Overexpression of Apolipoprotein A-IV Enhances Lipid Secretion in IPEC-1 Cells by Increasing Chylomicron Size*

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Intestinal apolipoprotein A-IV expression is highly regulated by dietary lipid in newborn swine, suggesting a role in lipid absorption. Constitutive overexpression of apoA-IV in newborn swine enterocytes enhances basolateral secretion of triacylglycerol (TG) in TG-rich lipoproteins 4.9-fold (Lu, S., Yao, Y., Meng, S., Cheng, X., and Black, D. D. (2002) J. Biol. Chem. 277, 31929–31937). To investigate the mechanism of this enhancement, IPEC-1 cells were transfected with a tetracycline-regulatable expression system (Tet-On). In cells incubated with oleic acid, a dose response relationship was observed between medium doxycycline concentration and basolateral apoA-IV and TG secretion. Similarly regulated expression of apoA-I did not enhance lipid secretion. The mean diameter of TG-rich apoA-IV and TG secretion. Similarly regulated expression of apoA-I did not enhance lipid secretion. The mean diameter of TG-rich lipoproteins secreted from doxycycline-treated cells was larger than from untreated cells (87.0 nm versus 53.4 nm). Basolateral apoB secretion decreased. Using the same expression system, full-length human apoA-IV (376 amino acids); a “pig-like” human apoA-IV, lacking the C-terminal EQQQ repeats (361 amino acids); and a “chicken-like” apoA-IV, further truncated to 343 amino acids, were expressed in IPEC-1 cells. With increasing protein secretion, cells expressing the full-length human apoA-IV displayed a 2-fold increase in TG secretion; in sharp contrast, cells expressing the pig-like human apoA-IV displayed a 25-fold increase in TG secretion and a 27-fold increase in lipoprotein diameter. When human apoA-IV was further truncated to yield a chicken-like protein, TG secretion was inhibited. We conclude that overexpression of swine apoA-IV enhances basolateral TG secretion in a dose-dependent manner by increasing the size of secreted lipoproteins. These data suggest that the region in the human apoA-IV protein from residues 344 to 354 is critical to its ability to enhance lipid secretion, perhaps by enabling the packaging of additional core TG into chylomicron particles. The EQQQ-rich region may play an inhibitory or modulatory role in chylomicron packaging in humans.

Apolipoprotein A-IV (apoA-IV) is a lipid-binding protein that is expressed predominantly in the mammalian small intestine (1–4). Although numerous functions have been ascribed to apoA-IV, including roles as a post-prandial satiety signal (5, 6), a lipoprotein anti-oxidant (7), a mediator of reverse cholesterol transport (8, 9), an anti-atherosclerotic factor (10, 11), and an anti-inflammatory factor (12), the preponderance of evidence points to a primary role in intestinal lipid absorption. Of all the intestinal genes associated with lipid absorption, the apoA-IV gene is the most responsive to intestinal lipid flux (13). In the enterocyte, apoA-IV is incorporated into nascent chylomicrons at an early stage of biogenesis in the ER and is secreted on the surface of chylomicrons at the basolateral membrane (14). Therefore, most apoA-IV dissociates from the chylomicron surface and in humans circulates predominantly as a lipid-free protein (15).

We have previously shown that apoA-IV is expressed in the small intestine of neonatal swine (4). In animals given a high fat intraduodenal lipid infusion for 24 h, jejunal apoA-IV mRNA expression increased 7-fold at mainly the transcriptional level in comparison to control animals given a low fat infusion (4, 16). This observation, coupled with the fact that this lipid responsiveness decreases as the piglets are weaned from a high fat breast milk diet, suggests that apoA-IV may play a role in facilitating intestinal lipid absorption in the suckling newborn.

To test the hypothesis that expression of apoA-IV enhances enterocyte chylomicron secretion in response to fatty acid absorption, we developed a stably transfected newborn swine intestinal epithelial cell line that synthesizes and secretes excess swine apoA-IV. We chose the IPEC-1 cell line for this purpose specifically, because we have extensively characterized the regulation of lipoprotein synthesis and secretion and apolipoprotein expression by lipid in these cells (17–20). These cells have relatively low lipid transport efficiency and low background apoA-IV expression, compared with native enterocytes, enabling us to observe marked increases in lipid export should they occur. Using this cell line, we previously demonstrated that when cultured with oleic acid, high level constitutive overexpression and secretion of apoA-IV enhanced radiolabeled basolateral triacylglycerol and cholesterol ester secretion by 4.9-fold, and phospholipid by 2-fold, mainly as lipoproteins in the chylomicron and very low density lipoprotein (VLDL) density range (21).

An acknowledged limitation of this approach is the fact that the level of constitutive apoA-IV overexpression was far higher than the normal physiologic range. To address this limitation, in the present studies,
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IPEC-1 cells were transfected with a tetracycline-regulatable transactivator and a full-length swine apoA-IV cDNA driven by the tetracycline response element, allowing regulation of apoA-IV expression and basolateral secretion in a physiologic range by culture medium doxycycline concentration. To identify protein structural components essential for this enhancement, we also performed similar experiments with human apoA-IV, as well as truncated mutants of human apoA-IV resembling swine and chicken apoA-IV.

