2012

Light-Limited Access to Fructose Alters Metabolic Function and Adipose Tissue Catecholaminergic Activity in Mice

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Light-Limited Access to Fructose Alters Metabolic Function and Adipose Tissue Catecholaminergic Activity in Mice

A thesis submitted in partial fulfillment
of the requirements for the degree
of Master of Science

By

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B.Pharm., Alfateh University, Tripoli, Libya 2004

2012
Wright State University
WRIGHT STATE UNIVERSITY
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Date: June, 11 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION
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And Adipose Tissue Catecholaminergic Activity In Mice” BE ACCEPTED IN PARTIAL

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Consumption of high levels of fructose produces glucose intolerance, sympathetic nervous system activation, and renal dysfunction. A diet high in fructose may play a role in the worldwide epidemic of obesity and diabetes. The aim of this study was to test the hypothesis that time (light/dark) limited access to fructose influences body fat, metabolic function, and adipose tissue catecholaminergic activity in mice. Male C57BL/6 mice were given standard chow and assigned to one of three groups: Control (n=10, water 24h); FL (n=11, 10% fructose solution during 12h light period); and FD (n=10, 10% fructose solution during 12h dark period). Metabolic parameters measured were body fat (BF, Echo-MRI), adipocyte cell size, and glucose tolerance as well as plasma glucose, adiponectin, insulin, leptin, triglycerides, and cholesterol. Catecholamine levels were measured directly in white adipose tissue (WAT) using high performance liquid chromatography (HPLC) with electrochemical detection. Immunocytochemistry was used for examining tyrosine hydroxylase (TH) staining in WAT and brainstem. Mice given fructose during the light phase (inactive period for mice) showed evidence for a diabetic like symptoms with prominent changes in adipose tissue fat. Results showed: 1) enhanced increase in BF and increased size of adipocytes in FL; 2) increased plasma insulin and leptin in FL without changes in glucose or glucose tolerance; 3) no change in cholesterol or triglycerides; 4) measurable amounts of norepinephrine (NE), epinephrine (EPI) in WAT without difference among groups; 5) enhanced staining for TH in WAT and brainstem locus coeruleus. The changes in adiposity occurred even though caloric intake was not different
among groups. In conclusion, results document that restriction of fructose access to the light period produced pathological effects on metabolic function along with catecholaminergic activation in WAT and brainstem. These data may have clinical implications since the timing of intake may be important in the control of adiposity and the development of metabolic syndrome.
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1. INTRODUCTION

1.1 Adipose tissue overview

White adipose tissue (WAT) forms the largest fat depot in the body, localized primarily in the abdomen and the subcutaneous layer. The fat is stored in adipocytes which form a mesh-like cellular network (Figure 1). The cells act as storage vessels for triglyceride (TG) which is metabolized to release free fatty acids (FFAs) into the circulation. FFAs in turn provide energy for bodily functions. WAT also contains preadipocytes, fibroblasts, mast cells, mesenchymal cells, endothelial cells, and stromovascular cells (Frayn, Karpe, Fielding, Macdonald, & Coppack, 2003). WAT is considered to be an endocrine organ because it produces the metabolic hormones, leptin, adiponectin, and resistin as well as the immunological modulators, IL-6 and TNF-α.
1.2. Leptin

