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Apolipoprotein A5 deficiency aggravates high fat diet induced obesity due to impaired central regulation of food intake

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FASEB journal **2013**, 27(8): 3354-3362

Abstract

Mutations in apolipoprotein A5 (APOA5) have been associated with hypertriglyceridemia in humans and mice. This has been attributed to a stimulating role for APOA5 in lipoprotein lipase-mediated triglyceride hydrolysis and hepatic clearance of lipoprotein remnant particles. However, due to the low APOA5 plasma abundance, we investigated an additional signaling role for APOA5 in high fat diet (HFD) induced obesity.

Wildtype (WT) and *Apoa5*$^{-/-}$ mice on chow diet showed no difference in bodyweight or 24h food intake (*Apoa5*$^{-/-}$, 4.5±0.6g, WT, 4.2±0.5g), while on HFD *Apoa5*$^{-/-}$ mice ate more in 24h (*Apoa5*$^{-/-}$, 2.8±0.4g, WT, 2.5±0.3g, $p<0.05$) and became more obese than WT mice. Also, intravenous injection of APOA5-loaded VLDL-like particles lowered food intake (VLDL-control, 0.26±0.04g, VLDL+APOA5, 0.11±0.07g, $p<0.01$). In addition, the HFD induced hyperphagia of *Apoa5*$^{-/-}$ mice was prevented by adenovirus-mediated hepatic overexpression of *APOA5*. Finally, intracerebroventricular injection of APOA5 reduced food intake compared to injection of the same mouse with artificial cerebral spinal fluid (aCSF, 0.40±0.11g, APOA5, 0.23±0.08g, $p<0.01$).

Introduction

Elevated plasma triglyceride (TG) levels and prolonged circulation of lipoprotein remnants are independent risk factors for cardiovascular disease [1-5]. In the post absorptive state, hypertriglyceridemia is a consequence of aberrant kinetics of VLDL, including hepatic overproduction and/or delayed clearance [6]. Apolipoprotein A5 (APOA5) has classically been described to be involved in the regulation of TG-rich lipoprotein metabolism [7]. APOA5 is a potent stimulator of lipoprotein lipase (LPL) [8, 9] and facilitates lipoprotein remnant clearance in a LDL-receptor dependent manner [10]. The function of APOA5 in lipid metabolism is supported by the fact that non-obese carriers of the *APOA5* -1131T>C polymorphism have a disturbed postprandial lipidemic response after a high fat meal [11, 12]. However, even though *in vitro* and animal studies suggest that APOA5 accelerates LPL mediated TG metabolism and the fact that APOA5 levels are positively associated with plasma TG levels, APOA5 does not directly affect postprandial VLDL kinetics in mild dyslipidemics or type 2 diabetics [13, 14].

In humans, the plasma concentration of APOA5 is low (114 to 258ng·ml^{-1} in normolipidemic subjects [15, 16]), possibly due to its low excretion rate [17, 18]. On a molar basis, APOA5 plasma levels are 1,000 and 10,000 fold lower when compared to Apolipoprotein B (APOB) and APOA1, respectively. Because of the low plasma concentration [19] and in light of the low secretion rate, APOA5 has been suggested to play a role in intracellular lipid metabolism. Intracellular APOA5 is associated with lipid droplets in liver [20] and adipocytes [21]. Interestingly, APOA5 shows a high protein similarity to perilipin-1 (PLIN1), a well-studied inhibitor of hormone sensitive lipase (HSL) activity [22]. Recently, intracellular APOA5 has been shown to co-localize with perilipin-1 in adipose tissue, and to actively regulate TG uptake, and thus may be involved in the development of obesity [21].

In addition to dyslipidemia, and in support of a role of APOA5 in the development of obesity, *APOA5* gene variants have been associated with a greater degree of obesity in a number of epidemiological studies involving Caribbean Hispanics [23], pediatric patients [24] and Brazilian elderly [25]. In part, this could be due to the proposed role of APOA5 in circulating lipid metabolism. Interestingly, it may also be due to altered food intake patterns, as the *APOA5* -1131T>C gene variant has been shown to be associated with higher fat intake [26] and to modulate the effects of dietary fat intake on body mass index and obesity risk [27, 28] and dyslipidemia [29].

The aim of this study was to assess the role of APOA5 in the regulation of food intake. First, we confirmed that *Apoa5*$^{-/-}$ mice are dyslipidemic and have an impaired lipid clearance. In addition, we further demonstrate that *Apoa5*$^{-/-}$ mice become more obese, and develop hepatic steatosis and become more insulin resistant on a high fat diet (HFD) than WT mice, due to a hyperphagic phenotype. Furthermore, the hyperphagic phenotype of *Apoa5*$^{-/-}$ mice is reduced upon adenoviral vector mediated overexpression of *APOA5*. Moreover, systemic (intravenous) as well as central (lateral ventricle) administration of APOA5 reduced food

intake by 60% and 45%, respectively, suggesting that APOA5 inhibits food intake, at least partly, via a central mechanism.

Materials and Methods

Animals and diets

Male wild type (WT) C57Bl/6Jico mice were obtained from Charles River laboratories (Charles River, Maastricht, The Netherlands) at an age of 10 weeks. After at least 2 weeks of acclimatization, mice were fed a HFD (45% energy as fat, 4.73kcal·g^{-1}, D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands). *Apoa5*$^{-/-}$ mice [30] were bred in-house at the Leiden University Medical Center and back-crossed on the C57Bl/6Jico background for at least 8 generations prior to homozygous breeding. Absence of the *Apoa5*$^{-/-}$ gene was confirmed by isolated tail DNA PCR (forward; TGGAGGGTCAAAGAAGGATG, reverse; GGTTGCTGGTCACAGGATTT). Animals were housed in a controlled environment (21°C, 40-50% humidity) under a 12h photoperiod (07:00–19:00). Food and tap water was available *ad libitum* during the whole experiment, unless otherwise indicated. In all experiments, male mice were used. All animals were weighed at least once every week. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures from the Leiden University Medical Center, Leiden, The Netherlands approved the protocol.

