Published in final edited form as:

Mol Nutr Food Res. 2018 July ; 62(13): e1800219. doi:10.1002/mnfr.201800219.

High fat diet dysregulates hypothalamic-pituitary axis gene expression levels which are differentially rescued by EPA and DHA ethyl esters

Saame Raza Shaikh^{a,b,*}, Patti R. Shaver^a, and Brian M. Shewchuk^{a,*}

^aDepartment of Biochemistry and Molecular Biology and the East Carolina University Diabetes and Obesity Institute, Brody School of Medicine, East Carolina University, Greenville, NC 27858

^bDepartment of Nutrition, Gillings School of Global Public Health and School of Medicine, The University of North Carolina at Chapel Hill, CB #7461 Chapel Hill, NC 27599

Abstract

Scope: Dietary fat composition can modulate gene expression in peripheral tissues in obesity. Observations of the dysregulation of growth hormone (GH) in obesity indicate that these effects extend to the hypothalamic-pituitary (H-P) axis. We thus determined whether specific high fat (HF) diets influenced the levels of *Gh* and other key gene transcripts in the H-P axis.

Methods and results: C57BL/6 mice were fed a lean control diet or a HF diet in the absence or presence of OA, EPA or DHA ethyl esters. Comparative studies were conducted with menhaden fish oil. The HF diet lowered pituitary *Gh* mRNA and protein levels, and cell culture studies revealed that elevated insulin and glucose could reduce *Gh* transcripts. Supplementation of the HF diet with OA, EPA, DHA, or menhaden fish oil did not improve pituitary *Gh* levels. The HF diet also impaired the levels of additional genes in the pituitary and hypothalamus, which were selectively rescued with EPA or DHA ethyl esters. The effects of EPA and DHA were more robust relative to fish oil.

Conclusion: A HF diet can affect H-P axis transcription, which can be mitigated in some genes by EPA and DHA, but not fish oil in most cases.

Keywords

obesity; n-3 PUFA; hypothalamic-pituitary axis; growth hormone

1 Introduction

Diet-induced obesity is associated with a range of metabolic abnormalities in response to nutrient overload, oxidative stress, and genetic factors. Dietary fat composition is a key

*To whom correspondence should be addressed: S. Raza Shaikh, Department of Nutrition, Gillings School of Global Public Health and School of Medicine, The University of North Carolina at Chapel Hill, CB #7461 Chapel Hill, NC 27599, Brian M. Shewchuk, Department of Biochemistry and Molecular Biology, Brody School of Medicine at East Carolina University, 600 Moye Blvd, Greenville, NC, shewchukb@ecu.edu, (252) 744-5096.

Conflict of Interest

The authors declare no conflict of interest

variable that influences the etiology of obesity in part by targeting gene expression in metabolic tissues such as the liver, skeletal muscle, and adipose. For example, mouse models show that high fat diets exacerbate inflammatory gene expression in adipose tissue, which potentially contributes to an impairment in glucose and insulin sensitivity [1, 2]. In contrast, inclusion of n-3 polyunsaturated fatty acids (PUFA) in a high fat diet improves adipose tissue inflammation and whole body metabolism [3, 4]. Therefore, understanding how dietary fat composition regulates gene expression is essential for establishing the mechanistic underpinnings by which obesity promotes metabolic aberrations.

Central to the control of essentially all physiological systems is the unitary function of the hypothalamic-pituitary (H-P) axis. In considering the full physiological effects of obesogenic markers, addressing the potential for obesity to affect the functions of the H-P axis becomes significant. Indeed, diet-induced obesity is associated with altered sensitivity of the hypothalamus to peripheral satiety and adiposity signals which may contribute significantly to the pathophysiology of the disease [5], and could conceivably perturb the function of the hypothalamus in regulating pituitary hormone production. The hypothalamus receives input from the periphery and the central nervous system in the form of cytokines, neurotransmitters, hormones, and different metabolites. In response to these signals, the hypothalamus secretes multiple hormone releasing factors or suppressors that control the synthesis and release of hormones from the anterior pituitary gland, which in turn impact peripheral tissue function in a wide range of systems. These hormones include growth hormone (GH), prolactin (PRL), thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), follicle stimulating hormone (FSH), and luteinizing hormone (LH), which are synthesized by cognate cell types in the anterior pituitary. Significantly, impairments in several of these hormone axes have been associated with obesity in human cohorts, most consistently in the case of GH, in which obesity has routinely been associated with reduced circulating GH levels [6–9]. Animal models have also shown impairments in GH expression, as well as reductions in expression of GH-releasing hormone receptor (GHRHR), which mediates the release of GH from pituitary somatotropes in response the hypothalamic GH-releasing hormone (GHRH), in association with obesogenic metabolism [6, 7]. Alterations in ACTH, thyroid hormones (T_3 and T_4 , regulated by pituitary TSH), and the gonadotropins (LH/FSH) have also been reported [9–14].

While there has been a steady accumulation of findings supporting a model in which markers of obesogenic metabolism can impinge on H-P axis function, some elements of the model require additional resolution and completion. For example, there are additional components in the GH regulatory axis that could also be affected by obesity. The release of GH from pituitary somatotropes is governed by hypothalamic GH releasing hormone (GHRH) and its cognate receptor (GHRHR) expressed in pituitary somatotropes. A reduction in the expression of either of these signaling components could blunt the release of GH. In addition, feedback regulation of GH levels is mediated by hypothalamic expression of the receptor for GH (GHR). It also remains unclear whether a high-fat diet (of which 45% of kcal are from fat) would be sufficient to induce these effects in wildtype mice, as previous mouse models typically employed a 60% fat diet [7]. How the presence of n-3 PUFA impinges on the outcomes of HF diet-induced obesity in the H-P axis has also not been fully addressed, which is significant in light of building evidence of the ability of n-3 PUFA to

ameliorate some of the dysfunction in peripheral tissues in obesity. This question is encompassed by the broader problem of identifying the causal agents of the obesogenic environment, which may include elevated glucose, insulin, or serum lipids, and the mechanisms by which they affect the H-P axis. In the case of GH, previous studies indicate the potential for glucose and insulin to influence the suppression of GH levels [6, 7].

