Elucidation of signaling mediators between adipose and neural tissue

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in
the Graduate School of The Ohio State University

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The Ohio State University
2014

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Abstract

Obesity and major mood disorders (MMD), namely bipolar disorder and major depressive disorder, have drastically increased in recent years in the United States adolescent population. Evidence indicates higher levels of circulating pro-inflammatory cytokines in both obesity and mood disorders. The effect of adipokines released by adipose tissue on the central nervous system (CNS) and inflammation in the progression of mood disorders remains understudied. In our study, we aimed to elucidate mediators between adipose and neural tissue that may link progression of mood disorders and metabolic imbalance. We hypothesized that adipose tissue can produce and secrete signaling molecules such as neuroadipokines or inflammatory cytokines that influence CNS function. We investigated 1) production and secretion of glia maturation factor β (GMFβ) in WT and Aldh1a1−/− adipocytes and 2) inflammatory and anti-inflammatory factors in mood disorder patients’ serum and their cumulative inflammatory impact on adipocytes measured by NF-κB, a master transcriptional regulator of inflammation. We found that GMFβ is produced and secreted by adipocytes and its expression depends on aldehyde dehydrogenase 1A1. Thus, GMFβ is a neuroadipokine that can function in adipose as well as neural tissue. In addition, we found increasing variability of NF-κB and its target cytokines if severity of MMD in patients increases. These data suggest that
both suppression and increases in inflammation may influence MMD progression that warrants studies in specific MMD populations.
Acknowledgments

I am deeply grateful for the support, encouragement, and guidance of my advisor, Dr. Ouliana Ziouzenkova. Inspired by her style of teaching in my Macronutrient Metabolism class and her pure passion for science, I visited her office to inquire whether she would take me as a member in her lab. I was hesitant because of my lack of previous lab experience, to which she replied, “It’s not like scientists were born with pipets in their hands!” Since then she has been there for me in my graduate school journey, advising me not only in an academic and scientific capacity, but in life matters as well. She always helped me to improve in many ways and I thank her for that.

I would also like to thank the Human Nutrition department, for accepting me as their student and allowing me to further my professional education, all teachers who have taken the time to teach me, and my lab members who have been an invaluable source of knowledge and assistance in my research experiences, and lastly thanks for the guidance of my committee members.
Dedication

I would like to dedicate this thesis to my parents, without their support I would not be the person I am today nor have had the opportunity to have this wonderful research experience.
Vita

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CHAPTER 1

Introduction

In the past 30 years, childhood obesity rates have doubled and adolescent obesity rates have quadrupled in the United States [1]. Obesity can result from 1) energy imbalance resulting in changes in adipose tissue mass and/or 2) hormone and signaling imbalance causing changes in adipose tissue mass with or without a difference in energy intake via redistribution of internal energy between tissues. Energy balance is a subject of regulation by humoral signaling and the CNS, depending on genetic and environmental factors [2, 3]. In addition to its energy storage function, adipose tissue has endocrine functions: 1) several hormones, such as cortisol, estrogen, and thyroid hormone, can be activated, generated, and/or deactivated in adipose tissues to regulate intrinsic processes; 2) hormones, such as adiponectin and leptin, and cytokines (pro- or anti-inflammatory), such as TNF-α and IL-6, can be produced in adipose tissue and released into the circulation for regulation of metabolism and immune functions [4, 5]. Impairment in hormone production circuits results in changes in adipose tissue mass and alters properties of adipose tissue independent of energy intake. There is an increase in the release of pro-inflammatory cytokines such as MCP-1, TNF-α, and IL-6 in the adipose tissue of obese patients that potentially promotes inflammation in other tissues [6]. Thus, altered inflammatory signaling in the adipose tissue of obese individuals may contribute to adverse physiological outcomes. In fact, obese youth are more likely to have a host of
health problems including sleep apnea, bone and joint problems, high cholesterol, pre-diabetes and high blood pressure, all of which are augmented by chronic inflammation associated with obesity [7-9]. Youth suffering from obesity are also more likely to be obese as adults, putting them at greater risk for developing cardiovascular disease, type II diabetes, stroke, osteoarthritis and some cancers [10, 11]. Furthermore, obesity has been linked with several mental diseases including major depressive disorder (MDD) and bipolar disorder (BD) [12-14]. MDD and BD are classified as major mood disorders in which the main symptom is a pervasive and persistent disturbance in an individual’s mood [15]. Mood disorders, like obesity, are also a serious problem among adolescents in the United States where 4.7% of 13-18 year olds have a severe mood disorder [16]. There is increasing evidence that major mood disorders are associated with higher levels of circulating pro-inflammatory cytokines and neuroinflammation [17-19]. Thus, altered inflammatory signaling presents a common risk factor in the pathology of obesity and mood disorders.

