

INCREASED EXPRESSION OF RECEPTORS FOR OREXIGENIC FACTORS IN
NODOSE GANGLION OF DIET-INDUCED OBESE RATS

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Running Title: high fat diet alters receptor expression in vagal neurons

Abstract

The vagal afferent pathway is important in short-term regulation of food intake and decreased activation of this neural pathway with long-term ingestion of a high fat diet may contribute to hyperphagic weight gain. We tested the hypothesis that expression of genes encoding receptors for orexigenic factors in vagal afferent neurons are increased by long-term ingestion of a high fat diet, thus supporting orexigenic signals from the gut. Obesity prone (DIO-P) rats fed a high fat diet showed increased body weight and hyperleptinemia compared to low fat fed controls and high fat fed diet-induced obese resistant (DIO-R) rats. Expression of the type I cannabinoid receptor and growth hormone secretagogue receptor 1a in the nodose ganglia was increased in DIO-P compared with low fat fed or DIO-R rats. Shifts in the balance between orexigenic and anorexigenic signals within the vagal afferent pathway may influence food intake and body weight gain induced by high fat diets.

INTRODUCTION

Regulation of food intake and body weight involves both peripheral and central nervous systems, together with endocrine pathways (7). Both short and long term regulation of food intake occurs via complex, hierarchical control that maintains body weight within a narrow range. Signals initiated in the gastrointestinal (GI) tract in response to food ingestion are generally thought to be involved in short term regulation of food intake. Thus, the presence of nutrients in the gut lumen decreases food intake by meal termination and reduction in the size of individual meals, but with little or no effect on overall daily food intake (21). Cholecystokinin (CCK), released by fat and protein digestive products in the proximal small intestine, decreases meal size, at least in part, via activation of CCK₁ receptors (CCK₁R) expressed by vagal afferent neurons terminating in the gut wall (3,9,8). Peptide YY₃₋₃₆ is an anorexigenic gut hormone released from the distal gut that reduces food intake following exogenous administration (2), possibly also acting at the level of vagal afferent nerve terminals (13).

However, there are hormones and bioactive molecules released by the GI tract that increase food intake and may stimulate appetite. These include ghrelin, a peptide released from the gastric corpus (14) and cannabinoids, such as anandamide (10). These factors are released during the inter-meal interval and plasma levels are highest right before meal initiation (7,22). There is good

evidence that exogenous ghrelin stimulates food intake, acting, at least in part, via the vagal afferent pathway (8), although this had not been confirmed in another study (1).

It has recently been shown that expression of peptide receptors by vagal afferent neurons is regulated by nutritional status. Prolonged fasting increases expression of receptors associated with anorexigenic mediators, the cannabinoid type 1 (CB1) and melanocortin concentrating hormone type 1 (MCH-1) receptors; this increase is reversed by feeding or administration of CCK (4,5). Thus, under normal conditions, GI-derived anorexigenic and orexigenic signals interact to regulate expression levels; this is likely to be important in control of food intake and energy homeostasis. However, there is no information on whether this balance between anorexigenic and orexigenic receptor expression is altered by long term changes in diet. Chronic ingestion of a high fat (HF) diet leads to hyperphagia and weight gain. Numerous studies have shown adaptation at the level of the hypothalamus after chronic ingestion of a HF diet (7,10), but there is evidence to suggest that adaptation to HF foods can also occur in the periphery at the level of the vagal afferent pathway. For example, ingestion of HF diet decreases vagal afferent activation in response to CCK and to intestinal lipid (121) and diet-induced obesity in mice leads to attenuation of the synergistic action of urocortins and CCK to decrease food intake and delay gastric emptying (12).

In the present study, we tested the hypothesis that adaptation to a HF diet alters expression of gut hormones receptors (CCK₁R, Y2 receptor, GHSR), leptin receptor (ObR), and CB1 receptor and fatty acid amide hydrolase (FAAH), both part of the endocannabinoid system, by vagal afferent neurons. We measured mRNA expression at the level of the vagal afferents by quantitative real-time RT-PCR. It is well established that distinct diet-induced obese prone (DIO-P) and resistant (DIO-R) phenotypes of Sprague Dawley rat can be revealed by maintenance on a HF diet (15,16). We measured changes in the metabolic profile (body weight, adiposity, food intake, plasma insulin and leptin) in rats fed a low fat (LF) control diet or a high fat (HF) diet for either one week or following maintenance on the diets for eight weeks.