In light of the correlation between obesity and impaired GH axis function and the potential for n-3 PUFA to modify the effects of obesity in other contexts, this study focused on how inclusion of n-3 PUFAs in a high fat diet influenced gene transcription of multiple components of the GH regulatory pathway in the H-P axis. The specific objective of this study was to first determine if a high fat diet enriched in milkfat impaired pituitary GH levels and if inclusion of the monounsaturated fatty acid oleic acid (OA) or the long chain n-3 PUFAs eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid ethyl esters could mitigate the effects. Parallel studies in cell culture addressed alternative factors that could influence GH levels. We subsequently determined if the high fat diets targeted the gene expression of additional components of H-P axis function and if the effects of EPA and DHA ethyl esters were distinct from those of dietary supplementation with menhaden fish oil.

2 Materials and Methods

2.1 Animals and diets.

Male C57BL/6 mice, approximately 5–6 weeks old, were fed experimental diets for 10 weeks; a lean control diet, a high fat diet (HF), or a high fat diet supplemented with either oleic acid (HF-OA), eicosapentaenoic acid (HF-EPA), or docosahexaenoic acid (HF-DHA) ethyl esters (Envigo). Ethyl esters, purchased from Cayman, were greater than 90% purity as verified by a third party using blinded samples. The control diet was a 5% fat by weight diet enriched with soybean oil. The high fat diets contained 45% of total kcal from fat with anhydrous milkfat as the primary fat source. OA, EPA, or DHA ethyl esters accounted for 2% of the total energy. This corresponds to levels of EPA and DHA that are achievable with dietary supplementation and used in clinical trials [15]. For some studies, the lean and HF diets were supplemented with menhaden fish oil that is enriched in n-3 PUFAs. For these experiments, 2% and a 1.3% of the total kcal were from EPA and DHA, respectively. The composition of the experimental diets was previously published [16]. The mice were multi-housed in hot washed cages using a cob bedding on a standard 12/12 light/dark cycle with temperature regulated at 23°C. Food and water were provided *ad libitum*. Given that n-3 PUFAs can oxidize, the diets (stored in aliquots with a nitrogen flush) were changed every other day to prevent oxidation. Mice were routinely checked by staff in the Department of Comparative Medicine. Failure to groom and/or loss of more than 20% body weight indicated potential sickness and thereby euthanasia.

At the end of the feeding period, mice were euthanized with CO₂ inhalation followed by cervical dislocation. Hypothalami were excised from brains, and intact pituitaries were removed. Mice were euthanized in accordance with the guidelines set forth by East Carolina University Brody School of Medicine for euthanasia and humane treatment (AUP#C059b).

2.2 RNA isolation and quantitative reverse transcription-PCR (qRT-PCR).

Hypothalami and pituitaries were homogenized immediately in Trizol (Life Technologies), and total RNA purified according to the manufacturer's protocol. qRT-PCR was performed using SYBR-green fluorescence (SYBR Supermix, BioRad). Relative transcript levels were determined by the C_t method using $\beta 2$ microglobulin ($\beta 2M$) mRNA as an internal control, converted to a linear function by a base-2 antilog transformation, and normalized to the control diet for each transcript. Primer sequences are shown in Table S1. Primer sequences and thermocycler parameters were optimized for 100% efficiency and unique product formation.

2.3 Western blot analysis of protein levels.

To detect GH, whole protein was isolated from pituitary homogenates according to the manufacturer instructions. For experiments with GH3 cells, whole cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA) supplemented with a protease inhibitor cocktail (Sigma). Twenty-five μ g protein was resolved on a 10% polyacrylamide (37.5:1 acrylamide:bis-acrylamide) minigel, and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in TBST (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20), and incubated with monkey anti-rGH (National Hormone and Peptide Program) overnight at 4° C. After multiple washes in TBST, the membrane was incubated with a horseradish peroxidase-conjugated rabbit anti-human IgG (DakoCytometrics) in 5% nonfat dry milk/TBST for 2 hours at room temperature. Immune complexes were detected with a chemiluminescent substrate (Pierce Super Signal). To control for protein loading, blots were stripped with 25 mM glycine-HCl, pH 2, 1% w/v SDS, washed with PBS and incubated with a mouse anti- β tubulin antibody (a gift of Fred Bertrand, University of Alabama at Birmingham), followed by an HRP-conjugated goat anti-mouse secondary antibody (Pierce). The same method was applied to the immunodetection of phospho-Akt, total Akt, phospho-ERK1/2, and total ERK1/2 (Cell Signaling). For these assays, an HRP-conjugated goat anti-rabbit secondary antibody (Pierce) was used.

2.4 Cell culture experiments.

The rat cell line GH3 (ATCC) was maintained in phenol red-free low glucose (5 mM) DMEM (Life Technologies) supplemented with charcoal stripped 10% fetal bovine serum (Atlanta Biologicals) and 1 $\times$ penicillin/streptomycin solution (Life Technologies) at 37° C in 5% CO₂. Ten million cells were plated in 6-well tissue culture plates in the above media and incubated overnight. After treatments as indicated, protein and RNA were harvested for analysis as described above. For glucose treatment experiments, cells maintained in 5mM glucose were washed with PBS and fresh DMEM containing either 5mM or 25mM glucose was added and incubated for the indicated times. For insulin treatments, recombinant human insulin was added to the indicated final concentrations from a commercial stock (Sigma).

2.5 Statistical analysis.

The rationale for the sample size was based on previous qRT-PCR studies [16, 17] which showed that five mice per diet was sufficient achieve a power of 0.8 with an alpha of 0.05.