Plasma and liver lipid analysis

Plasma was obtained via tail vein bleeding and assayed for TC and TG, using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA), respectively. Free fatty acids (FA) were measured using NEFA-C kit from Wako Diagnostics (Instruchemie, Delfzijl, the Netherlands). Human APOA5 levels were determined using a previously described ELISA [31].

Lipids were extracted from livers according to a protocol modified from Bligh and Dyer [32]. In short, a small piece of liver was homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1,800µl of CH_3OH-$CHCl_3$ (3:1 vol/vol) to 45µl of homogenate. The organic phase was dried and dissolved in 2% Triton X-100. Hepatic TG and TC concentrations were measured using commercial kits, as described above. Liver lipids were expressed per milligram of protein, which was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

Lipid clearance analysis

Glycerol tri(9,10(n)[^{3}H]oleate ([^{3}H]TO) and [1α,2α(n)-^{14}C]cholesteryl oleate ([^{14}C]CO) double radiolabeled VLDL-like emulsion particles (mean diameter 80nm) were prepared as described before [33]. Mice were anesthetized with 6.25mg·kg^{-1} Acepromazine (Alfasan, Woerden, The Netherlands), 6.25mg·kg^{-1} Midazolam (Roche, Mijdrecht, The Netherlands) and 0.31mg·kg^{-1} Fentanyl (Janssen-Cilag, Tilburg, The Netherlands) prior to particle injection via the tail vein. At time points 2, 5, 10 and 15min post-injection, blood was taken to determine clearance rates. After 15 minutes mice were sacrificed and organs were harvested to determine [^{3}H]TO and [^{14}C]CO uptake.

Determination of adipocyte differentiation capacity

Adipose tissue from the reproductive and subcutaneous region were harvested and kept in PBS. The tissue was minced and digested in 0.5g·l^{-1} collagenase in HEPES buffer (pH 7.4) with 20g·l^{-1} of dialyzed bovine serum albumin (BSA, fraction V, Sigma, ST Louis, USA) for 1h at 37°C. The disaggregated adipose tissue was filtered through a nylon mesh with a pore size of 236μm and the stromal vascular cells were isolated. Preadipocytes were differentiated in an adipogenic medium and differentiation capacity was determined as previously described [34].

Indirect calorimetry/metabolic cage analysis

WT and *Apoa5*$^{-/-}$ mice were subjected to indirect calorimetry/metabolic cage analysis (Phenomaster, TSE Systems, Bad Homburg, Germany). A period of 48 hours of acclimatization was included prior to the start of the experiment. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were determined at 9 minute intervals. Respiratory exchange ratio (RER) was calculated as the ratio between VCO_2 and VO_2. Energy expenditure (EE), fat oxidation (FAox) rate and carbohydrate oxidation (CHox) rate were calculated as previously described [35]. Food intake was monitored in real time. Data from the light and dark phase were averaged and tested separately to distinguish periods of high and low physical activity. In total the measurement period lasted 7.5 days (chow≈3 days and HFD≈4.5 days). The switch to the HFD was performed inside the metabolic cage system at 14:00 for all animals. For the long term experiment, mice were fed a HFD for a period of 4 weeks prior to analysis.

Adenovirus vector mediated overexpression of human APOA5 in Apoa5$^{-/-}$ mice

A separate group of *Apoa5*$^{-/-}$ mice were used for adenoviral vector gene transfer, and subjected to indirect calorimetry/metabolic cage analysis during the initial week of high fat feeding. A period of 24 hours of acclimatization was included prior to the start of the experiment and baseline measurements were performed for each individual animal. Subsequently, animals were randomized based on body mass and injected intravenously (*vena caudalis*) with mock LacZ or human *APOA5* expressing adenovirus vectors (≈2·10^{9}pfu). Calorimetric analysis was continued for 4 days after injection until the expected peak in transgenic expression [36, 37]. Since adenovirus vector mediated gene expression in itself may affect energy balance [38-40] data were analyzed per animal separately in a paired comparison setting (i.e. pre-injection vs. post-injection). Differences in the change in food intake over the 4 days were compared between groups.

Intravenous APOA5 injection and food intake test

WT mice were fed a high fat diet for a period of 1 week prior to analysis to ascertain habituation to the high fat diet. All mice were food restricted for 6 hours prior to injection, starting at 12:00, to induce the drive for food intake. To assess the effect of elevated circulating APOA5 levels on food intake, VLDL emulsion particles [41] were loaded with human APOA5 (100ng·µl^{-1}, Abnova, Jhongli, Taiwan). Human APOA5 protein was produced in an *in vitro* wheat germ expression system, which generally results in negligible endotoxin levels (Personal communication, V. Yu, Abnova, Jhongli, Taiwan). Analysis of the Sepharose purified preparation by SDS-PAGE and Coomassie staining revealed a single band. Non-loaded control and APOA5 loaded emulsion particles were intravenously injected in the tail vein at a dose of 6µg per mouse and injected 6h after food restriction. After injection, food intake was recorded up to 120 minutes post injection and expressed as gram food consumed per unit of 60 minutes.

