorporate cell surface apoA-I to form active HDL particles. Because cell surface apoA-I is depleted, it is possible that new apoA-I is synthesized to compensate for the cellular levels of apoA-I, thus explaining the increase in apoA-I synthesis.

In summary, we have demonstrated that in HepG2 cells, class A and class L peptides increase the secretion of apoA-I, phospholipids, and HDL-C. The higher apoA-I/apoB and PL/C ratios, considered good indicators of negative risk for coronary artery disease (40–44), and the for-

mation of preβ-HDL observed in this study suggest that 18L and 37pA might have antiatherogenic properties. We propose that the previously observed antiatherogenic properties of class A peptides could be attributable, in part, to their coordinated stimulatory effect on apoA-I and phospholipid secretion. Further studies are required to understand the mechanism by which the peptides modulate different lipid and protein levels in plasma membrane.

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