Synthetic Amphipathic Peptides Resembling Apolipoproteins Stimulate the Release of Human Placental Lactogen*

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Human placental lactogen (hPL) is a 22.5-kDa protein hormone synthesized and secreted by the placental syncytiotrophoblast which has striking homologies in chemical, biological, and immunological properties to growth hormone and prolactin (1, 2). The hormone is first detected in maternal blood at about 6 weeks of gestation and increases in concentration at a relatively linear rate until 34 weeks, reaching a maximal concentration of 6000–8000 ng/ml (3). The factors involved in the regulation of the synthesis and release of hPL, however, are poorly understood. We have recently demonstrated that high density lipoprotein (HDL) at physiologic concentrations stimulates the release of the protein hormone hPL from an enriched fraction of cultured trophoblast cells and that the activity is due to apolipoproteins A1, AII, and CI and not the lipid constituents on HDL (4). Since trophoblast membranes have receptors for HDL (5) and plasma levels of HDL and hPL increase concomitantly during pregnancy (6), these findings suggest a physiologic role for HDL in hPL secretion.

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‡The abbreviations used are: hPL, human placental lactogen; HDL, high density lipoprotein; apoA1, apolipoprotein A1; DMPC, dimyristoyl phosphatidylcholine.

Apolipoproteins are noncovalently bound to the outer layer of HDL and are integral to the organization and metabolism of the lipoproteins (7). The amphipathic helix is generally accepted to be the structural form of the lipid-associating domains of the exchangeable apolipoprotein classes A, C, and E (8). The amphipathic helix model defines a general α-helical domain containing opposing polar and nonpolar faces and a specific distribution of charged residues. Positively charged residues are distributed at the interface between the polar and nonpolar faces, and negatively charged residues occur along the center of the polar face. The presence of the amphipathic lysine and arginine residues at the polar/nonpolar face interface increases the amphipathicity of the amphipathic helical domains of the apolipoproteins. Interfacial basic residues distinguish the amphipathic helices in apolipoproteins from amphipathic helices in other classes of proteins.

Apolipoprotein A1 (apoA1) contains eight tandem 22-mer repeats at its carboxyl-terminal end, each of which has the properties of an amphipathic helical domain (9). To examine whether this characteristic structure of the apolipoproteins is also important in the stimulation of hPL release, we have examined the effects on hPL release of several synthetic peptides that were designed to mimic the apolipoproteins in their structure and properties.

MATERIALS AND METHODS

Peptide Synthesis and Apolipoprotein Preparation—Peptides were synthesized by the solid-phase method of peptide synthesis as previously described (10, 11). HDL was prepared by sequential flotation ultracentrifugation of normolipemic plasma (density 1.125–1.215 g/ml) as described by Shumaker and Puppione (12), then dialyzed against three changes of 0.15 M NaCl, 0.1 mM EDTA, pH 7.4, and stored at 4 °C. Apolipoproteins A1 and AII were prepared by DEAE molecular sieve chromatography (13) of delipidated HDL and similarly dialyzed. Analysis by overload (100 μg) sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated proteins were >98% purified.

Trophoblast Culture and hPL Release Studies—Enriched fractions of hPL producing trophoblast cells were prepared from term placentas using collagenase and hyaluronidase digestion and isopycnic centrifugation on 40% Percoll gradients as previously described (14). Dispersed cells were cultured in 24-well plastic plates coated with Vitrogen-100 at 0.5 million cells in 1 ml of RPMI 1640 medium with L-glutamine and sodium bicarbonate (GIBCO) and 5% fetal calf serum. After 24 h, the cells were washed with RPMI 1640 without fetal calf serum and the peptides added to triplicate wells. Aliquots of media were removed at the appropriate times, and media was assayed for hPL by specific homologous radioimmunoassay (15). Results are expressed as nanograms of hPL released/milliliter.

Perfusion Studies—Dispersed trophoblast cells were placed into an Endotronics Micro Module perfusion system, 10 million cells/chamber, and perfused with RPMI 1640 at 6 ml/h as previously described (16). After a 2-h equilibration period, the chambers were
perifused with media only or media containing HDL, apoAII, or 18A-Pro-18A for 15 min, then returned to control media. One- or 5-min fractions were collected and assayed for hPL as above.

**Synthetic Peptides**—Multilamellar vesicles of DMPC were prepared by vortexing DMPC in phosphate buffer (10 mM in 150 mM NaCl, pH 7.0) directly or after forming complexes with DMPC (1:2.5 peptide to DMPC weight ratio). The data on complex size and percent helicity has been published previously (11, 17). Displacement of native apolipoproteins from HDL was determined by incubation of 1:1 weight ratio of peptide and 125I-HDL overnight with quantification of free apolipoproteins by counting of electrophoretically separated proteins (10).