METHODS

Animals: Diets and Experimental Procedures

Male Sprague Dawley rats (6 weeks of age, Harlan, San Diego) were fed *ad libitum* a low fat diet (LF: Research Diets D12450B) or a high fat diet (HF: Research Diets D12451) for 1 or 8 weeks. The LF diet provided 3.85 kcal/g of energy (70% carbohydrate, 20% protein, 10% fat [SAT:25.1%-MUFA:34.7%-PUFA:40.2%]) and the HF diet provided 4.73 kcal/g of energy (35% carbohydrate, 20% protein, 45% fat [SAT:36.3%-MUFA:45.3%-PUFA:18.5%]). Rats were initially housed in pairs for 1 week, then housed individually in a temperature-controlled room with regular light conditions (lights on 06:00 and off 18:00). Water was freely available throughout the experiments and body weight was recorded daily. Food intake was measured daily for the first three weeks, every 2-3 days thereafter for the duration of the experiment. The HF group weighed 177 ± 4 g (n=15) and the LF group weighed 176 ± 7 g (n=10) at the beginning of the experiment (NS). All experiments were performed in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee, UC Davis.

Measurement of plasma insulin and leptin

Blood samples were collected from the tail (1mL in 30 μ L of heparin), following a 12h fast during the light cycle (6pm), at week 1 and week 8. On a different day, 12 hr fasted rats were gavaged with 1ml/100g of lipid emulsion (Intralipid 20%; Baxter HealthCare Corp., Deenfield) and blood samples were collected 2 hours after gavage. Blood was centrifuged (1,000g 10min), the

plasma removed and stored at -80°C. Leptin and insulin were measured by ELISA (Catalog No.: 22-LEP-E06 Lot No.: 090107 for Leptin, Catalog No.: 80-INSRTU-E01 Lot No.: 00208 ALPCO Diagnostics, Salem, NH).

Tissue Collection

After 1 or 8 weeks on the diets, rats were fasted for 12 hrs during the light cycle, gavaged with 1 mL/100g lipid emulsion (Intralipid 20%) and after 2 hours, deeply anesthetized with sodium pentobarbital (100 mg/kg IP; Nembutal, Abott Laboratories). The rationale for the use of lipid gavage in fasted rats 2 hours prior to euthanasia was to ensure that all animals were in the same nutritional status. A blood sample (2 mL) was obtained from the descending aorta. Tissues were dissected with instruments cleaned with RNA Zap (Ambion, Austin, TX) and collected into 2 ml tubes previously decontaminated with RNA Zap, flash frozen in liquid nitrogen and stored at -80°C. Left and right nodose ganglia were collected; epididymal, mesenteric and retroperitoneal fat pads were dissected and weighed individually. An adiposity index consisting of the summed weight of the three fat pads divided by the final body weight was calculated.

Quantitative real-time PCR

Right and left nodose ganglia were pooled from each individual rat. Samples (10 to 20 mg) were ground with a mortar and pestle under liquid nitrogen. Once powdered, RNA extraction was executed with the RiboPure Kit (Ambion, Austin, TX); samples

were extracted by adding 500 μ L of Tri-Reagent. Sample integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA) and RNA concentration assayed with a NanoDrop (Thermo Fisher Scientific, Wilmington, DE).

cDNA was synthesized by reverse transcription from 500ng total RNA using a cDNA Synthesis Kit Superscript III First Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using Taqman® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The Taqman® Gene Expression Assay consisted of a FAM™ dye-labeled TaqMan® MGB probe and two PCR primers formulated into a single tube for each target gene (GenBank Accession Number: CCK1-R: M88096, Y2-R: AY004257, CB1: X55812, FAAH: U72497, Fa: D84550, GHS-R: AB001982, and 18S ribosomal: X03205). 6 μ L of cDNA diluted 30-fold after reverse transcription were added to each well and let dry out overnight. Each PCR was run with 8 μ L of a mix containing PCR Mix TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and TaqMan® Gene Expression Assay on the ABI PRISM 7700 Sequence Detection System according to the manufacturer (Applied Biosystems, Foster City, CA, USA). Expected amplicon sizes were confirmed by high resolution agarose gel eletrophoresis.

