Apolipoprotein A-IV is involved in detection of lipid in the rat intestine

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Long chain triglyceride (>C12) in the intestinal lumen potently inhibits gastric emptying and acid secretion via the vagal afferent pathway. While the mechanism of inhibition involves the formation of chylomicrons, the essential role of the apolipoprotein apo A-IV is unclear. Using apo A-IV$^{-/-}$ mice, we tested the hypothesis that inhibition of gastric emptying and gastric acid secretion in response to dietary lipid is dependent upon apo A-IV. As measured by nuclear scintigraphy in awake mice, gastric emptying of an ingested whole-egg meal was significantly faster in apo A-IV$^{-/-}$ knockout *versus* A-IV$^{+/+}$ controls (34 ± 1 *versus* 54 ± 3 min, $P < 0.0001$). In anaesthetized A-IV$^{+/+}$ mice, meal-stimulated gastric acid secretion was 59% inhibited by intestinal lipid infusion; this was abolished in apo A-IV$^{-/-}$ mice. Oral gavage of lipid in awake mice activated neurones throughout the nucleus of the solitary tract (NTS) in A-IV$^{+/+}$ mice, measured by immunohistochemical localization of Fos protein expression. However, in the mid region of the NTS (bregma −7.32 to −7.76 mm), Fos expression in response to intestinal lipid was significantly decreased by 50% in apo A-IV$^{-/-}$ mice compared to A-IV$^{+/+}$ controls. We conclude that activation of the vagal afferent pathway and inhibition of gastric function in response to dietary lipid is partly dependent upon apo A-IV.

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Obesity is a growing global epidemic that involves genetic, environmental, and behavioural factors. In the United States it is estimated that 64% of the population is overweight or obese (Shortt, 2004). A diet high in fat content is an environmental factor that is positively correlated with the incidence of obesity (Lichtenstein *et al.* 1998), and dietary fat plays an important role in the regulation of food intake and body mass control (Wenk, 2004). Regulation of fat intake and fate is controlled by a number of mechanisms, including those that are sensitive to the amount of stored fat (Elmquist *et al.* 1999) and those that are sensitive to the macronutrient content of the food being consumed (Moran & Kinzig, 2004). The chemosensory transduction system in the gastrointestinal tract is the first point at which information regarding the macronutrient content of food can be monitored. The vagal afferent pathway is sensitive to luminal triglyceride, and aids in the control of meal size and the sensation of satiety (Berthoud & Neuhuber, 2000). The densest innervation of vagal afferents is at the duodenum (Berthoud *et al.* 1995); during the intestinal phase of digestion, signals from the duodenum act to tightly regulate feedback inhibition of gastric motility and secretion, stimulation of pancreatic secretion, gall bladder contraction, and relaxation of the sphincter of Oddi (Raybould, 2002). These feedback and feedforward responses allows for the matching of the digestive and absorptive capacity of the intestine with the entry of food from the stomach and secretions from the gall bladder and pancreas.

Chylomicron formation is required for the absorption of long chain fatty acids of carbon chain length C12 or greater, which are critical for lipid-induced inhibition of gastric function (Hunt & Knox, 1968) and the release of cholecystokinin (CCK) from endocrine cells (Isaacs *et al.* 1987; Matzinger *et al.* 2000). Apolipoprotein A-IV (apo A-IV) is a protein secreted by the enterocyte of the small intestine in humans (Elshourbagy *et al.* 1987) and by both the small intestine and the liver in rodents; however, the primary site of secretion in rodents is the intestine (Wu & Windmueller, 1979). Apo A-IV appears to be the only lipoprotein that is directly influenced by dietary lipid. Active lipid absorption stimulates apo A-IV expression, synthesis and release (Hayashi *et al.* 1990; Apfelbaum *et al.* 1987; Rodriguez *et al.* 1997) and stimulation of apo A-IV production by lipid feeding is associated with

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the formation of chylomicrons (Hayashi *et al.* 1990). Chylomicron formation and apo A-IV synthesis can be rapid (Tso *et al.* 2001), which lends itself to the idea of its involvement in signalling of intestinal lipid content to other organs. Apo A-IV seems to have important roles in lipid and lipoprotein metabolism, including the inhibition of lipid oxidation (Qin *et al.* 1998), the increase of circulating low-density lipoprotein cholesterol (Weinstock *et al.* 1997), and prevention against atherosclerotic lesions (Duverger *et al.* 1996; Cohen *et al.* 1997; Baroukh *et al.* 2001). However, in addition to effects on lipid fate and metabolism, apo A-IV has been shown to inhibit food intake (Fujimoto *et al.* 1992, 1993; Tso *et al.* 2001), inhibit gastric motility (Glatzle *et al.* 2002, 2003, 2004) and gastric emptying (Okumura *et al.* 1996) and gastric acid secretion (Okumura *et al.* 1994, 1995). Apo A-IV may be physiologically involved in the regulation of food intake; an increase in short-term food intake was observed in male apo A-IV knockout mice following an overnight fast. However, overall growth and *ad libitum* food intake were not significantly different between apo A-IV knockout mice and their wildtype controls (Weinstock *et al.* 1997). Apo A-IV acts to inhibit gastric motility via CCK-responsive vagal afferent fibre discharge and a CCK_1 receptor pathway (Glatzle *et al.* 2004), and this is consistent with a role for apo A-IV in lipid-induced inhibition of gastric emptying.

The present study tested the hypothesis that apo A-IV is involved in lipid sensing in the intestine. The specific aims were to demonstrate that: (1) inhibition of gastric emptying and gastric acid secretion in response to dietary lipid is dependent upon apo A-IV and (2) lipid-induced activation of the vagal afferent pathway is dependent upon apo A-IV. To establish a role for apo A-IV in the gastrointestinal response to lipid, we used a mouse strain with a deletional mutation of the apo A-IV gene, and their wildtype counterparts.