Lateral ventricle cannulation and ICV APOA5 injection and food intake test

WT mice were fed a high fat diet for a period of 1 week prior to analysis to ascertain habituation to the high fat diet. To assess the effect of central administration of APOA5 on food intake, a 25 gauge guide cannula was implanted into the left lateral ventricle as previously described [42, 43]. Similar to the IV injection experiment, mice were food restricted to induce the drive for food intake. All animals received a successive 1µl bolus injection of artificial cerebrospinal fluid (aCSF, Harvard Apparatus, Holliston, MA, USA) (7d post-surgery), APOA5 (10d post-surgery, 8ng/mouse) and neuropeptide Y (NPY, 14d post-surgery, 5µg/mouse) under light

isoflurane anesthesia. After injection, food intake was recorded during the first 120 minutes post injection and expressed as gram food eaten per unit of 60 minutes. Correct placement of the cannula was assessed in all mice in the fed state at 14d post-surgery by a bolus injection of neuropeptide Y at 0900 a.m. Cannulation was assumed to be correct if the animal consumed more than 300mg of food within 1 hour post NPY injection. One animal did not meet this criterion and was excluded from the experiment.

Hyperinsulinemic-euglycemic clamp in conscious mice

WT and *Apoa5*$^{-/-}$ mice were subjected to hyperinsulinemic-euglycemic clamp analysis as previously described [44]. In short, all mice were equipped with a single catheter in the right jugular vein for infusion. After surgery, mice recovered for a period of 8 days prior to the clamp analysis, in individual cages. Food intake and body weight returned to preoperative levels within 2-3 days. Food was removed 9 hours before the start of the experiment, but the animals had free access to water. Clamp quality control was determined by calculating the coefficient of variation (COV) of the glucose infusion rate (GIR) and plasma glucose levels over the last 30 minutes of the clamp. COV of the GIR and glucose levels were determined to be below 2% for both genotypes.

Statistics

All data are represented as mean ± standard deviation. Energy intake data was tested by unpaired t-test for normally distributed data or paired t test in case of the adenoviral and icv experiment. Threshold for statistical significance was set at 5%. Tests were performed using PASW (SPSS) Statistics, version 18.

Results

High fat fed Apoa5$^{-/-}$ mice are hyperlipidemic and have severely impaired lipid clearance

Plasma was isolated from food restricted *Apoa5*$^{-/-}$ and WT mice fed a HFD for 10 weeks, after which plasma cholesterol, TG, phospholipid and free fatty acid levels were determined. *Apoa5*$^{-/-}$ mice were hyperlipidemic (Table 1) and in line with these data, *Apoa5*$^{-/-}$ mice showed severely impaired lipid clearance. The plasma decay of injected [^{3}H]TO and [^{14}C]CO-labeled emulsion particles was slower in *Apoa5*$^{-/-}$ mice for the TO label (Figure 1 A, B) as well as the CO label (Figure 1 C, D), due to a significantly lower uptake in organs with a high metabolic activity (liver, heart, muscle, brown adipose tissue). In addition, CO label uptake was also lower in liver and brown adipose tissue, but not in heart and muscle. These data confirm previously published data (10;30) and demonstrate that the hyperlipidemic phenotype of *Apoa5*$^{-/-}$ mice is associated with an impaired lipid partitioning.

*Table 1: Plasma lipid levels of HFD fed Apoa5$^{-/-}$ and WT control mice. Data represent mean values ± SD, **=p<0.01.*

	Apoa5-/-	**WT**
Triglyceride(mmol/L)	5.7±2.8	1.1 ± 0.2**
Cholesterol (mmol/L)	6.8 ± 1.1	4.0 ± 0.6**
Phospholipids (mmol/L)	7.1 ± 1.0	4.3 ± 0.5**
Free fatty acids (mmol/L)	1.9 ± 0.9	1.0 ± 0.3**

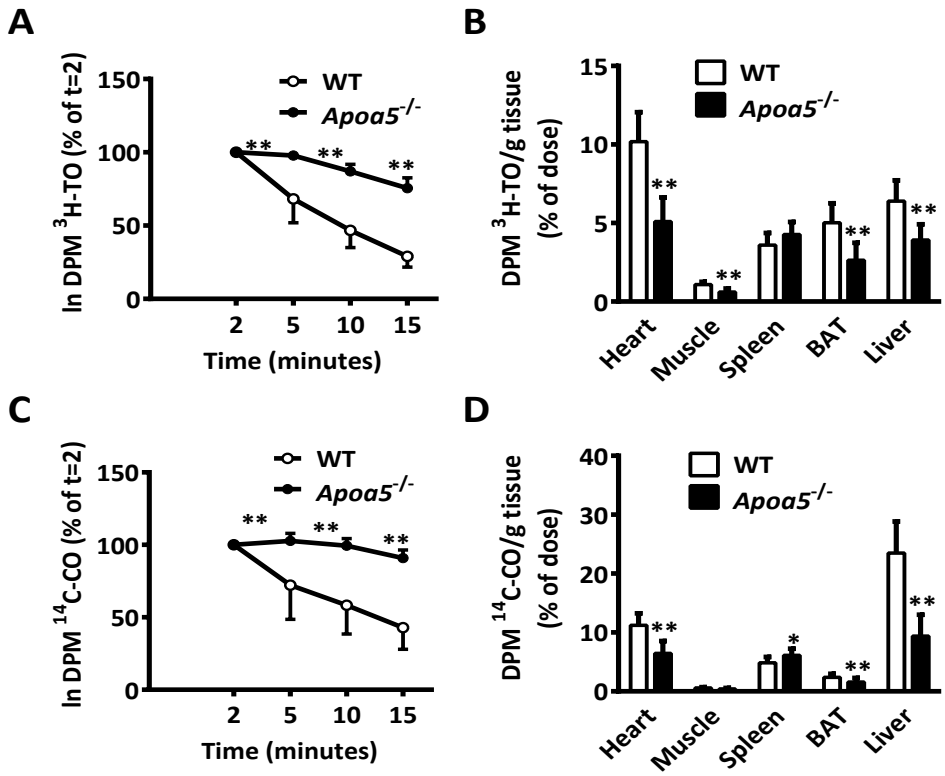

*Figure 1: Plasma decay curve (A) and tissue specific retention (B) of the ^{3}H-TO label and the decay curve (C) and tissue specific retention (D) label of the ^{14}C-CO label after mass lipid injection in Apoa5$^{-/-}$ and WT mice. Data represent mean values ± SD, *=p<0.05, **=p<0.01.*