Leptin is a 167-amino acid peptide (Brennan & Mantzoros, 2006; Zhang et al., 1997) produced almost exclusively in adipose tissue (Margetic, Gazzola, Pegg, & Hill, 2002). Its secretion is influenced by fat mass and changes in energy status (Considine et al., 1996). A reduction of leptin either in humans or rodents leads to increased body weight (BW) and body fat (BF). Leptin is particularly important in the obesity syndrome because it regulates food intake and energy expenditure (Zhang et al., 1994). Leptin binds to leptin receptors (ObRbs) located throughout the central nervous system (CNS) and peripheral tissues (Fei et al., 1997). The ObRb receptor is highly expressed in the hypothalamus, which is involved in energy homeostasis (Elmquist, Bjorbaek, Ahima, Flier, & Saper, 1998; Fei et al., 1997). After release from adipocytes, leptin is transported though the blood-brain barrier into the CNS, where it causes hunger inhibition and a sensation of satiety. Insulin and leptin, act together as peripheral signals to the CNS, regulating feeding behavior and metabolic homeostasis (Niswender & Schwartz, 2003). The nocturnal rise in leptin secretion is entrained to mealtime probably responding to the cumulative hyperinsulinemia which stimulates leptin secretion from adipose tissue (Sinha & Caro, 1998). Change or deletion in leptin receptors has the opposite effects, obesity and diabetes. An example of a pathological genetic model is the \emph{db/db} mouse which has a mutation of the Ob receptor (White & Tartaglia, 1996). A mutation in a human gene in similar manner produced obesity and glucose intolerance, (Licinio et al., 2004)

1.3. Leptin and insulin

Leptin has peripheral effects on insulin secretion and insulin action which leads to changes in metabolism of adipocytes, skeletal muscle, and other tissues. Leptin is considered to be an important regulator of peripheral glucose homeostasis. Like leptin, insulin also has receptors in
the hypothalamus with secretion proportional to fat stores (Baskin, Wilcox, Figlewicz, & Dorsa, 1988). Experimental studies have shown that prolonged intravenous leptin infusion increases glucose use and increases tissue sensitivity to insulin. Insulin plays an important role in the regulation of leptin secretion and vice versa for leptin and insulin secretion. It is thought that insulin increases leptin production by adipose tissue, whereas, leptin inhibits insulin secretion and insulin gene expression. The suppressive action of leptin on insulin production is regulated both by the autonomic nervous system and directly by influence on leptin receptors in B cells in pancreas.

1.4. Leptin and the sympathetic (SNS) nervous system

The interrelationship between leptin and the SNS is very strong. Leptin stimulates the SNS, seen by increased blood pressure (BP), thermogenesis (Scarpace, Matheny, Pollock, & Tumer, 1997), and increased NE turnover (Collins et al., 1996). Leptin infusion also increases renal sympathetic nerve activity (SNA) by increasing sympathetic outflow to the kidney (Haynes, Morgan, Walsh, Mark, & Sivitz, 1997). The sympathetic stimulatory effects were absent in Zucker rats (fa/fa) that have mutations in leptin receptors (Haynes et al., 1997). Likewise in ob/ob mice, leptin produced no increase in NE turnover in WAT, an effect produced by leptin resistance. The proposed scenario is that leptin which is released from adipose tissue activates the SNS (Haynes et al., 1997) with inhibition of leptin secretion by SNS activation as a feedback mechanism (Hardie, Rayner, Holmes, & Trayhurn, 1996; Ricci, Fried, & Mittleman, 2000). Experimental studies in rats and mice showed increase in leptin mRNA and in plasma leptin after blockade of NE synthesis (Rayner & Trayhurn, 2001; Sivitz et al., 1999). Data indicate a role of SNS in leptin gene expression modulation through a feedback loop to adipose tissue.
1.5. Sympathetic neural innervation of white adipose tissue

