Obesity induced by a high-fat diet down-regulates apolipoprotein A-IV gene expression in rat hypothalamus

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Running head: Apolipoprotein A-IV in high-fat diet-induced obese rats

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ABSTRACT

Apolipoprotein A-IV (apo A-IV) is an anorectic protein produced in the intestine and brain that has been proposed as a satiety signal. To determine whether diet-induced obesity alters apo A-IV gene expression in the intestine and hypothalamus, rats were fed a high-fat (HF), low-fat (LF) or standard chow diet (CHOW) for 2, 4, 6, 8, or 10 weeks. Rats fed the HF diet had significantly greater body weights than rats given the LF and CHOW diets. Intestinal and plasma apo A-IV levels were comparable across dietary groups and time. LF and CHOW rats had comparable hypothalamic apo A-IV mRNA across the course of the experiment. However, HF rats had a slow and progressive diminution in hypothalamic apo A-IV mRNA over time that became significantly lower than that of LF or CHOW rats by 10 weeks. Intragastric infusion of lipid emulsion to animals that were fasted overnight significantly stimulated hypothalamic apo A-IV mRNA in LF and CHOW rats, but had no effect in HF rats. These results demonstrate that chronic consumption of a HF diet significantly reduces apo A-IV mRNA levels and the response of apo A-IV gene expression to dietary lipids in the hypothalamus. This raises the possibility that dysregulation of hypothalamic apo A-IV could contribute to diet-induced obesity.

Key words: apolipoprotein, lipids; gene expression; hypothalamus; intestine
INTRODUCTION

The prevalence of obesity is increasing worldwide and rates have accelerated over the past decade. This fact indicates that the primary cause of obesity lies in environmental and behavioral changes although genetic factors contribute to the propensity of an individual to become obese. One well-established important environmental factor predisposing to obesity is the amount of fat in the diet. Epidemiological studies have identified a significant positive correlation between average dietary fat intake and the incidence of obesity (8; 9). Importantly, recent data suggest that obese animals have blunted satiety, raising the possibility that defective signaling of satiety from gastrointestinal tract and/or central nervous system (CNS) sources may contribute to the etiology of obesity (18).

Apolipoprotein A-IV (apo A-IV) is a protein secreted by the small intestine and liver in rats (1; 5; 20). Numerous studies have demonstrated that apo A-IV synthesis by small intestinal epithelial cells is stimulated by active lipid absorption (5; 13). Hayashi et al. reported that the stimulation of apo A-IV production by lipid administration is associated with the formation of chylomicrons because that such stimulation could be abolished by Pluronic L-81 (L-81), a potent inhibitor of the formation of chylomicrons (6). Recently, we have demonstrated that apo A-IV is expressed in rat hypothalamus. Like the regulation of apo A-IV in the intestine, hypothalamic apo A-IV gene expression is also depressed by fasting and restored by lipid re-feeding (10).

Although acute induction of apo A-IV synthesis in the intestine and hypothalamus by acute fat administration is well documented, the effects of chronic maintenance on a high-fat (HF) diet are less clear. In 1990, Weinberg et al. first reported the adaptation of plasma apo A-IV in response to prolonged fat feeding in humans (17). They demonstrated that after consuming a HF
diet for one week, human plasma apo A-IV was significantly elevated compared with baseline levels, but that it returned toward baseline after the second week on the HF diet. Those authors proposed an autoregulation of plasma apo A-IV at the level of catabolism (17). In their study, however, neither apo A-IV synthesis nor plasma metabolism were examined.

Kalogeris et al. (7) infused a fat emulsion intragastrically to overnight fasted rats for 0, 1, 2, 4, 8, or 16 days. They observed an initial 40% increase in plasma apo A-IV in response to intragastrically administered fat compared with saline-infused controls. However, with continued daily fat feeding, the plasma A-IV response declined progressively such that by 4 days, plasma A-IV levels were not different between fat- and saline-infused groups. Jejunal mRNA levels and mucosal apo A-IV synthesis had parallel time-dependent refractoriness to fat administration.