**Statistical Analysis**—Each experiment was performed in triplicate and statistical analyses were performed using analysis of variance. Contrasts were performed using Scheffe’s test.

**RESULTS**

The synthetic peptides used in this study were designed to examine how the amphipathic α-helical regions of apolipoproteins contribute to their lipid binding and biological function (11, 12). They have been well characterized in earlier works and have been shown to form complexes with phospholipids (17), exchange with native apolipoproteins on HDL (18), and serve as cofactors in the lecithin/cholesterol acyltransferase-mediated catalysis of cholesterol ester formation (19). The model peptide, peptide 18A, is 18-amino acid residues long and forms an α-helix with a polar and a nonpolar face and a specific distribution of charged residues. It has positively charged amino acid residues at the interface of the polar and nonpolar faces and negatively charged residues along the center of the polar face. The amino acid sequences of the synthetic peptides examined and a space filling model of peptide 18A are shown in Fig. 1.

When added to cultured trophoblast cells, the amphipathic peptides which mimic the properties of apoA1 stimulated a significant, dose-dependent increase in hPL release (Fig. 2). The model peptide, peptide 18A, stimulated an 8.5-fold increase in hPL release over control cells. Peptide 18A-Pro-18A stimulated more release than the single helical peptide 18A, with a maximal 18-fold increase in hPL release. This peptide has two model helices joined by a proline, giving it the potential for cooperativity between helices as the two helical segments can pivot around proline (an amino acid which does not permit helix formation). Increasing the hydrophobicity of 18A by substitution of glutamic acid residues for aspartic acid and leucine for alanine, compound (Glu18A-Leu16-17)18A, changed the shape of the dose response curve and increased the amount of hPL released at higher concentrations (10.5-fold increase at 150 μg/ml). Reverse-18A, which has the position of positive and negative charges reversed, had no effect on hPL release. ApoA1 stimulated a 3.1-fold increase in hPL release. Intact HDL stimulated 2.2-fold increase at concentrations of 1.0–1.5 mg protein/ml (data not shown). The dose response curves for apoA1 and peptides 18A and 18A-Pro-18A reached different maximum effects, but all three had the same half-maximal effective concentration of 25 μg/ml. The dose response curve for peptide (Glu18A-Leu16-17)18A did not reach a maximum effect at the doses tested.

Previous studies have shown that the affinity of a peptide for lipids can be represented by the diameter of the protein-lipid discoidal structures formed with phospholipids such as DMPC, with smaller complexes indicating greater affinity (10). As shown in Table I, 18A-Pro-18A and the more hydrophobic (Glu18A-Leu16-17)18A both form small complexes, whereas changing the distribution of the charged residues as in reverse-18A causes the formation of large complexes which are unstable. Peptide 18A-DMPC complexes are intermediate in size. The lipid affinity of these peptides correlates with the magnitude of maximal hPL release, with 18A-Pro-18A and (Glu18A-Leu16-17)18A stimulating the most release, reverse-18A being inactive, and 18A intermediate in stimulatory activity. Another characteristic of the apolipoproteins and other lipid-associating proteins is an increase in helicity upon interaction with phospholipids, as measured by circular dichroism (20). Peptide reverse-18A exhibits low percent α-helicity both in buffer and in the presence of DMPC, whereas peptide 18A

![Fig. 1. Space filling model of 18A amphipathic peptide. Stipped areas indicate positively charged amino acid residues, and striped areas indicate negatively charged residues. 18A: Asp-Tyr-Leu-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu-Lys-Leu-Lys-Glu-Ala-Phe. Model amphipathic representing the charged residue positions analogous to apolipoproteins. Reverse-18A: Lys-Tyr-Leu-Asp-Ala-Phe-Tyr-Lys-Asp-Val-Ala-Lys-Glu-Leu-Lys-Ala-Phe. Charged residue positions in 18A reversed. (Glu18A-Leu16-17)18A: Asp and Ala were substituted with Glu and Leu at positions indicated to increase the lipid affinity of 18A. 18A-Pro-18A: repeating units of 18A joined by Pro. This increases the lipid affinity by cooperativity between the two 18A amphipathic helical domains.](image-url)
The release of hPL by perifused trophoblasts exposed to equimolar amounts of HDL (C), apoAII (O), and peptide 18A-Pro-18A (●). Dispersed trophoblast cells were placed into a perfusion system, 10 million cells/chamber, and perifused with RPMI 1640 at 6 ml/h. After a 2-h equilibration period, the chambers were perifused with media only or media containing HDL (1 mg/ml), apoA11 (200 μg/ml) or 18A-Pro-18A (50 μg/ml) for 15 min, then returned to control media. One- or 5-min fractions were collected and assayed for hPL.

displays a doubling of percent α-helicity with phospholipids and (Glu^6-Leu^14^)18A increases 2.5-fold. Peptide 18A-Pro-18A is already 59% helical in buffer and shows little further increase with DMPC; this may be due to the two or more nonpolar faces self-associating in aqueous solution to minimize aqueous contact and thereby stabilizing the α-helical structure (17).

