Effect of leptin on intestinal apolipoprotein AIV in response to lipid feeding

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Received 11 September 2000; accepted in final form 4 May 2001

Doi, Takashi, Min Liu, Randy J. Seeley, Stephen C. Woods, and Patrick Tso. Effect of leptin on intestinal apolipoprotein AIV (apo AIV) in response to lipid feeding. Am J Physiol Regulatory Integrative Comp Physiol 281: R753–R759, 2001.—We determined apolipoprotein AIV (apo AIV) content in intestinal epithelial cells using immunohistochemistry when leptin was administered intravenously. Most of the apo AIV immunoreactivity in the untreated intestine was located in the villous cells as opposed to the crypt cells. Regional distribution of apo AIV immunostaining revealed low apo AIV content in the duodenum and high content in the jejunum that gradually decreases caudally toward the ileum. Intraduodenal infusion of lipid (4 h) significantly increased apo AIV immunoreactivity in the jejunum and ileum. Simultaneous intravenous leptin infusion plus duodenal lipid infusion markedly suppressed apo AIV immunoreactivity. Duodenal lipid infusion increased plasma apo AIV significantly (measured by ELISA), whereas simultaneous leptin infusion attenuated the increase. These findings suggest that leptin may regulate circulating apo AIV by suppressing apo AIV synthesis in the small intestine.

small intestine; lipid; immunohistochemistry; enzyme-linked immunosorbent assay

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The physiological roles of apo AIV include the inhibition of food intake (10, 11), the inhibition of gastric motility and gastric acid secretion (22), the inhibition of lipid oxidation (23), the increase of circulating low-density lipoprotein cholesterol (5, 28), and protection against atherosclerosis (5, 8). The formation and secretion of chylomicrons stimulate the synthesis and secretion of apo AIV (14). We have recently demonstrated that peptide YY also stimulates jejunal apo AIV synthesis and secretion in rats (17).

Leptin is synthesized in adipocytes, and its administration decreases food intake and increases energy expenditure (6, 9). Recently, it has been reported that the intravenous administration of leptin reduces the apo AIV mRNA levels of the small intestine after a fat load (21). While this is an important finding, the results were limited to apo AIV mRNA levels. Hence, it cannot be determined if intestinal mucosal apo AIV content and/or circulating apo AIV were also affected by leptin. The aims of the present study therefore were 1) to determine the normal distribution of apo AIV along the small intestine and how this distribution is affected by lipid absorption, and 2) to determine how leptin affects the intestinal mucosal apo AIV system during lipid absorption.

MATERIALS AND METHODS

Production and characterization of the antiserum to rat apo AIV. Rat apo AIV was purified according to the procedure of Hayashi et al. (14). Briefly, lipoproteins of density < 1.21 g/ml were isolated from rat plasma by density ultracentrifugation. The sample was delipidated, and proteins were separated by SDS-PAGE. The area of gel corresponding to apo AIV was cut, and apo AIV was isolated.

Antiserum against rat apo AIV was raised from rabbits (New Zealand White) as described by Hayashi et al. (14). The animals were injected at multiple subcutaneous sites with an emulsion of apo AIV (250 μg) and Freund’s complete adjuvant. Subsequent injections were administered with an emulsion of apo AIV and Freund’s incomplete adjuvant. Antisera were collected 1 wk after the third boost. The IgG fraction of the antisera was collected by ImmunoPure (G) IgG purification kit (Pierce, Rockford, IL)

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and was used for Western blot analysis and immunohistochemistry.

**Western blot analyses.** For Western blot analysis, either 0.5 μg of rat lipoprotein fraction or 50 μg of jejunal mucosal homogenate was electrophoresed in 10% SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was incubated overnight at 4°C with polyclonal antibody against apo AIV (diluted 1:30,000) in 0.1 M PBS containing 0.3% Triton X-100 (PBST with a pH of 7.4) and 1% BSA. The membrane was then washed and incubated for 2 h at room temperature with peroxidase-conjugated anti-rabbit IgG (diluted 1:500; DAKO, Carpinteria, CA) in 0.1 M PBST containing 1% BSA. The labeling was visualized by reacting with a mixture containing 0.02% 3,3'-diaminobenzidine (DAB), 0.0045% H₂O₂, and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.6).