Analysis of relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method (17) using 18S as mRNA loading control. Briefly, the C_T (threshold cycle when fluorescence intensity exceeds 10 times the standard deviation of the baseline fluorescence) value for the

target amplicon (CCK1-R, Y2-R, CB1, FAAH, GHS-R) and endogenous control (18S) are determined for each PCR reaction. Each PCR reaction was repeated in triplicate (17). The sample expression levels are expressed as an *n*-fold difference relative to those found in the average low fat (week1) nodose ganglia expression level.

Statistical Analysis.

Body weight and composition; plasma leptin and insulin. A two-way repeated measure ANOVA was performed with time, diet and time-diet interactions as independent variables. All analyses were conducted using SigmaStat (version 3.11, Systat Software, Inc.). Differences among group means were analyzed using multiple comparison procedures (Holm-Sidak method) and considered significant if $P < 0.05$. One HF week 1 and one LF week 8 rats died during gavage explaining the difference in group size between body weight data and gene expression data.

Quantitative Real-Time PCR. Data are presented as the adjusted mean $2^{-\Delta\Delta CT} \pm$ S.E.M. Statistical analysis of the differences in mRNA levels was performed by two-way ANOVA with time, diet and time-diet interactions as independent factors. Differences among group means were analyzed using multiple comparison procedures (Holm-Sidak method) and considered significant if $P < 0.05$ (SigmaStat version 3.11, Systat Software, Inc.).

Principal Component Analysis. Principal components analyses were performed on data sets using the Excel Add-In developed by the Bristol Centre for Chemometrics (<http://www.chm.bris.ac.uk/org/chemometrics/index.html>). Prior to performing PCA, quantitative results for each variable were transformed by vast scaling as described (23) using the following

equation:
$$x_{ij} = \frac{(x_{ij} - \bar{x}_i)}{s_i} \cdot \frac{\bar{x}_i}{s_i}$$

This transformation provided better separation of experimental groups than either autoscaling, i.e. data transformed to unit standard deviation, or pareto scaling, i.e. transformed to unit standard error of the mean.

RESULTS

Within the HF group, two distinct phenotypes emerged by week 3; in a subgroup of HF rats, there was a significant increase in body weight gain compared to LF rats (Fig 1A). HF rats with the highest terminal body weight were designated DIO-P; body weight was significantly greater in this group than both DIO-R and LF groups ($p < 0.05$). There were no differences in body weight between DIO-R and LF groups at any time point (NS).

There was no significant difference in the adiposity index between the LF or HF rats at week 1. At week 8, the DIO-P group had a higher adiposity index than the LF rats ($p = 0.005$), while DIO-R group did not ($p = 0.06$; Fig. 1B); there was no significant difference in the adiposity index between the DIO-P and DIO-R ($p = 0.3$). However, there were significant differences in the weights of individual fat pads between groups (Fig 1C). DIO-P rats had significantly larger mesenteric fat pad weight than either the DIO-R or LF rats (LF vs. DIO-P, $p < 0.001$; DIO-R vs. DIO-P, $p < 0.05$). Both DIO-R and DIO-P rats had significantly larger epididymal fat pad weight than LF rats ($p < 0.05$). There were no significant differences in the retroperitoneal fat pad weight between any groups ($p \geq 0.09$).

After 8 weeks on a HF diet, DIO-P rats had a significantly higher caloric intake than DIO-R or LF rats (kcal/day; 1 week LF vs. HF: 67 ± 4 vs. 60 ± 6 , $p = 0.04$; 8 weeks LF vs. DIO-R 73 ± 4 vs. 66 ± 9 , $p = 0.2$; LF vs. DIO-P: 73 ± 4 vs. 94 ± 7 , $p < 0.001$; $n = 5$ in each group).

There was no significant difference in fasting plasma level of leptin at week 1; however, after 8 weeks on the diets, DIO-R and DIO-P had higher fasting plasma leptin level compared to LF rats, consistent with higher adiposity (pg/ml; week 1; LF: 339 ± 80 ; HF: 431 ± 101 ; week 8: LF: 372 ± 49 ; DIO-R: 816 ± 67 ; DIO-P: 755 ± 136 ; $p = 0.003$). The increase in plasma leptin between the fasted state and two hours after gavage with a lipid load was significantly higher in the DIO-P rats compared to LF ($p < 0.05$) but not DIO-R rats ($p = 0.2$; Fig. 2).