EXPERIMENTAL PROCEDURES

Materials—[9,10-3H]oleic acid (26.3 Ci/mmoll and Pro-Mix® L-[35S]methionine/cysteine (>1000 Ci/mmoll) were purchased from Amersham Biosciences. Oleic acid (C18:1n-9, OA) and essentially fatty acid-free bovine serum albumin were purchased from Sigma.

Cloning of Full-Length Swine ApoA-IV and A-I cDNAs—The following PCR primers were generated from the published swine apoA-I (EMBL accession X69477) and apoA-IV (GenBank™ accession AJ222966) cDNA sequences: swine apoA-I, Forward: 5'-CACCACAGTCTTTCTCCTTGAAGGTGCTGAA-3' (sense, nt -19 to +1, before ATG); Reverse: 5'-CTGCTCCGGAACAGCTTATT-3' (antisense, nt +867 to +896, 51 bp after stop codon). Swine apoA-IV Forward: 5'-AGGTAGTCTGATCGTCAAGCTGACGGAC-3' (antisense, nt +1358 to +1377, 125 bp after stop codon).

Total RNA was extracted from domestic pig intestinal cells and IPEC-1 cells; 25 μg was used for cDNA synthesis. In the PCR reaction, one-tenth of the generated cDNA was used for high fidelity PCR. Platinum® Pfx PCR polymerase (Invitrogen) was used as described in the manufacturer’s protocol.

Cloning of the apoA-I and A-IV cDNA fragments was carried out using the vector Bluescript KS+ (Stratagene) and the manufacturer’s protocol. Kanamycin-resistant clones were analyzed by restriction enzyme digestion and DNA sequencing to verify sequences.

Cloning of Full-Length Human ApoA-IV and Mutants—Human apoA-IV cDNA was inserted into plasmid pTRE2hyg as follows: an apoA-IV cDNA plasmid, generated as described previously (22), was used as a template for PCR. The 5’ sense strand primer (5’-ATACGGAGATCTCTAGTAAGGCGGTGGTCCTGACCCCTGGCC-3’) contained a flanking BglII restriction site. The 3’ antisense primer (5’-TGACTTACCGTGTCATCGTCTGCGTGATGGAC-3’) contained a flanking MluI restriction site. The PCR product was digested with BglII and MluI and ligated to BamHII and MluI-digested pTRE2hyg. The resulting plasmid was named pTapoAIV. The pig-like variant of human apoA-IV (pTapoAIV-PL) was generated as described above with the exception that the following 3’ antisense mutagenic primer was used for PCR: 5’-TGACTTACCGTGTCATCGTCTGCGTGATGGAC-3’. The chicken-like variant of human apoA-IV (pTapoAIV-CL) was generated as described above with the exception that the following 3’ antisense mutagenic primer was used for PCR: 5’-TGACTTACCGTGTCATCGTCTGCGTGATGGAC-3’. The validity of each cDNA clone was confirmed by automated DNA sequence analysis.

IPEC-1 Cell Culture—The derivation of the IPEC-1 cell line has been described previously (17). Cells from passages 25 to 80 were used in these studies, and all cell culture was carried out at 37°C in an atmosphere containing 5% CO2. Undifferentiated IPEC-1 cells were maintained in serum-free medium in plastic culture flasks (75 cm2, Corning Glassworks, Corning, NY) in growth medium (GM): Dulbecco’s modified Eagle’s medium/F12 medium (Invitrogen), supplemented with 5% fetal bovine serum (Invitrogen), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml) (ITS Premix, BD Biosciences, Bedford, MA), epidermal growth factor (5 μg/liter) (BD Biosciences), penicillin (50 μg/ml), and streptomycin (4 μg/ml) (Invitrogen). To induce differentiation, undifferentiated cells were harvested by trypsinization, and 2 x 103 cells/well were plated on 75-mm diameter collagen-coated filters (3.0-μm pore size) in Transwell® culture plates (Costar, Corning, Inc.). Cells were maintained in serum containing GM for 48 h and then switched to the same medium containing 10−7 M dexamethasone (Sigma), but without FBS. Medium was then changed every 2 days. We have previously shown that after 10 days, IPEC-1 cells exhibit enterocytic features, including polarization with well defined microvilli facing the apical medium (17). Cellular membrane integrity was assessed by measurement of apical medium lactate dehydrogenase activity (Sigma).

Development of IPEC-1 Cells with Tetracycline-regulatable Apolipoprotein Expression—Undifferentiated IPEC-1 cells were plated and grown to 50% confluency in 60-mm culture dishes. After 24 h, cells were transfected with pTet-On® (BD Biosciences) using Lipofectamine 2000® (Invitrogen). Transfected cells were allowed to grow another 24 h and then plated in two 10-cm culture dishes, followed by G418 selection for 3 weeks. Clones were isolated and transferred to 24-well culture plates to allow an additional 2 weeks of growth. Individual clones were screened by luciferase assay with or without doxycycline after transfection with pTRE2hyg-Luc per the manufacturer’s protocol (BD Biosciences). The clone (Tet-19) demonstrating the highest luciferase induction by 1 μg/ml doxycycline was selected for generating Tet-On/apolipoprotein cell lines.