Methods

Animals

Experiments were performed using male C57BL/6J mice (JAX West, University of California, Davis), male apo A-IV knockout mice and the control strain (Weinstock *et al.* 1997). These mice were generated by Dr Jan Breslow (Rockefeller University, New York) using homologous recombination in embryonic stem cells. They were found to have normal lipid absorption, weight gain and food consumption up to 5 months of age. C57BL/6J mice were used in addition to the control strain for the apo A-IV knockout mice; the control strain (apo A-IV$^{+/+}$, referred to as wildtypes in the present study) and the apo A-IV mutant strain were found to be 98.15–99.07% genetically homologous with the C57BL/6J background. Mice were of initial weight 18–20 g (6 to 10 weeks of age), and were maintained on regular laboratory chow (Purina Laboratory Chow). Mice were fasted overnight but allowed water *ad libitum* prior to all experimental procedures. The institutional guidelines for care and use of laboratory animals were followed throughout the study.

Measurement of gastric emptying by nuclear scintigraphy

This method was described in detail in a previous publication (Whited *et al.* 2004). Briefly, during the experimental session, uncooked egg product was radioactively labelled with 15 MBq (0.4 millicuries) of Tc^{99m}-Mebrofenin (Amersham Health, Sacramento, CA, USA) per 25 ml egg product, and cooked in a microwave oven. The sample was weighed and the radioactivity of the sample measured using a gamma camera. Mice were allowed to freely feed on cooked egg product for 5 min. The initial radioactivity of the fed mouse was measured in order to permit calculation of amount of the diet consumed by the mouse. Mice were immediately placed in the restraints, and a series of images of the mice were obtained by collecting a dynamic series of images continuously for 60 min, then again at 120–125 min. The mice were imaged using a Technicare Omega 500 Gamma camera equipped with a high-resolution parallel hole collimator and Nuclear Mac 5.2.1 software was used.

Image analysis was facilitated using custom software developed using MATLAB 6.5. To aid in viewing and region of interest (ROI) selection, images were expanded by linear interpolation to 1024 × 1024 pixels. This image was then magnified to twice screen resolution, which allowed images of reasonable size from a single mouse to be viewed in cine format prior to ROI selection. Viewing the images from a single mouse in cine format aided the user in identifying anatomy by observing the temporal change in position of the meal during the acquisition sequence, and determining which images were free of subject motion. Beginning with the first image determined to be motion free, a circular region of interest was then manually positioned over each subsequent image that the user determined was free of motion, in a location that the user decided included only stomach. The sum of the pixel values in each region along with the time after feeding that the image was acquired were recorded in a table. These count rates were then corrected for the physical decay of Tc^{99m} and gastric half emptying time ($t_{1/2}$ min) was calculated.

Each mouse was used several times in different imaging sessions. At the end of all the sessions, mice were killed by an overdose of sodium pentobarbital (100 mg kg^{-1} I.P.), followed by bilateral thoracotomy.

Measurement of gastric acid secretion

Mice (apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$, $n = 5$ in each group) were anaesthetized initially with tribromoethanol

(Avertin, 250 mg kg^{-1}, 12.5 mg ml^{-1} I.P., Sigma, St. Louis, MO, USA) for induction and maintained with thiobutabarbital (Inactin, mg ml^{-1}, 50 mg kg^{-1} S.C., Sigma). A tracheal cannula was placed, and after a midline abdominal incision the pylorus was isolated and tied off. A double-lumen catheter to simultaneously perfuse and collect gastric perfusate was placed in the stomach via the oesophagus. A cannula was placed into the duodenum for perfusion of lipid and the abdominal incision was closed. Saline was infused (4 ml h^{-1}) into the gastric cannula, and the collected gastric acid perfusate was back-titrated to a pH of 7 with 0.001 M NaOH using a Radiometer Copenhagen ABU 901 Autoburette attached to a Radiometer Copenhagen PHM 290 pH meter. The preparation was allowed to stabilize for 30 min, after which basal gastric acid secretion was recorded for 15 min. From t_{15} to t_{150} (min), 8% peptone (Becton Dickinson, Franklin Lakes, NJ, USA) was infused continuously into the stomach to stimulate gastric acid secretion. From t_{90} to t_{150}, 6% Intralipid (2 ml h^{-1}) was infused into the duodenum. Following completion of the experiment, mice were euthanised by cervical dislocation.

Measurement of Fos protein expression in the NTS

This method has been described in detail previously (Sagar *et al.* 1988). Briefly, following treatment, mice were anaesthetized with sodium pentobarbital (100 mg kg^{-1} I.P., 50 mg ml^{-1} Western Medical Supply, Arcadia, CA, USA) and transcardially perfused with 20 ml of heparinized 0.9% saline (0.1 ml heparin (100 ml saline)$^{-1}$) followed by 25 ml 4% paraformaldehyde (Sigma). The brainstem was removed and postfixed in 4% paraformaldehyde for 1 h. Sections were cut at 100 μm using a vibratome. Sections were incubated for 1 h in goat serum-phosphate-buffered saline (PBS) (Chemicon, Temecula, CA, USA), incubated in primary antibody (1 : 2000 rabbit antifos; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h, followed by incubation with the secondary antibody (1 : 200 biotinylated goat anti-rabbit; Vector Laboratories, Burlingame, CA, USA) for 2 h. Tissue was incubated for 3 h in ABC solution (Standard *Elite* Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). DAB solution (Sigma) was added for a 5 min incubation, and then 50 μl H_2O_2-PBS (0.1 ml 30% H_2O_2 : 10 ml PBS) was added to catalyse the DAB reaction; the reaction was stopped with a PBS wash. Tissue was thoroughly washed between each incubation period.