While the significant co-occurrence of obesity and mood disorders is known [20], the interplay between adipose tissue and the progression of mood disorders at the molecular level is largely understudied. Although both mental diseases and obesity have higher levels of circulating pro-inflammatory cytokines, it is unclear whether the cytokines influence the diseases’ progression. It is also not known how the cytokines produced by neural and adipose tissue influence each other. Elucidating these mechanisms is an important step towards understanding future prevention and therapeutic options for these diseases.
Our overarching research hypothesis is that adipose tissue can produce signaling molecules such as neuroadipokines or inflammatory cytokines that influence central nervous system function. These cytokines can be identified by 1) comparison of neuroadipokines released from the adipose tissue in lean and obese mouse animal models and 2) investigating inflammatory cytokines in a cohort of patients with and without mood disorders in context of their BMI.
CHAPTER 2

Literature Review

2.1 Introduction: Increase in obesity in adult and adolescent populations.

Obesity rates have reached epidemic proportions in the United States where 35.7% of adults and 16.9% of children and adolescents are considered obese, and more than two thirds of adults are considered overweight or obese [1]. Obesity is measured by an individual’s body mass index (BMI), which is the quotient of his/her weight in kilograms divided by his/her height in square meters. An individual with a BMI between 25 and 29.9 is considered overweight and one greater than 30 is considered obese. It is estimated that the annual medical cost associated with obesity in the United States is $190.2 billion [21]. This high-cost of obesity is a result of the development of debilitating and chronic co-morbidities in obese patients.

2.2 Obesity as a risk factor for metabolic and mental diseases

Obesity increases the risk of developing many diseases including diabetes mellitus type 2, hypertension, stroke, heart disease, sleep apnea, some cancers and osteoarthritis [22, 23]. Obesity has also been linked with several mental diseases including MDD and BD [12-14, 24]. One study found that atypical MDD is a strong predictor of obesity [25]. Vice versa, another study found that among men there was a significant positive association between visceral adiposity and depressive symptoms [26]. While there is increasing evidence that obesity and depression are associated, the underlying
mechanisms of their development are not fully understood. Several studies suggest that dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis may be involved in the progression of depression [27-29]. The HPA-axis responds to stress with the release of cortisol, a hormone responsible for increasing energy release in the body and regulating appetite [30]. Adipose tissue is responsible for the conversion of cortisone into its active form, cortisol, that can potentially increase levels of this stress hormone in obese patients [31]. Adipokines such as adiponectin, resistin, and leptin, have been associated with depression and may negatively influence the CNS [32-35]. The amount and properties of adipokines, hormones and other bioactive metabolites in the circulation depend on the type of adipose tissue.

2.3 Adipose tissue classification based on function in energy homeostasis

Adipose tissue classification is divided into two major types: white adipose tissue (WAT) and brown adipose tissue (BAT) [36, 37].

WAT has lipogenic functions, storing energy in the form of triglycerides [38]. WAT was first categorized based on its location. The two subtypes of WAT are: 1) subcutaneous fat, which refers to WAT beneath the skin, and 2) visceral fat, which broadly refers to the fat surrounding the organs. The visceral fat is further classified into mesenteric and/or omental fat based on developmental origin [36].

BAT has thermogenic functions, utilizing triglycerides to produce heat. In human infants, BAT is found in cervical, axillary, perirenal and periadrenal regions and declines with age [39]. BAT is densely filled with uncoupling protein 1 (UCP-1) positive mitochondria and arises from a similar progenitor as skeletal muscle which expresses myogenic factor
UCP-1 dissipates heat in BAT [41]. This occurs when protons that are pumped out of the mitochondria by the electron transport chain are able to cross back over the mitochondrial inner membrane through UCP-1, thereby uncoupling oxidative phosphorylation and releasing energy as heat. Heat dissipation properties of BAT are physiologically beneficial [39]. Elevated amounts of BAT are associated with improved glucose sensitivity and increased resistance to obesity in human and mouse obesity models [41, 42]. In rodents exposed to cold or adrenergic stimulation there is an appearance of UCP-1 positive cells with a BAT-like morphology termed ‘beige’ or ‘brite’ adipocytes [43]. Inducing the ‘browning’ of WAT could be a viable target for therapeutic strategies against obesity [44].

2.4 The role of WAT in cytokine production and inflammation

In addition to its energy storage function, WAT depots are multiple organs with dissimilar endocrine functions secreting signaling molecules termed adipokines that regulate energy homeostasis, inflammation and numerous other body processes (Fig. 1) [6]. Specific fat depots have different developmental origins responsible for heritable genetic characteristics of adipocytes and thus varying, and sometimes opposing, functions [45]. Visceral fat depots have been associated with metabolic diseases while subcutaneous fat depots may have beneficial metabolic effects [46]. This has been demonstrated to depend on more pro-inflammatory cytokines produced in visceral fat than in subcutaneous fat (reviewed in [47]). Pro-inflammatory cytokines, such as TNFα, MCP-1, and IL-6, also contribute to insulin resistance [4, 5, 48, 49]. The transcription factor complex nuclear factor-kappa B (NF-κB) is a master regulator of inflammation. It
regulates both expression of these cytokines and mediates inflammation in response to these cytokines in visceral and subcutaneous fat [50-52]. The activation of NF-κB in adipocytes could be triggered by circulating cytokines, intra- and extra-cellular oxidative stress, pathogens, environmental and metabolic factors. Upon activation, the NF-κB complex translocates to the nucleus and binds to specific cognate DNA response elements (NF-κB-RE) to regulate transcription [50]. Elevation in cytokine expression through NF-κB in visceral fat may underlie adverse outcomes of obesity. NF-κB is activated in adipocytes by cytokines, leading to a further increase in expression of pro-inflammatory cytokines in a vicious circle enhancing inflammation and obesity. In contrast to visceral fat, subcutaneous fat produces anti-inflammatory adipokines such as insulin-sensitizing adiponectin [48].
Figure 1. Adipose tissue affects energy homeostasis and inflammation in other organs via communication by cytokines and other bioactive molecules