**Preparation of tissue for immunohistochemistry.** Male Sprague-Dawley rats weighing 275–300 g were maintained in a 12:12-h light-dark cycle (light, 0600–1800) and given free access to food and water. Food was withheld from the animals for 24 h before surgery. Under halothane anesthesia, the animals were killed, and the small intestine was removed. The duodenum, proximal jejunum, distal jejunum, proximal ileum, and distal ileum were excised and washed in cold fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB with a pH of 7.4) for 4 h at 4°C. After washing for 2 days in several changes of PB containing 15% sucrose at 4°C, the specimens were dipped in PB containing 10% gelatin for 6 h at 37°C. The gelatin-embedded specimens were immersed at 4°C for 2 h in PB containing 4% paraformaldehyde and then washed for 2 days in several changes of PB containing 15% sucrose at 4°C. Each intestinal segment was frozen in dry ice, and 12-μm-thick sections were cut. The sections were collected in PBS with Triton X-100 (PBST).

**Immunohistochemistry.** The sections, in a free-floating state, were treated with 0.5% H₂O₂ in PBST for 30 min to inactivate endogenous peroxidases. After washing, the sections were incubated for 2 days at 4°C with the rabbit polyclonal antibody against apo AIV (diluted 1:30,000) in PBST containing 1% BSA and washed. The sections were then incubated for 2 h at room temperature with biotinylated anti-rabbit IgG (diluted 1:1,000; Vector, Burlingame, CA) in PBST containing 1% BSA. The sections were again washed and incubated for 1 h at room temperature with the avidin-biotin-peroxidase complex (diluted 1:4,000, Vector Laboratories, Burlingame, CA) in PBST. For some sections, 2% normal rat serum was added to the solution of secondary antibody to get rid of nonspecific staining. Color was developed by reacting the sections for 5 min with a mixture containing 0.02% DAB, 0.0045% H₂O₂, and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.6). The stained sections were mounted on gelatin-coated glass slides, air-dried, counterstained with nuclear fast red, dehydrated, and placed under a coverslip.

To ensure that the immunostaining was specific for apo AIV, we performed immunoblotting of the antisera with apo AIV antigen before using the antisera for immunohistochemistry. Rat apo AIV was diluted in PBST at several concentrations ranging from 0.023 to 2.3 μM. The solutions were added to the polyclonal antibody against apo AIV with a working dilution. The mixture was then incubated overnight at 4°C before immunohistochemical application.

**Surgery.** The rats were implanted with a right atrial cannula for intravenous injection and a duodenal cannula for intraduodenal injection. Food was withheld from the animals for 24 h before surgery. Under pentobarbital sodium anesthesia (50 mg/kg ip), a silicone tube (0.9-mm OD) was inserted through the right external jugular vein with the inner end affixed just outside of the right atrium. The outer end of the silicone tube was attached to an L-shaped stainless steel tube and sewn to the skin on the back of the head. The tube was filled with nonheparinized polyvinylpyrrolidone (0.5 g/ml) according to the methods of Doi et al. (7). For the duodenal cannula, a silicone tube (2.1-mm OD) was passed through the fore stomach, extended 2 cm into the duodenum, and then secured in place with a gastric purse-string suture and a drop of cyanoacrylate glue. After surgery, rats were placed in restraining cages in a temperature-regulated chamber maintained at 30°C and allowed to recover for 24 h. During the recovery period, they received continuous duodenal infusions of a glucose-saline solution containing 145 mM NaCl, 4 mM KCl, and 0.28 M glucose at 3 ml/h.