Apo A-IV has been proposed as a dietary fat-elicited satiety signal (2-4). Thus, we hypothesize that in response to increased fat ingestion hypothalamic apo A-IV production increases and participates in the compensatory response to caloric excess. If this adaptive rise in apo A-IV were to become blunted in the setting of obesity, we further hypothesize that the response to caloric excess or fat ingestion may be blunted further potentiating obesity. In the current study we address the feasibility of this hypothesis in an established model of HF-induced obesity (19). The results indicate that HF-induced overeating and weight gain are associated with a change in the gene expression of apo A-IV in the hypothalamus.
METHODS

Animals. Male Long-Evans rats weighting 220-250 g (Harlan, Indianapolis, IN) were housed individually in plastic tub cages and maintained in a temperature-controlled (22 ± 1°C), humidity-controlled room on a 12:12 light-dark cycle. They received pelleted rat chow (Teklad Sterilizable Mouse/Rat Diet, Harlan) and tap water ad libitum for 1 week to allow for recovery from shipment and stabilization of body weight before being divided into dietary groups on the basis of comparable mean body weight. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati and were conducted in AAALAC-approved facilities.

Diets. Two pelleted semi-purified, nutritionally complete experimental diets (AIN-93M) prepared by Dyets, Inc. (Bethlehem, PA) were used. The HF diet contained 20 g fat/100 g diet by weight (19 g butter oil and 1 g soybean oil to provide essential fatty acids) and provided 19.34 kJ/g of diet, including 7.74 kJ/g as fat. The low fat (LF) diet contained 3 g butter oil and 1 g soybean oil/100 g diet by weight and had 16.12 kJ/g of diet, including 1.29 kJ/g as fat. The HF and LF diets differed only by fat and carbohydrate content, as described before (19), because protein and all of the essential minerals and vitamins were equalized to the amount required for rats per kJ (15).

Experimental Groups. HF rats received ad libitum access to the HF diet, and LF rats received ad libitum access to the LF diet. Because HF and LF rats consumed different amounts of energy each day, two specific control groups were used. One control group, the pair-fed HF group (PHF), was given the HF diet each day, but in an amount limited to the average daily
caloric consumption of the rats fed the LF diet ad libitum. That is, these rats received the same proportion of dietary fat as the HF rats, but had their energy intake yoked to that of the LF rats. Mean daily energy intake of LF rats was calculated every 3 days, and that precise amount of energy, provided as the HF diet, was administered to the yoked rats each day. Over the course of 10 weeks, the mean energy intake of the LF and the PHF rats was 83% of the energy intake of the HF rats.

Although PHF rats consumed the same amount of energy each day as LF rats, their food intake was limited and these animals should be considered to be chronically food-restricted. Since food restriction per sé, independent of dietary conditions, could influence important variables of interest, we included a second control group which consumed the LF diet, and which was energy restricted by the same proportion (i.e., -17%) each day as the PHF rats (i.e., the PLF group). We also included an additional group of rats that consumed a nonpurified chow (CHOW) diet (Teklad Sterilizable Mouse/Rat Diet, Harlan) ad libitum for providing a link to the numerous reports that used CHOW diets as the only control for a HF diet. The difference between CHOW and semipurified diets includes nutrient content and energy density. Therefore five (HF, LF, PHF, PLF and CHOW) groups were included.

Experiment-1: The rats in the different dietary groups were maintained for 2 (7 rats/group), 4, 6, 8 (5 rats/group) or 10 weeks (10 rats/group). Animals in each condition were deprived of food for 20 hours before sacrificed in order to stabilize apo A-IV levels. Following decapitation, the brains were quickly removed, and the whole hypothalamus was dissected according to the scheme depicted in the atlas of Paxinos and Watson (14). Jejunum and trunk blood were
collected. The intestinal tissues were immediately frozen in liquid nitrogen and stored at -80°C. Trunk blood was centrifuged and plasma was stored at -80°C. Separate aliquots of plasma were taken for assay of apo A-IV measured by ELISA and triglyceride (TG) using an enzymatic assay (Sigma Chemical, St. Louis, MO).