There was no significant difference in fasting insulin between LF rats or DIO-R and DIO-P rats at 8 weeks (ng/mL: 8 weeks LF vs. DIO-R vs. DIO-P: 0.97 ± 0.11 vs. 1.07 ± 0.18 vs. 1.01 ± 0.13 ; NS).

Nodose ganglion receptor expression

After 8 weeks on the LF or HF diet, there was an increased expression of CB1R (2 ± 0.7 -fold), CCK₁R (3 ± 1 -fold), (3 ± 1 -fold), and GHSR1a (5 ± 3 -fold), in the DIO-P group compared to either the DIO-R or LF rats (Fig 3). There was no significant difference in the gene expression of ObR and Y2R between any groups.

Discrimination of DIO-P and DIO-R phenotype

As shown above, multiple differences were identified between the experimental groups. By using the aggregated data set and the unsupervised multivariate analysis principle components analysis (PCA) to discriminate experimental groups, the relative weight each factor has in separating these groups was evaluated within a single analysis. Considering the entire data matrix, rats at week 1 could not be discriminated, despite maintenance on the different diets, but were distinct from rats at week 8 (Fig 4). The daily caloric intake, mass specific caloric intake (i.e. kcal/day/g body weight) and adiposity index were the most powerful discriminates of animals by age and diet, discriminating LF and DIO-R from DIO-P animals at 8 weeks. Moreover, the change in nodose ganglion expression profiles were powerful factors separating the experimental groups, and in this aggregate analysis, the variance within the data set after vast scaling was not different among groups (F-test, $p > 0.05$).

DISCUSSION

The results from the present study demonstrate that expression of receptors for gut-derived orexigenic factors (ghrelin and cannabinoids) in the nodose ganglion increase in rats chronically ingesting a high fat diet and prone to obesity, but not in rats fed the same diet but which are resistant to the obesigenic effects of the HF diet. This increase in receptor expression is accompanied by an increase in food intake in DIO-P rats compared to the DIO-R rats; these data suggest that an increase in expression of orexigenic receptors by vagal afferent neurons may increase food intake via peripherally acting ghrelin and endogenous cannabinoids. This increase in receptor expression is not dependent solely on the ingestion of a HF diet, but is only evident in rats susceptible to the obesigenic effects of ingesting high fat diets; that is, rats with an increase in body weight, circulating leptin and a significantly greater mesenteric fat pad mass. Whether this change in receptor expression is driving the hyperphagia and weight gain or is secondary to these metabolic changes is not clear from the present study. We have previously shown that rats fed a HF diet became hyperphagic on a high fat diet predominantly via an increase in meal size (18), suggesting a decreased satiation. The predominant effect of exogenous ghrelin on food intake is to decrease latency to feeding; ghrelin action is mediated at least in part via the vagal afferent pathway (8), although other investigators have not been able to confirm this finding (1). It has recently been shown that there is considerable short term regulation of receptor expression, both at the level of RNA and protein, in vagal afferents (4,5). Prolonged (48 hour) fasting increases expression of CB1 and MCH-1 receptors; this is reversed by feeding or by exogenous CCK acting via a CCK₁R mechanism.

Prolonged fasting did not change expression of GHSRs but administration of ghrelin counteracts the decrease in the CB1/MCH1Rs in response to refeeding or CCK (4). Thus, an increase in GHSR expression in response to high fat feeding might result in an increased orexigenic drive and an increase in food intake due to an increase in CB1R expression and possibly MCH-1R. Levels of protein were not measured in the current study; however, there is good agreement between levels of mRNA and protein for these receptors (4,5). It is interesting to note there is evidence from ghrelin or GHS-R null mice model to suggest that ghrelin signaling is required for development of the full phenotype of diet-induced obesity (24,25).