**Experimental procedure.** Twelve rats were divided equally into two experimental and one control groups. The control rats received continuous intraduodenal infusion of glucose-saline solution at 3 ml/h and intravenous infusion of saline at 90 μl/h for 4 h. One experimental group received a duodenal infusion of 5% Intralipid (Pharmacia, Clayton, NC) at 3 ml/h as well as a continuous intravenous infusion of saline. The other experimental group received a continuous duodenal lipid infusion plus an intravenous infusion of mouse recombinant leptin (Calbiochem, La Jolla, CA) dissolved in saline at a rate of 100 μg/h for 4 h. The concentration of leptin was 1.11 μg/ml, and the dose of leptin was based on its ability to increase plasma leptin sufficiently to suppress food intake (4, 15). At the conclusion of the experiment, rats were killed, and the tissues of the small intestinal tract were prepared for immunohistochemistry. Blood was collected for apo AIV quantitation by ELISA.

**Quantitative morphometric analysis.** Quantification of immunostaining was carried out in a personal computer-based computer connected to the microscope through a high-resolution video camera (DAGE, MTI, Michigan City, IN). The image package, Image-Pro Plus (Media Cybernetics, Silver Spring, MD), allowed us to determine the optical density of a known area of intestinal mucosa in optical density units. The integrated intensity and the area of mucosa were measured for three sections from each segment of the small intestine harvested from each rat and averaged. Statistical analysis was carried out by unpaired t-test.

**Measurement of plasma apo AIV.** Plasma apo AIV concentration was measured by an ELISA. A 96-well high-binding ELISA plate (Corning, Corning, NY) was coated with 100 μl of goat antiserum against rat apo AIV (1:300 diluted in 0.1 M citrate buffer, pH 3.5) and incubated overnight at 4°C. After washing in 10 mM PBS containing 0.05% Tween 20 (PBS-Tween 20), nonspecific protein binding sites were blocked by the addition of 1% BSA in PBS-Tween 20. One hundred microliters of standard (0–35 ng/ml apo AIV) and plasma samples (1:10,000 diluted in PBS-Tween 20 containing 1% BSA) were added in duplicate to the coated wells and incubated overnight at 4°C. After washing, 100 μl of rabbit polyclonal antibody against rat apo AIV (1:3,000 diluted in PBS-Tween 20 containing 1% BSA) were added and incubated for 2 h at 37°C. After washing, 100 μl of peroxidase conjugated anti-rabbit IgG (1:200 diluted in PBS-Tween 20 containing 1% BSA, DAKO) were added and incubated for 1 h at 37°C. Color development was initiated by the addition of 200 μl of O-phenylenediamine dihydrochloride peroxidase substrate (Sigma, St. Louis, MO). After 30 min in the dark at room temperature, 50 μl of 3 M HCl were added to stop the reaction, and the absorbance at 492 nm was measured. Statistical analysis was carried out by unpaired t-test.
RESULTS

Specificity of polyclonal antibody against rat apo AIV. The specificity of the rabbit polyclonal antibody against rat apo AIV was examined by Western blot analysis (Fig. 1). The antibody detected a single band in the lipoprotein fraction as well as the jejunal homogenate. The estimated molecular weight was ~44,000, concurring with the reported molecular weight of rat apo AIV (3).

Apo AIV distribution in rat small intestine. Figure 2 depicts apo AIV immunostaining along the rat small intestinal tract in the non-lipid-stimulated condition. In the duodenum, weak apo AIV immunostaining was observed in epithelial cells but not in the goblet or the enteroendocrine cells of the villi (Fig. 2A). Significantly stronger apo AIV immunostaining was observed in the jejunum than in the duodenal epithelial cells, with staining exclusively in the epithelial cells of the villi but not in the cells of the crypts (Fig. 2, B and C). There was a gradient of immunostaining along the villus, with most staining concentrated in the top and tapering off toward the crypt. In the proximal ileum (Fig. 2D), apo AIV immunostaining was observed to be weaker in intensity than in the jejunum. In the distal ileum (Fig. 2E), a few weak immunoreactive epithelial cells were observed on the top of the villi. A few cells in the lamina propria displayed weak immunostaining but were probably nonspecific since the staining was subsequently abolished when incubating 2% normal rat serum with the secondary antibody before utilizing the secondary antibody in the immunohistochemistry procedure.

To ensure that the observed immunostaining in the intestinal epithelial cells was specific, we performed
the immunoabsorption test. We first preabsorbed the polyclonal antibody with 2.3 μM of apo AIV. This treated antibody failed to detect any apo AIV staining in the rat proximal jejunal section except for a few cells in the lamina propria identified as nonspecific staining (Fig. 2F).

Figure 3 summarizes the results of the image analysis of the intensity of apo AIV staining in the different regions of the small intestine. The intensity of immunostaining was low in the duodenum, highest in the jejunum, and decreased toward the ileum. The intensity was extremely low in the distal ileum.

Apo AIV immunostaining during lipid absorption. Apo AIV immunostaining in the proximal jejunum and the proximal ileum after constant intraduodenal infusion of an Intralipid solution is depicted in Fig. 4. Immunostaining increased markedly in both the jejunum (Fig. 4, B and E) and the ileum (Fig. 4H) after lipid infusion compared with controls (Fig. 4, A, D, and G). The upper and middle part of the villus in the jejunum had moderate staining for apo AIV. After lipid infusion, staining increased markedly in the entire villus. In the ileum, apo AIV immunostaining was moderate in the upper villi and weak in the lower villi before lipid infusion. After lipid infusion, the immunostaining spread throughout the whole villi. Under higher magnification, dotted staining was observed in the supranuclear portion of the cytoplasm in epithelial cells exposed to glucose saline, and positive staining was observed with goblet cells (Fig. 4D). Therefore, staining increased in magnitude and was more widespread after lipid infusion (Fig. 4E).

Figure 5 depicts the quantitative analysis of the immunostainings. Lipid infusion increased the apo AIV immunostaining by 6-fold in the proximal jejunum and by ~30-fold in the proximal ileum (P < 0.01 for each).

Effect of leptin infusion on apo AIV immunostaining. Intravenous infusion of murine leptin reduced the intensity of apo AIV staining in the different regions of the small intestine of the rat, finding that the highest levels are in the jejunum, with lower levels in the duodenum and ileum. We also demonstrated that the amount and extent of this distribution is increased by intraduodenal lipid absorption. In parallel with these observations, plasma apo AIV levels are also increased by lipid infusion. Finally, we observed that the simultaneous administration of leptin at a dose that has no impact on intestinal cells in the absence of a lipid infusion attenuates the apo AIV response to lipid.

DISCUSSION

Using immunohistochemistry, we determined the normal distribution of apo AIV along the small intestine of the rat, finding that the highest levels are in the jejunum, with lower levels in the duodenum and ileum. We also demonstrated that the amount and extent of this distribution is increased by intraduodenal lipid absorption. In parallel with these observations, plasma apo AIV levels are also increased by lipid infusion. Finally, we observed that the simultaneous administration of leptin at a dose that has no impact on intestinal cells in the absence of a lipid infusion attenuates the apo AIV response to lipid.

Polyclonal antibody against rat apo AIV raised from rabbit antisera was used in this study. To be certain of the conclusions indicated by the data, specificity of the antibodies for apo AIV was imperative. We therefore determined specificity by Western blot analysis and immunoabsorption testing. Using Western blot, this antibody recognized the protein, which was determined to have a single band with a molecular mass of ~44 kDa in both the lipoprotein samples (lipoproteins isolated from plasma with a density < 1.21 g/ml) and the intestinal jejunal homogenate. Immunooabsorption testing resulted in the elimination of apo AIV immunostaining in the proximal jejunum after preabsorption of the apo AIV antibody with apo AIV. Only a few cells in the lamina propria had nonspecific staining, and the staining in these cells was totally abolished by the addition of 2% normal rat serum to the solution of secondary antibody. These results indicate that the polyclonal antibody was highly specific for apo AIV.

Kalogeris et al. (16) reported that apo AIV synthesis increased in the jejunum after the infusion of a modest load of lipid but did not increase in the ileum. This is probably because most of the lipid infused was absorbed in the jejunum before reaching the ileum (16). When lipid was infused directly into the ileum, ileal apo AIV synthesis increased (18). Those findings imply
that a relatively large dose of lipid is needed if apo AIV synthesis in the jejunum and the ileum is to occur and be measurable simultaneously. Consequently, in the present experiment we infused a total of 600 mg of triacylglycerol over a 4-h period to ensure that lipid reached both the jejunum and the ileum. This rate of lipid infusion is about four times the typical rate employed in most of our previous studies (14, 18). It is important to note that this relatively high rate of triacylglycerol infusion was not associated with steatorrhea. Because infused lipid reached the proximal ileum in all of the rats but only reached the distal ileum in some of the rats, we analyzed only the proximal ileum.

Apo AIV is known to be synthesized mainly by the intestinal epithelial cells (13, 30), and apo AIV synthesis is higher in the jejunum than in the ileum (1). The distribution of apo AIV observed in the present study concurs with these reports. In the jejunum, all of the epithelial cells of the villi were found to synthesize apo AIV, such that lipid infusion increased the apo AIV content in these cells. Importantly, no apo AIV immunostaining was observed in the cells of the crypt. In the ileum in the control condition, apo AIV was observed mainly in cells occupying the upper portion of the villi. After lipid infusion, apo AIV was observed in all epithelial cells of the upper and lower villi. These results suggest that all epithelial cells in the jejunal and ileal villi have the capability to synthesize apo AIV and that this synthesis is markedly stimulated by the absorption of fat. The distribution of apo AIV observed along the small intestine would suggest that most of the lipid absorption normally occurs in the jejunum. When the intestinal epithelial cells were examined under high magnification, apo AIV existed as granules concentrated mainly in the supranuclear region of the cell. After lipid infusion, apo AIV staining spread throughout the entire cytoplasm. These findings probably re-

fluctuates in response to dietary changes, indicating a role of leptin in apo AIV physiology. Further studies are necessary to determine which of these mechanisms or other mechanisms(s) mediate leptin's action on intestinal AIV protein expression.

Perspectives

Apo AIV is a very interesting peptide. Its synthesis and secretion from the intestine are closely linked to the absorption of lipids, and many of its known hormonal actions suggest a role in the regulation of lipid metabolism and homeostasis (5, 8, 22, 24). The recent observations that exogenous apo AIV reduces food intake whether administered systemically or centrally (10, 11), that the central administration of apo AIV antibodies increases food intake (11, 19), and that apo AIV is synthesized in the hypothalamus (19) add a new dimension to its profile. Hence, apo AIV is one of the newest members of a group of gut-brain peptides that are important in the regulation of energy homeostasis and body adiposity (25, 29). Although no data are yet available to address it, a reasonable hypothesis would be that apo AIV is more closely related to the ingestion of fats than to other macronutrients.

It is not surprising that the administration of lipids into the intestine would increase the synthesis and secretion of apo AIV. More surprising is the observation that leptin attenuates this response. Although considerable additional work will be necessary to sort out the role of leptin in apo AIV physiology, the implication is that as one becomes more obese (and/or consumes a higher proportion of dietary fat), the elevated levels of leptin cause them to secrete proportionately less apo AIV from the intestine. Although this might be seen as a natural brake helping to limit, curtail, or otherwise alter some aspect of lipid metabolism, it is curious that it might also lead to the ingestion of even

![Graph showing quantitative analysis of apo AIV immunostaining in rat proximal jejunum and proximal ileum after lipid and leptin infusion.](Image)

**Table 1. Plasma apo AIV levels after lipid and leptin infusion**

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<tr>
<th>Treatment</th>
<th>Plasma APO AIV, µg/ml</th>
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<tr>
<td>Glucose-saline ID + saline IV</td>
<td>137.3 ± 3.0</td>
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<tr>
<td>Lipid ID + saline IV</td>
<td>162.0 ± 3.8*</td>
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<tr>
<td>Lipid ID + leptin IV</td>
<td>145.5 ± 5.6†</td>
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Values are means ± SE; n = 4 in each group. apo AIV, apolipoprotein AIV; ID, intraduodenal; IV, intravenous. *P < 0.01 compared with glucose-saline ID + saline IV group. †P < 0.05 compared with lipid ID + saline IV group.
more calories by reducing a normal satiety signal. At present all that can be said is that apo AIV physiology interacts in multiple ways with the regulation of energy homeostasis.

We thank S. Zheng for technical assistance. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-54504, DK-56863, and DK-56910.

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