Tet-19 cells were transfected with the pTRE plasmids containing apolipoprotein cDNA using the same protocol as was used to develop the Tet-19 cell line. Both G418 (400 μg/ml) and hygromycin (300 μg/ml) were used for clone selection. After selection and expansion of clones demonstrating the highest induction of mRNA and protein secretion, as determined by real-time PCR and Western blot, the cells were used for triacylglycerol radio labeling experiments. In preliminary experiments, induction of apolipoprotein secretion by differentiated cells was maximal at 6 h of incubation with each dose of doxycycline (data not shown).

Analysis of Swine and Human ApoA-IV and Swine A-I mRNA by Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted from cells (23), and aliquots (2–10 μg) were treated with 0.5 units of DNase RQ1 (Promega) at 37°C for 60 min in 50 μl of 10 mM Tris- HCl, pH 7.5, 6 mM MgCl2, 10 mM NaCl, 1 mM dithiothreitol, 20 units of RNase inhibitor (RNAsin, Promega). The RNA was then sequentially extracted with phenol-chloroform and chloroform, precipitated with ethanol, washed once (with 70% ethanol), and resuspended in 20–40 μl of H2O. For cDNA synthesis, 2 μg of total RNA was used with the Omniscript® RT kit (Qiagen, Valencia, CA) with oligo-dT nucleotides at 37°C for 1 h.

The QuantiTect SYBR green PCR kit (Qiagen) was used for real-time PCR using the following primers: β-actin: Forward, 5’-CTCCTCCTCTTGGGCATGGA-3’ (sense, nt 426–443); Reverse: 5’-CAGACCTCATGATCGAAGTT-3’ (antisense nt 470–490, swine apoA-I: Forward: 5’-CGGTATGTTGATGCGCATAAAA-3’ (sense, nt. 117–138); Reverse: 5’-CCAGTTGGTCCAGAGAGTCCAGA-3’ (antisense nt 199–220); swine apoA-IV: Forward 5’-GGGACACCTGACGTCTCCT-3’ (sense, nt 1011–1028); Reverse 5’-CCTTCTGAAGGGTGCTGAA-3’ (antisense, nt 1061–1081). Human apo-A-IV and mutants: Forward 5’-TGCCACCGAGCTGCGTAA-3’ (sense nt 248–267); Reverse 5’-TGACCTCCTCGGGCTTCTTC3’ (antisense, nt 738–757).
Following reverse transcription, real-time quantitative PCR was performed using the Applied Biosystems Prism 7900HT Sequence Detection System (Applied Biosystems, Foster, CA). The following PCR profile was used: 2 min at 50 °C, 10 min at 90 °C, and 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. The amount of SYBR Green was measured at the end of each cycle. Analyses were performed in duplicate. To generate the standard curves for apolipoproteins and β-actin, serially diluted cDNA was prepared, and real-time PCR was performed as above. Standard curves were generated by plotting the relative ratio of apolipoprotein gene expression.

Western Blot Analysis of Apolipoproteins—Rabbit anti-swine apoA-IV and A-I antibodies were generated as described previously (4, 24). The antisera were subjected to ammonium sulfate precipitation and used directly as a 1:1000 dilution for Western blot analysis.

1 ml of basolateral culture medium was collected with added protease inhibitors (Complete®, Roche Applied Science) on ice, and medium was concentrated 4:1 (Centriplus YM-10®, Millipore, Austin, TX). Samples were electrophoresed on an 8% (apoA-I and A-IV) or 3–20% (apoB) SDS-polyacrylamide electrophoresis (PAGE) gel followed by transfer to nitrocellulose filters. Western blotting was conducted using the ECL Western blot kit according to the manufacturer’s protocol (Amersham Biosciences). The swine apoA-IV antibody was used for the human apoA-IV and mutant blots. The blots were scanned, and digital densitometry was carried out using NIH Image software.

ApoB and A-IV Radiolabeling—At day 10 after plating on Transwell filters, differentiated cells were washed with Hank’s Balanced salt solution, followed by incubation in methionine- and cysteine-free medium for 1 h. Radiolabeling was initiated by the addition of 500 μCi of ProMix® 1-[14C]methionine/cysteine with 0.8 mCi oleic acid/albumin complex and with or without doxycycline to induce apoA-IV expression. Incubation was carried out for 24 h at 37 °C. Basolateral medium was then harvested for apoB and A-IV immunoprecipitation, and protease inhibitors (Complete®) were added.

ApoB and A-IV Immunoprecipitation—5-ml aliquots of basolateral culture medium were preclarified by incubation with 0.2 ml of IgGsorb® (10% solution, w/w; The Enzyme Center, Boston, MA) for 1 h at 4 °C with constant agitation. The samples were then centrifuged, and the supernatant was collected. The amount of human apoB and swine apoA-IV antibody added to the supernatant was determined to be in excess by re-immunoprecipitation of samples. Swine apoA-IV antibody was used to immunoprecipitate human apoA-IV and mutants. Samples were incubated for 18 h at 4 °C with gentle agitation. The antigen-antibody complexes were harvested by the addition of 50 μl of protein A-Sepharose (10%, w/v; Amersham Biosciences) and incubation for 2 h, followed by centrifugation to harvest the pellet. The pellet was washed six times with immunoprecipitation buffer (10 mM NaH2PO4, 5 mM (Na2)EDTA, 100 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine) followed by suspension of the pellet in 50 μl of reducing buffer. Samples were heated at 95 °C for 5 min and centrifuged, and the supernatant was subjected to SDS-PAGE using a 3–20% acrylamide gradient under reducing conditions (25). After electrophoresis, gels were fixed in methanol-acetic acid-water (40:7.5:3, v/v) for 30 min, washed three times with distilled water, soaked in Amplify (Amersham Biosciences) for 30 min, and dried at 80 °C for 4 h. Autoradiography was performed by exposing the gels to Kodak X-Omat AR film for 2–7 days. Apolipoprotein bands were identified by comparison to coelectrophoresed molecular weight standards (Invitrogen).