High fat diet feeding aggravated obesity development in Apoa5$^{-/-}$ mice

Bodyweight was monitored in WT and *Apoa5*$^{-/-}$ mice fed a HFD. Diet induced weight gain was more pronounced in *Apoa5*$^{-/-}$ mice, which thereby became more obese compared to WT (Figure 2). White adipose tissue mass was significantly higher in *Apoa5*$^{-/-}$ mice in both the reproductive (*Apoa5*$^{-/-}$, 2.4±0.3g, WT, 1.7±0.5g, p<0.01) and subcutaneous (*Apoa5*$^{-/-}$, 1.7±0.6g, WT, 1.1±0.3g, p<0.01) white adipose tissue depot. Due to the large infiltration of pancreatic tissue, the mesenteric fat depot could not be isolated in a reproducible manner [45]. Heart, liver, spleen, lung, or kidney mass did not differ between genotype (data not shown). Since APOA5 has been described to regulate fatty acid uptake in adipocytes [21] and thus possibly affect adipogenesis, the differentiation capacity of pre-adipocytes isolated from the reproductive and subcutaneous fat pads of HFD fed *Apoa5*$^{-/-}$ and WT mice was studied by Nile red assay. Differentiation capacity did not differ between genotypes for either of the

isolated fat pads (p=0.70 and p=0.69 for reproductive and subcutaneous fat, respectively (Supplemental Figure S1). Together, these data demonstrate that HFD fed *Apoa5*$^{-/-}$ mice become more obese than WT mice and that this is not due to an intrinsic difference in the adipogenic capacity of the white adipose tissue.

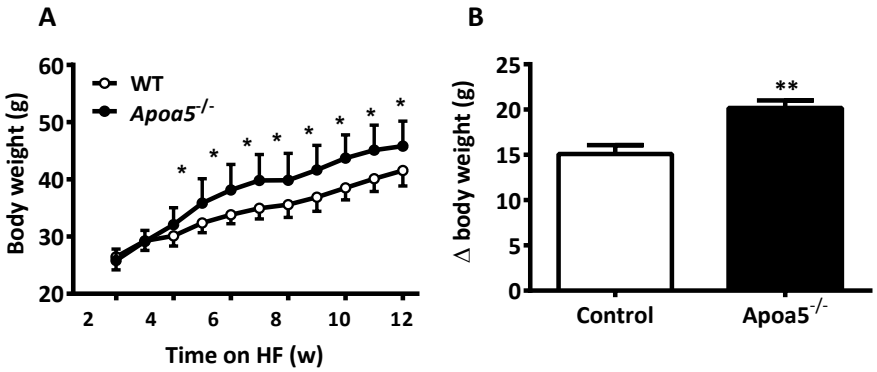

Figure 2: Weight development of Apoa5$^{-/-}$ *mice and WT controls during high fat diet intervention. Data represent mean values ± SD, *=p<0.05, **=p<0.01.*

Apoa5$^{-/-}$ *develop aggravated hepatic steatosis and insulin resistance upon HFD feeding*

Ectopic fat accumulation in the liver of *Apoa5*$^{-/-}$ and WT control mice was assessed. Although total hepatic mass did not differ between groups, hepatic TG content was 50% higher in HFD fed *Apoa5*$^{-/-}$ mice (*Apoa5*$^{-/-}$, 0.47±0.16μmol·mg^{-1} protein, WT, 0.31±0.17μmol·mg^{-1} protein, p=0.056). Hepatic cholesterol and phospholipid accumulation did not differ between groups (data not shown).

Since HFD fed *Apoa5*$^{-/-}$ mice become more obese and develop hepatic steatosis upon HFD feeding, tissue specific insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp analysis in conscious mice (Supplemental Figure S2). GIR was lower in *Apoa5*$^{-/-}$ mice (*Apoa5*$^{-/-}$, 335±64μmol· (kg·min)$^{-1}$, WT, 408±60μmol·(kg·min)$^{-1}$, p<0.05, (Supplemental Figure S2a)), indicating a reduction in insulin sensitivity. In addition, the hyperinsulinemic circulating glucose level at stable GIR was significantly higher in *Apoa5*$^{-/-}$ mice (*Apoa5*$^{-/-}$, 6.6±1.2mmol·l^{-1}, WT, 5.5±0.7mmol·l^{-1}, p<0.05 (Supplemental Figure S2b)). To correct for this difference in circulating glucose pool, metabolic clearance rates (MCR) were determined. MCR was significantly reduced in *Apoa5*$^{-/-}$ mice (*Apoa5*$^{-/-}$, 77.0±15.4ml·(kg·min)$^{-1}$WT, 101.0±18.1ml·(kg·min)$^{-1}$, p<0.05 (Supplemental Figure S2c)). Collectively, these data show that *Apoa5*$^{-/-}$ mice, in addition to obesity, develop hepatic steatosis and insulin resistance when fed a HFD.

Apoa5$^{-/-}$ mice are hyperphagic when fed a high fat diet

Given the association of APOA5 with fat intake (27;28), we determined whether the change in energy balance in *Apoa5$^{-/-}$* mice was due to a defect in oxidative metabolism (which may arise from the reduced lipid clearance capacity) or food intake (FI). At the start of the experiment, when animals were fed a chow diet, bodyweight did not differ significantly between groups (*Apoa5$^{-/-}$*, 25.3±0.8g, WT, 26.2±1.4g, p=0.2). Furthermore, FI measured over a period of 24 hours did not differ between groups when animals were fed a chow diet (*Apoa5$^{-/-}$*, 4.5±0.6g, WT, 4.2±0.5g). In addition, respiratory exchange ratio (RER) and energy expenditure (EE) were similar between groups (Data not shown).