Images were taken on a Provis microscope and analysed using Paint Shop Pro, Edition 7. A stereotaxic mouse brain atlas was used to determine the location of the nucleus of the solitary tract (NTS) in each section of tissue (Paxinos & Franklin, 2001). A region of interest was drawn around the NTS) and the area postrema (AP) and all activated neurones in the NTS region of interest were counted. Neurones were determined to be immunopositive (above threshold) by their colour and size. Representative sections were chosen to represent regions of the NTS pre- (bregma −8.00 to −7.92 mm), at (−7.76 to −7.32 mm) and post-AP (−7.08 to −6.48 mm). Three sections were chosen for each region for a total of nine sections per mouse. The numbers of labelled neurones per section were summed for each region for each mouse; this value was used in subsequent statistical analyses.

Measurement of plasma triglyceride

C57BL/6J mice/apo A-IV$^{+/+}$ ($n = 13$) and apo A-IV$^{-/-}$ ($n = 12$) mice were used for these experiments. C57BL/6J mice were used in addition to the control strain for apo A-IV$^{-/-}$ mice, because the apo A-IV$^{+/+}$ mice were found to be 98.15–99.07% genetically homologous with the C57BL/6J background. Fasted mice or fasted mice gavaged with 0.2 ml 20% Intralipid (15 min pretreatment, 0.04 g lipid) were deeply anaesthetized with sodium pentobarbital (100 mg kg^{-1} I.P., 50 mg ml^{-1} Western Medical Supply, Arcadia, CA, USA). The abdominal cavity was opened and the inferior vena cava was isolated. Using a heparinized syringe, blood was collected from the inferior vena cava, centrifuged, and the plasma was separated and stored at −80°C. Plasma triglyceride (TAG) levels were assessed using a TAG kit (Sigma).

CCK immunohistochemistry

Fed mice (apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$, $n = 3$ in each group) were anaesthetized with sodium pentobarbital (100 mg kg^{-1} I.P., 50 mg ml^{-1} Western Medical Supply, Arcadia, CA, USA) and transcardially perfused with 20 ml heparinized 0.9% saline (0.1 ml heparin (100 ml saline)$^{-1}$) followed by 25 ml 4% paraformaldehyde (Sigma). The duodenum was removed and postfixed in 4% paraformaldehyde for 1 h. The tissue was transferred to a 25% sucrose solution with 1% sodium azide (Sigma) for 2 h at room temperature, and then refrigerated overnight. Sections were cut at 12 μm (longitudinal gut) or 20 μm (transverse gut) using a cryostat. Sections were incubated for 1 h in goat serum-PBS (Chemicon, Temecula, CA, USA), incubated in primary antibody (1 : 5000 rabbit X CCK; AB1972, Chemicon, Temecula, CA, USA) for 3 h, followed by incubation with the secondary antibody (1 : 200 Alexa 488 goat anti-rabbit; Molecular Probes, Eugene, OR, USA) for 2 h. Tissue was thoroughly washed between each incubation period. Images were taken on a Provis microscope, and a representative image was selected from each mouse strain (apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$) for both transverse and longitudinal sections of the duodenum, in order to compare endocrine cell morphology.

Experimental protocols

Effect of intestinal lipid on gastric emptying. Apo A-IV$^{+/+}$ ($n = 9$) and apo A-IV$^{-/-}$ ($n = 13$) mice were used for these experiments. Mice were acclimatized to restraint and to the experimental diets during training sessions 2–3 times a week for 2 weeks prior to the first experimental session. Each mouse participated in four imaging sessions; egg white, whole egg, or egg yolk, and whole-egg diet plus CCK (20 min prior to the whole-egg meal, 22 pmol I.P., Sigma; Mantella *et al.* 2003). The mice were offered approximately 1 g labelled egg product and allowed to freely feed for 5 min prior to the imaging session.

Effect of intestinal lipid on Fos expression in the NTS. Apo A-IV$^{+/+}$ ($n = 20$) mice and apo A-IV$^{-/-}$ ($n = 20$) mice were used for these experiments. Fasted mice were gavaged with 0.2 ml 20% Intralipid (0.04 g lipid, Fresenius Kabi, Germany) or 0.9% saline, or treated with CCK (22 pmol I.P., Sigma). A group of mice was pretreated with the CCK_1 receptor antagonist, devazepide (15 min; 100 μg kg^{-1} I.P.; Whited *et al.* 2004) followed by either 0.2 ml saline or 20% Intralipid. The dose of 0.2 ml Intralipid was used because it contains approximately the same amount of lipid as is in the average amount of whole egg consumed by the mice (0.04 g TAG in 0.2 ml Intralipid/0.035 g TAG in 0.31 mg whole egg). After 120 min, mice were deeply anaesthetized with sodium pentobarbital (100 mg kg^{-1} I.P., 50 mg ml^{-1} Western Medical Supply, Arcadia, CA, USA), transcardially perfused with fixative, and tissue removed.

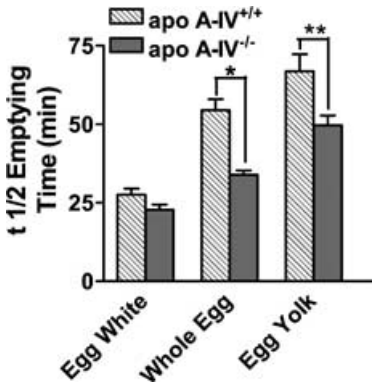

Figure 1. Lipid-induced inhibition of gastric emptying is significantly attenuated in apo A-IV knockout mice
Gastric emptying, expressed as half emptying time, of test meals differing in fat content was measured in freely fed apo A-IV wildtype and knockout mice using nuclear scintigraphy. In wildtype mice, the rate of gastric emptying was significantly inhibited by an increase in the fat content of the ingested test meals. In apo A-IV$^{-/-}$ mice, the inhibitory effect of lipid on gastric emptying was significantly attenuated (*$P < 0.0001$ and **$P < 0.01$, wildtype *versus* knockout, $n = 9$ apo A-IV$^{+/+}$ and $n = 13$ apo A-IV$^{-/-}$). Error bars are S.E.M.