The ability of the peptide analogs to associate with lipids can perhaps be best summarized by their capacity to displace native apolipoproteins from the surface of lipoprotein particles. Table I shows that the analogs displace iodinated apolipoproteins from HDL with rank order ability 18A-Pro-18A > (Glu^6-Leu^14^)18A > 18A > reverse-18A. Maximum stimulation of hPL release by peptide analogs shows the same rank order. These findings indicate that the same compounds that are the most potent stimulators of hPL release also bind most avidly with phospholipids. The characteristics of high α-helicity, nonpolar face hydrophobicity and a specific charge distribution appear important to both hPL release and phospholipid association.

As shown in Fig. 3, the time course of the stimulation of hPL release by 18A-Pro-18A was identical to that of HDL and apolipoprotein AII. Trophoblast cells perifused with equimolar* amounts of 18A-Pro-18A, apoAII, or native HDL all released hPL within 5 min of exposure, and the pattern of response of the three compounds was essentially identical. The release of hPL in response to the amphipathic peptides is not due to a "non-specific" detergent effect of the peptides since the increase in hPL release was not accompanied by the leakage of the cytosolic enzymes lactid dehydrogenase and alkaline phosphatase and nonhelicical detergents have no effect on hPL release (data not shown). For example, deoxycholate in a molar concentration 10-fold greater than that of 18A-Pro-18A caused no increase in hPL secretion. Triton X-100 in 10-fold excess did cause an increase in hPL release but the increase was accompanied by the leakage of cystolic enzymes into the medium. Peptide 18A-Pro-18A stimulated a specific release of hPL, i.e. without release of intracellular enzymes.

**DISCUSSION**

These studies indicate that synthetic peptides which mimic the structure of the lipid-associating domains of the exchangeable apolipoproteins cause a specific, rapid, dose-dependent release of hPL from trophoblast cells with an identical time course as the apolipoproteins. These findings suggest that the secondary structure of an amphipathic α-helix is the functional unit in the HDL-stimulated release of hPL from trophoblast cells. Variations of the model peptide indicate that hydrophobicity, α-helicity, and a specific charge distribution all contribute to both the hPL stimulatory activity and to the ability to associate with phospholipids. The parallel nature of these requirements indicates that lipid binding may be a necessary function of an active analog of the amphipathic helix and suggests that these peptides may be working through interaction with cell phospholipids, most likely those of the plasma membrane.

The finding that the ability to stimulate hPL release is governed by secondary structure rather than a specific amino acid sequence and that several variations of the model peptide are very effective stimulators argues against a "lock and key" type of protein ligand-receptor interaction as the first step in stimulation. The correlation of hPL stimulation and phospholipid affinity suggests that a protein-lipid interaction may instead be important. Indeed, the amphipathic α-helix portions of the apolipoproteins are believed to be the lipid-binding domains (8). The ability of the analog peptides to stimulate greater amounts of hPL release than apoA1 may indicate that these membrane-interactive portions are more accessible in the smaller compounds. Since the nonpolar face of the apolipoproteins is believed to be associated with lipid-protein phospholipids in the intact HDL moiety, part of or all of the apolipoprotein molecule would theoretically need to

**TABLE I**

| Analog peptide | Diameter of peptide: DMPC complex (Å)* | % helicityb | % Displacement apolipoproteins from HDL* | Maximal % stimulation of hPL release |
|----------------|-----------------------------------------|-------------|----------------------------------------|--------------------------------------|
| 18A-Pro-18A    | 148 ± 19                                | 49          | 21.0                                   | 1840                                 |
| [Glu^6-Leu^14^]18A | 123 ± 46                               | 22          | 12.7                                   | 1050                                 |
| 18A            | 206 ± 30                                | 15          | 11.3                                   | 850                                  |
| Reverse-18A    | 257 ± 26                                | 11          | 2.0                                    | 128                                  |

* Diameter of peptide-DMPC complex determined by electron microscopy after incubation at 1:5 weight ratio mixture.

b Measured by circular dichroism where percent helix = (O_222 + 3000/3900) × 100 where O_222 is the mean residue ellipticity at 222 nm.