WAT is a complex tissue comprising of endothelial cells, fibroblasts, preadipocytes, mature adipocytes, macrophages, neural precursors and stem cells. Arrow between energy homeostasis and inflammation is double-headed because each one influences the other. For example, in the case of obesity, increased adipose tissue mass may produce increased levels of pro-inflammatory cytokines leading to increased inflammation. The inflammation may exacerbate energy regulating processes leading to more disruption of energy homeostasis, and so on.

2.5 Aldh1a1/- mice: a model to elucidate differences in WAT depots formation and discovery of adipokines

Retinoic acid (RA) is produced predominantly by Aldh1a1 by conversion of retinaldehyde (precursor is retinol) to RA in visceral fat and by Aldh1a1 and Aldh1a3 in subcutaneous fat [53]. RA is able to regulate transcription of target genes by binding to retinoid x receptors (RXRs) and retinoic acid receptors (RARs) [54], mediating numerous processes such as adipogenesis [55]. Mice deficient in Aldh1a1 (a1KO) resisted diet-induced obesity and insulin resistance and had a higher metabolic rate, reaction quotient and body temperature [56]. Because RA mediates numerous processes through binding to
nuclear receptors and adipose tissue is the second largest storage site for vitamin A [57], a1KO is an excellent model for investigation of inflammation and energy homeostasis. A previous study from our laboratory elucidated changes in protein expression that may be responsible for resistance to HF diet-induced obesity in a1KO mice. Utilizing differential in gel electrophoresis (DIGE) to make a proteomic comparison between visceral adipose tissue of WT and a1KO mice, several proteins were found to exhibit altered expression, including glia maturation factor beta (GMFβ) [58]. GMFβ expression increased by almost 10-fold in a1KO mice adipose tissue. This was an unexpected finding, because GMFβ has mainly been shown to be produced by astrocytes in the brain and secreted in the brain and spinal cord to regulate growth of neurons and glia [59].

WAT is a complex tissue comprising of endothelial cells, fibroblasts, preadipocytes, mature adipocytes, macrophages, neural precursors and stem cells. To elucidate GMFβ’s function in WAT, it is critical to understand whether GMFβ is expressed in adipocytes or is produced in neural tissue in WAT.

2.6 Study hypothesis and objective

Understanding the connection between adipose tissue and the CNS could potentially help explain the link between obesity and mental disorders. This in turn may provide potential targets for the prevention or treatment of obesity, mental disorders, or both.

Our overarching research hypothesis is that WAT can produce neuroadipokines that influence CNS function. We aimed to identify neuroadipokines by 1) comparison of cytokines released from adipocytes obtained from lean a1KO and obese wild type (WT)
mice or 2) investigating inflammation factors relevant to WAT in a cohort of lean and overweight patients with and without mood disorders.

We hypothesized that GMFβ is a neuroadipokine secreted by adipocytes. The first research aim was to investigate the production and secretion of GMFβ in WT preadipocytes and a1KO preadipocytes (Fig. 2).

In addition to identifying novel neuroadipokines, it is important to examine the relationship between adipose tissue, inflammation and progression of CNS diseases in humans. The contribution of inflammation to the progression of mood disorders in lean and overweight patients is understudied. The second research aim was to investigate inflammatory and anti-inflammatory factors in mood disorder patients’ serum and their cumulative inflammatory impact on adipocytes. We hypothesized that adolescent patients affected by more severe mood disorders would exhibit increased inflammatory and oxidative stress factors in their serum compared to control subjects that could be assessed in NF-κB reporter adipocytes.
Figure 2. Elucidating the effect of adipose tissue on the CNS via a clinical and basic research approach
CHAPTER 3

Production and secretion of GMFβ by adipocytes

3.1 Introduction

Identifying novel adipokines that can play a role in the CNS may provide potential targets for treatment or prevention of mood disorders and obesity. In the brain, growth and differentiation of neurons and glia is under control of several growth factors [59]. GMFβ is a protein found predominantly in the brain and was first isolated from bovine brain tissues and characterized by Lim and associates [59]. The GMFβ gene is located on human chromosome 14 (International Human Genome Sequence Consortium, 2001). Its sequence is highly conserved across species. GMFβ mRNA is mainly expressed in the brain and spinal cord although trace amounts have been found in other tissues, namely ovary and testis where its function remains unknown [60]. GMFβ is mainly found in cells of neural origin [61]. Using immunofluorescence on cultured astrocytes [62] and cultured Schwann cells [63], it was determined that GMFβ is mainly a cytoplasmic protein that can be secreted to regulate growth of neurons and glia [59]. Secreted GMFβ was detected in human serum [64].