Therefore, a minimum of five mice per diet were planned for the gene expression studies, which were executed with six to eight mice as indicated in the figure legends. Mice were sacrificed in cohorts to cohort effects. All data sets displayed normal distributions. Therefore, significant differences in gene expression among the control, high-fat, and n-3 PUFA supplemented diets were determined by ANOVA followed by *post hoc* Bonferroni and Tukey HSD multiple comparison tests ($\alpha=0.05$) using SPSS software (IBM), which yielded the same assignments of significance. Highly significant differences between diets are denoted by distinct labels in the data figures and as described in the figure legends. A post-hoc power analysis of the data indicated that a 2-fold difference was associated with a power of 90–100%.

3 Results

3.1 High fat diet lowers pituitary GH levels, which are not improved by dietary supplementation with OA, EPA, or DHA ethyl esters.

We first tested the hypothesis that high fat diet-induced obesity would result in reduced *Gh* mRNA, and that dietary fat type would further influence pituitary *Gh* levels (Fig. 1). The high fat diet was supplemented with ethyl esters of OA, EPA, or DHA. The OA ethyl ester was included as a monounsaturated control. At the transcript level, the HF diet lowered *Gh* mRNA by 56% relative to the lean control (Fig. 1A). The addition of OA, EPA, or DHA ethyl esters to the HF diet did not influence pituitary *Gh* mRNA levels, which remained low compared to the control diet (Fig. 1A). We specifically confirmed that the HF diet also reduced pituitary GH protein levels by Western blot, which showed a significant reduction with the HF diet compared to the lean control (Fig. 1B).

3.2 Insulin and glucose lower GH levels in a somatotrope cell line.

Administration of the HF diet increased insulin and glucose levels in the mice used in this study, indicating reduced glucose control [17]. Given that dietary fat composition did not influence pituitary *Gh* mRNA levels in the obese HF diet-fed mice, we conducted cell culture studies to determine whether somatotrope GH levels could instead be modulated by insulin and glucose. Administration of insulin to the pituitary somatotrope cell line GH3 resulted in a robust canonical insulin signaling response. AKT phosphorylation was detectable within 5 minutes of insulin administration and peaked by 60 minutes (Fig. 2A). ERK1/2 phosphorylation was also detectable by 60 minutes after insulin administration (Fig. 2A). These results are consistent with the potential for insulin to elicit effects in the somatotrope lineage where GH is expressed. With respect to an effect on *Gh*, insulin lowered relative *Gh* mRNA levels in GH3 cells in a dose-dependent manner (Fig 2B). Similarly, exposure to 25 mM glucose reduced the level of *Gh* mRNA compared to 5mM glucose, which was further reduced in the presence of 10 nM insulin (Fig. 2C). Nearly identical effects on GH protein levels were observed (Fig. 2D). These findings suggest that the observed effect of HF diet induced obesity on GH levels may be mediated by elevated circulating insulin and glucose concentrations and are less dependent on the composition of dietary fat.

3.3 Additional components of the GH regulatory axis are affected by high fat diet and rescued with EPA or DHA supplementation.

We determined whether the HF diet and ethyl esters of OA, EPA or DHA could affect their levels of expression. The HF diet resulted in a significant reduction in the levels of pituitary *Ghrhr* (Fig. 3A) as well as hypothalamic *Ghrh* and *Ghr* mRNA (Fig. 3B), consistent with a potential blunting of GH release and feedback control. Both EPA and DHA restored pituitary *Ghrhr* (Fig. 3A) and hypothalamic *Ghr* and *Ghrh* (Fig. 3B) mRNA to levels similar to those observed in lean controls, in contrast to the lack of effects on *Gh* mRNA (Fig. 1A). This effect was not observed with OA supplementation.

3.4 High fat diet alters transcript levels of multiple H-P axis hormones which are differentially rescued by EPA or DHA.

Components of the ACTH, TSH, and LH/FSH signaling pathways were selected to expand the potential effects of dietary fat composition on gene expression in the hypothalamus and pituitary. In the case of the ACTH system central to the control of adrenal glucocorticoid production, mice consuming the HF and HF+OA diet showed elevated pituitary levels of the transcript for pro-opiomelanocortin (*POMC*), the precursor peptide for ACTH (Fig. 3A). The HF diet supplemented with EPA or DHA abrogated the HF diet-associated increase in *Pomc* expression (Fig. 3A).

In the context of the thyroid axis, we assayed the levels of pituitary mRNA for both subunits of thyroid-stimulating hormone (TSH); the unique TSH β subunit, and the pituitary glycoprotein hormones alpha subunit (CG α) common to chorionic gonadotropin (CG), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and TSH. The levels of *Cga* mRNA was increased by the HF and the HF+OA diets by almost 2-fold, and this effect was abrogated by EPA and DHA (Fig. 3A). The levels of pituitary *Tsh β* mRNA were unchanged across all experimental groups (Fig. 3A). In the case of the gonadotropin subunits *Lhb β* and *Fsh β* , the HF diet was associated with a trend toward reduced mRNA levels and a correction by EPA and DHA, but the differences did not reach statistical significance (Fig. 3A). However, the level of mRNA for gonadotropin releasing hormone (*Gnrh*), which modulates pituitary LH and FSH release, was significantly reduced in the hypothalamus of HF diet-fed mice. This suppression was significantly reversed by EPA, but the trend toward reversal by DHA was not statistically significant (Fig. 3B). We also determined whether the expression of this central regulator of anterior pituitary transcription POU1F1 is affected by the HF diet. Interestingly, mice fed the HF and HF+OA diets showed increased levels of *Pou1f1* mRNA that was abrogated by EPA and DHA (Fig. 3A).