* Displacement of apolipoproteins determined by incubation of 1:1 weight ratio of peptide and iodinated HDL overnight with quantification of free apolipoproteins by counting of electrophoretically separated proteins.

HPL stimulation was performed as described under "Materials and Methods." The cells were exposed to peptide for 1 h and results expressed as percent increased release over control.

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*Molecular weights used were: 18A-Pro-18A 400; apoAII 17,400; and HDL 74,000 (assuming an average of two apoAII and one apoAII/HDL moiety) (7).
Reassociate with the cell membrane phospholipids. The lack of previous phospholipid binding may explain why the delipidated apolipoprotein is effective at a 10-fold lower concentration than intact HDL. The dose response curves for apoA1 and peptides 18A and 18A-Pro-18A reached different maximum effects, suggesting that there were a limited number of sites on the membrane at which these compounds could act, and that the peptides with higher lipid binding affinity had more sites available for interaction. The similar half-maximal effective doses suggests that they are all interacting with the membrane through the same mechanism. Peptide (Gu4-Leu118)18A appears to have a biphasic dose response curve, with the increase in hydrophobicity allowing greater membrane interaction at higher concentrations.

Association of the 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. Alterations in membrane permeability causing, for example, influx of calcium ions could stimulate second messenger production, such as an increase in cAMP levels. Alternatively, a change in membrane fluidity or lipid bilayer organization could activate a membrane-bound phospholipase activity with subsequent release of phosphoinositols, diacylglycerol, or other intracellular regulators. These mechanisms would be analogous to the action of polycations, such as poly-L-lysine, which are believed to exert their biological actions through electrostatic interactions of their positively charged amino acids with the negatively charged surface of cells (21).

Poly-L-lysine has been found to alter membrane permeability (22) and to stimulate a calcium-dependent phospholipase (23). Changes in membrane permeability could also be affected by the creation of ion-conducting pores. Ion channel proteins are composed of multiple homologous subunits, each of which appears to contain numerous membrane-spanning amphiphilic α-helices, and 21-residue synthetic peptides designed to form amphiphilic α-helices which have a high degree of hydrophobicity have recently been shown to form ion channels (24).

It is also possible that the apolipoprotein analogs stimulate hPL release by binding to a membrane protein that has a general affinity for amphiphilic helices. Calmodulin is known to bind with high affinity a variety of peptides which share a potential amphiphilic α-helix structure, such as melittin (25), mastoparan (26), chicken gizzard myosin light chain kinase fragments (27), β-endorphin, and β-endorphin analogs (28). A similar membrane protein binding phenomenon where the secondary structure of an amphiphilic α-helix is the determining characteristic could mediate the action of the variety of apolipoprotein analogs and therefore of the various apolipoproteins.

There are marked similarities between the apolipoprotein analogs and several peptide hormones. Several authors have proposed the existence of a category of protein hormones that, despite diverse biological functions and nonhomologous sequences, share a number of structural similarities (29, 30). These hormones are single stranded, of intermediate length (10–50 amino acid residues), lack a definite tertiary structure, have hydrophobic residues regularly spaced every 3 to 4 residues giving the potential for amphiphilic helix formation, and form stable complexes with phospholipids. Examples of this class of peptide hormones include glucagon, calcitonin, the fully active parathyroid hormone fragment 1–34, and β-endorphin. Synthetic peptide analogs with conservative substitutions through the potential amphiphilic helix areas show retention of binding and biological activity, indicating that the amphiphilic α-helix rather than the specific amino acid sequence is the critical factor (29). Corticotropin-releasing factor is another biologically active peptide which is believed to fold into two amphiphilic α-helical domains during interaction with the cell membrane (31).

Although this class of hormones and the apolipoproteins share an amphiphilic helical nature, the hormones mentioned have segments which cannot tolerate substitutions, usually at the amino terminal end. This required region is believed to be important in high affinity receptor interaction, as in amino acid residues 1–5 of β-endorphin (30), whereas the amphiphatic helical areas of this family of hormones is postulated to interact with plasma membrane phospholipids and thereby to aid in proper positioning of the hormone to the receptor (32). The apolipoprotein analogs, in contrast, are fully substituted, again suggesting that they may not require interaction with a receptor moiety.

In conclusion, these studies of the apolipoprotein analogs indicate that the amphiphatic α-helix is important not only in apolipoprotein-lipid association but in the interaction of apolipoproteins and intact cells. The analogy with other peptide hormones illustrates the general biological importance of the amphiphatic α-helix configuration. The correlation of hPL stimulatory ability and phospholipid binding affinity, and the lack of an amino acid-specific region, suggest that the analog peptides and therefore the apolipoproteins themselves may influence hPL release by direct interaction with plasma membrane phospholipids.

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