GMFβ has many functions in neural tissues:

1) Anti-tumorigenic: Several tumor cells not limited to neural origin were treated with recombinant GMFβ and it showed a strong anti-proliferative effect [65]. This effect was most pronounced in glioma, neuroblastoma, and Schwannoma cells
2) Neuroprotective and neurotrophic: Viral expression of GMFβ in C6 glioma cells increased expression of the neurotrophic factors brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and increased presence in conditioned medium [66]. When NGF and BNDF from conditioned medium was applied to PC12 rat pheochromocytoma cells, there was increased neurite out-growth and resprouting [66]. In addition, cerebellar granule neurons were protected against ethanol toxicity when treated with this conditioned medium [67].

3) Regulates axonal growth: A study showed that a collagen gel mixture containing GMFβ improved axonal growth compared to controls in rat sciatic nerves that were transected and repaired via an entubulation technique. Those that were treated with the GMFβ mixture had significantly more regrowth [68].

4) Inflammation: Research suggests a possible association between up-regulation of GMFβ and brain cytotoxicity though inflammation. Overexpression of GMFβ in astrocytes was responsible for the death of primary oligodendrocytes via interactions between purified cultures of astrocytes, microglia and oligodendrocytes [69]. This occurred partially through activation of NF-κB, a master regulator of inflammation in the body regulating transcription of genes involved in the inflammatory response. Activating NF-κB resulted in higher levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IP-10 which became cytotoxic to oligodendrocytes [69]. Another study showed that ventricular infusion of human amyloid beta peptide 1-42 in mouse brains resulted in higher levels of glial activation, GMFβ, and induction of pro-
inflammatory cytokines [70]. When the same infusion was given to GMFβ knockout mice, they showed suppressed production of pro-inflammatory cytokines and reduced glial activation [70].

The function of GMFβ in tissues other than the CNS is unknown. Our previous work found that GMFβ is also expressed in the visceral adipose tissue of WT and a1KO mice (Fig. 3) [58]. Adipose tissue is comprised of many cell types such as endothelial cells, fibroblasts, preadipocytes, mature adipocytes and macrophages, neural precursors and stem cells. Whether GMFβ is produced by adipocytes and secreted as an adipokine is unknown. Understanding the capacity in which GMFβ functions in adipose tissue could help link energy homeostasis, adipogenesis, and CNS functions. This knowledge could provide targets for the prevention and treatment of obesity, mood disorders or both. Future studies will also improve the understanding of possible neuro-inflammatory effects of GMFβ secreted by adipose tissue.

We hypothesized that GMFβ is a neuroadipokine that is produced and secreted by adipocytes in an Aldh1a1-dependent manner.
Figure 3. Enhanced GMFβ protein levels in the visceral fat (VF) of male a1KO compared to WT mice [58]
(A) WT and a1KO male and female mice were fed a HF diet. Female and male a1KO mice were resistant to HF diet induced obesity. (B) VF of male and female mice were analyzed via Difference gel electrophoresis (DIGE) proteomic comparison (male WT compared to male a1KO gel shown) to identify possible genes in the VF responsible for resistance to obesity of a1KO mice. (C) Expressions of several genes involved in FA metabolism were altered. In addition, GMFβ expression was found increased by almost 10-fold in the male a1KO vs. WT mice and 8-fold in female a1KO mice.

3.2 Methods

3.2.1 Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) and cell culture media from Invitrogen (Carlsbad, CA) unless otherwise indicated.

3.2.2 Cell differentiation

WT (C57Bl6/J) mouse preadipocytes and a1KO mouse preadipocytes from previously
isolated stromal vascular fraction from subcutaneous fat of 16- and 11-month old WT and a1KO female mice on a regular chow diet [58, 71]. Preadipocytes were immortalized as described before [58]. Preadipocytes were cultured and maintained in standard culture medium (high glucose DMEM containing 10% calf serum and 1% penicillin-streptomycin) [58]. Differentiation was induced on day 0 (d0) in confluent preadipocytes using a differentiation cocktail medium containing 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), insulin (1.7 µM), dexamethasone (1 µM), 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in DMEM (differentiation media I) [72]. Differentiation media II contained the same reagents as differentiation media I except for the dexamethasone and IBMX, and was added on d2, d4. Media was harvested on d0, d2, d4, d6 and then cells were lysed with RIPA buffer.

3.2.3 Protein assay

The protein content in RIPA cell lysates was measured using a BCA assay kit (Thermo Fisher Scientific; Rockford, IL). The absorbance (562 nm) was measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader.

3.2.4 Western blot

To concentrate proteins between 10-50 kD 5 mL of harvested media was filtered with Amicon Ultra-15 Centrifugal Filter Units (Millipore) by centrifugation in a GR412 centrifuge (Jouan) at 4000 rpm for 1 min at RT. Media filtrates used for Western blotting had equal unfiltered starting volume. Cell lysates each contained 28 µg/µL protein content. Media filtrates and protein lysates were separated on 4-20% polyacrylamide gels under reducing conditions and transferred to a polyvinylidene fluoride membrane.
(Immobilon-P; Millipore). GMFβ protein on membranes was detected using primary polyclonal antibody rabbit anti-GMFβ (ProteinTech) at 1:1000 dilution in Odyssey blocking buffer (LI-COR Biosciences). The secondary antibody used was green goat anti-rabbit (Odyssey). For quantification, housekeeping beta-actin was analyzed on the lysate same membranes using rabbit anti-β-actin (ProteinTech) in a 1:1000 dilution. Membranes were analyzed using an Odyssey Infrared Imaging System. Bands were quantified using ImageJ software. The GMFβ/β-actin ratios for lysate membranes were normalized across membranes by loading 28 µg/µL of standard containing 10 µL from each lysate sample.