Incubation of Cells with Oleic Acid—Differentiated cells (10 days post-plating Transwell filters in serum-free medium) were prepared, and fresh medium was added to both the apical and basolateral Transwell compartments. The apical medium contained 0.8 mM oleic acid complexed with albumin (4:1 molar ratio) (26). This fatty acid concentration is in the physiologic range, and above this concentration the basolateral secretion of triacylglycerol begins to plateau in IPEC-1 cells (17). For Tet-On induction, doxycycline was added to the culture medium at the same time that the experimental incubation was started. Cells were incubated for 24 h followed by harvest of culture medium and cells. For measurement of protein and mRNA, cell lysates and basolateral medium were prepared and processed as described above.

Triacylglycerol Radiolabeling with [3H]Oleic Acid—Cells were incubated for 24 h with 12 μCi of [3H]oleic acid/well, and cell lysates and basolateral medium were collected and processed as described above. The total lipid in the lysates and medium was extracted (18), the extracts applied to silica gel G plates, and subjected to thin-layer chromatography using petroleum ether-diethyl ether-acetic acid 80:20:1 (V/V/V). The bands corresponding to triacylglycerol, cholesterol ester, and phospholipid were identified by comparison to co-chromatographed standards by exposure to iodine vapor and scraped off the plate for liquid scintillation counting. Cellular content and secretion of radiolabeled lipid were expressed as DPM/well/24 h.

Lipoprotein Size Determination by Negative Staining and Transmission Electron Microscopy—To isolate triacylglycerol-rich lipoproteins, comprising chylomicrons and very low density lipoproteins (d ≤ 1.006 g/ml), basolateral culture medium was subjected to density ultracentrifugation using a Beckman SW 41 Ti rotor (Beckman Instruments, Palo Alto, CA) at 17 °C (18). Lipoprotein suspensions were placed on Formvar® carbon-coated grids and allowed to partially dry for 20 min. Filter paper was used to remove any solution left on the grid. The grids were then placed on a drop of 2% phosphotungstic acid, pH 6.0, for 2 min. The grids were viewed on a JEOL 1200 EX Transmission Electron Microscope (JEOL, Peabody, MA) at 60 kV. Images were captured at 20,000× using an AMT 2K digital camera (Advanced Microscopy Techniques, Danvers, MA). Particle diameter measurements were made in a blinded manner using the digital imaging software from AMT.

Lipoproteins secreted by IPEC-1 cells incubated with oleic acid and overexpressing pig-like human apoA-IV were so large that they could not be negatively stained. Therefore, they were visualized with an osmium vapor technique. Briefly, 5 μl of the sample was placed on a carbon-coated copper grid. Three 500-μl drops of 2% osmium tetroxide were placed in close proximity to the sample. A small glass Petri dish was placed on top of all solutions to create an osmium-rich atmosphere. After 20 min, the grids were blotted with filter paper and allowed to dry before transmission electron microscopy and measurement as described above.

Statistical Analysis—Data in experimental groups were analyzed by Student’s unpaired t test. Statistical significance was set at a two-tailed p value of < 0.05.

RESULTS

Doxycycline-regulatable Swine ApoA-IV mRNA Expression and Basolateral Secretion of ApoA-IV Protein and Labeled Triacylglycerol in IPEC-1 Cells—As shown in Fig. 1A, mRNA from IPEC-1 cells expressing doxycycline-regulatable swine apoA-IV demonstrated a progressive increase in apoA-IV mRNA levels as measured by real-time PCR with increasing medium doxycycline concentration. As shown in Fig. 1A, basolateral secretion of apoA-IV protein generally paralleled the increase in mRNA as the medium doxycycline concentration increased, although apoA-IV protein secretion reached a plateau above 800 ng/ml.
doxycycline. Fig. 1B shows that as secretion of apoA-IV increased with increasing doxycycline concentration, basolateral secretion of labeled triacylglycerol paralleled secretion of apoA-IV. The secreted porcine apoA-IV protein appears as a 42-kDa band in the Western blots of basolateral media (Fig. 1C).

**Doxycycline-regulatable Swine ApoA-I mRNA Expression and Basolateral Secretion of ApoA-I Protein and Labeled Triacylglycerol in IPEC-1 Cells**—To determine whether the enhancement of basolateral labeled triacylglycerol secretion was specific for apoA-IV, IPEC-1 cells were transfected with a regulatable porcine apoA-I construct. As shown in Fig. 2A, as with the apoA-IV construct, apoA-I mRNA expression increased in a dose-dependent manner with increasing medium doxycycline concentration. Likewise, basolateral apoA-I protein secretion increased in parallel with the increasing mRNA expression (Fig. 2A).