After the chow period, all animals were switched to the HFD (Figure 3). During the first 24 hrs after the switch to the HFD, FI was significantly higher when compared to the chow diet in both groups (*Apoa5$^{-/-}$*, 4.7±0.3g, WT, 4.7±0.1g, p<0.01 for both groups vs. chow), but did not differ between groups (p=0.20). After that, FI declined in both groups, but remained higher in the *Apoa5$^{-/-}$* group. Food intake during the last 24 hours was significantly higher in *Apoa5$^{-/-}$* mice during the dark period (*Apoa5$^{-/-}$*, 2.1±0.3g, WT, 1.9±0.7g, p<0.05) but did not differ between groups during the light period (*Apoa5$^{-/-}$*, 1.2±0.5g, WT, 1.1±0.4g). Total FI during the first 96 hours of HFD intervention was higher in the *Apoa5$^{-/-}$* group (*Apoa5$^{-/-}$*, 16.5±1.2g, WT, 14.2±0.7g, p<0.01). The HFD induced a gradual shift from carbohydrate to fat metabolism in both groups. RER and absolute carbohydrate oxidation declined and absolute fat oxidation increased, but this switch was less pronounced in *Apoa5$^{-/-}$* mice (Figure 3).

Similar results were found after 4 weeks of HFD intervention, where food intake was significantly higher in *Apoa5$^{-/-}$* mice during a period of 24 h (*Apoa5$^{-/-}$*, 2.8±0.4g, WT, 2.5±0.3g, p<0.05). Oxidative fat metabolism was still reduced in *Apoa5$^{-/-}$* mice compared to controls (Supplemental Table S1). These data show that HFD fed *Apoa5$^{-/-}$* mice remain hyperphagic, and have reduced relative and absolute fat oxidation rates after prolonged high fat feeding.

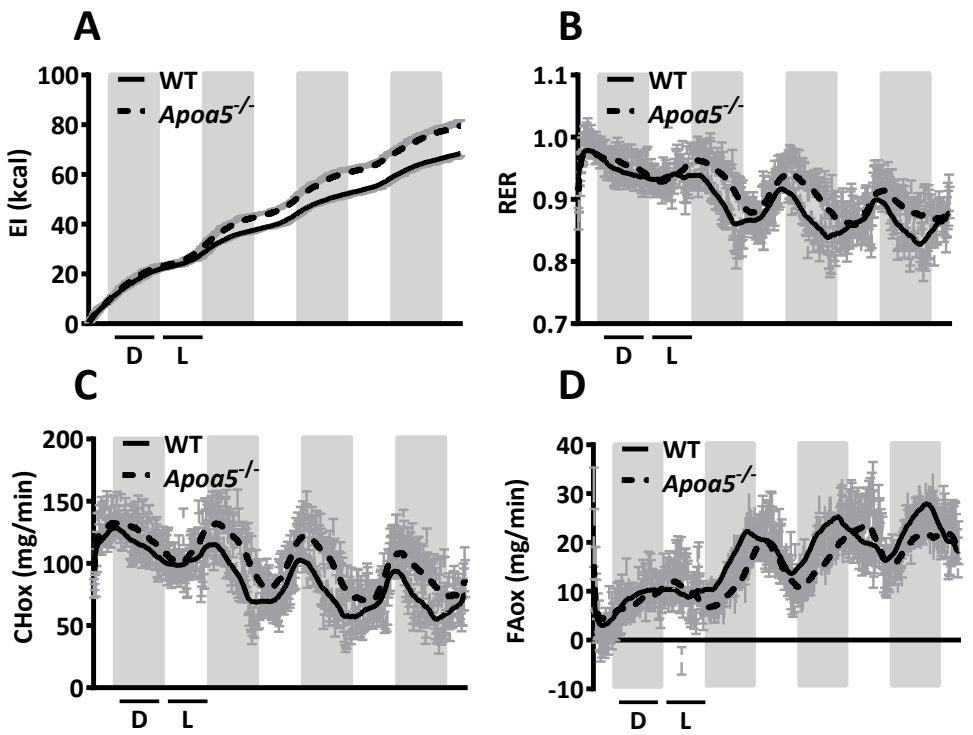

Figure 3: Indirect calorimetry data from Apoa5$^{-/-}$ (dashed line) and WT (solid line) mice during the first week of high fat diet intervention in the light (L) and dark (D) periods of the day. A) Energy intake (EI),B) Respiratory exchange ratio (RER), C) Carbohydrate oxidation (CHox) and D) Fat oxidation (FAox). All graphs depict the smoothed average ± SEM.

Adenovirus mediated gene transfer of APOA5 to Apoa5$^{-/-}$ mice reduces food intake

To assess whether hepatic expression of *APOA5* rescues the hyperphagic phenotype, *Apoa5*$^{-/-}$ mice were injected with adenovirus vectors expressing either LacZ or human *APOA5*. At the time of injection, body mass did not differ significantly between the groups (*APOA5*, 22.7±1.8g, LacZ, 24.2±3.0g, p=0.3). Basal FI, determined at day 0, did not differ between groups during the light (*APOA5*, 0.66±0.23g, LacZ, 0.53±0.16g, p=0.2) or dark period (*APOA5*, 1.43±0.50g, LacZ, 1.54±0.23g, p=0.6). At the fourth day after virus injection, FI was compared to basal levels in both groups. In LacZ mice, FI was significantly higher compared to basal levels during the light (0.80±0.23g, p<0.01) as well as the dark period (2.01±0.72g, p<0.01), whereas in *APOA5* injected animals, FI during the light and dark period was similar to basal values (Light, 0.85±0.39g, p=0.08 and dark period 1.41±0.59g, p=0.46, respectively). Hepatic overexpression of *APOA5* resulted in human APOA5 plasma levels of 57.6±16.6µl·ml^{-1}. These data show that *APOA5* overexpression is sufficient to reduce food intake.