Statistical analysis

Scintigraphy. Decay-corrected counts *versus* time were analysed using non-linear regression, and fitted to a one-phase exponential decay curve, and the half-emptying time ($t_{1/2}$) calculated. Significant differences in $t_{1/2}$ emptying between treatment groups were calculated using an unpaired *t* test and a one-way ANOVA followed by Bonferroni's multiple comparison test. $P < 0.05$ was taken as significantly different. All reported results are the $t_{1/2}$ emptying time ± S.E.M.

Fos protein expression in the NTS. Significant differences in Fos protein expression between treatment groups were calculated using a one-way ANOVA followed by Bonferroni's multiple comparison test. $P < 0.05$ was taken as significantly different. All reported results are the number of Fos-positive neurones ± S.E.M.

Gastric acid secretion and plasma triglyceride levels. Significant differences between treatment groups were calculated using an unpaired *t* test. $P < 0.05$ was taken as significantly different. All reported results are the number of positive neurones, meq gastric acid secretion min^{-1} ml^{-1} or mg plasma triglyceride ml^{-1} ± S.E.M.

Results

Lipid-induced inhibition of gastric emptying in apo A-IV$^{+/+}$ and apo-IV$^{-/-}$ mice

Increasing amounts of lipid in the ingested diet produced a significant slowing of gastric emptying in apo A-IV$^{+/+}$ as previously demonstrated in C57BL/6J mice (Whited *et al.* 2004). However, in apo –IV$^{-/-}$ mice, there was a significant attenuation in the inhibition of gastric emptying induced by lipid (Fig. 1). The difference in mean $t_{1/2}$ emptying time between the three diets for both apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$ mice was significantly different for whole egg ($P < 0.0001$) and egg yolk ($P < 0.01$), but not for egg white.

There was no significant difference between apo A-IV$^{+/+}$ or apo A-IV$^{-/-}$ mice in the amount of test diet eaten (egg white, whole egg and egg yolk, Table 1).

Effect of exogenous CCK on gastric emptying

In order to determine whether the attenuated inhibitory response to lipid in apo A-IV$^{-/-}$ mice was not dependent on a decrease in sensitivity to CCK, $t_{1/2}$ was measured in apo A-IV$^{-/-}$ mice in response to exogenous CCK. CCK (22 pmol I.P., 20 min prior to the whole-egg meal) significantly inhibited gastric emptying of whole egg in apo A-IV$^{-/-}$ mice (vehicle *versus* CCK: $t_{1/2}$ 34 ± 1 min *versus* 57 ± 3 min, $n = 13$, $P < 0.0001$). Gastric emptying rate was not altered by exogenous administration of

Table 1. Amount of test diet eaten and corresponding lipid content in gastric emptying studies

	Egg white	Whole egg	Egg yolk
Apo A-IV$^{+/+}$	0.22 ± 0.05 g	0.27 ± 0.06 g	0.35 ± 0.05 g
$n = 9$ mice	0.00 g lipid	0.03 g lipid	0.11 g lipid
Apo A-IV$^{-/-}$	0.30 ± 0.04 g	0.35 ± 0.04 g	0.42 ± 0.05 g
$n = 13$ mice	0.00 g lipid	0.04 g lipid	0.13 g lipid

There was no significant difference in the amount of test diet eaten (egg white, whole egg and egg yolk) between apo A-IV wildtype and knockout mice. In addition, there was no significant different in the amount of test diet eaten by Apo A-IV wildtype and knockout mice for all three test diets.

CCK in apo A-IV$^{+/+}$ ($t_{1/2} = 54 \pm 3$ min *versus* 63 ± 3, respectively, $n = 9$, not significant (n.s.)). There was no significant effect of CCK treatment on the amount of whole egg consumed between apo A-IV$^{+/+}$ mice (0.38 ± 0.04 g, $n = 9$) and apo A-IV$^{-/-}$ mice (0.47 ± 0.03 g, $n = 13$, n.s.).

Lipid-induced inhibition of gastric acid secretion

In apo A-IV$^{+/+}$ mice, intragastric perfusion with peptone significantly stimulated gastric acid secretion ($n = 5$, $P < 0.0001$, Fig. 2). Intestinal perfusion with lipid inhibited peptone-stimulated gastric acid secretion in apo A-IV$^{+/+}$ mice by 59% ($n = 5$, $P < 0.0001$, Fig. 2). In apo A-IV$^{-/-}$ mice, basal and peptone-stimulated gastric acid secretion were significantly higher compared to apo A-IV$^{+/+}$ mice (basal; $P < 0.0001$, peptone stimulated; $P < 0.01$, Fig. 2). However, in apo A-IV$^{-/-}$ mice, in contrast to A-IV$^{+/+}$ controls, intestinal lipid had no significant effect on peptone-stimulated gastric acid secretion ($n = 5$, n.s., Fig. 2).