However, in contrast to the behavior of cells transfected with apoA-IV, the increase in apoA-I secretion had no significant effect on basolateral-labeled triacylglycerol secretion (Fig. 2B). The corresponding Western blot showing a 26-kDa band of secreted porcine apoA-I protein is shown in Fig. 2C.

**Electron Microscopy of Triacylglycerol-rich Lipoproteins Secreted by IPEC-1 Cells Overexpressing Swine ApoA-IV**—Fig. 3 shows the size distribution of triacylglycerol-rich lipoproteins secreted into the basolateral medium by IPEC-1 cells with and without increased apoA-IV expression. Cells were incubated for 24 h with 0.8 mM oleic acid, either without doxycycline treatment or with 800 ng/ml of doxycycline added to the culture medium. Triacylglycerol-rich lipoprotein particles secreted from the doxycycline-treated cells (Fig. 3B) were larger and had a wider size distribution than those secreted from untreated cells (Fig. 3A).
The mean diameter of the particles from the treated cells, 87.0 ± 2.9 nm (mean ± S.E.), was significantly greater than that of the particles from the untreated cells, 54.3 ± 0.8 nm (p < 0.0001). Size distribution histograms show that the lipoproteins from the untreated cells formed a single discrete population (Fig. 3C), whereas lipoproteins from the treated cells comprised a more heterogeneous population (Fig. 3D).

Calculation of mean particle volumes from the diameter values provides information on the capacity of the particles to accommodate core neutral lipid. The particles secreted from the untreated cells had a mean volume of 83,830 nm³, and those from the treated cells had a mean volume of 344,791 nm³. The treated/untreated particle volume ratio is 4.1, a value very close to the 4.4-fold increase in triacylglycerol secretion that was induced with doxycycline treatment.

Influence of Swine ApoA-IV Expression on Basolateral ApoB Secretion—To examine the impact of induction of swine apoA-IV expression on apoB secretion in differentiated IPEC-1 cells, the parent IPEC-1 cell line (Tet-19) expressing the tetracycline-regulatable transactivator alone, as well as the cell line (AIV-6) expressing both the transactivator and tetracycline response element driving the apoA-IV insert, were incubated with 0.8 mM oleic acid in the apical medium and were either untreated or treated with 800 ng/ml doxycycline for 24 h. Protein was labeled with [35S]methionine/cysteine, followed by apoB and A-IV immunoprecipitation from the basolateral medium. As shown in Fig. 4, basolateral secretion of labeled apoB-48, B-100, and A-IV by the Tet-19 cells was relatively unchanged by doxycycline treatment. In contrast, AIV-6 cells treated with doxycycline secreted proportionally less labeled apoB-48 in the face of enhanced apoA-IV. Although these cells secrete little apoB-100, the faint band from the AIV-6 cells also appears reduced in the face of increased apoA-IV secretion. It should be noted that in the AIV-6 clone, basal apoA-IV secretion is higher than that of the Tet-19 parent cell line that expresses apoA-IV at very low levels. This is because of background constitutive apoA-IV expression. As shown in Fig. 4, the cellular content of apoB declined with increasing apoA-IV expression. In light of the reduction in intracellular and secreted apoB in the face of increased apoA-IV and triacylglycerol secretion, the increase in triacylglycerol secretion induced by apoA-IV appears to be because of an increase in the size, rather than number, of particles secreted from the cells (27).

Doxycycline-regulatable Expression of Human ApoA-IV and Mutant mRNA and Basolateral Secretion of Protein in IPEC-1 Cells—The C-terminal amino acid sequences of human and swine apoA-IV are shown in Fig. 5. As compared with swine apoA-IV, human apoA-IV has a hydrophilic EQQQ-rich region. A pig-like human apoA-IV mutant was derived by deleting the EQQQ-rich region (Fig. 5). Further deletion of an additional eleven amino acids resulted in a chicken-like apoA-IV (Fig. 5). Human apoA-IV and these two mutants were expressed in IPEC-1 cells using the TET-on system. As shown in Fig. 6, quantitative
RT-PCR analysis of the mRNA from IPEC-1 cells expressing doxycycline-regulatable native swine apoA-IV demonstrated a progressive increase in mRNA levels as measured by real-time PCR with increasing medium doxycycline concentration. Increases were comparable, especially at the lower doxycycline concentrations. As shown in Fig. 7, basolateral secretion of human apoA-IV and mutant proteins increased as the medium doxycycline concentration increased. In sharp contrast, cells expressing the pig-like human apoA-IV displayed a 25-fold increase in TG secretion. However, when apoA-IV was further truncated to yield a chicken-like protein, TG secretion was inhibited below baseline levels. For comparison, data from cells expressing native swine apoA-IV and A-I are included in the figure. Fig. 10 shows the basolateral secretion of triacylglycerol, cholesteryl ester, and phospholipid in cells expressing the pig-like human apoA-IV mutant as the medium doxycycline concentration was progressively increased. Core triacylglycerol secretion increased at a much greater rate than surface phospholipid, consistent with secretion of these lipids in a progressively larger spherical particle.