Experiment-2: Five groups of rats were maintained on the different diets for 10 weeks. Before the final day, the animals were deprived of food for 20 hours. On the final day, the fasted rats in each group were divided into two sub-groups (6 rats/group) of equal body weight. One sub-group received physiological saline by gavage (5 ml/rat), and the other received lipid (Intralipid, containing 20% lipid, 5 ml/rat). Four hours after the gastric load, the rats were killed by decapitation and treated as described above.

RT-PCR for apo A-IV mRNA. Total RNA from hypothalamus and intestine were isolated with Tri Reagent (Molecular Research Center, Inc Cincinnati, OH) according to the manufacturer’s suggested protocol. Total RNA concentration was determined spectrophotometrically at 260 nm. To ensure uniform and reproducible reaction conditions, both RT and PCR reaction regents were prepared as master mixes and aliquotted into individual tubes prior to using them for each batch of reactions. Hypothalamic apo A-IV mRNA level was measured by competitive RT-PCR (10). RT was carried out with 200 ng of hypothalamic total RNA digested by DNase together with varying concentrations of apo A-IV competitor RNA, generated as previously described (10) in a volume of 50 µl with random hexamer primer and Moloney murine leukemia virus (MMLV) reverse transcriptase according to manufacturer’s guidelines (Amersham Pharmacia Biotech, Piscataway, NJ). The cDNAs were then amplified with 29 cycles. One round of amplification
consisted of 30 sec at 92°C, 45 s at 60°C and 45 sec at 72°C, where the final extension step lasted 7 min. Intestinal apo A-IV mRNA was determined by relative RT-PCR. The process for the relative RT-PCR was the same as described above except that a 10 ng sample of total RNA and no apo A-IV competitor RNA were used in the RT reaction. The sequences of the primers for apo A-IV mRNA are depicted in Table 1. To determine whether equal amounts of total RNA had been added to the RT-PCT reaction, the housekeeping gene for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, primers in Table 1) was used as an internal control.

PCR products were separated on 1.5% agarose gel containing Gelstar staining (FMC, Rockland Maine). The intensities of the bands were quantified using a PhosphorImager (Molecular Dynamics, USA). For competitive RT-PCR, the intensity of the bands and the log of the ratio of amplified target PCR products (hypothalamic apo A-IV) to standard PCR products (apo A-IV competitor) were graphed as a function of the known amount of standard RNA added to the reaction (10). For relative RT-PCR, the level of amplified apo A-IV mRNA of intestine was expressed as a ratio of GAPDH mRNA amplified in the same RT-PCR assay.

The following control experiments were performed to ensure the specificity of the RT-PCR reaction. First, the in vitro transcribed RNAs (target and standard) were amplified without random hexamer primer for reverse transcript to ensure that the amplification products obtained derived only from the RNAs and not from remaining DNA sequences caused by incomplete digestion of the templates. Second, a no-template negative control reaction was carried out to control for contamination of the PCR components. In both cases no PCR product was detectable by GelStar staining (data not shown).
Assay of apo A-IV by ELISA. Plasma apo A-IV was measured by the ELISA antibody sandwich method. 100 µl of standard (0 ~ 35 ng/ml apo A-IV) and plasma samples diluted (1:10,000) with phosphate-buffered saline containing 0.05% Tween-20 (PBSTw) and 1% BSA were added in duplicate to the coated 96-well plates by 100 µl of goat antiserum against rat apo A-IV, which was diluted in 0.1 M citrate buffer (pH 3.5). The plates were incubated overnight at 4°C. Then 100 µl of rabbit polyclonal antibody against rat apo A-IV (1:2,000 diluted in PBSTw 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 PBSTw containing 1% BSA) was added and incubated for 1 h at 37°C. Color was developed by adding 200 µl of OPD Peroxidase Substrate (Sigma, St. Louis, MO). After 30 min in the dark at room temperature, 50 µl of 3 M HCl was added to stop the reaction and the optical density was measured at 492 nm.