3.2.5 Statistical analysis

Data were shown as mean ± SD based on 3 independent experiments. The group comparison was performed using Student t-test and Mann-Whitney U-test if data were not normally distributed.

3.3 Results

To investigate GMFβ protein expression and secretion during adipocyte differentiation and the influence of Aldh1a1 on this process, WT and a1KO preadipocytes were differentiated and lysates and media were collected on differentiation days 0, 2, 4, and 6. Western blot analysis of adipocyte lysates revealed a similar GMFβ protein expression pattern in lysates of WT adipocytes over the time course of differentiation (Fig. 4). Day 0 and 2 showed significantly different levels of GMFβ protein expression (d₀: WT=1.53, a1KO=2.52, d₂: WT= 1.98, a1KO=3.06). The highest protein expression of GMFβ occurred on day 2 WT. GMFβ protein expression in a1KO was higher (WT 92-131%,
a1KO 152-202% than in WT adipocytes throughout differentiation. The highest protein expression of GMFβ occurred on day 2 (WT 131%, a1KO 202%). After day 4, GMFβ protein expression in a1KO lysates increased while expression decreased in WT lysates. The WT and a1KO lysates differed significantly by Mann-Whitney U-test (U(16)= 0.02, z= 2.3).

In the media of WT adipocytes, the levels of secreted GMFβ were nearly under detection limits during the time course of differentiation (Fig. 5). a1KO adipocytes showed significantly increased levels of secreted GMFβ on day 6. The WT and a1KO media did not differ significantly by Mann-Whitney U-test (U(10)= 0.69, z= 0.58).
Figure 4. GMFβ expression in WT and a1KO adipocytes shows a similar pattern over time-course of differentiation from preadipocytes to adipocytes

GMFβ in cell lysates were collected during differentiation of WT adipocytes and a1KO adipocytes. Lysates were collected on days 0, 2, 4 and 6. 28 µg protein from lysates were analyzed using polyclonal anti-GMFβ. Data were normalized by β-actin. WT data are represented as open squares and a dashed line, a1KO are represented as solid squares and a solid line. Kinetics data of GMFβ protein expression during differentiation are shown as % ± SD normalized to GMFβ/β-actin ratio seen in WT preadipocytes on day zero set to 100%.

Insert shows 1 representative Western blot of GMFβ and β-actin protein expression in WT and a1KO adipocytes on different days. 3 independent experiments were performed.
Figure 5. Increased levels of secreted GMFβ in a1KO but not in WT adipocytes over time course of differentiation

GMFβ protein levels were detected by Western blotting using media collected during differentiation of WT adipocytes and a1KO adipocytes. Media was collected on days 0, 2, 4 and 6 of differentiation. Proteins between 10-50 kD were filtered from 5 mL media and protein from media were analyzed using polyclonal anti-GMFβ. WT data are represented as open squares and a dashed line, a1KO are represented as solid squares and a solid line. Kinetics data of secreted GMFβ protein expression during differentiation shown as band density on membranes. Data are shown as mean band density ± SD.

Insert shows 1 representative Western blot of protein expression of GMFβ secreted from WT and a1KO adipocytes on different days. 3 independent experiments were performed.
3.4 Discussion

Obesity and mood disorders are serious diseases affecting an increasingly large portion of the population [1, 73]. Increased inflammatory factors are implicated in either of these diseases, although key cytokines mediating crosstalk between adipose and CNS and influencing the diseases’ progression remain to be elucidated. Deregulation of signaling molecules secreted from adipose tissue can increase inflammation that could negatively influence the CNS. Identifying novel adipokines and their function on the CNS is important in understanding the interaction between adipose tissue and neural tissue. Our results indicate that GMFβ is an adipokine because it is produced and secreted by adipocytes. GMFβ protein expression and secretion is increased in a1KO compared to WT adipocytes, suggesting Aldh1a1-dependent inhibition of GMFβ protein levels in adipose. Given that GMFβ was established as a neurokine influencing various aspects of CNS biology, GMFβ could be considered a neuroadipokine. [66]. Future studies are needed to investigate effects of GMFβ 1) in adipocytes and 2) on crosstalk between adipocytes, neural cells and axons locally in adipose tissue and systemically. In addition, studies have shown that overexpression of GMFβ lead to increased activation of NF-κB and levels of pro-inflammatory cytokines [69, 70]. This effect of GMFβ could be important in the progression of mood disorders and obesity and warrants future study.
CHAPTER 4

Immune response in an adolescent cohort of lean and overweight patients a with wide spectrum of mood disorders