The increase in CCK₁R expression was surprising given functional data to suggest a decrease in activation of the vagal afferent pathway by CCK when rats are maintained on a high fat diet (21). It is possible that there is altered coupling between receptor and intracellular signaling pathways or changes in synaptic activity at the level of the nucleus of the solitary tract where vagal afferents terminate. There is data to suggest decreased fatty acid amide hydrolase (FAAH) expression and/or activity as a result of obesity or high-fat intake (10); here we show that FAAH is correlated with CB1 expression in the nodose ganglia. It is interesting to note the expression of FAAH in the nodose ganglia suggesting that there may be regulation of the endocannabinoids at the level of the vagal afferents in conjunction with the CB1 receptor.

It is well-documented that Sprague-Dawley rats fall into two broad groups, those that gain significant weight on a HF diet and those that seem relatively resistant to the obesigenic effects of a HF diets (15,16). In the current study, we present an original way to discriminate DIO-P from DIO-R rats beyond using body weight gain alone. We performed a PCA using a variety of phenotypic outcomes successfully differentiating cohorts, highlighting that phenotypic “signatures” may be used as a tool to predict diet-induced obesity. It is interesting to note that there were no significant differences in the overall adiposity of the two groups of HF rats, which are both significantly increased over the LF rats. However, if the different fat depots are examined separately, it can be seen that there is an increase in mesenteric, but not epididymal or retroperitoneal fat mass in the DIO-P animals. Mesenteric fat is thought to be a source of inflammatory mediators that can influence the GI tract and distant organs (6). It is also interesting to note that fasting plasma levels of leptin do not reflect the overall increase in adiposity; in the DIO-R rats, there is a tendency for an increase in plasma levels of leptin as would be expected with an increase in fat pad mass, however, this did not reach significance. There is no difference in fasting plasma levels of insulin in either high fat fed group, suggesting that the animals were not insulin resistance after 8 weeks on the diets. Although the mechanism by which receptor expression in vagal afferent neurons is changed in response to the HF diets in DIO-P is not the focus of the present study, it is interesting to speculate on the role of inflammatory mediators released from the mesenteric fat on vagal afferent nerve terminals.

Taken together, these data suggest that the gut-brain axis, in addition to metabolic changes associated with a chronic ingestion of a high-fat diet, is modified by the diet, leading to altered short-term control of food intake towards an orexigenic system and an increase in food intake. The increase in the expression of orexigenic receptors CB1 and GHSR 1a might lead to an increase peripheral hunger signals and thus contribute to hyperphagia when ingesting a high fat diet. The data also show a paradoxical increase in expression of CCK₁Rs; however, it remains to be determined whether this is associated with altered receptor signaling as several studies have shown a decrease in vagal afferent response to CCK in rats maintained on high fat diets.

Acknowledgements: Work supported by NIHDK 41004 (HER), USDA/ARS Intramural CRIS 5306-51530-016-00D (SHA, JWN), and USDA/ARS Postdoctoral Fellowship (TK).

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FIGURES LEGENDS:

Figure 1: Effect of ingestion of HF on body weight gain, adiposity and fat pad mass in LF and HF rats after 1 and 8 weeks on respective diets. A. DIO-P rats had a significant increase in body weight (expressed as % of initial body weight) at 3-8 weeks compared to DIO-R or LF rats (LF or DIO-R vs. DIO-P, $p < 0.05$). B. Adiposity index calculated as sum of fat pads expressed as % of body weight. Note at week 1, rats divided into two groups, either LF or HF as cannot be discriminated into DIO-R and DIO-P groups. There is a significant increase in adiposity in DIO-R and DIO-P rats compared to rats maintained on LF diet (LF vs DIO-R or DIO-P $p < 0.05$). C. Mass of different fat pads; there is a significant difference in mesenteric fat pad mass between DIO-R and DIO-P after 8 weeks on a HF diet ($p < 0.05$). Data expressed as mean \pm SEM; LF n=10, DIO-R n=7, and DIO-P n=8, . Different letters denote significant differences between columns.

Figure 2: The increase in plasma leptin concentration between fasted state and 2 hours after oral lipid gavage was significantly higher in DIO-P rats than in LF or DIO-R rats after 8 weeks on a HF diet. Data expressed are mean \pm SEM (week 1, LF n=5, HF n=5; week 8, LF n=4, DIO-R and DIO-P n=5). Groups with different letters are significantly different at $p < 0.05$.