Statistical analyses. All values are expressed as group mean ± SEM. Data were analyzed by parametric statistics (repeated measures ANOVA with time as the repeated measure, followed by planned t-test). Differences were considered significant when the probability of the difference occurring by chance was less than 5 in 100 (P < 0.05).
RESULTS

*Body weight and plasma lipids.* Body weight of the 5 groups of rats differed after 2 weeks on the different diets, and after 10 weeks the HF rats were significantly heavier, as depicted in Figure 1, than all other groups (Fig. 1). As expected, PHF rats weighed less than HF rats ($P < 0.05$) and were not different from the LF rats ($P = 1.005$). PLF rats weighed less than LF rats, but the difference was not significant ($P = 0.546$). These results are comparable to what we have previously observed with these diets (19).

Plasma TG levels were higher in LF rats than in any of the other four groups over the entire course of the experiment (Table 2). The difference reached significance at Week 8 compared with PLF ($P = 0.019$) and PHF ($P = 0.029$) rats, and at week 10 compared with PLF rats ($P = 0.032$). TG levels in HF rat plasma were slightly lower than that in LF rats, and slightly higher than those in CHOW, PLF and PHF, but the difference among these five groups was not significant (Table 2).

*Intestinal apo A-IV mRNA.* We determined intestinal apo A-IV mRNA levels from rats in each of the five groups at any time period. There were no significant differences in gene expression of apo A-IV. Also intestinal apo A-IV mRNA did not significantly correlate with body weight gain in any group of animals (data not shown).

*Plasma apo A-IV.* Plasma apo A-IV protein levels measured by ELISA were not affected by different diets over the course of the experiment. No significant correlations were found
between plasma TG and intestinal or plasma apo A-IV mRNA levels, indicating that higher plasma TG in LF and HF rats were not associated with peripheral apo A-IV levels.

*Hypothalamic apo A-IV mRNA.* The effect of dietary fat on apo A-IV gene expression in the hypothalamus was dependent on the length of the dietary treatment. The HF rats had a slow and progressive diminution in hypothalamic apo A-IV gene expression, compared to the LF or CHOW animals that attained significance after 10 weeks (Fig. 2). After 10 weeks on the diets, apo A-IV mRNA levels in the hypothalamus of the HF rats were reduced by 31% compared to the LF group ($P < 0.05$) and 43% compared with the CHOW animals ($P < 0.01$).

*The response of hypothalamic apo A-IV gene expression to the stimulation of dietary lipid.* As depicted in Fig. 3, intragastric infusion of lipid emulsion to fasted animals significantly increased hypothalamic apo A-IV mRNA content in all groups other than the HF rats ($P < 0.05$); i.e., hypothalamic apo A-IV in the HF rats is less responsive to dietary lipids than the other dietary groups.
DISCUSSION

As we have previously reported (19), rats with free access to the HF diet consumed more energy and became obese relative to rats with free access to a diet containing the same constituents but a lower proportion of fat. The purpose of these experiments was to test the hypothesis that in response to increased fat ingestion hypothalamic and intestinal apo A-IV production increases and participates in the compensatory response to caloric excess. However, this hypothesis has not been tested directly. What we found are that hypothalamic apo A-IV gene expression decreased as a function of time on the HF diet, and that the difference from controls was reliable by 10 weeks. Interestingly, neither intestinal nor circulating apo A-IV levels were significantly altered, which are consistent with data in humans where after 2 weeks of consuming a high-fat diet, the plasma apo A-IV did not respond to lipids (8). Further, Kalogeris et al. (7) demonstrated that intragastric infusion of a fat emulsion to overnight-fasted rats increased intestinal expression and plasma apo A-IV levels only during the first few days. With continued daily fat infusion, these responses had a rapid and progressive diminution.