4.1 Introduction

Research suggests an association between obesity, MDD and BD [12-14]. MDD and BD are classified as major mood disorders in which the main symptom is a pervasive and persistent disturbance in an individual’s mood. Mood disorders, like obesity, are a serious problem in the United States where 9.5% of the adult population has a mood disorder and 4.7% of 13-18 year old patients have a severe mood disorder [16, 73]. Three major interrelated mechanisms were proposed to contribute to increasing rates of mood disorders. Research suggests that major mood disorders are associated with inflammation, specifically with higher levels of circulating pro-inflammatory cytokines and neuroinflammation [17-19]. However it remains uncertain what cytokines or their combinations can trigger disease and serve as markers for mood disorder development. Neuropsychiatric disorders have also been linked to oxidative stress and metabolic dysfunction, but there is a gap in knowledge about mechanisms underlying the development and progression of mood disorders specifically in relation to adipose tissue [74, 75]. It is possible that adipose tissue inflammatory cytokines and adipokines play a pivotal role in this process. There is evidence that those affected by MDD have higher levels of pro-inflammatory cytokines in the circulation and cerebral spinal fluid, namely
C-reactive protein, TNF-α, and IL-6 [17, 19, 76]. Pro-inflammatory cytokines in the circulation may activate the transcription factor complex NF-κB, a master regulator for immune response and inflammation [50, 52] in adipocytes and other tissues. It regulates several pro-inflammatory cytokines, such as MCP-1 and IL-6 by binding directly to its response element (NF-κB-RE) in respective promoters and enhancers [77, 78]. NF-κB activation leads to the transcription of pro-inflammatory cytokines, further enhancing inflammation [50]. The contribution of pro-inflammatory cytokines from adipose tissue to the progression of mood disorders remains understudied. In addition to pro-inflammatory cytokines, the blood contains many natural anti-inflammatory factors such as IL-1ra, IL-4, and IL-10 (Reviewed in [79]). Vitamin D (Fig. 6) is also circulated in the blood and has some immune-modulating and anti-inflammatory functions (Reviewed in [80]). In addition to its role in bone homeostasis and others, vitamin D regulates inflammation directly through its effects on immune cells. Vitamin D regulation occurs through its binding to the vitamin D receptor (VDR) which translocates to the nucleus and forms a heterodimer with RXR. This heterodimer then binds to the vitamin D response element (VDRE) in the target gene promoter to up-regulate transcription of genes [81]. VDR bound to vitamin D is also able to decrease NF-κB activation by binding directly to IKKβ, which phosphorylates inhibitors of NF-κB, a key step in NF-κB’s activation [82]. Furthermore, evidence indicates an inverse association between vitamin D levels and obesity possibly due to regulation of inflammation [83, 84].

It is unclear whether pro- and anti-inflammatory factors dominate the regulation of the progression of mood disorders. Understanding such mechanisms could lead to strategies
for their prevention. Although the measurement of specific pro- and anti-inflammatory cytokines is important, it is difficult to assess cumulative inflammation in patients with mood disorders. In addition to pro-inflammatory cytokines, NF-κB can be activated through signaling cascades by fatty acids, ceramides, lipopolysaccharide, modified LDL, and others through innate pattern recognition receptors [85]. Information about cumulative inflammatory signals can be obtained through a measurement of the activation of the NF-κB. Our lab has developed an NF-κB-RE reporter bioassay (Fig. 7) for measuring inflammation related to activation of NF-κB by non species-specific factors [86]. To assess the multifactorial impact on the development of mood disorders, I applied this NF-κB-RE reporter bioassay to study cumulative inflammatory response in a cohort of patients affected by mood disorders that were obtained by Dr. Gracious (Nationwide Children’s Hospital, Columbus OH) in context with measured levels of MCP-1, IL-6, autoantibody to oxLDLs, and vitamin D concentrations in plasma. We hypothesized that patients affected by more severe mood disorders and/or with elevated BMI would have more inflammation and oxidative stress factors in their serum compared to control subjects.
4.2 Methods

4.2.1 Patients

Patients (n=63, age range 10 to 17, age mean 13.71 ±1.86, 78% male, 64% Caucasian, BMI mean 25.29 ±7.47) consisted of individuals with no mood disorders (n=12), with an acute mood disorder episode (n=10), with moderate mood disorders (n=16) and with severe mood disorders (n=25). Groups were assigned based on consultation with collaborating statistician and psychiatrist. Sample collection was performed by Dr.

Figure 6. Vitamin D has metabolic, neuroprotective, bone homeostatic and immune functions

Vitamin D action is mediated through its binding to VDR which translocates to the nucleus to form a heterodimer with RXR. This VDR-RXR complex binds to VDREs on target promoters which up-regulate transcription of target genes.
Gracious (Nationwide Children’s Hospital, Columbus OH). Blood serum was collected and provided in a double-blind fashion as part of the NIMH LAMS-2 study examining mood symptom changes over time in children, as well as vitamin D, BMI, IL-6 data. [87]. IL-6 was measured via Immulite 1000 automated chemiluminometer and 25-hydroxyvitamin D (25-OHD) was measured via liquid mass spectrometry detection. The BMI, IL-6 and vitamin D experiments were performed in the laboratory of Dr. Gracious and provided for analysis in my study.

4.2.2 Materials
Reagents were purchased from Sigma-Aldrich (St. Louis, MO) and cell culture media from Invitrogen (Carlsbad, CA) unless otherwise indicated.