Peripheral and central APOA5 protein administration reduce food intake

To determine whether hepatic *Apoa5* expression, or increased circulating APOA5 protein levels mediates reduction in food intake (FI), APOA5 protein was loaded onto VLDL like emulsion particles and injected intravenously in WT mice. Injection of APOA5-loaded emulsion particles significantly reduced food intake (-60%) during the first 60 minutes post injection as compared to unloaded (control) emulsion particles (control, 0.26±0.04g, APOA5, 0.11±0.07g, p<0.01, Figure 4A). During the second hour after the injection, APOA5-loaded emulsion particles did not lower food intake compared to control particles (control, 0.18±0.11g, APOA5, 0.13±0.08g). These data show that increased circulating APOA5 protein levels are capable of reducing food intake.

A peripheral increase of circulating APOA5 protein may affect FI via upregulation of LPL mediated lipolysis (45) or a direct peripheral to central signaling pathway. To determine whether APOA5 protein is capable to reduce FI directly via a central mechanism, aCSF (as a negative control) or aCSF+APOA5 protein was injected into the lateral ventricle of food deprived WT mice (Figure 4B). APOA5 injection significantly lowered FI during the first 60 minutes post injection (40-50%) compared to aCSF (aCSF, 0.40±0.11g, APOA5, 0.23±0.08g, p<0.01) (Figure 4B). FI tended to be reduced during the second hour after APOA5 injection (aCSF, 0.17±0.06g, ApoA5, 0.11±0.10g, p=0.1). These data show that central APOA5 protein administration directly reduces food intake, even at nano-molar concentrations and suggest that the food intake-reducing effect of APOA5 may be regulated, in part, via a central mechanism.

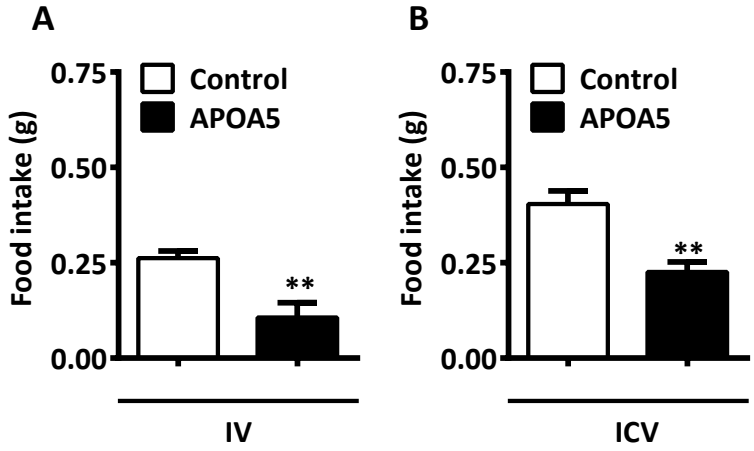

*Figure 4: Food intake during the first 60 min. after (A) intravenous injection of unloaded VLDL-like emulsion particles (white bars) or APOA5 loaded VLDL-like emulsion carrier particles (black bars) and (B) intracerebroventricular injection of aCSF with (black bars) or without (white bars) APOA5 injection. Data represent mean values +/- SD, **=p<0.01.*

Discussion

Here, we confirm previous reports that APOA5 plays a central role in lipid metabolism and energy balance. *Apoa5*$^{-/-}$ mice are hypertriglyceridemic and hypercholesterolemic and display impaired lipid partitioning. Furthermore, *Apoa5*$^{-/-}$ mice develop obesity, hepatic steatosis and insulin resistance when fed a HFD. In addition, we show that *Apoa5*$^{-/-}$ mice are hyperphagic, and that adenoviral overexpression of *APOA5* reverses this phenotype. Furthermore, intravenous as well as central injection of purified APOA5 protein reduced food intake in WT mice (60% and 45%, respectively), suggesting a central effect of APOA5 on the regulation of food intake. Collectively, we confirm that APOA5 plays a crucial role in lipid clearance but we also demonstrate a novel role of APOA5 in the regulation of food intake. Therefore, we speculate that peripheral to central translocation of APOA5 is a satiety signal acting perhaps independently of the effects of APOA5 on LPL-mediated lipolysis.

Food intake is known to be variable and time dependent in C57Bl/6 mice switched to a HFD [46]. After an initial phase of hyper caloric intake, food intake decreases and hypothalamic expression of pro- opiomelanocortin (POMC) is up regulated, possibly as a defensive mechanism against the development of obesity [46]. Here, we confirm that food intake upon high fat feeding was only transiently elevated in control C57Bl/6 mice. In contrast, food intake was significantly higher in *Apoa5*$^{-/-}$ mice upon HFD intervention when compared to controls, and, interestingly, the decline in food intake was largely absent in *Apoa5*$^{-/-}$ mice. Food intake of *Apoa5*$^{-/-}$ mice remained higher compared to both WT mice and baseline values for a period of at least 5 weeks. In addition to hyperphagia, the oxidative metabolism of *Apoa5*$^{-/-}$ mice was characterized by a low rate of fat oxidation. It is, however, unlikely that the increased obesity grade in the *Apoa5*$^{-/-}$ mice is due to a reduced fat oxidation, as peak oxidation rates (during the light period, after 4 weeks of HFD feeding) did not differ from controls. Together, these data clearly show that, compared to controls, *Apoa5*$^{-/-}$ mice have a higher caloric intake when fed a HFD, but do not match fat oxidation to this excess, ultimately leading to a more positive fat balance, and thus obesity.

To assess the hyperphagic phenotype in more detail, human *APOA5* was overexpressed in the liver via an adenovirus. Four days after *APOA5* virus injection, the hyperphagic phenotype of *Apoa5*$^{-/-}$ mice was prevented compared to LacZ virus injected animals. The established human APOA5 plasma concentration was substantially higher (100-300 fold) than the endogenous mouse APOA5 concentration, which is in the same range as earlier experiments [8, 47]. Thus, supraphysiological APOA5 levels reduced food intake. Subsequently, we investigated whether low plasma levels of APOA5 could also reduce food intake. Therefore protein free VLDL like emulsion particles were loaded with 6µg purified APOA5 and injected intravenously to mimic the postprandial hypertriglyceridemic situation. Addition of APOA5 to the VLDL particles resulted in a large, but transient reduction in food intake, which is in line with a putative function of APOA5 as a satiation signal. These data show that APOA5 is involved in the regulation of food intake, either directly via a central mechanism and/or

indirectly by its known effect on peripheral lipolysis. The liberation of free fatty acids at the vessel wall during peripheral lipolysis may in itself induce meal termination, as hypothalamic sensors monitor energy status and adjust food intake [48]. Recent studies showed that central administration of oleic acid results in a reduction of food intake [45] in a melanocortin-4-receptor (MC4R) dependent manner, and that this is dependent on nutritional status [49]. Therefore, it could be that the effect of APOA5 on food intake is indirect and involves the stimulation of peripheral lipolysis, and subsequent transport of fatty acids to the brain. In previous studies, however, it has been shown that although APOA5 levels rise concomitantly with TG levels in the postprandial period, this does not affect postprandial VLDL kinetics [13, 14], arguing that the satiating effect of APOA5 may be independent of its effect on lipolysis.

Human cerebrospinal fluid (CSF) contains spherical lipoproteins that resemble HDL in plasma [50, 51]. Furthermore, it has been described that active endocytosis of lipoproteins occurs at the blood brain barrier [52]. A number of apolipoproteins have been identified in human CSF. APOE and APOA1 are the major apolipoproteins; with APOA2, A4, C1, D, C2, C3, C4, J and H present as well [50, 51, 53]. Interestingly, it has been shown that the concentration of APOA4 in CSF increases as a result of lipid feeding [54]. To be able to act as a nutritional sensor, the peripheral-to-central transition of the signal must be rapid. The transcytosis time of lipoprotein particles at the blood brain barrier has been shown to be short (≈15 minutes). Thus, if lipoprotein bound APOA5 acts as a satiety signal, the transition time is likely not rate limiting in the potential of APOA5 to mediate satiation.

Interestingly, central APOE is involved in food intake in rats via a melanocortin 3/4 receptor dependent mechanism [55]. Furthermore, APOA4, the closest structural homolog of APOA5, has also been shown to be expressed in the hypothalamus [56] and to decrease food intake in a cholecystokinin dependent manner [57], also partly via a melanocortin regulated central mechanism [54, 58]. These findings indicate that the effect of APOA4 and APOE on food intake may share a common signaling route. APOA5 is often described as being exclusively expressed in the liver [59, 60]. However, a recent report demonstrated that *APOA5* is expressed in the duodenum and colon in mice and human subjects, and that this expression can be modulated by fatty acids as well as fibrates [61]. Furthermore, active transcription of *Apoa5* in the brain has been shown in birds [62] and mice [63]. More specifically, in mice, central transcription of *Apoa5* was determined to be located at the hypothalamus [64]. In humans, microarray analysis of post mortem brain material revealed that *APOA5* is expressed in the brainstem and near the paraventricular nuclei of the hypothalamus [65]. To assess whether the hyperphagic food intake of *Apoa5*$^{-/-}$ mice could be a direct consequence of the loss of central APOA5 signaling rather than a consequence of impaired peripheral lipolysis, purified APOA5 was injected in the lateral ventricle of HFD fed mice. Central administration of purified APOA5 also largely reduced food intake. Although we cannot formally exclude the presence of contaminants in the commercially produced APOA5, the endotoxin-free production of the protein and the further 12-fold dilution in aCSF, make it unlikely that the

observed effect of ICV administered APOA5 on food intake is non-specific. Possibly, APOA5 mediates energy balance by its association with lipid particles, some of which are able to be transported across the blood brain barrier. It has been shown that in particular LDL and HDL can cross the blood brain barrier via receptor mediated transport [66, 67]. A possible site of peripheral to central translocation of APOA5 containing lipoproteins could be the median eminence of the basal hypothalamus, adjacent to the arcuate nucleus [68]. The arcuate nucleus is crucially involved in the regulation of energy balance and is subject to plastic remodeling when dietary fat content is modulated [69]. Interestingly, low, but significant, APOA5 protein levels have been detected at the lateral ventricle wall in humans [70], which is directly adjacent to the arcuate nucleus.

Interestingly, the inhibiting effect of APOA5 on food intake after intracerebroventricular injection was already achieved at a dose of 8ng/mouse. This dose was approximately 500 fold lower compared to the doses of APOE [55] and APOA4 [54] that are able to reduce food intake. As mentioned before, APOE and APOA4 decrease food intake in a dose dependent manner, which is mediated by the melanocortin system [55, 56]. Since the anorexigenic effects of central oleic acid injection are also mediated via the melanocortin system [45], it is possible that the effect of APOE, APOA4 and APOA5 on food intake are mediated by a regulation of brain lipid metabolism rather than by a direct effect of the individual apolipoproteins themselves. This is further strengthened by the observation that LDL receptor knock out (*Ldlr*$^{-/-}$) mice become more obese compared to controls when fed a 60% HFD [71] and that the LDL receptor mediates, at least in part, the effect of APOE on obesity [72]. Interestingly, similar to *Apoa5*$^{-/-}$ mice, *Ldlr*$^{-/-}$ mice do not reduce their caloric intake when switched from a chow to a HFD [71].