Electron Microscopy of Triacylglycerol-rich Lipoproteins Secreted by IPEC-1 Cells Overexpressing Pig-like Human ApoA-IV—Fig. 11 shows the size distribution of triacylglycerol-rich lipoproteins secreted into the basolateral medium by IPEC-1 cells with and without increased pig-like human apoA-IV expression. Cells were incubated for 24 h with 0.8 mM oleic acid, either without doxycycline treatment or with 800 ng/mL of doxycycline added to the culture medium. Triacylglycerol-rich lipoprotein particles secreted from the doxycycline-treated cells (Fig. 11B) were strikingly larger than those secreted from untreated cells (Fig. 11A). The mean diameter of the particles from the treated cells, 3721 ± 144 nm

**FIGURE 4.** Cellular and secreted radiolabeled apoB and A-IV in differentiated IPEC-1 cells with tetracycline-regulatable native swine apoA-IV expression. Parent Tet-19 cells transfected only with the tetracycline-regulatable transactivator or AIV-6 cells derived from the Tet-19 cells and transfected with the tetracycline response element driving the swine apoA-IV cDNA were incubated for 24 h with 0.8 oleic acid (OA) and [35S]methionine/cysteine with or without doxycycline (Dox), followed by immunoprecipitation, SDS-PAGE, and autoradiography of labeled apolipoproteins.

**FIGURE 5.** C-terminal sequences of human apoA-IV and truncation mutants. To identify structural elements that might mediate enhancement of chylomicron secretion by human apoA-IV, a pig-like mutant was generated lacking the C-terminal EQQQ repeat domain, and a chicken-like mutant was produced by further truncating 11 amino acids. Native pig apoA-IV is shown at the bottom.

**FIGURE 6.** Regulation of native and mutant human apoA-IV mRNA expression in IPEC-1 cells treated with doxycycline. Each point represents mRNA abundance (log y-axis; determined by real-time PCR as apolipoprotein/β-actin ratio) at each concentration of doxycycline (x-axis) divided by the values with no added doxycycline. Data points represent the mean of values from duplicate experiments.

**FIGURE 7.** Regulation of native and mutant human apoA-IV basolateral protein secretion in IPEC-1 cells treated with doxycycline as determined by protein radio-labeling and immunoprecipitation followed by SDS-PAGE and autoradiography.
(mean ± S.E.), was significantly greater than that of the particles from the untreated cells, 139 ± 6.1 nm (p < 0.0000001). Note that the particles from the treated cells were so large that they could not be negatively stained, as were the untreated cells. Therefore, the much larger particles were visualized with an osmium vapor technique. Size distribution histograms show that the lipoproteins from the untreated and treated cells formed single discrete populations (Fig. 11, C and D).

Calculation of mean particle volumes from the diameter values provides information on the capacity of the particles to accommodate core neutral lipid. The particles secreted from the untreated cells had a mean volume of 1,406,187 nm³, and those from the treated cells had a mean volume of 26,997,759,775 nm³. The treated/untreated particle volume ratio is ~20,000, a value that would easily accommodate the increased core lipid, even in the face of far fewer particles.

Cellular and Secreted ApoB in IPEC-1 Cells Expressing Human ApoA-IV and Mutants—Fig. 12 shows secreted apoB-48 and native and human mutant apoA-IV, as well as cellular apoB-48, with oleic acid incubation with and without doxycycline treatment in IPEC cells. As expected, treatment with doxycycline resulted in an increase in basolateral apoA-IV secretion. Native human apoA-IV expression resulted in an increase in cellular apoB-48, but a decrease in secreted apoB-48, similar to that observed with native swine apoA-IV. Expression of the pig-like mutant caused a decline in cellular apoB-48, but little change in the secreted protein. Finally, expression of the chicken-like mutant resulted in an increase in both cellular and secreted apoB-48.

DISCUSSION

These data confirm our previous observation that basolateral secretion of newly synthesized triacylglycerol-rich lipoproteins in IPEC-1 cells is enhanced by the constitutive overexpression of swine apoA-IV at supraphysiologic levels (21), but also provide further insight into the mechanism of the apoA-IV enhancement effect. First, these data suggest that the observed effects of swine apoA-IV on triacylglycerol-rich lipoprotein secretion are physiologically relevant, because the secretory enhancement occurred in a dose-response manner over a range of apoA-IV secretion levels. Second, these data suggest that the enhancement in triacylglycerol-rich lipoprotein secretion is specific for apoA-IV, because it was not observed with swine apoA-I. Third, these data demonstrate that the increased basolateral triacylglycerol secretion induced by apoA-IV expression is characterized by secretion of larger lipoprotein particles with an average volume that can account for the magnitude of the increased amount of core neutral lipid. Fourth,
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because secretion of apoB decreased with increased native swine and human apoA-IV expression, it appears that the ability of apoA-IV to enhance triacylglycerol transport involves solely an increase in the size, rather than the number, of triacylglycerol-rich particles secreted. Finally, data from the human native and mutant apoA-IV experiments suggest that the region in the human apoA-IV protein from residues 344 to 354 is critical to its ability to enhance enterocyte lipid secretion. The data also suggest that the EQQQ-rich region may play an inhibitory or modulatory role in chylomicron secretion in humans. Finally, data from the human apoA-IV mutant experiments suggest variable effects on apoB depending on the magnitude of lipid secretory enhancement.