4.2.3 Cell differentiation
Mouse NF-κB/GFP biosensor preadipocyte cell line was derived from 3T3-L1 preadipocyte cell line that were stably transfected with an NF-κB-RE/GFP lentiviral reporter vector and an mCherry lentiviral control vector as described in [86]. The inflammatory response in these cells were characterized by selecting clones which showed the highest GFP/mCherry fluorescence ratio after stimulation with LPS ([86], Chapter 2.4). Cells were cultured and maintained in standard culture medium (high glucose DMEM containing 10% calf serum and 1% penicillin-streptomycin). Differentiation was induced on day 0 (d₀) in confluent preadipocytes using a differentiation cocktail medium (differentiation media I) containing 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), insulin (1.7 µM), dexamethasone (1 µM), 10% fetal

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bovine serum (FBS) and 1% penicillin-streptomycin in DMEM. *Differentiation media II* contained the same reagents as *differentiation media I* except for the dexamethasone and IBMX, and was replaced every 48 h (d2, d4). Cells were stimulated (d6) with 300 µL DMEM containing 1% penicillin-streptomycin and 1% serum collected from patients affected by mood disorders. A standard (STD) containing 5 µL of serum from each patient was used to stimulate cells separately for normalization. Experiment was performed in a ‘blind’ fashion with coded patients’ information. Cells were lysed with RIPA buffer and harvested on d8. 80 µL of cell lysates for each well were transferred to a 96-well plate and GFP fluorescence was measured at wavelengths Ex/Em 485/528 using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). The ratio of GFP fluorescence/protein concentration of lysates represented NF-κB activation. The experiments that were performed on different days were normalized to the STD. Identification of coded patient disease statuses was released after experiments and data analyses were completed.

**4.2.4 Protein assay**

The protein content in RIPA cell lysates was measured using a BCA assay kit according to manufacturer’s instruction (Thermo Fisher Scientific; Rockford, IL). The absorbance (562 nm) was measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader.

**4.2.5 Enzyme-linked immunosorbent assay (ELISA)**

MCP-1 concentrations in the serum of patients affected by mood disorders were analyzed using an MCP-1 ELISA Kit (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. The absorbance (450 nm) was measured using a Synergy H1
Hybrid Multi-Mode Microplate Reader and quantified based on standard concentration curve.

Human IgG autoantibodies to oxidized low-density lipoprotein (LDL) in the serum of patients affected by mood disorders were analyzed using an Anti-Oxidized LDL (oLAB) ELISA Kit (Biomedica, Austria) according to the manufacturer’s instructions. The absorbance (450 nm) was measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader.

Figure 7. NF-κB-RE reporter bioassay measures cumulative inflammation signal in serum from adolescent children
4.2.6 Statistical analysis

Data are shown as mean ± SD based on 3 independent experiments for each patient serum sample. Correlation was determined using Pearson correlation analysis (SPSS Windows 7).

4.3 Results

To assess cumulative inflammatory factors in the serum of patients affected by a wide range of mood disorders, we utilized an NF-κB-RE reporter bioassay. We found that variability in NF-κB activation increases as the severity of mood disorder increases (Fig. 8) (Acute mood group= 40-124%, Moderate mood group= 33-211%, Severe mood group= 34-315%, %’s presented compared to No mood group mean NF-κB activation set to 100%). Several measures were taken to decrease variability related to the biological reporter assay. In agreement with the NF-κB-RE reporter bioassay, plasma levels of an NF-κB-regulated cytokine, MCP-1, also increases in variability as severity of mood disorder increases (Fig. 9) (Acute mood group= 91-119%, Moderate mood group= 78-115%, Severe mood group= 77-123%, %’s presented compared to No mood group mean MCP-1 set to 100%).

There was a significant correlation between vitamin D level and anti-oxLDL (r=0.28, p=0.03) (Fig. 10) and a significant inverse correlation between BMI of subjects and vitamin D (r=−0.40, p=0.001) (Fig. 11). There was a significant correlation between BMI and IL-6 levels (r=0.29, p=0.02) and a significant inverse correlation between IL-6 and vitamin D (r=−0.28, p=0.02) (Table 1). Correlations between all other parameters were not significant (Table 1).
<table>
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<tr>
<th>Variables</th>
<th>(1) Vitamin D</th>
<th>(2) BMI</th>
<th>(3) anti-oxLDL</th>
<th>(4) IL-6</th>
<th>(5) MCP-1</th>
<th>(6) NF-kB</th>
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<td>(1) Vitamin D</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>(6) NF-kB</td>
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<td>.395</td>
<td>.255</td>
<td>.556</td>
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**Table 1. Correlation matrix of variables**

Correlations of variables based on 2-tailed significance are shown in cohort of patients with and without mood disorders. Pearson correlations are shown in parenthesis with any two-tailed significance below .05.
Figure 8. Variability in NF-κB activation in response to blood increases in patients with severe mood disorders compared to combined groups of healthy subjects and subjects with differing severities of mood disorders.