Lipid-induced activation of neurones in the NTS

The number of Fos-positive NTS neurones was analysed with respect to region within the NTS (pre-, at- and post- AP) (Fig. 3). In apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$ mice gavaged with saline, expression of Fos protein in neurones in the NTS was not significantly different between the two groups in all regions of the NTS (n.s., $n = 4$ mice in each group, Figs 3 and 4). In apo-IV$^{+/+}$ mice, gavage with Intralipid significantly increased the number of Fos-positive neurones in all regions of the NTS compared to saline gavage (pre-AP, $P < 0.05$; at-AP, $P < 0.001$; post-AP, $P < 0.001$, $n = 4$ mice in each group, Figs 3 and 4). However, in apo A-IV$^{-/-}$ mice, gavage with Intralipid significantly increased Fos expression in the region of the NTS at- and post-AP, but not in pre-AP (Intralipid *versus* saline; at-AP, $P < 0.001$; pre-AP, NS; post-AP, $P < 0.01$, $n = 4$ mice in each group, Figs 3 and 4). The increase in the number of Fos-positive neurones in the NTS at-AP in response to Intralipid gavage was significantly different between apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$ mice ($P < 0.001$, $n = 4$ mice in each group, Figs 3 and 4*B*). There was no significant difference in the regions of the NTS pre- and post-AP between apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$ mice in response to Intralipid gavage (n.s., $n = 4$ mice in each group, Fig. 4*A* and *C*).

In apo A-IV$^{+/+}$ mice, administration of the CCK_1 receptor antagonist, devazepide (15 min pretreatment; 100 μg kg^{-1} I.P.) with Intralipid gavage (0.04 g lipid) significantly decreased the number of Fos-positive neurones in the NTS (at-AP, post-AP) in comparison to Intralipid alone (pre-AP, n.s.; at-AP, $P < 0.001$;

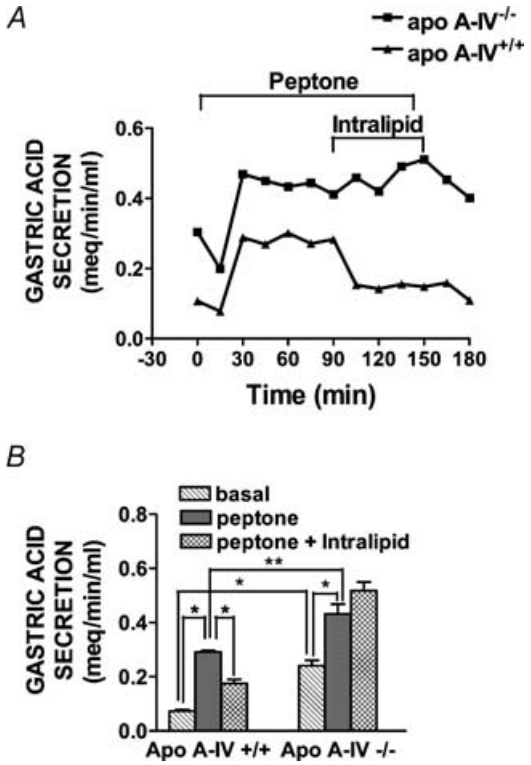

Figure 2. Lipid-induced inhibition of gastric acid secretion is abolished in apo A-IV knockout mice

A, time course changes in gastric acid secretion in individual apo A-IV wildtype and knockout mice. Gastric acid secretion in anaesthetized mice was continuously measured by back titration in response to intragastric perfusion with peptone (t_0-t_{150}) and duodenal perfusion with Intralipid (t_{90}-t_{150}). In both the wildtype mouse and knockout mouse, intragastric peptone stimulated gastric acid secretion, but duodenal lipid infusion inhibited meal-stimulated gastric acid secretion only in the wildtype control. *B*, intestinal lipid inhibition of gastric acid secretion in apo A-IV knockout ($n = 5$) and wildtype controls ($n = 5$) during basal, intragastric peptone and intragastric peptone, together with intestinal lipid perfusion. In apo A-IV$^{-/-}$ mice, basal and peptone-stimulated gastric acid secretion were significantly higher compared to apo A-IV$^{+/+}$ mice (basal; $^*P < 0.0001$, peptone stimulated; $^{**}P < 0.01$). In both wildtype and apo A-IV knockout mice, intragastric peptone significantly stimulated gastric acid secretion, but duodenal lipid infusion inhibited meal-stimulated gastric acid secretion only in the wildtype control ($^*P < 0.0001$). Error bars are S.E.M.

post-AP, $P < 0.01$, $n = 4$ mice in each group, Fig. 4*A*–*C*). However, administration of the CCK_1 receptor antagonist devazepide had no significant effect on Fos expression in response to intestinal lipid in apo A-IV$^{-/-}$ mice at any region of the NTS (n.s., $n = 4$ mice in each group, Fig. 4*A*-*C*). Thus, after devazepide treatment, there was no significant difference in Fos expression in response to Intralipid gavage between apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$ mice.

Administration of CCK (15 min pretreatment; 22 pmol I.P.) significantly increased Fos expression in the NTS of both apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$ mice; the increase was not significantly different between the two groups at any region of the NTS (pre-AP: 73 ± 6 *versus* 61 ± 7 neurones, n.s.; at-AP: 275 ± 26 *versus* 239 ± 16 neurones, n.s.; post-AP: 75 ± 13 *versus* 76 ± 9 neurones, n.s., $n = 4$ mice in each group).

Plasma TAG assay

In apo A-IV$^{+/+}$/C57BL/6J and apo A-IV$^{-/-}$ mice, Intralipid gavage significantly increased plasma TAG in comparison with fasting TAG levels ($P < 0.008$ and $P < 0.03$, respectively, $n = 4$–9; Table 2). However, there was no significant difference between fasting or lipid-fed TAG levels in apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$ mice.