In summary, we demonstrate that *Apoa5*$^{-/-}$ mice are hyperphagic and become more obese and insulin resistant on a HFD when compared to WT mice. Furthermore, we show that APOA5 reduces food intake after central administration. Peripheral injected APOA5 reduces food intake by 60% while ICV injection reduced food intake by 40%, suggesting that the effect of APOA5 on food intake may, at least in part, be mediated via a central mechanism. We propose that the low circulating levels of APOA5 seen in rodents and humans signify a satiety-related signaling function, possibly in addition to an effect of APOA5 on triglyceride hydrolysis.

Acknowledgments

This work was supported by grants from the Center of Medical Systems Biology (CMSB), the Netherlands Consortium for Systems Biology (NCSB) established by The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NGI/NWO), and the Netherlands Diabetes Foundation (DFN2007.00.010 to PCN Rensen). PCN Rensen is Established Investigator of the Netherlands Heart Foundation (grant 2009T038). Furthermore, we would like to thank Rick Havinga (University Medical Center Groningen) for surgical and analytical assistance.

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Supplemental data

*Table S1: Indirect calorimetry data from long term HFD fed Apoa5$^{-/-}$ and WT controls. Data represent mean ± SD, *p < 0.05*

	Light period		Dark period	
	Apoa5$^{-/-}$	**WT**	**Apoa5$^{-/-}$**	**WT**
Respiratory exchange rate	0.798 ± 0.011	0.793 ± 0.011	0.847 ± 0.010	0.830 ± 0.010*
Energy expenditure (kcal/h)	0.583 ± 0.052	0.554 ± 0.031	0.657 ± 0.045	0.656 ± 0.026
Carbohydrate oxidation rate (mg/h)	54.3 ± 11.2	47.1 ± 5.7	88.7 ± 8.8	79.0 ± 8.3
Fat oxidation rate (mg/h)	40.0 ± 1.9	39.8 ± 3.6	34.2 ± 4.2	37.9 ± 2.2*
Activity (beam breaks)	118 ± 26	130 ± 36	284 ± 90	476 ± 140*

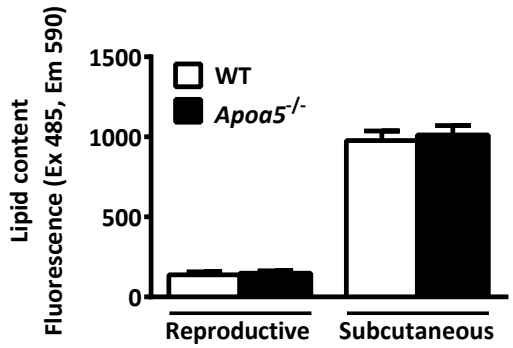

Figure S1: Data representing the differentiation capacity of adipocytes from reproductive and subcutaneous fat depots. Data represent mean ± SD.

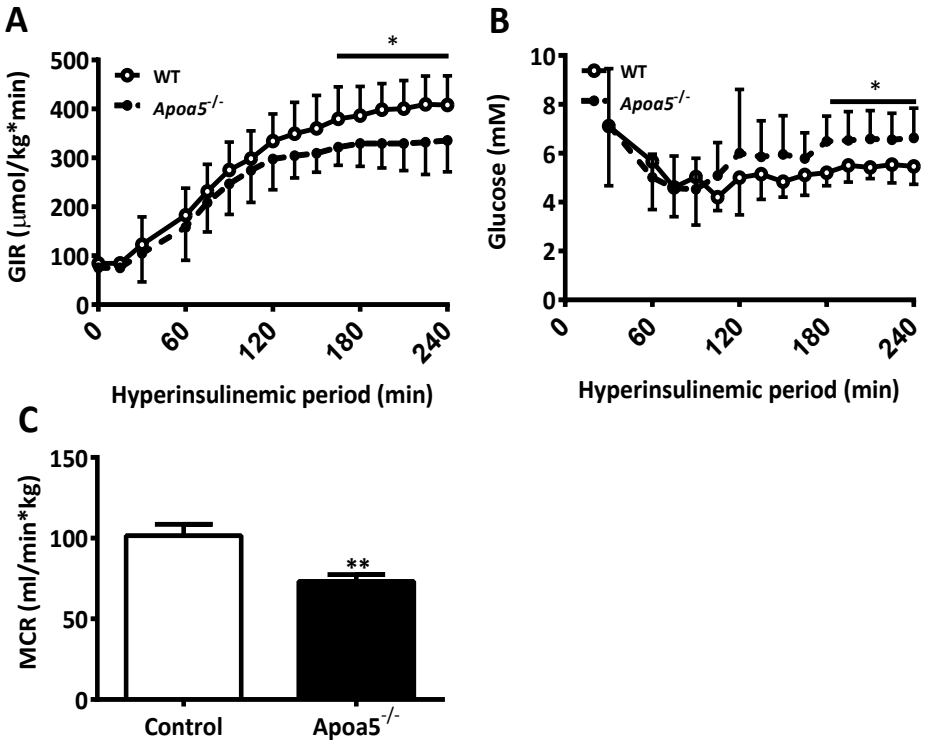

*Figure S2: Glucose infusion rates (A), plasma glucose levels (B) and hyperinsulinemic metabolic clearance rate (C) during the hyperinsulinemic-euglycemic clamp analysis of Apoa5$^{-/-}$ and WT represent mean ± SD, *p < 0.05, **p < 0.01.*