Obesity is associated with defects in the sensitivity of the hypothalamus to peripheral signals, particularly leptin and insulin, which may contribute to its etiology. Thus, we determined the effects of fat composition on transcripts encoding the leptin receptor (*Lepr*) and the insulin receptor (*Insr*) in the hypothalamus. *Lepr* mRNA was not affected with the HF or HF+OA diets relative to the control. However, EPA, but not DHA, increased *Lepr* mRNA levels compared to the control, HF, and HF+OA diets (Fig. 3B), indicating a potential sensitivity to lipid composition. *Insr* mRNA levels in the hypothalamus reduced by

50% with all of the HF diets relative to the control (Fig. 3B), consistent with a reduced cellular sensitivity to insulin.

3.5 Fish oil supplementation modifies the changes in *Ghrhr* gene expression driven by a high fat diet.

We next determined if administration of fish oil to lean or HF fed mice had similar effects as observed for the EPA/DHA ethyl ester model. Analyses of *Gh* levels revealed no effect of fish oil on lean or obese animals (Fig. 4A). None of the pituitary transcripts assayed were affected by fish oil in lean mice (Fig. 4B). Furthermore, pituitary transcript levels that were affected by the HF diet were generally not improved with the addition of fish oil (Fig. 4B). The only notable exception was *Ghrhr* mRNA, which was increased by fish oil relative to its suppression by the HF diet (Fig. 4B). In the case of hypothalamic transcripts, the HF diet showed the same effects as measured in the previous model system (Fig. 3B) except leptin receptor levels, in which the reduction compared to lean controls reached statistical significance (Fig. 4B). However, fish oil had no beneficial effects in either lean or obese mice on hypothalamic transcripts, although the improvement in *Ghrh* mRNA mediated by fish oil was nearly significant (Fig. 4B). Overall, these cohorts recapitulate the previously observed effects of high fat diet-induced obesity on the levels of the tested pituitary and hypothalamic transcripts, and indicate that fish oil displayed less robust effects than the EPA and DHA ethyl esters.

4 Discussion

The findings presented here extend the understanding of diet-induced obesity by demonstrating that dietary fat composition, notably the levels of EPA and DHA, can influence the expression of several hypothalamic-pituitary axis genes that are sensitive to the effects of a high fat diet. These results provide new mechanistic targets by which EPA and DHA could exert their beneficial metabolic effects. Overall, the results of this study indicated that obesity in mice resulting from 10 weeks of exposure to a milkfat-based HF diet was associated with both increases and decreases in the expression of multiple H-P axis hormones and regulatory peptide transcripts (Fig. 5). The gene-specific positive and negative effects of the HF diet indicate a likely diversity in the underlying regulatory processes and the involvement of specific molecular mechanisms. Future studies will address the underlying mechanistic basis for the gene-specific effects of the HF diet and n-3 PUFAs, which may involve both direct actions of dietary lipids and their metabolites, and the indirect actions of interacting pathways in the obesogenic environment.

4.1 Multiple components of the GH signaling axis were affected by a high fat diet.

Observations in humans have identified an inverse correlation between body mass index and plasma GH levels, suggesting a mechanistic link between the obesity and GH axis suppression. The point in the GH regulatory pathway at which the suppression occurs, and the underlying mechanisms, have not been fully characterized. Several hypotheses have been proposed involving both suppression of hypothalamic GHRH synthesis and release, and the cognate somatotrope response through changes in GHRHR expression and GH expression and release. Increased expression and release of hypothalamic somatostatin (SST), which

suppresses GH secretion, and its cognate somatotrope receptor (SSTR) could also be involved in the altered GH levels seen in obesity [8, 23], although evidence in mice suggests that SST levels are unaffected by obesity [7]. These changes, each of which can affect obesity-associated GH deficiency or act in concert to exacerbate the phenotype, can be ultimately due to multiple causal agents, such as elevated free fatty acids and glucose [6, 24, 25], or to changes in the levels of additional endocrine signals such as leptin and insulin [8, 23].

In the present study, we observed a significant reduction in GH mRNA and protein levels in HF diet-fed obese mice, consistent with effects at the level of *Gh* gene transcription. Interestingly, this effect was independent of effects of POU1F1, which increased in mRNA levels in the obese cohort. POU1F1 (also known as Pit-1) is a pituitary-specific transcription factor essential for the activation of multiple anterior pituitary hormone genes, including *Gh*, *Tsh β* and prolactin (*Prl*), as well as the auto-activation of the *Pou1f1* gene itself [18–22]. Previous studies have similarly observed GH reduction independent of effects on Pit-1 [6]. Measurements of plasma insulin levels and glucose control in glucose tolerance tests showed the development of hyperglycemia and hyperinsulinemia in these mice [17], which could underlie the reduced GH expression. Experiments in cultured somatotropes confirmed a sensitivity to insulin as indicated by canonical insulin-stimulated AKT and ERK1/2 phosphorylation, and that both insulin and elevated glucose could additively repress GH expression at the mRNA and protein levels. Thus, elevated plasma glucose and insulin levels associated with obesity may contribute to GH deficiency by directly repressing *Gh* gene expression. These data in part reinforce findings in other systems of a blunting of pituitary *Gh* expression by insulin [6, 7]. Interestingly, recently published work reported a connection between dietary n-6 and n-3 PUFA levels and both positive and negative changes in hypothalamic transcripts that appear to be mediated by insulin signaling [26], suggesting a potentially broader role for insulin in mediating the effects of dietary lipids in the H-P axis.

While reduced *Gh* expression would be sufficient to suppress circulating GH levels, we also observed a reduction in pituitary *Ghrhr* mRNA, indicating the potential for a reduced sensitivity to hypothalamic GHRH, which could blunt the stimulation of pituitary GH release. Significantly, we also observed a reduction in expression of the hypothalamic *Ghrh* ligand itself, which has not been seen in previous studies. The reduced GHR expression observed in hypothalamus of obese mice further indicates the potential dysfunction of regulatory feedback circuits in the H-P axis, and has also not been previously observed. Significantly, the findings in our system were consistent with observations in primate and mouse models, which also pointed toward a repressive effect of lipid exposure and insulin on GHRHR expression [6, 7]. Taken together, these observations reinforce a model in which reductions in the expression of multiple components of the GH signaling axis may contribute to obesity-associated GH deficiency.