The unique structure of apoA-IV provides clues to the mechanism by which it can enhance triacylglycerol transport. The amphipathic α-helices in human apoA-IV are very hydrophilic and have a radial charge distribution that precludes deep penetration into lipid monolayers (28). These helical domains are also present in pig apoA-IV, although they are somewhat less hydrophilic compared with those of the human protein (29). Weinberg et al. (28) have proposed that the presence of these hydrophilic helices enables apoA-IV to adopt an elastic, pressure sensitive conformation at lipid interfaces. During chylomicron assembly, these hydrophilic α-helices would be recruited into the expanding nascent particle surface, and by maintaining constant interfacial elasticity would stabilize particle growth, and enable the packaging of more core lipid molecules per particle.

During the second step of VLDL and chylomicron assembly, large amounts of core triacylglycerol are added to the nascent lipoprotein particles, and they expand to attain sizes as large as 10,000 Å. In hepatic VLDL assembly, the C-terminal α-helical domains of apoB-100 adapt their surface conformation in response to the increase in particle diameter (30). However, in the intestine, the activity of the RNA editing enzyme apobec-1 truncates apoB-100 to form apoB-48, which lacks these expansile domains. We speculate that in the intestine, apoA-IV compensates for the absence of the C-terminal domains in apoB-48, and that the presence of apoA-IV in the ER during chylomicron assembly, either through developmentally increased synthesis (as in the newborn piglet) or by genetically manipulated overexpression (as in the present study), enhances the efficiency of triacylglycerol absorption by enabling secretion of larger lipoprotein particles.

The observations that expression of native swine apoA-IV increased both the mean particle diameter and the width of the particle size distribution support the concept that apoA-IV enhances lipid secretion by facilitating packaging of more core lipid per particle. Data from the present and previous studies show that swine apoA-IV overexpression does not significantly affect intracellular triacylglycerol synthesis (21). Furthermore, if under the experimental conditions of the present studies, each secreted lipoprotein particle contains only one molecule of apoB, the decrease in apoB secretion with increased apoA-IV expression indicates that the number of particles secreted may have actually decreased (27). A possible mechanism for a reduction in apoB secretion with a concomitant decrease in the number of secreted particles would be the production of particles containing apoA-IV, but devoid of apoB. Alternatively, there may be multiple copies of apoA-IV on the apoB-containing chylomicrons. In support of the decreased apoB secretion, we demonstrated a concomitant decrease in intracellular apoB mass with increasing doxycycline dose. Also, we previously showed that constitutive overexpression of swine apoA-IV does not affect apoB mRNA abundance in IPEC-1 cells (21). Therefore, the decrease in cellular and secreted apoB protein with increasing swine apoA-IV expression in the present studies likely represents increased apoB degradation, which is a potential mechanism of apoB regulation in the small intestine by channeling lipid into particle expansion at the expense of particle number, creating a preponderance of underlipidated apoB that is susceptible to degradation (31). The exact cellular mechanism of this decrease in apoB is the subject of ongoing studies.

The finding that swine apoA-I did not enhance triacylglycerol secretion, and actually depressed secretion below basal levels, suggests that the enhancement of chylomicron secretion may be a unique property of apoA-IV. At present the region of the swine apoA-IV molecule that mediates the enhanced packaging of core lipid into larger particles is unknown. Native human apoA-IV enhanced basolateral triacylglycerol secretion ~2-fold, less than the 5-fold augmentation seen with swine apoA-IV. However, when the C-terminal EQQQ-rich region of human apoA-IV was deleted, the increase in triacylglycerol secretion was 25-fold. This suggests an inhibitory or modulatory role of this region in humans. Indeed, Pearson et al. (32, 33) found that the C-terminal region in human apoA-IV moderates its lipid avidity and rate of lipid binding, which may be important in allowing the assembly and
lipidation of larger chylomicrons. Of particular interest, the further deletion of 11 amino acids (344 to 354) in the chicken-like mutant resulted in no enhancement of secretion. It is doubtful that this small region is involved in lipid binding, since it is not part of an amphipathic helical repeat. However, we speculate that it may be crucial in influencing the tertiary conformation of the protein through effects on protein folding or interaction with the amino terminus or other regions of the protein. The structure, conformation and interfacial behavior of apoA-IV are sensitive to even the most conservative modification of the C terminus (34, 35). Furthermore, recent studies have shown that the C-terminal truncation of human apoA-IV alters its lipid affinity, binding rate and elasticity at hydrophobic interfaces (32, 36, 37). Hence, the finding that the chicken-like mutant did not enhance triacylglycerol secretion is in keeping with the elastic barostat hypothesis, which posits that the stabilization and growth of larger triacylglycerol-rich emulsion particles require the presence of highly elastic and conformationally flexible apolipoproteins. In this regard, we have shown that human apoA-IV possesses just such interfacial properties, whereas chicken apoA-IV does not (28, 38). It is interesting that native chicken apoA-IV is a much less elastic protein than human apoA-IV (38), and that avian intestine does not synthesize large chylomicrons (39).