Figure 3: CCK1R, GHSR, CB1, and FAAH expression in the nodose ganglia are significantly increased in DIO-P but not DIO-R rats. No change was observed for Y2R and ObR. Receptor expression expressed relative to LF rats at week 1 as a control group. Data expressed are mean \pm SEM (week 1, LF n=5, HF n=5; week 8 LF n=4, DIO-R n=5, DIO-P n=5).

Figure 4: Principle component analysis of all measured parameters from all rat groups after vast scale transformation of the data reveals the phenotypic shifts responsible for group changes in a single evaluation. Within group variance was equivalent after transformation and experimental groups are clearly discriminated. The first two principle components accounted for 86% of the variance in the data set. The 8wk HF DIO-R animals differ from the 8 wk LF group in PC 2 ($p = 0.03$) but not PC1 ($p = 0.6$), while the 8wk HF DIO-P group differed from the LF group in both components ($p < 0.001$).

Figure 5: Correlations between variables were assessed in a Pearson's correlation matrix with all measured variables. Notably, fasting leptin was strongly correlated with epididymal fat mass, but weakly correlated with other adipose depots measured. Also of specific interest, the change in CCK₁R expression in nodose ganglion was strongly correlated with the fasting to 2hr-post lipid challenge leptin.

Figure 1:

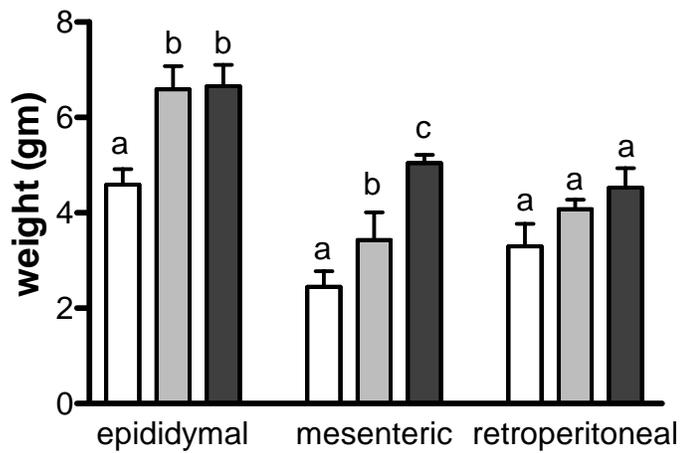
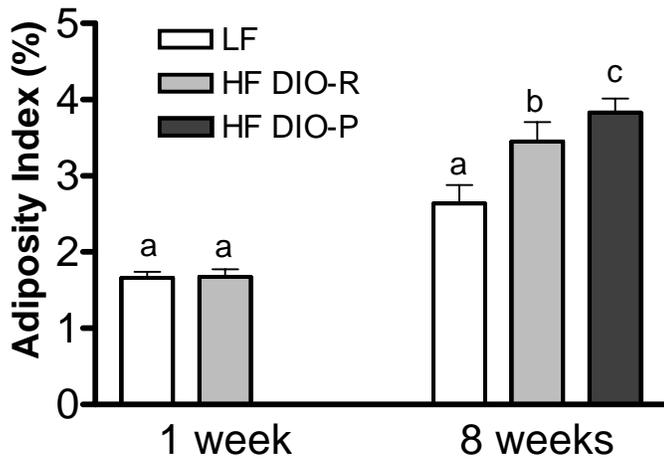
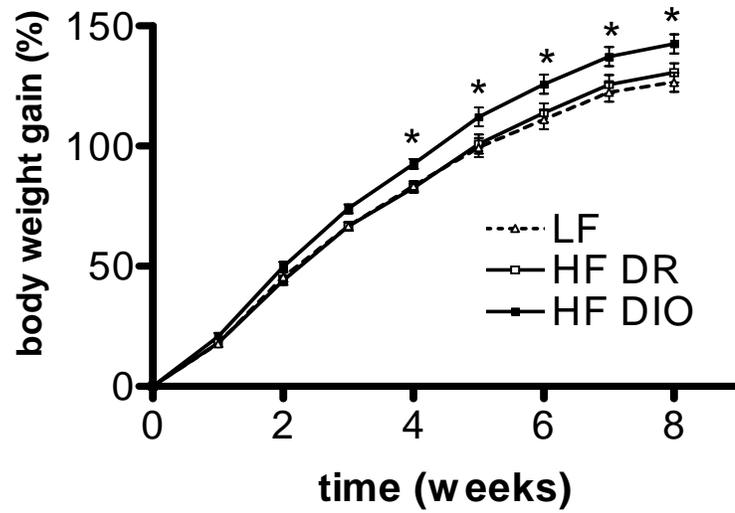