4.2 A high fat diet induced changes in mRNA levels in multiple pituitary hormone axes.

Sufficient material remained to expand our analysis to additional pituitary hormone axes that have been shown to be altered with obesity in human or animal models. In the context of the adrenal axis, the transcript for POMC (the precursor to ACTH) was elevated significantly in

HF diet-fed mice (Fig. 3). The roughly 4-fold increase was the greatest magnitude of effect that we observed among the genes assayed and indicates a significant activation of this gene by the obesogenic environment. POMC-expressing neurons in the hypothalamus and other brain regions play a role in the regulation of energy balance, such that an obesity-associated alteration of *Pomc* gene expression could indicate the potential for significant metabolic outcomes, in addition to impinging on the adrenal axis [27]. POMC-derived peptides have also been shown to blunt the negative feedback of hypothalamic CRH and pituitary ACTH release by adrenal glucocorticoids, which could exacerbate ACTH-mediated adrenal activation [27]. An increase in POMC transcripts as we observed in response to the HF diet-induced obesity could thereby amplify ACTH and subsequent glucocorticoid release.

In the case of the gonadotropic axis, HF diet-induced obesity was associated with a reduction in hypothalamic gonadotropin releasing hormone (*Gnrh*) mRNA, indicating a potential blunting of GnRH-stimulated pituitary FSH and LH release. In addition, of the genes for the FSH β and LH β subunits of FSH and LH (each of which are heterodimers sharing a CG α subunit in their active forms), *Lh β* mRNA was reduced in obese cohorts while *Fsh β* was unaffected by the HF diet. Interestingly, *Cga* mRNA levels increased in the obese animals. Thus, despite effects on the levels of multiple transcripts, not all genes assayed were affected by the HF diet, and the nature of the effect was gene-specific. This observation points toward a specific transcriptional or epigenetic mechanism that selectively affects gene transcription. In further support of specificity, the levels of mRNA for the TSH β subunit of TSH were also unaffected by the HF diet.

4.3 A high fat diet reduced hypothalamic *Insr* and *Lepr* mRNA levels.

The development of resistance to peripheral signals that play a central role in the regulation of food intake and energy expenditure, in particular leptin and insulin, has emerged from studies of the molecular basis for hypothalamic dysfunction in obesity as a likely major contributor to its etiology. Through this mechanism, a blunted hypothalamic response to circulating satiety and adiposity signals could exacerbate the trend toward obesity. In the present study, HF diet-fed obese mice showed reduced *Insr* and *Lepr* transcript levels, consistent with the potential for reduced sensitivity of the hypothalamus to circulating insulin and leptin (Fig. 3B, 4C).

4.4 EPA and DHA ethyl esters rescue HF diet-mediated changes in specific hypothalamic and pituitary gene transcripts.

Gene expression is a major mechanistic target of n-3 PUFAs. For example, n-3 PUFAs can function as ligands for peroxisome proliferator-activated receptors (PPARs), which are DNA-binding factors that regulate multiple metabolic pathways [28–30]. They may also function as ligands for specific G-protein coupled receptors (GPCRs), which can signal downstream transcriptional responses [31–33]. The rationale for studying the effects of EPA and DHA was driven by data showing that n-3 PUFAs can mitigate some of the pathophysiology of obesity and neuroinflammation, in part by the suppression of pro-inflammatory pathway gene expression in the hypothalamus [34–36]. These previous findings suggested the potential for general effects on gene expression in the H-P axis.

While we observed the suppression of pituitary *Gh* expression by HF diet-induced obesity, we did not find pituitary *Gh* mRNA levels to be influenced by specific fat composition within the HF diet. While the effects of HF diet on *Gh* levels were consistent with increasing evidence of dysregulation in the H-P axis associated with obesity in human cohorts and rodent models, resolution of the mechanisms underlying these connections remains incomplete. Despite the apparent insensitivity of *Gh* mRNA levels to n-3 PUFAs in our mouse system, the cell culture studies suggested that elevated levels of glucose and insulin may be driving the reduction in GH, which is supported by the impaired glucose control exhibited by these mice [17].

In contrast to the negative findings with *Gh*, our data showed that EPA or DHA abrogated the HF diet-mediated suppression of hypothalamic *Ghrh*, *Ghr*, and *Gnrh* transcript levels. In the case of *Lepr*, while the HF diet did not suppress expression, the presence of EPA significantly increased the level. The parallel actions of EPA in the HF diet on *Gnrh* and *Lepr* expression are notable, as *Gnrh* has been proposed to be regulated by leptin signaling through NPY neurons, in a manner that is blunted in obesity [14]. In the pituitary, while n-3 PUFA had no effect on the *Gh* mRNA level, EPA and DHA mitigated the effects of HF-diet on *Ghrh*, *Pou1f1*, and *Cga* transcripts. These observations indicate that the presence of n-3 PUFA can influence the effects of a HF diet on transcription of multiple physiologically significant H-P axis genes. While this initial study focused on key genes, informed by observed correlations between obesity and changes in specific pituitary hormone axes in humans and experimental animals, an unbiased transcriptome-wide approach would allow a more complete analysis of potential common pathways that could identify the discrete mechanisms of high fat diet and n-3 PUFA effects in the H-P axis.

Consistent with the potential for direct actions of n-3 PUFA in the H-P axis, studies in rodents show that dietary administration of n-3 PUFAs increases the levels of EPA and DHA in the hypothalamus and other brain regions [26, 34, 37–43]. Given that we had very limited tissue from each animal, we were unable to assay for EPA and DHA levels in the pituitary and hypothalamus, although this is a future experimental goal. An additional limitation of this study was the lack of information regarding circulating hormone levels, which is also an important future objective.