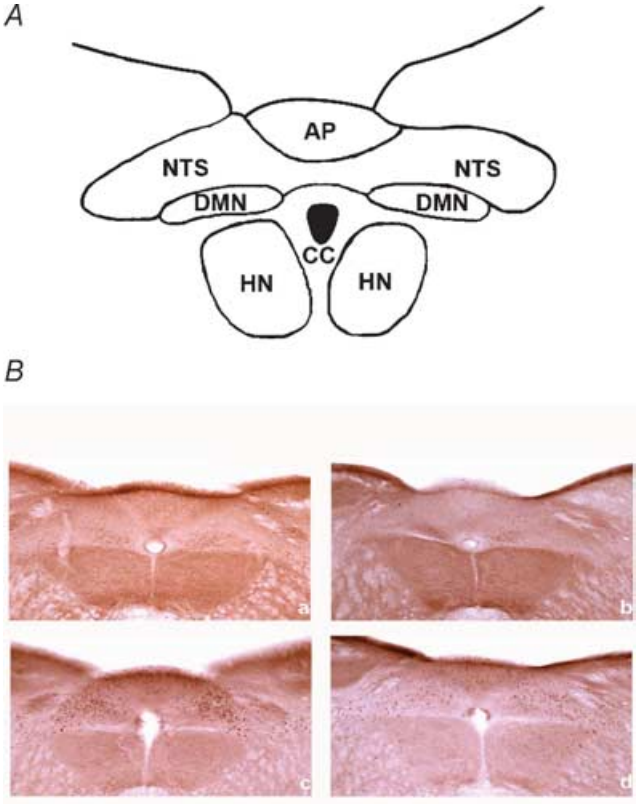

Figure 3. Lipid-induced Fos protein expression in the nucleus of the solitary tract (NTS) is attenuated in apo A-IV knockout mice
A, cytoarchitecture map for photomicrographs of mice brainstem. NTS = nucleus of the solitary tract; CC = central canal; AP = area postrema; DMN = dorsal motor nucleus of vagus; HN = hypoglossal nucleus. *B*, photomicrographs of mouse brainstem showing activation of Fos expression in neurones in the nucleus of the solitary tract at the area postrema in apo A-IV wildtype control and knockout mice following saline or Intralipid gavage. *a*, apo A-IV$^{+/+}$ saline; *b*, apo A-IV$^{-/-}$ saline; *c*, apo A-IV$^{+/+}$ Intralipid *d*, apo A-IV$^{-/-}$ Intralipid.

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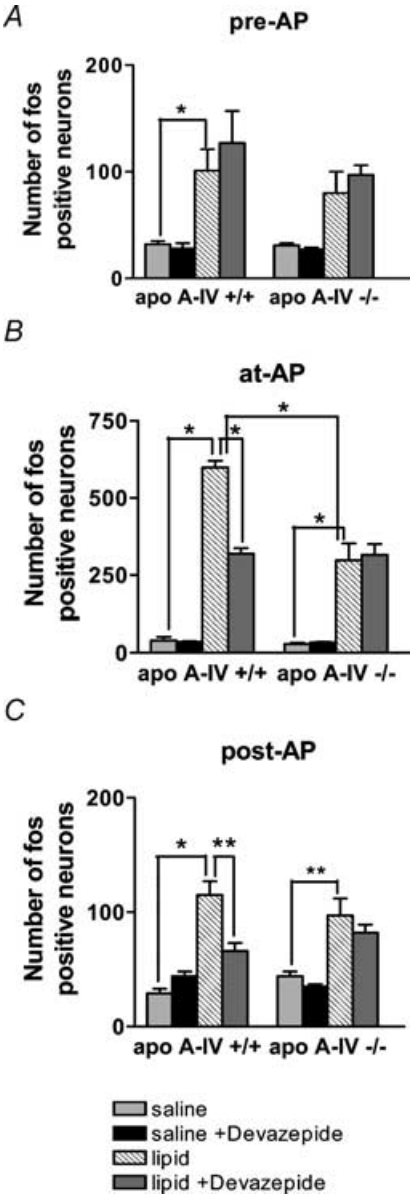

Figure 4. Lipid-induced Fos protein expression in the nucleus of the solitary tract (NTS) is attenuated in apo A-IV knockout mice
Activation of Fos expression at different levels of the NTS in apo A-IV knockout and wildtype control mice with saline ($n = 4$), saline and devazepide I.P. ($n = 4$), Intralipid gavage ($n = 4$) or Intralipid gavage and devazepide I.P. ($n = 4$). *A*, pre-area postrema (*$P < 0.05$); *B*, at-area postrema (*$P < 0.001$, note a change in axis scale for this graph); *C*, post-area postrema (*$P < 0.001$, **$P < 0.01$). Error bars are S.E.M.

Table 2. Plasma levels of triglyceride

	Fasted	Intralipid
Apo A-IV$^{+/+}$	67 ± 3 ($n = 4$)*	91 ± 5 ($n = 9$)*
Apo A-IV$^{-/-}$	51 ± 5 ($n = 5$)**	77 ± 7 ($n = 7$)**

There was no significant difference in lipid absorption between Apo A-IV wildtype and knockout mice. Intralipid gavage significantly increased fasting plasma triglyceride levels (mg TAG (ml plasma)$^{-1}$) in comparison with fasting TAG levels in both wildtype (*$P < 0.008$) and knockout (**$P < 0.03$) mice. However, there was no significant difference between fasting or lipid-fed TAG levels in wildtype and knockout mice.

CCK immunohistochemistry

Representative images chosen for transverse and longitudinal gut sections were compared to determine if there were any gross morphological differences in endocrine cell expression between apo A-IV$^{+/+}$ ($n = 3$) and apo A-IV$^{-/-}$ mice ($n = 3$). CCK-expressing endocrine cell distribution and overall morphology appears to be the same between the two strains of mice (Fig. 5).

Discussion

The present results support the hypothesis that lipid-induced stimulation of intestinal feedback is mediated, at least in part, by apolipoprotein A-IV. We have demonstrated that inhibition of gastric emptying and gastric acid secretion induced by lipid in the small intestine was markedly attenuated in the apo A-IV$^{-/-}$ mice compared to the wildtype controls. In addition, the data show that activation of the vagal afferent pathway, as determined by activation of neurones within the NTS, in response to intestinal lipid was significantly reduced in apo A-IV$^{-/-}$ mice. It is well established that intestinal lipid activates vagal afferents via a CCK_1 receptor-dependent mechanism, resulting in activation of NTS neurones and reflex changes in gastric motor function and gastric acid secretion (Lloyd *et al.* 1992; Hölzer *et al.* 1994; Zittel *et al.* 1994; Glatzle *et al.* 2002, 2003). We have previously shown that apo A-IV acts to inhibit gastric motor function via activation of CCK-responsive vagal afferent fibres, and by a mechanism dependent on CCK_1 receptors (Glatzle *et al.* 2004). Taken together, these data suggest that active lipid absorption results in apo A-IV release from enterocytes, which in turn acts to stimulate release of CCK from enteroendocrine cells in the intestinal epithelium, followed by activation of vagal afferents via the CCK_1 receptor.

The first aim of this study was to determine whether inhibition of gastric motor and secretory function in response to dietary lipid is dependent upon apo A-IV. We were able to quantify the gastric emptying rate of three test meals (egg white, whole egg, and egg yolk) under basal conditions, and have previously demonstrated that the rate of gastric emptying is proportional to the fat consumed (Whited *et al.* 2004). In apo A-IV$^{-/-}$ mice, the inhibitory effect of lipid on gastric emptying was significantly attenuated. This observation is in agreement with our previous data showing that administration of exogenous apo A-IV can inhibit proximal gastric motor function in rats (Glatzle *et al.* 2004). A decrease in intraluminal pressure in the proximal stomach will decrease gastric emptying by decreasing the delivery of chyme to the antral pump (Moragas *et al.* 1993). It is unlikely that the attenuated response to lipid is explained by a reduced sensitivity to CCK; administration of exogenous CCK was able to inhibit gastric emptying in the knockout mice.

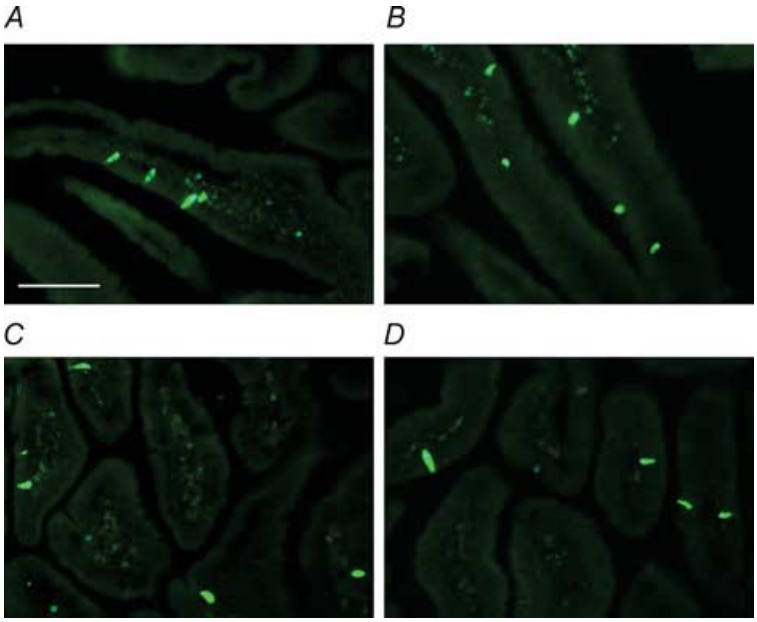

Figure 5. No gross difference in expression of duodenal CCK endocrine cells in apo A-IV wildtype and knockout mice
Photomicrographs of mice duodenum showing distribution of CCK-expressing endocrine cells in a chow-fed apo A-IV wildtype control and knockout mouse. *A*, apo A-IV$^{+/+}$ transverse section; *B*, apo A-IV$^{-/-}$ transverse section; *C*, apo A-IV$^{+/+}$ longitudinal section; *D*, apo A-IV$^{-/-}$ longitudinal section. Scale bar = 100 μs.

Administration of exogenous CCK in wildtype mice had no significant effect on the emptying rate of whole egg; this is presumably because in these animals, whole egg released endogenous CCK, which activated vagal afferents resulting in inhibition of gastric emptying. The effectiveness of CCK in the apo A-IV$^{-/-}$ mice suggests that the difference in $t_{1/2}$ gastric emptying time between apo A-IV$^{-/-}$ and apo A-IV$^{+/+}$ mice in response to lipid challenge is due to the lack of apo A-IV gene expression in the knockout mice, rather than altered sensitivity to CCK. Further evidence to support this conclusion is provided by the results from the studies determining activation of the vagal afferent pathway using expression of Fos in the NTS (see later).

Inhibition of gastric acid secretion in response to dietary lipid was completely absent in apo A-IV$^{-/-}$ mice. It has been previously shown that intracisternal injection of apo A-IV inhibits gastric acid secretion through α_2-adrenergic receptors (Okumura *et al.* 1994, 1995). Through the use of transgenic mice expressing human apo A-IV (hapo A-IV), Vergnes *et al.* (1999) showed that the overexpression of apo A-IV in these mice decreases gastric acid secretion without alteration of the gastric mucosa. Consistent with these published observations, in the present study, we observed that basal gastric acid secretion was significantly higher in apo A-IV$^{-/-}$ mice in comparison with apo A-IV$^{+/+}$ animals. Gastric acid secretion stimulated in response to intragastric peptone was also higher; however, the increase in response to peptone was not different between wildtype and knockout mice. The reason for the increase in basal gastric acid secretion is not clear, but might involve central apo A-IV rather than the intestinal apo A-IV, since central administration of apo A-IV inhibits gastric acid secretion. It is interesting to note that lipid-induced inhibition of gastric acid was completely absent in the apo A-IV$^{-/-}$; in contrast, gastric emptying of whole egg or egg yolk was still slower than for egg white in these mice, suggesting residual lipid-induced inhibition of gastric emptying. It is possible that the gastric secretory response and motor response are mediated by different pathways. This is unlikely because it has been shown that lipid-induced inhibition of gastric acid secretion is also mediated by a vago-vagal reflex CCK_1 receptor-dependent pathway, similarly to inhibition of gastric motor function (Lloyd *et al.* 1992). The reason for this discrepancy is unclear; however, two different lipids were used in these two studies; in the gastric emptying studies, we used egg products containing different amounts of lipid but also other nutrients, including protein. In the gastric acid secretion studies, we were able to use a lipid emulsion. This difference may in part account for the different degree of attenuation seen in the knockout mice.

The second aim of this study was to determine whether activation of the vagal afferent pathway by intestinal lipid is dependent upon apo A-IV. We measured Fos protein expression in neurones in the NTS as a measure of activation of the vagal afferent pathway. Stimulation of neurones induces transcriptional and translational activity of the c-*fos* oncogene, and results in the production of intracellular regulatory factors like Fos protein, whose expression is an indicator of neuronal activation. Duodenal perfusion with lipid emulsion significantly increases Fos protein expression in the NTS of the rat (Zittel *et al.* 1994). In the mid-region of the NTS (at the AP), the region where vagal afferents from the duodenum terminate, Fos expression in response to intestinal lipid was significantly reduced in apo A-IV$^{-/-}$ mice compared to apo A-IV$^{+/+}$ mice. Importantly, there was no significant difference between apo A-IV$^{-/-}$ mice treated with lipid and wildtypes treated with lipid in the presence of the CCK_1 receptor antagonist. This suggests that the attenuated response to lipid in apo A-IV$^{-/-}$ mice is due to an alteration in the ability of lipid to activate the CCK_1 receptor pathway. This observation, taken together with the observation that CCK was equally effective in activating NTS neurones in both wildtype and knockout mice in response to I.P. CCK injection, strongly suggests that apo A-IV knockout mice possess a functional afferent neuronal pathway, and are able to respond to CCK challenge in a similar manner to their wildtype counterparts. It is interesting to note that there is a residual response to lipid in both wildtypes and knockouts in the presence of the CCK_1 receptor antagonist; this suggests a CCK_1 receptor-independent pathway by which lipid activates vagal afferents. This may involve activation of vagal afferents by gastric distention or post-absorptive signals from the liver.

Apo A-IV is synthesized not only in the intestine, but also in the hypothalamus, a site intimately involved in the integration of signals for regulation of food intake and energy metabolism (Liu *et al.* 2001) and the deficit in reflex regulation of gastric function between apo A-IV$^{+/+}$ and apo A-IV$^{-/-}$ mice may be due, in part, to a lack of apo A-IV synthesis in the brain. However, the observation that activation of the vagal afferent pathway is attenuated in apo A-IV$^{-/-}$ mice suggests that the defect is in detection of lipid in the gut wall and activation of vagal afferent pathway.

The mechanism by which apo A-IV is involved in release or the action of CCK on vagal afferents is unclear. It is possible that apo A-IV stimulates CCK release from intestinal endocrine cells, but this has yet to be demonstrated. The mechanism by which endocrine cells release CCK in response to long-chain triglyceride is unclear. It has previously been demonstrated that infusion of long-chain fatty acids or long-chain triglyceride emulsions increases plasma levels of CCK (Liddle, 1994), specifically fatty acids of chain length C12 or longer (McLaughlin *et al.* 1998). This carbon chain length requirement suggests that chylomicron formation

is involved, as well as apo A-IV, because stimulation of apo A-IV production by lipid feeding is associated with the formation of chylomicrons (Hayashi *et al.* 1990). A direct effect of long-chain triglyceride has been shown in the enteroendocrine cell model, STC-1 cells, although whether this occurs in native endocrine cells in not known (McLaughlin *et al.* 1998; Sidhu *et al.* 2000). It is possible that apo A-IV mediates release of CCK, but the residual response to lipid for inhibition of gastric emptying and activation of NTS neurones may be mediated by a direct effect of free fatty acid on endocrine cells and release of CCK. Alternatively, it is possible that free fatty acid directly stimulates vagal afferent nerve terminals. It has been shown that short-chain fatty acids can directly stimulate mesenteric nerve fibres, but this has not been demonstrated for long-chain triglyceride; indeed, the ability of long-chain triglyceride to stimulate vagal afferent activity is completely blocked by the CCK_1 receptor antagonist, devazepide (Lal *et al.* 2001). The ability of apo A-IV to directly influence endocrine cell function is unknown. Measurement of plasma levels of CCK is confounding, because the action of CCK on vagal afferent nerve terminals is not an endocrine action and therefore not dependent on appearance in plasma, but occurs at the level of the lamina propria. Our observations suggest that there is no gross difference in expression of CCK endocrine cells in the apo A-IV$^{-/-}$ mice, but a detailed understanding of the role of apo A-IV in release of CCK remains to be determined.

It is unlikely that the differences observed in the present study between apo A-IV$^{-/-}$ and +/+ mice are associated with postabsorptive differences; the increase in plasma TAG in response to lipid was not significantly different between the two groups, confirming the observation that there is no difference in lipid absorption between the wildtype and knockout mice (Weinstock *et al.* 1997).

In summary, the present data provide evidence for a role for apo A-IV in the detection of lipid in the intestinal wall. This is the first site in the body at which intake of macronutrients can be monitored. Detection of nutrients in the wall of the gut initiates changes in function of the gastrointestinal tract and also causes decrease in food intake, processes that are important in digestion and absorption, and possibly in body weight regulation.

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