Lines in chart represent mean values (No mood= 136±38, Acute mood= 134±33, Moderate mood= 129±53, Severe mood= 135±84). Dots represent individual patients.
Figure 9. Variability in MCP-1 levels was increased with increasing severity of mood disorder in combined groups of healthy subjects and subjects with differing severities of mood disorders across increasing severity of mood disorders. Lines in chart represent mean values (No mood= 7.3±0.63, Acute mood= 7.5±0.51, Moderate mood= 7.2±0.77, Severe mood= 7.4±0.74). Dots represent individual patients.
Figure 10. Serum 25-hydroxyvitamin D and autoantibody to oxLDL are positively correlated in combined groups of healthy subjects and subjects with differing severities of mood disorders
Figure 11. Serum 25-hydroxyvitamin D levels were inversely correlated with BMI in combined groups of healthy subjects and subjects with differing severities of mood disorders.
4.4 Discussion

As a measure of the body’s cumulative inflammatory signals, inflammatory factors in serum from patients affected by mood disorders was assessed through an NF-κB-RE reporter bioassay. In our study there was more variability in NF-κB activation as severity of mood disorder increased suggesting extreme manifestations of immune responses when affected by a severe mood disorder. To validate the NF-κB activation measurement, we measured MCP-1 as a downstream target cytokine of NF-κB and found increased variability in MCP-1 levels as severity of mood disorder increased. The variability seen is likely due to analysis of a non-homogenous range of mood disorders rather than a homogenous sample. Inflammation leads to oxidative stress and oxidative damage of cellular membrane and lipoproteins. These oxidized lipid epitopes are recognized by autoantibodies such as anti-oxLDL [88]. As an additional measurement to compare the effects seen in NF-κB activation to oxidative stress, we measured anti-oxLDL. The correlation between anti-oxLDL and vitamin D was not dependent on presence of mood disorder. We found that higher BMI correlates with lower vitamin D similar to other reported findings in humans [84, 89]. This BMI increase may be associated with more adipose tissue producing IL-6, which possibly leads to higher serum IL-6. This relationship is supported by the correlation between BMI and serum IL-6. The negative correlation between IL-6 and vitamin D may possibly be due to inhibition of IL-6 transcription by vitamin D. Vitamin D is able to inhibit transcription of target genes of NF-κB, one target of which is IL-6, by binding to VDR and this complex binding to and inactivating IKKβ, thus preventing activation of NF-κB [82].
Taken together, our results indicate that within each patient group inflammation occurs at different extents and it is triggered by different mechanisms. Moreover, it is possible that mood disorder conditions could result from elevated as well as suppressed inflammation as indicated by inflammation present in two opposite extremes in the most severe mood disorder group. Specific subgroups including patients with bipolar disorder may have a different inflammatory response, possibly due to modification from treatment effects or other unknown genetic factors.
Epilogue

Tissues communicate in the body through signaling molecules. For example, releasing cytokines from one tissue could signal a pathogen attack on the body, after which appropriate immune responses will be employed to return the body to health. Moreover, releasing certain hormones maintains energy homeostasis, signaling tissues to store energy when it is in surplus or to release energy from storage for use. There are many more functions that cytokines and hormones have, some of them known and some unknown. The interplay between these signaling molecules and body processes can get quite complicated, especially in the progression of diseases.

With complex profiles of hormones and cytokines circulating in the body sending different messages at any given moment, changing environments and stimulations can become chaotic. Facilitating homeostasis and a return to a stable state after any immune disruption is the job of the CNS. Without input from peripheral tissues through humoral signaling and electrical messages, the CNS would be disconnected and the body would cease to function properly. The CNS receives messages from peripheral tissues and sends responses to maintain order. Some diseases are manifestations of disrupted cross talk between the immune system, CNS, and other tissues in the body. For example, when adipose tissue biology is changed due to energy imbalance or changes in signaling due to genetics or environment, this disrupts homeostasis. Whether this disruption was caused
by faulty signaling originating from the CNS or adipose tissue sending disruptive messages to the CNS is important to know.

Adipose tissue plays a large endocrine role in the body by producing and releasing adipokines; and producing, activating and/or deactivating hormones. With the current obesity epidemic it is becoming critically important to elucidate the role that adipose tissue signaling plays in whole body energy homeostasis and the pathology of diseases. With a better understanding of these processes, prevention of obesity and its associated diseases becomes more possible. In our research, we investigated 1) the production and secretion of GMFβ from adipocytes, and 2) the inflammation factors in the serum of a cohort of adolescents affected by varying severities of mood disorders through an NF-κB-RE adipocyte reporter bioassay. We found that GMFβ is produced and secreted by adipocytes and its expression is Aldh1a1-dependent. The role of GMFβ in adipose tissue and its function on other tissues is unknown. Since GMFβ has neurotrophic functions in the brain and spinal cord, future studies can investigate the process of innervation in adipose tissue in the context of GMFβ.

We also found that NF-κB activation in adipocytes and MCP-1 levels in serum increased in variability as severity of mood disorder increased. There was a range of mood disorders represented in the cohort of patients investigated in this study, which may have skewed results. In future studies a more homogenous representation of a mood disorder should be studied to elucidate possibly clearer associations between inflammatory factors and the disease.
The significance of these findings in light of my career aspirations is highly relevant. My desire to be a physician has been a driving factor in my pursuit of education. Having done this research bolsters my skills and ability to practice medicine effectively. Not only did I have the chance to learn about molecular biology on a micro scale with my cell culture of adipocytes, but I was able to learn about clinical applications of biology on a macro scale through investigation of mood disorder patients as well. Together, these experiences will add to my repertoire of knowledge that will help me move healthcare forward in the future.
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