4.5 EPA and DHA ethyl esters were more effective than fish oil in targeting gene expression.

There is increasing evidence that one major limitation in the translation of n-3 PUFAs into the clinic for a range of metabolic and inflammatory diseases is driven in part by limited evidence of differential effects of EPA and DHA [44]. Our data highlight the notion that EPA and DHA individually are not identical as seen with at least two genes in which EPA, but not DHA, abrogated the HF-diet-mediated effect (*Gnrh* and *Lepr*). This is likely due to the different structures of these n-3 PUFA that influence downstream production of lipid mediators and targeting of transcription factors that can mediate gene expression. GnRH and LEPR are of high interest for future causal studies given that our recent studies suggest that long-term administration of EPA, but not DHA, improves fasting insulin and the HOMA-IR index [45]. An additional advancement from this study is that in contrast to the pure n-3

PUFA ethyl esters, fish oil was not as effective in mitigating the changes observed with the HF diet, as only *Ghrhr* transcript levels were affected by fish oil. Given that fish oil has ingredients outside of EPA and DHA, it is possible these agents are preventing the effects of EPA and DHA. Alternatively, the EPA/DHA (3/2) ratio in fish oil may not be adequate to elicit a response.

In conclusion, data from this study indicate that HF diet-induced obesity can modify pituitary hormone regulatory pathways, and indicate the potential for effects on multiple peripheral systems. These include GH signaling in liver and skeletal muscle, leptin and insulin feedback to the hypothalamus, and control of the thyroid, adrenal gland, and gonads (Fig. 5). These effects are differentially modified by the presence of the n-3 PUFAs EPA and DHA in the HF diet, which had incompletely overlapping effects on a subset of the genes. Fish oil did not replicate the actions of EPA/DHA, but had a more limited range of effects on the analyzed genes. These observations may underlie some of the correlations observed between obesity and altered pituitary hormone levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH R01AT008375 to S.R.S. S.R.S. and B.M.S designed experiments and wrote the manuscript; P.R.S and B.M.S. conducted experiments

List of abbreviations:

DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
OA	oleic acid
PUFA	polyunsaturated fatty acids

5 References

- [1]. Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J, Dorfman R, Wang Y, Zielenski J, Mastronardi F, Maezawa Y, Drucker DJ, Engleman E, Winer D, Dosch HM, Normalization of obesity-associated insulin resistance through immunotherapy. *Nat Med* 2009, 15, 921–929. [PubMed: 19633657]
- [2]. Winer S, Paltser G, Chan Y, Tsui H, Engleman E, Winer D, Dosch HM, Obesity predisposes to Th17 bias. *European Journal of Immunology* 2009, 39, 2629–2635. [PubMed: 19662632]
- [3]. LeMieux MJ, Kalupahana NS, Scoggin S, Moustaid-Moussa N, Eicosapentaenoic acid reduces adipocyte hypertrophy and inflammation in diet-induced obese mice in an adiposity-independent manner. *The Journal of nutrition* 2015, 145, 411–417. [PubMed: 25733455]
- [4]. Pahlavani M, Razafimanjato F, Ramalingam L, Kalupahana NS, Moussa H, Scoggin S, Moustaid-Moussa N, Eicosapentaenoic acid regulates brown adipose tissue metabolism in high-fat-fed mice and in clonal brown adipocytes. *J Nutr Biochem* 2017, 39, 101–109. [PubMed: 27833050]
- [5]. Thaler JP, Schwartz MW, Minireview: Inflammation and obesity pathogenesis: the hypothalamus heats up. *Endocrinology* 2010, 151, 4109–4115. [PubMed: 20573720]

- [6]. Luque RM, Gahete MD, Valentine RJ, Kineman RD, Examination of the direct effects of metabolic factors on somatotrope function in a non-human primate model, *Papio anubis*. *J Mol Endocrinol* 2006, 37, 25–38. [PubMed: 16901921]
- [7]. Luque RM, Kineman RD, Impact of obesity on the growth hormone axis: evidence for a direct inhibitory effect of hyperinsulinemia on pituitary function. *Endocrinology* 2006, 147, 2754–2763. [PubMed: 16513828]
- [8]. Maccario M, Grottoli S, Procopio M, Oleandri SE, Rossetto R, Gauna C, Arvat E, Ghigo E, The GH/IGF-I axis in obesity: influence of neuro-endocrine and metabolic factors. *Int J Obes Relat Metab Disord* 2000, 24 Suppl 2, S96–99. [PubMed: 10997620]
- [9]. Makimura H, Stanley T, Mun D, You SM, Grinspoon S, The effects of central adiposity on growth hormone (GH) response to GH-releasing hormone-arginine stimulation testing in men. *The Journal of clinical endocrinology and metabolism* 2008, 93, 4254–4260. [PubMed: 18765508]
- [10]. Bose M, Oliván B, Laferrere B, Stress and obesity: the role of the hypothalamic-pituitary-adrenal axis in metabolic disease. *Current opinion in endocrinology, diabetes, and obesity* 2009, 16, 340–346.
- [11]. Dandona P, Dhindsa S, Update: Hypogonadotropic hypogonadism in type 2 diabetes and obesity. *The Journal of clinical endocrinology and metabolism* 2011, 96, 2643–2651. [PubMed: 21896895]
- [12]. Dandona P, Dhindsa S, Chaudhuri A, Bhatia V, Topiwala S, Mohanty P, Hypogonadotropic hypogonadism in type 2 diabetes, obesity and the metabolic syndrome. *Curr Mol Med* 2008, 8, 816–828. [PubMed: 19075678]
- [13]. Reinehr T, Obesity and thyroid function. *Molecular and cellular endocrinology* 2010, 316, 165–171. [PubMed: 19540303]
- [14]. Teerds KJ, de Rooij DG, Keijer J, Functional relationship between obesity and male reproduction: from humans to animal models. *Hum Reprod Update* 2011, 17, 667–683. [PubMed: 21546379]
- [15]. Kim W, McMurray DN, Chapkin RS, n-3 polyunsaturated fatty acids--physiological relevance of dose. Prostaglandins, leukotrienes, and essential fatty acids 2010, 82, 155–158.
- [16]. Kosaraju R, Guesdon W, Crouch MJ, Teague HL, Sullivan EM, Karlsson EA, Schultz-Cherry S, Gowdy K, Bridges LC, Reese LR, Neuffer PD, Armstrong M, Reisdorph N, Milner JJ, Beck M, Shaikh SR, B Cell Activity Is Impaired in Human and Mouse Obesity and Is Responsive to an Essential Fatty Acid upon Murine Influenza Infection. *J Immunol* 2017, 198, 4738–4752. [PubMed: 28500069]
- [17]. Teague H, Harris M, Fenton J, Lallemand P, Shewchuk BM, Shaikh SR, Eicosapentaenoic and docosahexaenoic acid ethyl esters differentially enhance B-cell activity in murine obesity. *Journal of lipid research* 2014, 55, 1420–1433. [PubMed: 24837990]
- [18]. Chen RP, Ingraham HA, Treacy MN, Albert VR, Wilson L, Rosenfeld MG, Autoregulation of pit-1 gene expression mediated by two cis-active promoter elements. *Nature* 1990, 346, 583–586. [PubMed: 2142999]
- [19]. DiMattia GE, Rhodes SJ, Kronen A, Carriere C, O'Connell S, Kalla K, Arias C, Sawchenko P, Rosenfeld MG, The Pit-1 gene is regulated by distinct early and late pituitary-specific enhancers. *Dev Biol* 1997, 182, 180–190. [PubMed: 9073460]
- [20]. Lin SC, Li S, Drolet DW, Rosenfeld MG, Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development* 1994, 120, 515–522. [PubMed: 8162852]
- [21]. Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG, Swanson LW, Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev* 1990, 4, 695–711. [PubMed: 2379827]
- [22]. Shewchuk BM, Asa SL, Cooke NE, Liebhaver SA, Pit-1 binding sites at the somatotrope-specific DNase I hypersensitive sites I, II of the human growth hormone locus control region are essential for in vivo hGH-N gene activation. *J Biol Chem* 1999, 274, 35725–35733. [PubMed: 10585453]
- [23]. Scacchi M, Pincelli AI, Cavagnini F, Growth hormone in obesity. *Int J Obes Relat Metab Disord* 1999, 23, 260–271. [PubMed: 10193871]

- [24]. Barb CR, Kraeling RR, Rampacek GB, Glucose and free fatty acid modulation of growth hormone and luteinizing hormone secretion by cultured porcine pituitary cells. *J Anim Sci* 1995, 73, 1416–1423. [PubMed: 7665372]
- [25]. Renier G, Serri O, Effects of acute and prolonged glucose excess on growth hormone release by cultured rat anterior pituitary cells. *Neuroendocrinology* 1991, 54, 521–525. [PubMed: 1684230]
- [26]. Fernandes MF, Tache MC, Klingel SL, Leri F, Mutch DM, Safflower (n-6) and flaxseed (n-3) high-fat diets differentially regulate hypothalamic fatty acid profiles, gene expression, and insulin signalling. Prostaglandins, leukotrienes, and essential fatty acids 2018, 128, 67–73.
- [27]. Mountjoy KG, Pro-Opiomelanocortin (POMC) Neurones, POMC-Derived Peptides, Melanocortin Receptors and Obesity: How Understanding of this System has Changed Over the Last Decade. *J Neuroendocrinol* 2015, 27, 406–418. [PubMed: 25872650]
- [28]. Bassaganya-Riera J, Hontecillas R, CLA and n-3 PUFA differentially modulate clinical activity and colonic PPAR-responsive gene expression in a pig model of experimental IBD. *Clin Nutr* 2006, 25, 454–465. [PubMed: 16698153]
- [29]. Di Nunzio M, Danesi F, Bordoni A, n-3 PUFA as regulators of cardiac gene transcription: a new link between PPAR activation and fatty acid composition. *Lipids* 2009, 44, 1073–1079. [PubMed: 19859757]
- [30]. Zuniga J, Cancino M, Medina F, Varela P, Vargas R, Tapia G, Videla LA, Fernandez V, N-3 PUFA supplementation triggers PPAR-alpha activation and PPAR-alpha/NF-kappaB interaction: anti-inflammatory implications in liver ischemia-reperfusion injury. *PLoS One* 2011, 6, e28502. [PubMed: 22174823]
- [31]. Oh DY, Olefsky JM, Omega 3 fatty acids and GPR120. *Cell Metab* 2012, 15, 564–565. [PubMed: 22560206]
- [32]. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM, GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* 2010, 142, 687–698. [PubMed: 20813258]
- [33]. Talukdar S, Olefsky JM, Osborn O, Targeting GPR120 and other fatty acid-sensing GPCRs ameliorates insulin resistance and inflammatory diseases. *Trends Pharmacol Sci* 2011, 32, 543–550. [PubMed: 21663979]
- [34]. Liu Y, Chen F, Li Q, Odle J, Lin X, Zhu H, Pi D, Hou Y, Hong Y, Shi H, Fish oil alleviates activation of the hypothalamic-pituitary-adrenal axis associated with inhibition of TLR4 and NOD signaling pathways in weaned piglets after a lipopolysaccharide challenge. *The Journal of nutrition* 2013, 143, 1799–1807. [PubMed: 24005609]
- [35]. Orr SK, Trepanier MO, Bazinet RP, n-3 Polyunsaturated fatty acids in animal models with neuroinflammation. Prostaglandins, leukotrienes, and essential fatty acids 2013, 88, 97–103.
- [36]. Robinson LE, Buchholz AC, Mazurak VC, Inflammation, obesity, and fatty acid metabolism: influence of n-3 polyunsaturated fatty acids on factors contributing to metabolic syndrome. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 2007, 32, 1008–1024.
- [37]. Armitage JA, Pearce AD, Sinclair AJ, Vingrys AJ, Weisinger RS, Weisinger HS, Increased blood pressure later in life may be associated with perinatal n-3 fatty acid deficiency. *Lipids* 2003, 38, 459–464. [PubMed: 12848294]
- [38]. Begg DP, Sinclair AJ, Weisinger RS, Thirst deficits in aged rats are reversed by dietary omega-3 fatty acid supplementation. *Neurobiology of aging* 2012, 33, 2422–2430. [PubMed: 22226487]
- [39]. Chen HF, Su HM, Exposure to a maternal n-3 fatty acid-deficient diet during brain development provokes excessive hypothalamic-pituitary-adrenal axis responses to stress and behavioral indices of depression and anxiety in male rat offspring later in life. *J Nutr Biochem* 2013, 24, 70–80. [PubMed: 22818715]
- [40]. Jiang LH, Liang QY, Shi Y, Pure docosahexaenoic acid can improve depression behaviors and affect HPA axis in mice. *European review for medical and pharmacological sciences* 2012, 16, 1765–1773. [PubMed: 23208960]
- [41]. Levant B, N-3 (omega-3) polyunsaturated Fatty acids in the pathophysiology and treatment of depression: pre-clinical evidence. *CNS & neurological disorders drug targets* 2013, 12, 450–459. [PubMed: 23574159]

- [42]. Li D, Weisinger HS, Weisinger RS, Mathai M, Armitage JA, Vingrys AJ, Sinclair AJ, Omega 6 to omega 3 fatty acid imbalance early in life leads to persistent reductions in DHA levels in glycerophospholipids in rat hypothalamus even after long-term omega 3 fatty acid repletion. Prostaglandins, leukotrienes, and essential fatty acids 2006, 74, 391–399.
- [43]. Weisinger HS, Armitage JA, Sinclair AJ, Vingrys AJ, Burns PL, Weisinger RS, Perinatal omega-3 fatty acid deficiency affects blood pressure later in life. Nat Med 2001, 7, 258–259. [PubMed: 11231597]
- [44]. Shaikh SR, Wassall SR, Brown DA, Kosaraju R, N-3 Polyunsaturated Fatty Acids, Lipid Microclusters, and Vitamin E. Curr Top Membr 2015, 75, 209–231. [PubMed: 26015284]
- [45]. Sullivan EM, Pennington ER, Sparagna GC, Torres MJ, Neuffer PD, Harris M, Washington J, Anderson EJ, Zeczycki TN, Brown DA, Shaikh SR, Docosahexaenoic acid lowers cardiac mitochondrial enzyme activity by replacing linoleic acid in the phospholipidome. J Biol Chem 2018, 293, 466–483. [PubMed: 29162722]

Figure 1. High fat diet lowers pituitary *Gh* mRNA levels independent of the inclusion of OA, EPA, or DHA.

(A) Effects of high fat (HF) diet alone or supplemented with ethyl esters of OA, DHA, or EPA on growth hormone (*Gh*) mRNA levels in mouse pituitary. Transcript levels determined by qRT-PCR were normalized to the control diet level. (B) Immunoblot for Gh in total protein extract from intact mouse pituitary. Each of the three control and three HF samples is a pool of whole pituitary extract from two mice. Blot was stripped and re-probed for beta tubulin as a loading control. Non-matching letters indicate statistical significance ($p < 0.05$).

A **B** **C** **D**

Figure 2. GH mRNA and protein levels are reduced by elevated glucose and insulin exposure in the rat somatotrope cell line GH3.

(A) GH3 cells are responsive to insulin through canonical signaling pathways. Immunoblot for phosphorylated AKT and ERK1/2 in lysates of GH3 cells treated with 10 nM insulin for the indicated time. (B) Suppression of *Gh* mRNA levels by insulin is dose-dependent. qRT-PCR for *Gh* mRNA, normalized to the level observed with 5 mM glucose, no insulin (C) Elevated glucose concentration reduces *Gh* mRNA level independent of insulin. (D) Elevated glucose and insulin independently reduce GH protein levels. Immunoblot for GH in

whole cell lysates. Beta tubulin serves as a loading control. Non-matching letters indicate statistical significance ($p < 0.05$; $n = 6$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. High fat diet impairs transcript levels of select hypothalamic and pituitary genes which are improved with EPA and DHA ethyl esters.

(A) Pituitary RNA and (B) hypothalamus RNA. Levels were normalized to the control diet mean for each transcript and indicate the mean $\pm$ SEM (n=8 animals). Non-matching letters indicate statistical significance ($p < 0.05$).

Figure 4. Fish oil supplementation to a high fat diet only improves growth hormone releasing hormone receptor levels.

(A) HF diet-induced suppression of pituitary *Gh* mRNA level is not influenced by fish oil.

(B) Effects of high fat diet alone or supplemented with fish oil on transcript levels of selected genes expressed in the pituitary and (C) hypothalamus. Levels were normalized to the control diet mean for each transcript, and indicate the mean $\pm$ SEM (n=6 animals). Non-matching letters indicate statistical significance ($p < 0.05$).

Figure 5. Proposed model.

HF-diet induced obesity caused changes in the levels of hypothalamic and pituitary transcripts (arrows) involved in multiple hypothalamic-pituitary-peripheral gland axes. Some of these changes were mitigated by n-3 PUFA in the diet. These observations indicate the potential for downstream effects on multiple corresponding peripheral tissues, and in the sensitivity of the H-P axis to endocrine feedback.