It is also possible that the ability of apoA-IV to facilitate packaging of additional core lipid involves an interaction with other key proteins in the particle assembly process, such as apoB or microsomal triglyceride transfer protein (MTP). In this regard, Gallagher et al. (22) used KDEL-tagged human apoA-IV to block its ER export in COS cells cotransfected with MTP and truncated apoB mutants, and found that when apoB-41 was transfected with KDEL-tagged apoA-IV, secretion of apoB-41 was reduced by 80%. Similar results were obtained in McA-RH7777 cells transfected with apoB-25. They interpreted these data to suggest that apoA-IV may physically interact with apoB within the cell. They postulated that such an interaction could delay the transit of apoB-containing triglyceride-rich particles through the lipidation sites in the secretory pathway, thus allowing time for additional lipidation. Our observation that the secretion of apoB was inhibited by increased native swine apoA-IV secretion in doxycycline-treated cells, supports the concept that these two key apolipoproteins may interact within the secretory pathway.

In contrast to the effect of native swine and human apoA-IV expression on retarding apoB secretion in IPEC-1 cells, expression of human apoA-IV pig-like and chicken-like mutants demonstrated a more complex influence on apoB secretion that may be related to the magnitude of lipid secretion and possible regulatory effects on apoB. Expression of native human apoA-IV resulted in a decrease in apoB-48 secretion and an increase in cellular apoB-48. One possible explanation for this observation is that an interaction between apoA-IV and B retards the secretion of apoB-containing particles, as postulated for native swine apoA-IV, with retention of the apoB-48 that has been shunted away from degradation. Pig-like apoA-IV appeared to have little influence on apoB-48 secretion, but reduced cellular apoB. The tremendous triacyl-

FIGURE 11. Transmission electron microscopy of triacylglycerol-rich lipoproteins secreted by differentiated IPEC-1 cells with tetracycline-regulatable pig-like human apoA-IV expression. Cells were incubated for 24 h with 0.8 oleic acid and with or without 800 ng/ml medium doxycycline, followed by isolation of d ≤ 1.006 g/ml basolateral medium lipoproteins for negative staining (A) or osmium vapor staining because of the large particle size (B) and electron microscopy. Panel A, particles from cells not treated with doxycycline. Panel B, particles from cells treated with doxycycline. Note the different scales used in panels A and B. Panel C, histogram of particle sizes from untreated cells. Panel D, histogram of particle sizes from doxycycline-treated cells.
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glycerol secretory enhancement induced by this mutant peptide may have reduced the need for apoB for chylomicron packaging in the ER, thus shunting nascent apoB into the degradation pathway. The total lack of enhancement and even slight inhibition of triacylglycerol secretion by expression of the chicken-like apoA-IV may result in an increased need for apoB for chylomicron assembly and secretion, thus increasing the ER pool, as well as basolateral secretion. Finally, it is important to remember that these human apoA-IV experiments, in contrast to the native swine apoA-IV experiments, involved expressing a human polypeptide in a swine intestinal cell, which may influence intracellular interactions of human apoA-IV with other swine proteins, including apoB. Future studies will address these issues.

These data may appear to conflict with the observations from transgenic mouse models indicating that lipid absorption was unaffected either by overexpression of human apoA-IV (40) or targeted disruption of the apoA-IV gene (41). However, neither of these studies was performed under conditions of sustained lipid absorption that approached the rate-limiting capacity of the enterocyte. Moreover, inactivation of apoA-IV synthesis in transgenic animals may induce alternate compensatory pathways that maintain normal levels of intestinal lipid absorption. In this regard, the suckling rat does not efficiently transport dietary lipid in lymph chylomicrons, and indirect evidence suggests that the portal venous route may be important in this species and possibly all rodents, including mice, during the suckling period (42). Nonetheless, lymphatic transport is an important pathway for lipid absorption in the suckling swine (24), a species whose intestinal development more closely resembles the human (43, 44). Thus, IPEC-1 cells, with their characteristic profile of efficient fatty acid uptake and re-esterification, but inefficient basolateral secretion, may be a better model to delineate the mechanisms by which apoA-IV modulates lipid absorption.

In summary, the present studies confirm and extend our previous observations that apoA-IV can enhance basolateral triacylglycerol-rich lipoprotein secretion in newborn swine enterocytes (21). By using a tetracycline-regulatable expression system, the present studies establish that physiologic levels of native swine and human apoA-IV secretion enhance chylomicron transport by inducing the secretion of larger chylomicron/VLDL particles, either by biophysically stabilizing their surface, or possibly by interacting with apoB to slow their passage through the cellular compartments where second-step expansion occurs. Taken together, our data suggest that although, unlike apoB, apoA-IV is not obligatory for intestinal lipoprotein assembly, it may facilitate maximally efficient lipoprotein secretion. This function may be especially important in the newborn, where the intestine must rapidly and efficiently absorb the large lipid load provided by breast milk. Human apoA-IV mutant studies establish the importance of the C-terminal sequences of apoA-IV in the enhancement of chylomicron secretion probably by influencing particle assembly and/or lipidation in the endoplasmic reticulum. The exact biophysical properties of apoA-IV and the intracellular mechanisms by which it mediates this effect are the focus of ongoing studies.

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