Figure 2:

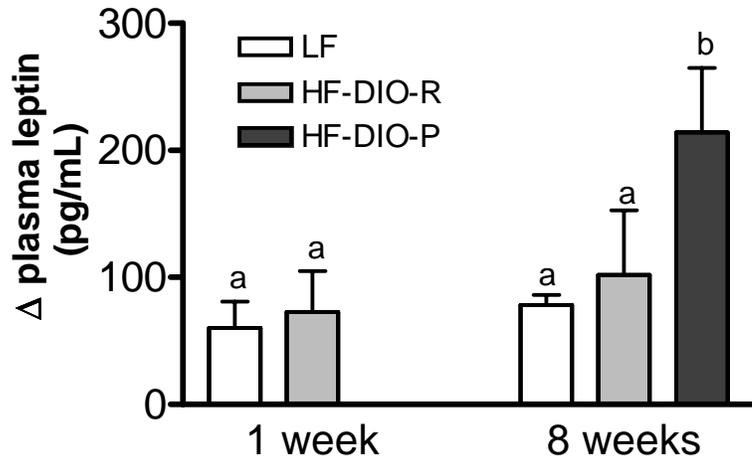


Figure 3:

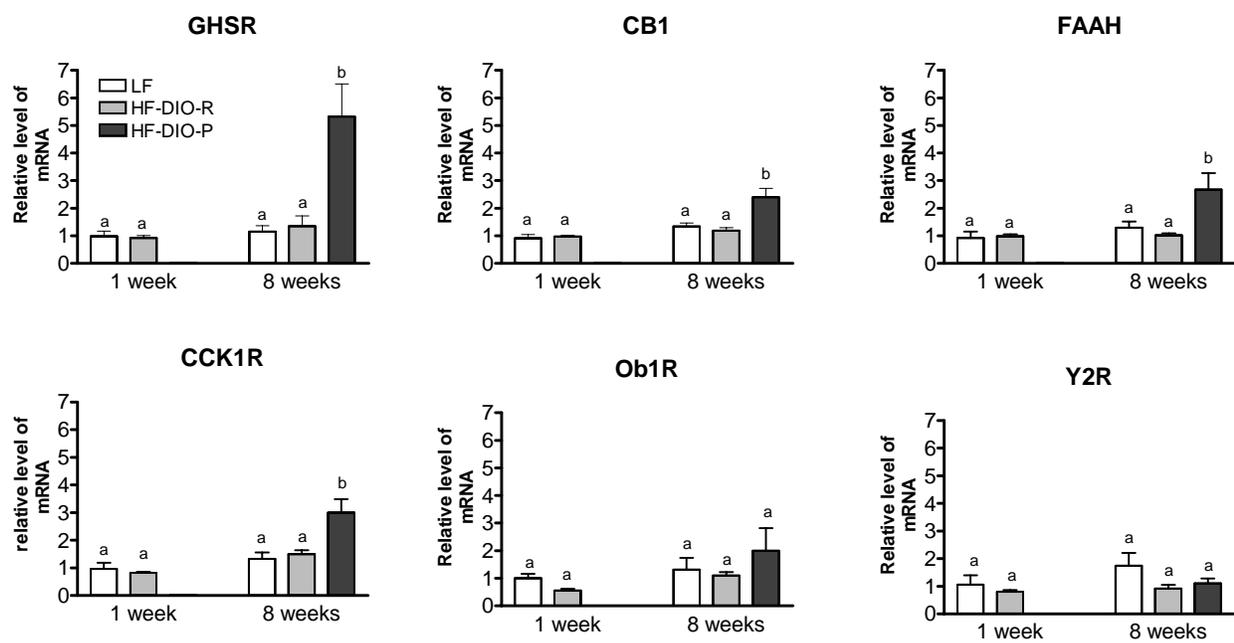


Figure 4:

