THE EFFECT OF HIGH FRUCTOSE INTAKES IN THE RAT DIET ON SERUM GHRELIN AND RECEPTOR EXPRESSION IN THE ARCUATE NUCLEUS

by

JESSICA LYNN BURGE

(Under the Direction of Silvia Giraudo)

ABSTRACT

This study investigated the effect of high fructose intakes in the rat diet on serum glucose, insulin, triglycerides, and ghrelin over the course of four weeks. We also examined expression of the ghrelin receptor, GHSR-1α, in the rat arcuate nucleus. No significant differences were found in body weight, food intake, serum triglycerides, or ghrelin during the four week period. Serum glucose was consistently higher in the fructose-fed group opposed to the dextrose group and the difference was significant at week 1 (p=.047), week 2 (p=.009), and week 4 (p=.009) measures. Serum insulin levels were also consistently greater in the fructose-fed group, but were only significant on the week 2 measurement (p=.0515, one way ANOVA) indicating the development of insulin resistance within a week of fructose feeding. Ghrelin receptor expression in the arcuate nucleus was significantly greater in rats fed a dextrose diet, opposed to the fructose-fed group (p=.0262). This may indicate an alteration in ghrelin receptor expression with high fructose intakes.

INDEX WORDS: ghrelin, fructose, ghrelin receptor, GHSR-1α, receptor expression.

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DEDICATION

This thesis would be incomplete without a mention of the support, guidance, and love provided by my parents, for whom this thesis is dedicated.

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CHAPTER 1

INTRODUCTION

Within the past 30 years, the obesity epidemic has emerged as one of the most widespread health issues in the United States. Research indicates that approximately 66% of adults in the U.S are either overweight or obese and approximately 17% of children and adolescents are termed overweight [1, 2]. The average American lifestyle, which lacks adequate physical activity and exceeds caloric needs, has augmented this predicament. As a consequence, the prevalence of metabolic disorders such as atherogenic dyslipidemia, insulin resistance, hyperinsulinemia, glucose intolerance, and hypertension has increased [1]. The American diet has been scrutinized as a contributing factor to this health crisis, particularly total carbohydrate intake and sugar consumption. Both total fructose and high fructose corn syrup consumption has increased in conjunction with the rise in obesity[3].

The etiology of the obesity epidemic is both complex and multi-factorial, but more research is needed to determine the mechanisms that are involved in the development of this condition. The role of fructose in the obesity epidemic and the metabolic effects that result from fructose consumption has been a growing area of research. Changes in plasma glucose, insulin, and triglycerides are among the most common effects reported in these studies. It should be noted that the metabolism of glucose and fructose differ markedly. Of particular importance is the fact that the pathway used by fructose to generate lipid synthesis substrates is considerably easier and less regulated than that of glucose [4]. Acute doses of fructose do not stimulate insulin secretion [5], which may be a potential mechanism for weight gain with fructose consumption [6] since insulin is an important long-term regulator of food intake [7]. Several

studies find that long-term ingestion of fructose leads to glucose intolerance, insulin resistance, and elevated fasting plasma insulin concentrations [8] [9].

The impact of fructose on obesity can also be explored by examining the effect of fructose intakes on satiety signals and hormones, such as ghrelin. Ghrelin is a 28 amino acid endocrine peptide that has most recently become known as an orexigenic peptide [10]. The functional ghrelin receptor (GHSR-1α) is a G-protein coupled receptor that has been identified in a variety of locations throughout the brain, particularly the arcuate nucleus [11-13]. Energy status appears to be indicative of fasting serum ghrelin levels [14] and insulin has been implicated as a possible moderator of serum concentration [15]. However, it remains uncertain how factors, such as fructose intake, might influence serum ghrelin or receptor expression.

This thesis seeks to examine the effect of high fructose intakes in the rat diet on serum glucose, insulin, triglycerides, and ghrelin over the course of four weeks. In addition, expression of the ghrelin receptor (GHSR-1α) in the arcuate nucleus of the hypothalamus was quantified. This information could be a beneficial addition to what is already known about factors that regulate eating behaviors. As an alteration in serum ghrelin levels or receptor expression may implicate high fructose intakes with decreased satiety and perhaps subsequent overeating.

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CHAPTER 2

LITERATURE REVIEW

Within the past 30 years, the obesity epidemic has emerged as one of the most widespread health issues in the United States. The National Health and Nutrition Examination Survey (NHANES) estimate that approximately 66% of adults in the U.S are either overweight or obese. This survey also indicates that approximately 17% of children and adolescents, age 2-19, are categorized as overweight. These percentages are based on body mass index (kg/ $m^{2)}$, which is the most common tool for measuring body fat. This corresponds to a BMI of 25-29.9 for overweight individuals and ≥ 30 for obese [1-3]. The average American lifestyle, which lacks adequate physical activity and exceeds caloric needs, has augmented this predicament. As a consequence, the prevalence of metabolic disorders such as atherogenic dyslipidemia, insulin resistance, hyperinsulinemia, glucose intolerance, and hypertension has increased [3].

Fructose Consumption and Metabolism

The American diet has been scrutinized as a contributing factor to this health crisis, particularly total carbohydrate intake and sugar consumption. On average, Americans receive approximately 16% of their total energy intake from added sugars, and the adolescent age group (12-17) has the highest intake. Non-diet soft drinks are the greatest source, as roughly one third of added sugars are obtained from these beverages [4]. Sweet corn-based syrups, such as high fructose corn syrup (HFCS), now account for approximately 40% of all added caloric sweeteners and this popularity is largely due to their ease of production, transport, and profitability [5]. Fructose is present naturally in fruits and vegetables and found in free form in HFCS [6]. Both

total fructose and high fructose corn syrup consumption has increased in conjunction with the rise in obesity. HFCS was first introduced to the market in 1970 as a cheaper alternative to sucrose [7] and is now most commonly used in soft drinks in the place of sucrose. Since its introduction, HFCS consumption has increased from 0.8g/d in 1970 to 91.6g/d in 2000 [5], with total fructose consumption increasing by 26%. During this same time period HFCS as a percent total of caloric sweeteners has increased from 0.4% to 42%, while the use of sucrose in products has fallen dramatically[5] [8].

There are several types of high fructose corn syrup, but the formula that is most commonly found in products within the United States is called HFCS 55. It is made by an enzymatic isomerization of dextrose to fructose [9] [8]. This compound consists of monosaccharides and is 55% fructose, 42% glucose, and 3% other sugars. HFCS differs from the disaccharide sucrose, which is composed of 50% fructose and 50% glucose and is bound by a weak glycosidic bond. Absorption differs among monosaccharides and disaccharides[5] [10]. Sucrose is cleaved by disaccharidases in the intestine and a sodium-glucose cotransporter absorbs the resulting glucose. Fructose absorption occurs in the duodenum and jejunum via a non-sodium-dependent process. Post absorption, glucose and fructose are carried to the liver by way of portal circulation [11]. The majority of fructose is metabolized by the liver and the small remaining percentage enters general circulation where it is taken up by muscle and adipose tissue [11-13]. In small quantities fructose has been shown to be important to liver metabolism. Petersen et al. found a correlation between low-dose fructose infusions and enhanced hepatic glycogen synthesis, which appears to be mediated by increased stimulation of glycogen synthase [14] .

The metabolism of glucose and fructose also differ markedly. Glucose is taken up by a variety of GLUT transporters that differ according to the tissue, for example in fat and muscle glucose is taken up by insulin-dependent GLUT 4 transporters and in the liver by non-insulin dependent GLUT 2 transporters [15]. Once inside the cell, the glucose is phosphorylated by the glucokinase enzyme, forming glucose-6-phosphate. This molecule can later be converted to glycerol and used as a backbone for triacyglycerol synthesis, but is also negatively regulated by phosphofructokinase [8]. The phosphofructokinase enzyme is the rate-limiting step of glycolysis and is inhibited when ATP and citrate levels are high. Thus, inhibition results in decreased glucose uptake by cells, and substrates that can be used for glycogen synthesis and the pentose phosphate pathway [8]. Fructose is taken up by way of GLUT 5 transporters, which are non-insulin dependent. These transporters are not found in pancreatic B-cells or in the brain, so fructose can not stimulate insulin or participate in central satiety signaling. When fructose enters the cell it is phosphorylated to fructose-1-phosphate by fructokinase. This compound is easily cleaved by aldolase B to form the two trioses glyceraldehyde and dihydroxyacetone phosphate [8]. These compounds can then be converted to glyceraldehyde-3-phosphate and serve as a backbone for both phospholipids and triacylglycerol synthesis. Since the two triose phosphates by-pass the phophofructokinase step of glycolysis, there is no negative feedback in fructose metabolism. This means that the products of fructose metabolism, which are glucose, glycogen, lactate, and pyruvate, can continually enter the glycolytic pathway. It should also be noted that all the fructose products are able to form the glycerol and acyl portions of the acyl glycerol molecule. Thus, the pathway used by fructose to generate lipid synthesis substrates is considerably easier and less regulated than that of glucose [11].

Fructose and Metabolic Disorders

The role of fructose in the obesity epidemic and the metabolic effects that result from fructose consumption has been the focus of numerous studies. Changes in plasma insulin, glucose, and triglycerides are among the most common effects reported. Acute doses of fructose do not stimulate insulin secretion [16], which may be a potential mechanism for weight gain with fructose consumption [8] since insulin is an important long-term regulator of food intake [17]. Insulin plays an important role in food intake control both directly with the CNS and through leptin secretion [18]. The interaction with the CNS appears to be a fast response, whereas leptin stimulation may take many hours[17]. Thus, leptin is considered a long-term regulator of food intake [19]. In a study by Bruning et al, insulin receptor knockout mice displayed hyperphagia and obesity [20].

Many studies find that long-term ingestion of fructose leads to glucose intolerance, insulin resistance, and elevated fasting plasma insulin concentrations [21]. Thorburn et al. found decreased insulin sensitivity in rats fed 35% of energy as fructose over a 4-week time span. Three diets groups were formed consuming 69% carbohydrate (34.5% starch, 34.5% fructose or glucose) and compared with a high starch control [22]. In a 15 month study conducted by Blakely and colleagues, an increase in fasting serum insulin and glucose concentrations was observed in rats fed 15% of energy as fructose and compared with a corn starch control diet. No significant differences were found in food intake or body weight between the groups [21].

The effect of fructose intake on triglyceride levels has been examined extensively in both human and rodent studies. An increase in triglyceride levels in human males with hyperinsulinemia was observed in a 5 week study using 0%, 7.5%, and 15% energy from fructose [23]. Similarly, a 24 hour human study in women consuming 30% of energy as fructose

with 3 meals found increased postprandial and next day triglycerides [24]. Whereas, a 4-week study conducted on healthy males, a moderate fructose intake was found to increase fasting total triacylglycerol, VLDL-triacylglycerol, and glucose concentrations without causing insulin resistance [25]. Shapiro et al. conducted a long-term fructose feeding study (60% fructose) on rats and found significantly higher serum triglyceride levels in the fructose group opposed to the control group. No difference was found in serum cholesterol, fasting glucose, fasting serum insulin, body weight, total body fat, or food intake [26]. Smaller quantities of fructose have been found to elicit similar results. A 8 week study found that doses as low as 10% fructose in drinking water can induce symptoms of metabolic syndrome in rats, including hypertension, hyperuricemia, and hypertriglyceridemia [27].

Ghrelin and its receptors

The impact of fructose on obesity can also be explored by examining the effect on satiety signals and hormones, such as ghrelin. Ghrelin is a 28 amino acid endocrine peptide. It was first established as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), but has more recently become known as an orexigenic peptide [28]. Ghrelin is mostly synthesized in the stomach and gastrointestinal tract, but a small quantity is also produced in brain areas such as the hypothalamus and pituitary [29]. Two forms of ghrelin are found in the blood, one is acylated at serine 3, most commonly by the medium chain fatty acid n-octanoic, and the other des-acyl ghrelin [30]. The n-octanoylation is essential for ghrelin to function as a growth hormone ligand, although the orexigenic effects of ghrelin are independent of GH stimulation [30]. Plasma ghrelin levels fluctuate throughout the day, and are highest before meals and fall postprandially [31]. In a study investigating the 24-hour pattern of ghrelin in fasting humans, a spontaneous rise and fall of ghrelin levels were found during times of normal meals, suggesting a circadian

fluctuation of the hormone. No correlation was found with growth hormone, insulin, or blood glucose [32].

The functional ghrelin receptor (GHSR-1α) is a G-protein coupled receptor that has been identified in a variety of locations throughout the brain. Both mRNA [33, 34] and autoradiography [35] studies have confirmed receptor expression in the arcuate nucleus, paraventricular nucleus (PVN), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH) and lateral hypothalamic area (LHA). Receptors have also been identified in the perifornical area [36]. Wren et al. found the microinjection of ghrelin into the ARC to illicit the greatest food intake response, opposed to other hypothalamic sites [37]. The ARC contains neurons that synthesize neuropeptide Y (NPY), agouti-related protein (AgRP), Melanin-concentrating hormone (MCH) and orexins [38]. Ghrelin is thought to communicate with orexigenic neurons through the hypothalamic hormones NPY and AgRP, both of which are hypothalamic neuropeptides that stimulate food intake [39]. Animal studies have shown that following ghrelin administration, both NPY and AgRP mRNA expression is elevated in the arcuate nucleus. The orexigenic effect of ghrelin administration is sustained in NPY-knockout mice, which suggests some redundancy of action among the neuropeptides [40]. This is further supported by the fact that the orexigenic effect of peripherally administered ghrelin is eliminated in double NPY and AgRP knock-out mice [41]. Studies conducted by Solomon et al. highlighted the importance of the lateral hypothalamus (LH) and perifornical area regions (PFA) in ghrelin satiety signaling. Increased c-fos positive neurons in the LH and PFA were observed when an orexigenic state was induced using subcutaneous injections of insulin and 2-deoxyglucose. The orexigenic effects were later reversed using an anti-ghrelin antibody and a decrease in c-fos neuron activation was observed in the LH and PFA [36].

Ghrelin and Obesity

An inverse relation has been established between plasma ghrelin and body weight. Obese individuals exhibit relatively low levels, while high levels are found in persons with anorexia. This is thought to be reflective of the energy stores in these individuals and also correlates to the function of ghrelin, as an individual in negative energy balance would need higher ghrelin levels to stimulate food intake [42]. In rodent studies, exogenous ghrelin has been shown to both initiate feeding and when chronically administered cause weight gain [37, 43]. The increase in body weight appears to be mediated by an increase in adipogenesis, decreased energy expenditure, fat catabolism, and lipolysis [44]. All of which suggests ghrelin's role in body weight regulation and meal initiation [43]. Gastric distention does not appear to be essential for ghrelin regulation; Overduin et al. found that isolated nutrient infusions into the stomach, duodenum, and jejunum were all equally effective in suppressing ghrelin. This indicates that the mechanism for postprandial suppression is not exclusive to the stomach or duodenum. However, ghrelin release is altered in individuals who have undergone Roux-en-Y gastric bypass surgery, which may be a result of the removal of large amounts of ghrelin producing tissue [45]. All macronutrient types seem capable of suppressing ghrelin postprandially, however glucose and amino acids were found to have a more rapid and prolonged effect than lipids. Given ghrelin's role as an orexigenic peptide, a failure of lipids to effectively suppress ghrelin may implicate a high fat diet alone with weight gain [46].

Studies have shown that insulin is important for ghrelin suppression. However, the insulin action may not be essential postprandially, as was originally thought. Instead, nutrient sensing, ghrelin-producing cells are thought to be responsible for ghrelin suppression when nutrients are ingested that do not stimulate insulin, such as fat [47]. When ghrelin is

administered intravenously it has been found to stimulate insulin and gastrin secretion [48]. Ghrelin has also been found to stimulate gastric acid secretion in rats, which may be via stomach gastrin secretion [49]. Interestingly, exogenous administration of gastrin and insulin also appears to stimulate ghrelin [50]. In a study conducted by Saad et al., intravenous insulin infusions were found to significantly suppress ghrelin in human subjects by as much as 64% [51]. However, contrary to these findings, Caixas et al. found subcutaneous short acting insulin injections in human subjects to have no effect on ghrelin concentrations [52]. This contradiction could be due the route of insulin administration or type of insulin used, but nonetheless illustrates the complex nature of ghrelin regulation [48] [51].

In a study conducted by Teff et al, a significant decrease in both glucose and insulin levels, 66% and 65% respectively, were observed after a high fructose meal. Although no significant differences were found in fasting ghrelin levels, postprandial ghrelin suppression was found to be lower in the fructose-fed group. The fructose was delivered in the form of a beverage, was consumed with a meal, and compared with a high glucose beverage group. The diet consisted of 55% of kcal as carbohydrates, 30% kcal were either free fructose or free glucose and 25% complex carbohydrates. However, the study included only female subjects who were tested under each condition, with one administration of the diet each time. Thus, the experiment only represents short term data [53]. Melanson et al. compared the effects of HFCS and sucrose in beverages that constituted 30% kcals of subject's intake. The study concluded that in the context of HFCS, fructose does not alter fasting glucose, insulin, leptin, or ghrelin levels. Again this study only included female subjects and obtained samples after one exposure [18]. Akhaven and colleagues conducted a study that compared the metabolic effects of four different sugar solutions of varying glucose-to-fructose ratios; including HFCS, sucrose, and a 50:50

mixture of glucose and fructose (in monosaccharide form). The data showed no difference in insulin, blood glucose, and ghrelin between the HFCS, sucrose, and 50:50 mixtures [54]. The varied results from these studies indicate a need for further investigation of the effect of fructose intake on ghrelin concentration.

Several studies have been examined during the development of this thesis, and the findings of these studies serve as a basis for this project. The ability of fructose to induce insulin resistance in rats has been demonstrated repeatedly. In a study conducted by Zavaroni et al., rats were fed a diet of 66% fructose for 7 days. Plasma insulin levels were found to be significantly higher ($p<0.05$-0.01) in the fructose-fed group at all time points measured. Steady state plasma glucose levels, a parameter used to determine insulin's ability to stimulate glucose disposal, were also significantly greater ($p<0.001$) in the fructose-fed group. This implies that insulin action of the fructose-fed rats was partially inhibited [55]. A follow-up study to investigate the mechanism of the previously observed fructose induced insulin insensitivity was then conducted. The study found increased glucose efflux in perfused livers of fructose-fed rats and concluded that an alteration in hepatic carbohydrate metabolism was responsible for these effects. It was also speculated that insulin's decreased ability to suppress hepatic glucose output could be the result of altered function of enzymes critical to glycolysis [56]. Thus, fructose-fed rats are found to have decreased glucokinase [57] and increased glucose-6-phosphatase activity [58].

The length of this study was based upon research conducted by Thorburn and colleagues, where rats were fed a diet of 69% carbohydrate from which 34.5% of kcals were either glucose or fructose and 34.5% of kcal were starch. The study was conducted for 30 +/- 1 days. Insulin levels were assessed using a hyperinsulinemic-euglycemic clamp procedure and were shown to increase in both groups throughout the study. The glucose infusion rate, a parameter used to

determine insulin resistance, was significantly reduced in the fructose group by 7.2 mg kg $^{-1}$ min^{-1}($p<0.01$), in comparison to the glucose group when measured at day 30. Fructose-fed rats had higher triglyceride levels at week two and three in comparison to the glucose-fed group. Comparison studies were conducted to investigate the relationship between triglyceride levels and glucose infusion rates; it was determined that higher triglyceride levels were associated with a decreased ability of insulin to suppress hepatic glucose efflux [22]. A similar timeframe was used in a study conducted by Lee et al. For 30 days, rats were fed either a high fat, low protein, or control AIN-76A diet, and subsequent ghrelin mRNA expression and plasma levels were measured. Both mRNA and plasma ghrelin levels were significantly lower in the high fat diet group and significantly higher in the low protein diet group [48].

The importance of ghrelin's role in food intake and glucostatic signaling is highlighted in a study conducted by Solomon et al [36]. A hyperphagic model was induced in rats by subcutaneously injecting either insulin or 2-deoxyglucose. Insulin caused hypoglycemia, while 2-DG caused hyperglycemia. Immunohistochemistry procedures revealed an increase in c-fos positive neurons due to insulin and 2-DG, in both the lateral hypothalamus and perifornical area. Subsets of the insulin and 2-DG groups were then injected with an anti-ghrelin antibody. Administration of the anti-ghrelin antibody inhibited the orexigenic signal induced by insulin and 2-DG and decreased c-fos neuron activation in the LH and PFA [36].

Summary

Ghrelin, the endogenous ligand for the growth hormone secretagogue receptor, is a hormone known for its ability to stimulate food intake [28]. Energy status appears to be indicative of fasting serum ghrelin levels [42] and insulin has been implicated as a possible moderator of serum concentration [51]. However, it remains uncertain how factors, such as

fructose intake, might influence serum ghrelin concentration or receptor activation. If the data indicate that serum ghrelin and GHSR-1α expression is altered in rats consuming a diet high in fructose, this information would be a vital addition to what is already known about factors that regulate eating behaviors. An alteration in serum ghrelin levels may implicate high fructose intakes with decreased satiety and perhaps subsequent overeating.

Hypothesis

It is hypothesized that elevated fructose intakes in the rat diet will increase serum ghrelin secretion and subsequent ghrelin receptor expression in the hypothalamus.

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CHAPTER 3

THE EFFECT OF HIGH FRUCTOSE INTAKES IN THE RAT DIET ON SERUM GHRELIN AND RECEPTOR EXPRESSION IN THE ARCUATE NUCLEUS

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ABSTRACT

This study investigated the effect of high fructose intakes in the rat diet on serum glucose, insulin, triglycerides, and ghrelin over the course of four weeks. We also examined expression of the ghrelin receptor, GHSR-1α, in the rat arcuate nucleus. No significant differences were found in body weight, food intake, serum triglycerides, or ghrelin during the four week period. Serum glucose was consistently higher in the fructose-fed group opposed to the dextrose group and the difference was significant at week 1 ($p=.047$), week 2 ($p=.009$), and week 4 ($p=.009$) measures. Serum insulin levels were also consistently greater in the fructose-fed group, but were only significant on the week 2 measurement ($p=.0515$, one way ANOVA) indicating the development of insulin resistance within a week of fructose feeding. Ghrelin receptor expression in the arcuate nucleus was significantly greater in rats fed a dextrose diet, opposed to the fructose-fed group ($p=.0262$). This may indicate an alteration in ghrelin receptor expression with high fructose intakes.

INTRODUCTION

Ghrelin is a 28 amino acid endocrine peptide that was initially identified as an endogenous ligand for the growth hormone secretagogue receptor, but has more recently become known as an orexigenic peptide [1]. Ghrelin is primarily synthesized in the stomach and gastrointestinal tract, but a small quantity is also produced in brain areas such as the hypothalamus and pituitary [2]. Two forms of ghrelin can found circulating in the blood, but only the octanoylated form is considered to be active and therefore able communicate centrally with its receptor [3]. The functional ghrelin receptor (GHSR-1α) is a G-protein coupled receptor that has been identified in a variety of locations throughout the brain including the arcuate nucleus, paraventricular nucleus, ventromedial hypothalamic nucleus, dorsomedial hypothalamus nucleus, and lateral hypothalamic area [4-6]. Receptors have also been identified in the perifornical area [7]. Interestingly, the microinjection of ghrelin into the ARC has been found to illicit the greatest food intake response, opposed to other hypothalamic sites [8]. The ARC contains neurons that synthesize neuropeptide Y (NPY), agouti-related protein (AgRP), Melanin-concentrating hormone (MCH) and orexins [9]. Furthermore, ghrelin is thought to communicate with orexigenic neurons through the hypothalamic hormones NPY and AgRP, both of which are hypothalamic neuropeptides that stimulate food intake [10].

An inverse relation has been established between plasma ghrelin and body weight. Obese individuals exhibit relatively low levels, while high levels are found in persons with anorexia[11]. Exogenous ghrelin has been shown to both initiate feeding and when chronically administered cause weight gain [8, 12]. The increase in body weight appears to be mediated by an increase in adipogenesis, decreased energy expenditure, fat catabolism, and lipolysis [13]. All of which supports ghrelin's role in body weight regulation and meal initiation [12]. Energy status appears

to be indicative of fasting serum ghrelin levels [11] and insulin has been implicated as a possible moderator of serum concentration [14].

The prevalence of obesity in the United States and worldwide has increased the demand for research investigating factors that effect food intake and energy balance. As a result, there has been immense interest in ghrelin since its discovery as the first circulating hunger hormone. However, much remains unknown about ghrelin, particularly what factors regulate secretion and suppression of the hormone. In addition, the role of fructose in the obesity epidemic and the metabolic effects that result from fructose consumption is also a growing area of research. This study seeks to examine the effect of high fructose intakes in the rat diet on serum glucose, insulin, triglycerides, and ghrelin over the course of four weeks. In addition, expression of the ghrelin receptor (GHSR-1α) in the arcuate nucleus of the hypothalamus was quantified. This information could be a beneficial addition to what is already known about factors that regulate eating behaviors. As an alteration in serum ghrelin levels may implicate high fructose intakes with decreased satiety and perhaps subsequent overeating.

METHODS

Animals

Twenty-four Male Sprague Dawley rats weighing 225-240g were obtained from Harlan (Prattville, AL) and were acclimated to housing (21-22°C with lights on 12h/day from 7:00 a.m. to 7:00 p.m.) and diet, with free access to food and water. The animals were housed individually in clear plastic shoebox cages attached to the BioDAQ Food Intake Monitoring System (Research Diets, New Brunswick NJ). The animals were divided into two groups of 12 animals each, and given free access to their assigned diet. The dextrose group (control) received a diet consisting of 21% kcal protein, 12% kcal fat, 68% kcal carbohydrate: 17% kcal corn starch and

51% kcal dextrose (Research Diets, New Brunswick NJ, #D08082501). The second group (treatment) received a diet containing: 21% kcal protein, 12% kcal fat, 68% kcal carbohydrate: 17% kcal corn starch, 40% fructose, and 10% kcal dextrose (Research Diets, New Brunswick NJ, #D08082502). The calorie content of the two diets were identical (3.90 kcal/g). The animals were given one week to acclimate to the diet before the experiment began and food intake was recorded. Guidelines for animal procedures are in accordance with the University of Georgia Institutional Animal Care and Use Committee.

Design

Rats were raised on regular rodent chow diet by the breeder until approximately 2 months of age. Upon arrival, 12 rats were switched to a dextrose control diet and 12 rats to a fructose treatment diet. The experiment was run in 2 cohorts, due to a limited number of cages in the feeding system. During the experiment the animals were fed ad libitum and daily food intakes were recorded using the BioDAQ Food Intake Monitoring System. The animals were weighed twice a week at 11 a.m. The evening prior to blood draws, animals were fasted for approximately 15 hours by closing the access gate to the food hopper. Blood was collected using the tailbleed method on day 1, day 7, day 14, and day 21. Animals were perfused over the course of 2 days, either at the end of day 24 or 25, and were fasted approximately 15 hours prior to euthanasia. The evening prior to perfusion at 6:30 p.m., food hopper gates were closed and final body weights were recorded. On the following day at 9 a.m., in groups of 2 animals, one from each diet group, the animals were euthanized with Buthenasia® (100mg/kg body wt ip). The animal was perfused via the aorta with 100 ml saline, followed by 500ml ice-cold 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and placed in 4% paraformaldehyde with

sucrose solution for 2 hour postfix, then transferred to 30% sucrose in PBS solution for 48 hours. Samples were stored at -80°C until analysis.

Blood Sampling

Animals were fasted approximately 15 hours prior to each collection. Blood samples were taken at day 1, 7, 14, and 21 using the tail bleed method. Samples were centrifuged for 20 min 1500G at 4ºC (Sorvall RC5C Plus). Samples were divided into microcentrifuge tubes and stored at -20 ºC until analysis. All samples were run in duplicate. Serum glucose was measured by the glucose oxidase method with reagents from Pointe Scientific, INC (Canton, MI). Serum triglycerides were measured by the glycerol phosphate oxidase method with reagents from Pointe Scientific, INC (Canton, MI). Serum ghrelin samples were analyzed by radioimmunoassay with a kit from Linco Research, Inc (GHRT-89HK, St. Charles MO). Serum insulin levels were analyzed by radioimmunoassay with a kit from Linco Research Inc, (RI-13K, St. Charles MO)

Immunohistochemistry

The following immunostaining procedures were used to determine the effect of the diets on ghrelin receptor expression in the arcuate nucleus of the hypothalamus. The brains were cut coronally using a cryostat (Jung FrigoCut 2800E) in 30 μm sections. The Rat Brain Atlas (Paxinos & Watson 4^{th} edition) was used to locate the arcuate nucleus (bregma -2.12 to -4.52mm). Free-floating sections were rinsed with PBS solution and incubated in 30% H_2O_2 for 60 minutes. Sections were then incubated for 2 hours in 4% normal rabbit serum, and afterwards were incubated in GHSR-1α antibody at 4ºC for 72 hours (goat polyclonal anti-GHSR-1 α antibody (Calbiochem) diluted 1:500 in 1% normal rabbit serum). Seventy-two hours later the sections were rinsed with PBS with triton X-100 3x20min and incubated in secondary antibody diluted 1:500 in (biotinylated rabbit anti-goat) for 1 hour. The sections were then rinsed with

PBS with triton X-100 3x20min and incubated in Avidin/Biotin complex for 1 hour (Vector Labs). Sections were then rinsed with PBS with triton X-100 once and subsequently rinsed twice with PBS without triton X-100. Sections were then reacted with 3, 3'-diaminobenzidine tetrahydrochloride solution (DAB) 1-2 minutes. Sections were mounted on gelatin coated slides and dried at room temperature overnight. Sections were dehydrated in ethanol, soaked in xylene, and coversliped using permount mounting medium (Fischer Scientific). The values for individual rats were averaged to determine the mean number of GHSR-1α- positive cells per section for each treatment group. The immunohistochemically-characterized neurons of the arcuate nucleus were quantified using Q Capture Suite (Quantitative Imaging Corp, Surrey BC) and Nikon Eclipse E400 microscope (Nikon Instruments, Melville NY).

Statistical Analysis

Statistical analysis were performed with programs of SPSS, Inc. 16.0 (Chicago, IL). For body weight, food intake, serum glucose, insulin, and ghrelin within-group comparisons were performed with repeated measures ANOVA. When the main effect for time was significant a Bonferroni post-hoc test was applied to determine individual differences between means. When the between subjects effect was significant, a one-way ANOVA was conducted to detect differences. Serum triglycerides and ghrelin receptor expression were analyzed using a one-way ANOVA. A value of $P < .05$ was considered statistically significant. Results are presented as mean ± SEM.

RESULTS

Body weight and food intake. At approximately 2 months of age and weight range 225g-236g the animals were divided into two groups and provided either a dextrose or fructose diet. Rats in both diet groups steadily gained weight at similar rates over the next 4 weeks (Table 3.1 and

Figure 3.1). The dextrose group had slightly higher body weights initially, though not significant in comparison to the fructose group. By the final week there was no significant difference between the groups. The average final body weight of the dextrose group was 330.96 ± 5.39g and 330.33 ± 6.06g for animals in the fructose-fed group. Food intake was also similar in both groups for all weeks. The cumulative food intake for the 4 week duration of the study for the dextrose group was 413.2 ± 9.35, compared to 418.3 ± 11.20 in the fructose-fed group (Table 3.1 and Figure 3.2). It is important to note that the week 1 and 4 total food intake quantities are smaller than week 2 and 3, because the time period in which the quantities measured were shorter (5-6 days in week 1 and 4, opposed to 7 days in week 2 and 3) Overall, the dextrose and fructose fed rats had no significant differences in body weight or food intake over the four-week period.

Serum glucose, insulin, triglycerides, and ghrelin. All blood parameters were taken after an overnight fast of approximately 15 hours using the tail bleed method. Serum glucose levels in both the dextrose and fructose-fed groups steadily increased over the course of the 4 weeks, and the change over time was found to be significant (p=.000, repeated measures ANOVA). Serum glucose was consistently higher in the fructose-fed group opposed to the dextrose group. The treatment effect was significant (p=.003, repeated measures ANOVA) and the difference between the two groups was significant at week 1 (p=.047, one-way ANOVA), week 2 (p=.009, one-way ANOVA), and week 4 (p=.009, one-way ANOVA) measures (Table 3.2 and Figure 3.3). The change over time was found to be significant for serum insulin levels (p=.005, repeated measure ANOVA) and the treatment effect was significant (p=.014, repeated measures ANOVA). Serum insulin levels were also consistently greater in the fructose-fed group. The treatment effect was significant (p=.014, repeated measures ANOVA) but the difference between groups

were only significant on the week 2 measurement (p=.0515, one way ANOVA) indicating the development of insulin resistance within 2 weeks of fructose feeding (Table 3.2 and Figure 3.4). It is important to note that a trend in insulin values could be seen, although these values do not meet the criteria for significance (week 1 p=.0976, week 3 p=.1554, week 4 p=.0666). Although many studies have found increased serum triglyceride levels with ingestion of a high fructose diet, this study found no significant differences in triglyceride levels between the two diet groups (p=.3511) (Table 3.2). Serum ghrelin levels in both the dextrose and fructose-fed groups steadily increased over the course of the 4 weeks, and the change over time was found to be significant (p=.004, repeated measures ANOVA). Ghrelin levels at the week 1 measure for the dextrose group were 2862.84 ± 276.24pg/ml and 2932.47 ± 236.18pg/ml for the fructose-fed group, compared to levels at week 4 4964.84 ± 1,059.35pg/ml for the dextrose group and 4058.68 ± 311.31pg/ml for the fructose-fed group (Table 3.3). However, the values were similar between the two groups on each week and no significant differences were detected between the groups.

Ghrelin receptor expression in the arcuate nucleus

The number of cells expressing the ghrelin receptor, GHSR-1α, in the arcuate nucleus was significantly greater in rats fed a dextrose diet, opposed to the rats fed a fructose diet (Table 3.4 and Figure 3.5 & 3.6). The average number of cells positive for the receptor, per section, in the dextrose group was 101.16 ± 9.29, opposed to 72.03 ± 7.68 in the fructose group (p=.0262).

DISCUSSION

The primary findings of this study are that high fructose intakes in the rat diet significantly increase both fasting concentrations of serum glucose and insulin, opposed to rats fed a high dextrose diet. Blood glucose was significantly greater at weeks 1, 2, and 4, while serum insulin was consistently greater in the fructose-fed group, but was only significantly

greater at week 2. It is important to note that a significant trend in insulin values could be seen, although these values did not meet the criteria for significance (week 1 p=.0976, week 3 p=.1554, week 4 p=.0666). The effect of fructose intake on serum glucose and insulin varies dramatically with acute and chronic doses. It has been established that acute doses of fructose cause smaller increases in glucose and insulin, opposed to glucose feeding [15]. This study is among many to report glucose intolerance and insulin resistance with chronic fructose feeding [16-18].

A variety of mechanisms have been proposed for the development of insulin resistance, many of which involve elevations in serum cholesterol, triglycerides, and free fatty acids[19-21]. Insulin resistance has been correlated with an increase in nonesterified fatty acid concentrations [19], that may alter carbohydrate metabolism by increasing hepatic glucose production [22, 23], and/or damage B-cell function [24]. An increase in nonesterified fatty acid concentration has also been correlated with an increase in triglycerides levels, particularly VLDL concentration [21]. However, this study did not find significant differences in serum triglyceride levels at the week 4 measure and technical complications prevented the measurement of triglyceride concentrations at all time points. Furthermore, since this study measured total triglyceride levels and not specific fatty acids or lipoproteins, it is unknown whether a difference existed in these parameters between the two diet groups. A meta-analysis conducted by Hollenbeck concluded that studies investigating the effect of fructose intakes at approximately 20% of total energy were much more consistent concerning LDL concentrations, opposed to effects on triacylglyerol concentrations. Finally, may have been difficult to detect significant differences in triglyceride levels without a standard chow diet group as a comparison [25].

It is well known that adipose tissue accounts for only a small percentage of glucose

disposal, in comparison to other tissues [26] [27]. In a study investigating the mechanism of insulin resistance in fructose-fed rats, it was determined that the liver, opposed to adipose tissue or skeletal muscle, is the primary organ involved in the "decline of insulin-induced uptake of glucose". The study noted significantly greater outflow of glucose from livers of the fructose fed animals and attributed the cause of insulin resistance in these animals to a failure of insulin to adequately suppress hepatic glucose output. This effect is likely due to regulatory changes in carbohydrate metabolism including glycogenesis, glycogenolysis, and/or gluconeogensis [28]. Animals deprived of dietary sources of glucose have been shown to have increased levels of glucose-6-phosphatase, which could correspond to increased gluconeogenesis [29, 30]. Other enzymes noted to have increased activity with high fructose feeding include: phosphor-gluconate dehydrogenase, adolase, fructokinase, and malate dehydrogenase [30].

No significant changes were reported in body weight or food intake over the course of four weeks. The effect of high fructose feeding on food intake varies greatly depending on acute or chronic feeding. Acute doses are more likely to result in increased food intakes, possibly due to smaller rises in glucose and insulin[31]. However, numerous studies that have compared a high fructose diet and a fructose-free counterpart found no significant difference in body weight [32-34] or food intake [35] with chronic feeding. Thus, the findings of this study are consistent with existing research regarding body weight and food intake.

In addition, this study found no significant differences in fasting serum ghrelin levels. In a human study conducted by Teff et al., no difference was found in fasting plasma ghrelin levels between high glucose and high fructose beverage groups. However, it was noted that postprandial ghrelin suppression was significantly lower in the fructose-fed group [36]. It appears that postprandial ghrelin suppression varies according to the macronutrient composition

of a diet, as both human and rodent studies have shown greater suppression with high carbohydrate meals, compared to high fat meals [37] [38]. Furthermore, a diminished postprandial ghrelin suppression has been correlated with a higher hunger rating in hyperlipidemic meal groups [37]. These findings are particularly interesting, since no significant differences in fasting serum ghrelin levels were detected between the two diet groups in this study. However, the effect of these diet groups on postprandial ghrelin suppression was not investigated and thus is unknown. It is also important to note that total ghrelin levels were measured in this study, instead of the active form of ghrelin. The octanoylation of ghrelin is required for activation and subsequent binding of the molecule to the GHSR-1α receptor [3].

Finally, this study also found significant changes in the number of cells expressing the ghrelin receptor in the arcuate nucleus of the rat brain, with high fructose intakes. Ghrelin receptor expression in animals fed a high fructose diet was significantly lower than their dextrose-fed counterparts ($p=.0262$). Although fasting serum ghrelin levels were not significantly different, it is possible that an alteration in postprandial ghrelin suppression may have led to the alteration of GHSR-1α expression. Attenuated postprandial ghrelin suppression, leading to a prolonged elevation of serum ghrelin levels, may have caused a down regulation of the ghrelin receptor.

In summary, the metabolic effect of high fructose intakes in the rat diet and subsequent influence on GHSR-1α receptor expression is a complex process. A high fructose diet does induce glucose intolerance and insulin resistance in the absence of significantly elevated fasting serum triglyceride levels. Furthermore, no difference was found in fasting serum ghrelin levels, despite significant alterations in cells expressing the ghrelin receptor in the arcuate nucleus.

CONCLUSION

The high fructose diet used in this study was found to have no effect on body weight, food intake, serum triglycerides, or serum ghrelin levels. However, the high fructose diet was found to increase fasting glucose levels, as well as insulin. The number of cells expressing the ghrelin receptor GHSR-1α in the arcuate nucleus was significantly greater in the dextrose fed group, which could be due to a failure of fructose to adequately suppress ghrelin postprandially. Further research is still needed to determine the mechanism involved in the alteration of ghrelin receptor expression with large intakes of fructose.

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Table 3.1: Effects of a fructose or dextrose diet on body weight and food intake

Time/Diet	Body Weight (g)	Food Intake (g)	
		Average Weekly Intake	Average Daily Intake
Week 1			
Dextrose	262.14 ± 1.91	82.59 ± 1.74	16.52
Fructose	255.88 ± 3.28	82.44 ± 1.61	16.49
Week 2			
Dextrose	312.12 ± 7.65	121.20 ± 3.03	17.31
Fructose	297.74 ± 4.10	122.57 ± 3.26	17.51
Week 3			
Dextrose	321.73 ± 5.02	120.19 ± 3.16	17.17
Fructose	319.50 ± 5.21	122.91 ± 3.89	17.56
Week 4			
Dextrose	330.96 ± 5.39	89.25 ± 3.59	16.23
Fructose	330.33 ± 6.06	91.81 ± 3.57	16.69
Total			
Dextrose		413.2 ± 9.35	
Fructose		418.3 ± 11.20	

Data are means ± SEM. No significant differences were found between the two groups.

Table 3.2: Effects of a dextrose or fructose diet on serum glucose, insulin, and triglycerides

Time/Diet	Glucose mg/dl	Insulin ng/ml	Triglycerides mg/dl
Week 1			
Dextrose	104.14 ± 2.96	.144 ± .036	
Fructose	115.03 ± 4.23*	.303 ± .0841	
Week 2			
Dextrose	111.31 ± 2.40	.107 ± .025	
Fructose	123.23 ± 3.40*	.216 ± .047*	
Week 3			
Dextrose	130.56 ± 4.29	.224 ± .0393	
Fructose	139.75 ± 4.25	.323 ± .055	
Week 4			
Dextrose	120.43 ± 3.81	.260 ± .0406	59.14 ± 4.23
Fructose	142.86 ± 6.83*	.451 ± .090	53.99 ± 3.37

Data are means ± SEM. The asterisk indicates a significant difference between the two groups (glucose week 1 p=.047, week 2 p=.009, week 4 p=.009, insulin week 2 p=.0515).

Table 3.3: Effects of a dextrose or fructose diet on serum ghrelin levels

Serum Ghrelin pg/ml		
Time	**Dextrose**	**Fructose**
Week 1	2862.84 ± 276.24	2932.47 ± 236.18
Week 2	3585.44 ± 413.16	3870.47 ± 417.31
Week 3	4589.56 ± 575.41	4542.13 ± 225.15
Week 4	4964.84 ± 1,059.35	4058.68 ± 311.31

Data are means ± SEM. No significant differences were found between the two groups.

Table 3.4: Effects of a dextrose or fructose diet on ghrelin receptor (GHSR-1α) expression in the arcuate nucleus.

Average Number of GHSR-1α Positive Neurons Per Section	
Dextrose	101.16 ± 9.29
Fructose	72.03 ± 7.68

Data are average number of GHSR-1α positive neurons per section ± SEM p=.0262.

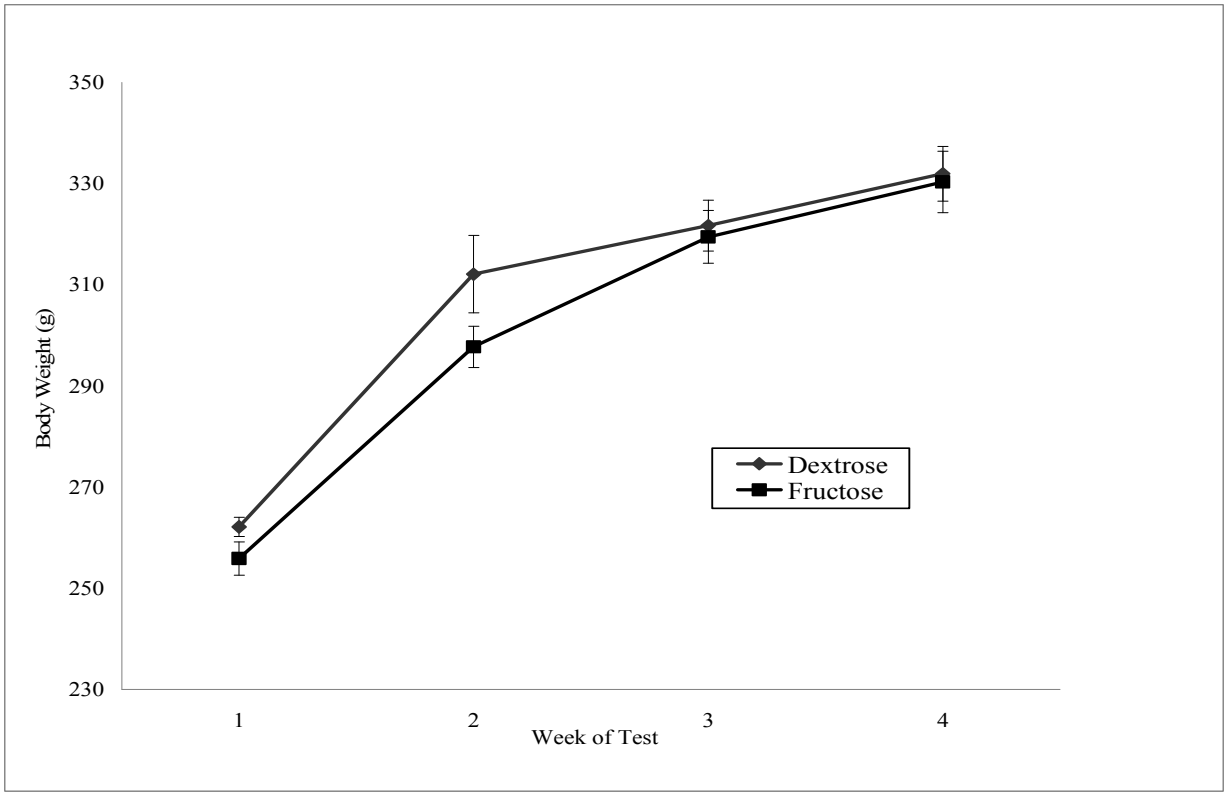

Figure 3.1: Effects of a fructose or dextrose diet on body weight over a four week period. Data are means ± SEM. No significant differences were found between the two groups.

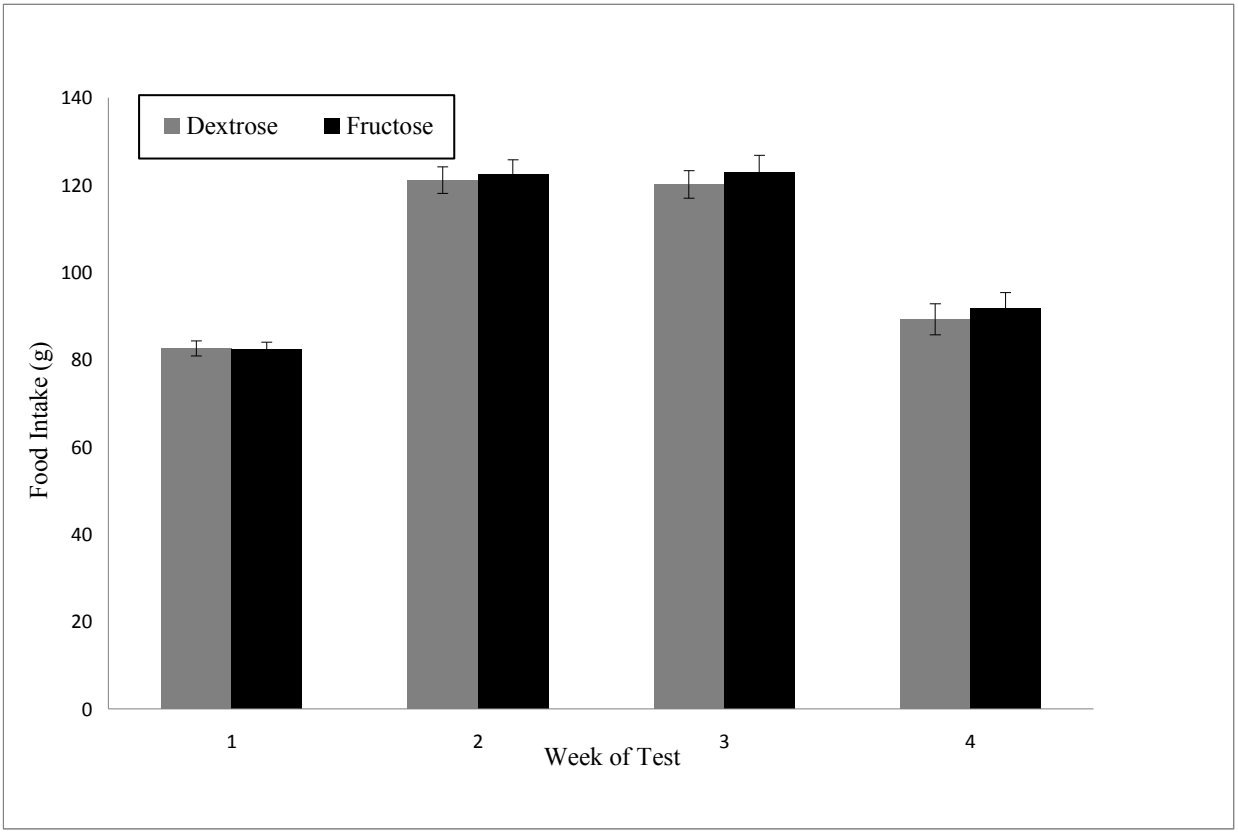

Figure 3.2: Effects of a fructose or dextrose diet on food intake.

Data are means ± SEM. No significant differences were found between the two groups.

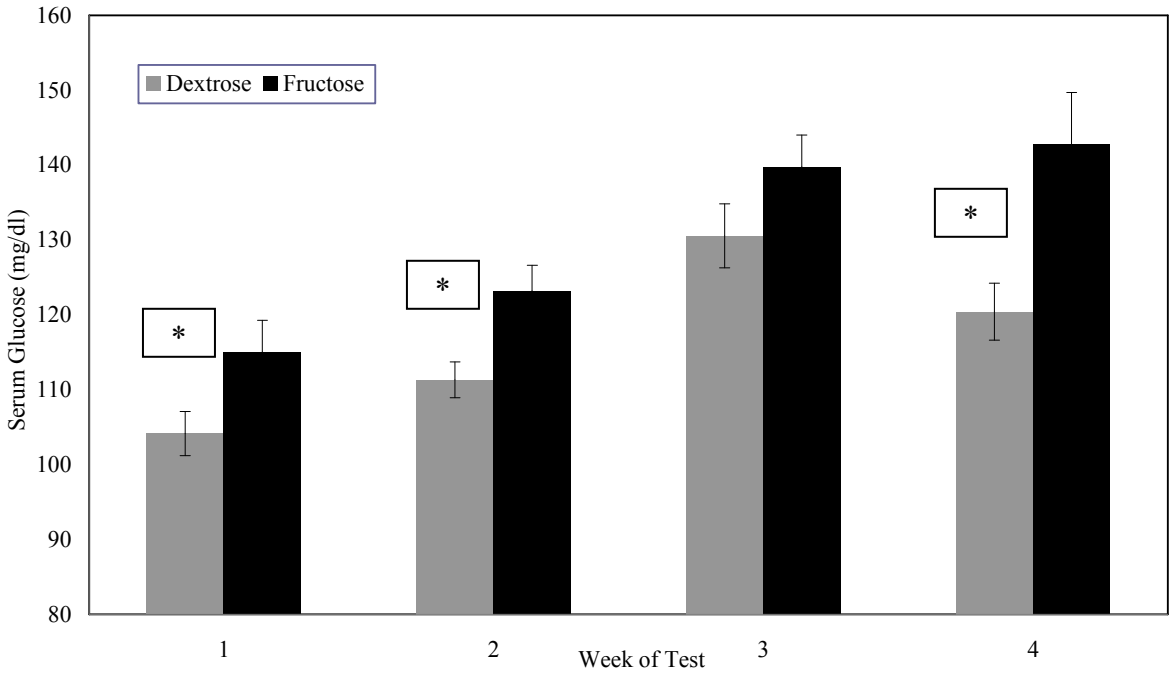

Figure 3.3: Effects of a dextrose or fructose diet on serum glucose.

Data are means ± SEM. The asterisk indicates a significant difference between the two groups (week 1 p=.047, week 2 p=.009, week 4 p=.009).

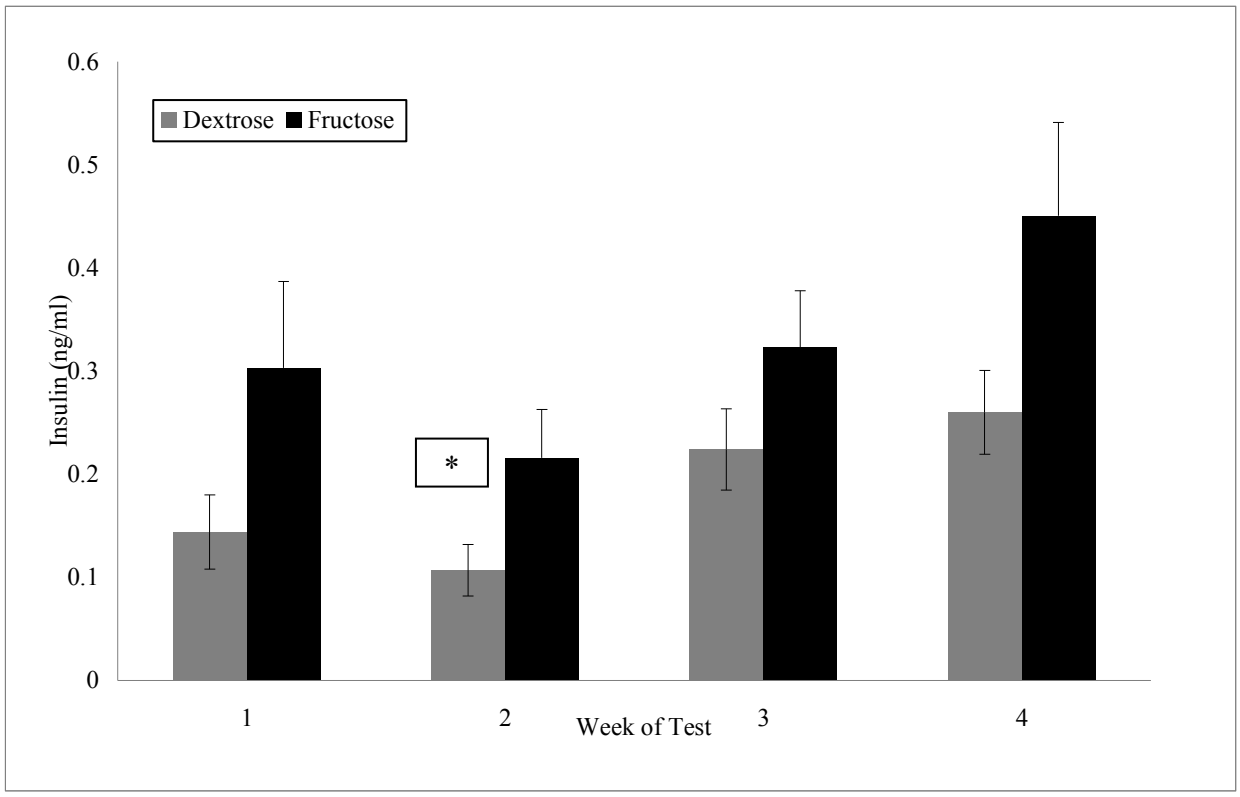

Figure 3.4: Effects of a dextrose or fructose diet on serum insulin.

Data are means ± SEM. The asterisk indicates a significant difference between the two groups (week 2 p=.0515).

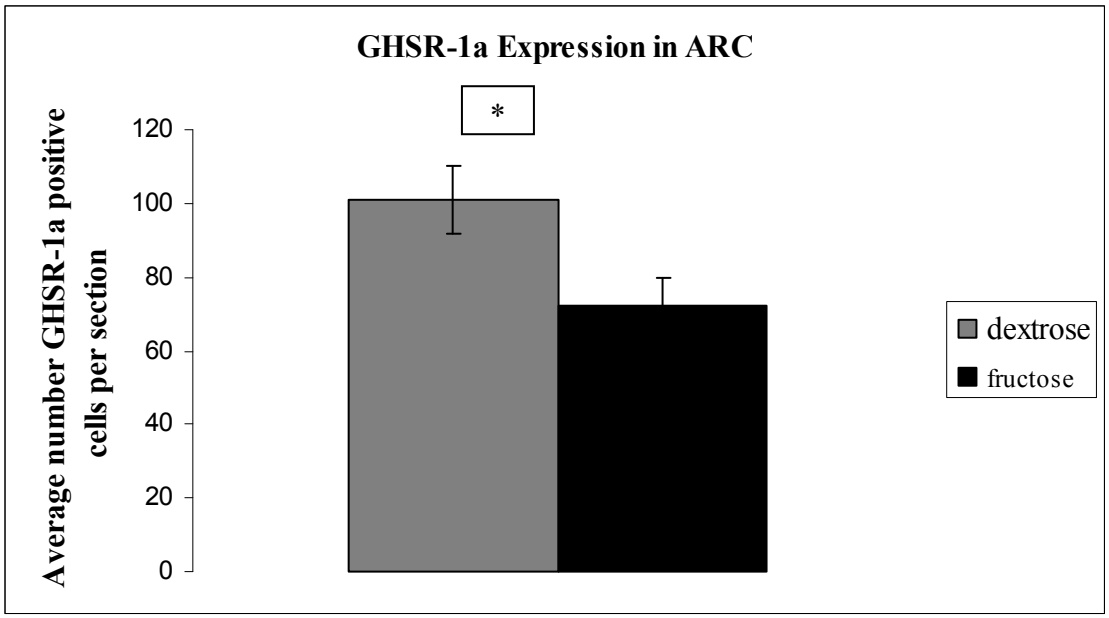

Figure 3.5: Effects of a dextrose or fructose diet on cells expressing the ghrelin receptor (GHSR-1α) in the arcuate nucleus.

Data are average number of GHSR-1α positive cells per section ± SEM, p=.0262.

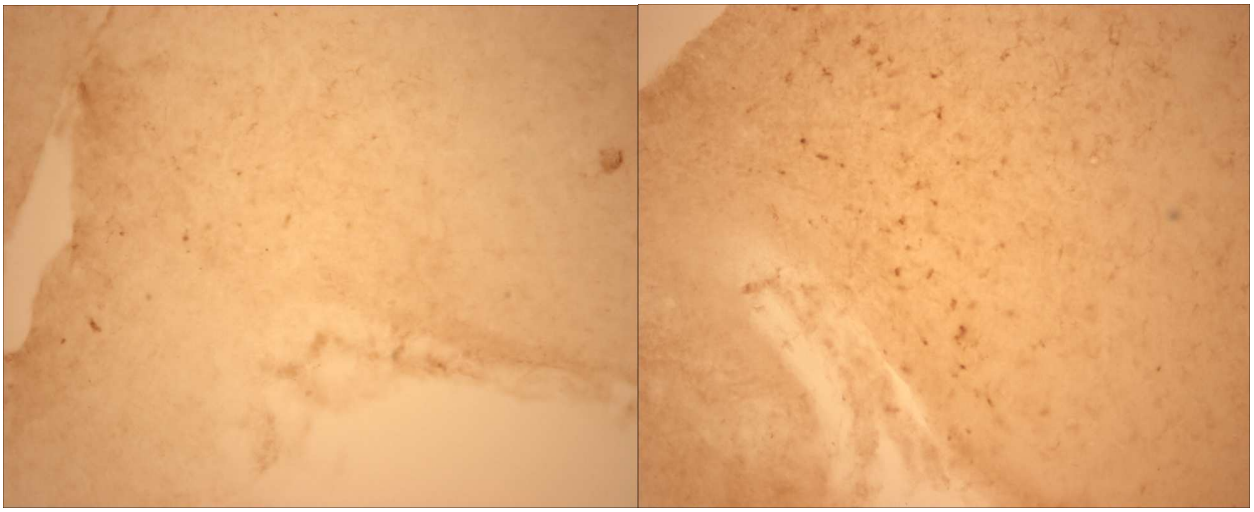

Figure 3.6: Expression of GHSR-1α positive cells in the arcuate nucleus of fructose-fed and dextrose-fed rats.

Immunohistochemistry demonstrating expression of cells positive for GHSR-1a in fructose-fed (left) and dextrose-fed (right) rats at 20X.

CHAPTER 4

CONCLUSION

This thesis illustrates the complexity of ghrelin regulation in both the periphery and the brain. This study investigated the effect of high fructose intakes in the rat diet, in comparison to a high dextrose diet, on serum glucose, insulin, triglycerides, and ghrelin. The high fructose diet increased both fasting glucose and insulin levels. However, no significant differences were found in food intake, body weight, serum triglycerides, or ghrelin. The number of cells expressing the ghrelin receptor GHSR-1α was found to be significantly greater in the dextrose diet group.

The purpose of this study was to determine the effects of a high fructose diet on both serum ghrelin and ghrelin receptor expression in the brain. Circulating blood levels of ghrelin fluctuate throughout the course of the day, as they are highest before meals when in the fasted state and fall postprandially [1]. Although this study did not find any significant differences in fasting serum ghrelin levels, it is possible that postprandial ghrelin suppression was altered with high fructose intakes. In a human study conducted by Teff et al., no difference was found in fasting plasma ghrelin levels between high glucose and high fructose beverage groups. However, it was noted that postprandial ghrelin suppression was significantly lower in the fructose fed group [2].

While many of the factors that influence ghrelin regulation remain unknown, insulin has been suggested as a potential modulator or the hormone. In a study conducted by Saad et al., intravenous insulin infusions were found to significantly suppress ghrelin in human subjects by as much as 64% [3]. This study is among many to report glucose intolerance and insulin

resistance with chronic fructose feeding [4-6]. A variety of mechanisms have been proposed for the development of insulin resistance, many of which include elevations in serum cholesterol, triglycerides, and free fatty acids [7-9]. However, since no significant differences were detected in serum triglyceride levels in this study it is unlikely that an alteration in free fatty acid concentrations induced insulin resistance. Long-term fructose feedings have been shown to alter hepatic enzymes involved in carbohydrate metabolism. Thus, it is more likely that changes in hepatic carbohydrate metabolism that resulted in greater hepatic glucose efflux and a failure of insulin to adequately suppress hepatic glucose output are responsible for the insulin resistance observed in the fructose-fed rats.

Future studies should focus on determining the effect of high fructose intakes on postprandial ghrelin suppression as this was not investigated here and has not been determined. In addition, it is important to determine the factors that regulate and alter ghrelin receptor expression.

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