Apo A-IV is an apolipoprotein stimulated in response to the ingestion of lipids (6; 10). Several lines of evidence suggest that brain apo A-IV plays an important role in the regulation of feeding behavior. Intracerebroventricular administration of apo A-IV significantly inhibits food intake in a dose-dependent manner without eliciting signs of toxicity (2; 4; 11). Blocking the action of endogenous apo A-IV with a specific neutralizing antibody increases meal size, implying that endogenous apo A-IV exerts an inhibitory tone on feeding (3; 10). Centrally administered NPY stimulates hypothalamic apo A-IV gene expression, suggesting that multiple factors interact to regulate apo A-IV levels in the hypothalamus (12). If brain apo A-IV is a
physiological regulator of fat intake, the reduced hypothalamic apo A-IV levels in the HF animals observed in the present study would be predicted to reduce the satiety response and thereby contribute to their hyperphagia and/or fat preference.

An important question is whether the change in hypothalamic apo A-IV gene expression is a result of exposure to the HF diet or the resulting obesity. Our results showed the rats that consumed a restricted caloric intake of HF diet did not become obese, and did not have decreased apo A-IV gene expression within the hypothalamus. We therefore conclude that the decrease in apo A-IV gene expression observed in the HF rats is secondary to increased caloric consumption and the consequent obesity rather than mere exposure to the HF diet itself. A very interesting question for future studies is whether one can increase the hypothalamic apo A-IV gene expression by diet restriction in animals that are already obese.

A change in hypothalamic apo A-IV gene expression could be caused by several factors. Given the sensitivity of hypothalamic apo A-IV gene expression to ingested lipids (10), the most obvious factor would be increased TG levels in the plasma of HF rats. This mechanism cannot be directly addressed in our study because our animals were fasted for 20 hours prior to measurement. An alternative cause of decreased hypothalamic apo A-IV gene expression would be increased peripheral apo A-IV levels, which could negatively feedback to regulated apo A-IV gene expression in the hypothalamus. However, this did not occur because peripheral apo A-IV levels were not significantly altered after HF feeding. Future experiments will need to investigate the mechanism(s) by which increased calorie consumption and/or obesity decreases hypothalamic apo A-IV gene expression.
The alteration in apo A-IV mRNA in the hypothalamus is not the only factor, which could contribute to the obesity caused by chronic HF feeding. Reduced apo A-IV signaling could also be the result of decreased response of hypothalamic apo A-IV to the dietary lipid. To test this hypothesis, we measured the hypothalamic apo A-IV mRNA levels after lipid infusion in all five groups of animals. We found that, unlike LF- or CHOW- fed rats, the response of hypothalamic apo A-IV gene expression to a lipid infusion in 10-week HF fed rats was completely absent in HF rats. Although requiring further study, one implication of these data is that a manipulation to prevent hypothalamic apo A-IV level from diminution could be useful to treat increased weight that results from exposure to a diet that mimics the macronutrient content of the average American (8; 9).

It is worth noting that the response of animals to the HF diet is a subtle but cumulative effect, because it took over 10 weeks period for the HF rats to accumulate 50% more body fat than LF rats while consuming just 17% more calories (19). The increase in obesity in our society is also the consequence of cumulative changes, which cause individuals to be in a mild positive energy balance year after year. Therefore, we may need to search for subtle effects such as those observed here to investigate the mechanisms by which specific diets increase body weight.

In conclusion, we have demonstrated that the chronic consumption of a HF diet significantly reduces apo A-IV gene expression and the response of apo A-IV to dietary lipids in the hypothalamus of rats. Since Apo A-IV suppresses food intake, this response of hypothalamic apo A-IV could contribute to diet-induced obesity. Future studies will be needed to clarify the
mechanisms by which increased body weight inhibits hypothalamic apo A-IV and to elucidate the specific hypothalamic areas where these changes occur.
ACKNOWLEDGEMENT

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DISCLOSURES

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REFERENCES


Figure legends:

Figure 1. Mean body weight (g) of the 5 groups of rats over the duration of the experiment. Values are means ± SEM, n = 7 (week 2), n= 5 (week 4, 6 and 8), n = 10 (week 10). *P < 0.05, compared with the other groups at week 10.

Figure 2. Mean hypothalamic apo A-IV mRNA level, as determined by competitive RT-PCR, in rats on the 5 diets over the course of the experiment. Results are expressed as means ± SEM, n = 7 (week 2), n= 5 (week 4, 6 and 8), n = 10 (week 10). *P < 0.05, compared with LF group, *P < 0.01, compared with the other groups at week 10.

Figure 3. Mean apo A-IV mRNA levels in the hypothalamus (fmol/μg total RNA) following saline or lipid infusion in the 5 groups of rats after 10 weeks, as determined by competitive RT-PCR. Results are expressed as means ± SEM (n = 6). **P<0.01; *P<0.05, compared to fasted animals fed with the same diet.
Table 1. *Primers used in RT-PCR reaction*

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers</th>
<th>Fragment size</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Apo A-IV | Forward: 5’- CTTTGCAACGAGCTAAAGG -3’  
Reverse: 5’- GCTGCTTGTCAGGTCTTCC -3’ | 343 bp        | Liu, 2001 (10)  |
| GAPDH  | Forward: 5’- TGCCCTCTCTTGTGACAAAGTTG -3’  
Reverse: 5’- CATTGCTGACAATCTTGAGGGAG -3’ | 386 bp        | Tatemoto, 1982 (16) |
Table 2. Plasma TG (mg/dl) in the 5 dietary groups over the course of the experiment

<table>
<thead>
<tr>
<th>Diets (n)</th>
<th>Week-2</th>
<th>Week-4</th>
<th>Week-6</th>
<th>Week-8</th>
<th>Week-10</th>
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<tr>
<td>Chow (n = 7)</td>
<td>78.2 ± 4.0</td>
<td>73.3 ± 5.3</td>
<td>75.9 ± 7.5</td>
<td>74.5 ± 10.3</td>
<td>75.7 ± 5.7</td>
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<tr>
<td>PLF (n = 5)</td>
<td>87.2 ± 11.5</td>
<td>79.3 ± 9.7</td>
<td>78.7 ± 4.7</td>
<td>68.9 ± 9.7</td>
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<td>LF (n = 5)</td>
<td>111.2 ± 9.8</td>
<td>120.6 ± 7.9</td>
<td>116.5 ± 10.8</td>
<td>117.9 ± 6.2*</td>
<td>117.5 ± 10.7#</td>
</tr>
<tr>
<td>PHF (n = 5)</td>
<td>84.4 ± 12.2</td>
<td>84.2 ± 9.0</td>
<td>79.5 ± 4.1</td>
<td>71.3 ± 6.2</td>
<td>77.7 ± 11.9</td>
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<tr>
<td>HF (n = 10)</td>
<td>103.2 ± 13.8</td>
<td>105.3 ± 13.3</td>
<td>102 ± 4.1</td>
<td>106.6 ± 9.7</td>
<td>104.1 ± 11.8</td>
</tr>
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Values are means ± SEM. *P < 0.05, compared with PLF and PHF groups at week 8; #P < 0.05, compared with PLF group at week 10.
Figure-1

![Graph showing body weight (g) vs. time (weeks) for different diets (HF, PHF, LF, PLF, CHOW). The graph demonstrates a trend of increasing body weight over time for all diets, with significant differences indicated by asterisks.](image-url)
Figure-2

Apo A-IV mRNA (fmol/μg total RNA) vs Time (weeks)

- HF
- PHF
- LF
- PLF
- CHOW

* peak
# trough
Figure-3

Groups

Apo-A-IV mRNA (fmol/μg total RNA)

CHOW  PLF  LF  PHF  HF

Fasting  Fasting+Lipid

**  **  *  **

0  20  40  60  80  100  120  140  160  180  200