One of the first studies to show direct WAT innervation by the SNS (postganglionic SNS fibers) was performed in hamsters (Youngstrom & Bartness, 1995). Using histofluorescence, catecholaminergic fibers could easily be visualized in WAT (Rebuffe-Scrive, 1991; Slavin & Ballard, 1978). Immunostaining for tyrosine hydroxylase (TH), a specific marker of sympathetic noradrenergic nerves, (Wilson et al., 1988) (Flatmark, 2000) also provided evidence for adrenergic innervation of WAT. The pathway for innervation was documented using pseudorabies virus for track tracing (Bamshad, Aoki, Adkison, Warren, & Bartness, 1998). SNS outflow from the brain to different depots of WAT is well documented (Bamshad et al., 1998; Bowers et al., 2004). After injection of pseudorabies (a transport marker) in WAT, there was widespread labeling in brainstem regions which control food intake and contain leptin receptors (Mercer, Moar, & Hoggard, 1998). In the hypothalamus, there was viral infection in the paraventricular nucleus (PVN), a region involved in sympathetic responses including blood pressure elevation and induction of lipolysis (Bartness & Bamshad, 1998). There was little labeling in the ventromedial hypothalamus even though this region is also involved in sympathetic responses and appetite control. TG storage is known as lipogenesis while secretion is known as lipolysis. High levels of lipogenesis cause obesity which is associated with hypertension, insulin resistance, and inflammation (Gustafson et al., 2009). WAT is heavily innervated by sympathetic fibers emanating from the CNS. The sympathetic terminals are involved in the control of fat secretion via activation of β-adrenergic receptors. In addition to adrenergic input, pancreatic insulin has prominent actions in WAT including inhibition of lipolysis (Chakrabarti & Kandror, 2011).
1.6. Energy balance in white adipose tissue

The action of the SNS in WAT is mediated by β-adrenoceptors (β-AR) and α₂-adrenoceptors (α₂-AR) (Lafontan et al., 1995). Catecholamines, NE and EPI, are released from the adrenal medulla or sympathetic nerve terminals and stimulate lipolysis by activating β receptors (Chu, Huddleston, Clancy, Harris, & Bartness, 2010; Carpene, Bousquet-Melou, Galitzky, Berlan, & Lafontan, 1998; Nonogaki, 2000; Bartness & Bamshad, 1998). Catecholamines also exert antilipolytic effects through stimulation of α₂-AR. EPI and NE have a higher affinity for α₂-AR than for β-AR indicating a role for the α₂ adrenergic pathway in the control of lipolysis (Chen et al., 1996). If the activation of β-AR in WAT is stronger than the α-AR, then lipolysis is activated. If α-AR predominate, then lipolysis is inhibited (Bartness & Bamshad, 1998). Insulin has an inhibitory effect on lipolysis by reducing the number of adrenergic binding sites available by translocation of insulin receptors to the intracellular compartments. This causes a desensitization of ARs to catecholamine-stimulated lipolysis (Engfeldt, Hellmer, Wahrenberg, & Arner, 1988).

1.7. Pathophysiological effects of fructose

Fructose is found naturally in a variety of foods, such as fruits, honey, and vegetables. Fructose has a chemical formula (C₆H₁₂O₆) similar to glucose. In glucose, an aldehyde group is attached at carbon 1, while in fructose the keto group is attached to carbon 2. The specificity and time course of fructose metabolism in the liver is related to the high maximum velocity of an enzymatic reaction (Vm) and low dissociation constant of the enzyme-substrate complex (Km) of fructokinase (Heinz, Lamprecht, & Kirsch, 1968; Adelman, Ballard, & Weinhouse, 1967). In contrast to glucose, hepatic metabolism of fructose is not regulated by insulin. In the first step of fructose metabolism in the liver, phosphorylation of fructose to fructose -1 phosphate occurs rapidly by
the action of fructokinase enzyme. This step occurs independently of insulin and without ATP feedback (Bruynseels et al., 1999; Cortez-Pinto et al., 1999). The rapid phosphorylation of fructose in the liver produces substrates for gluconeogenesis and lipogenesis.

The dramatic increase in fructose consumption in the last decades is thought to bear a relationship with the increased incidence of metabolic syndrome. In 2007, fructose intake in adults averaged almost 65 gm/day, mainly in the form of high fructose corn syrup (HFCS) (Tappy & Le, 2010). This global increase in fructose consumption is correlated with increased prevalence of metabolic syndrome, and type 2 diabetes mellitus (Montonen, Jarvinen, Knekt, Heliovaara, & Reunanen, 2007; Palmer et al., 2008), and obesity (Lustig, 2006; Isganaitis & Lustig, 2005). These syndromes are also associated with hypertension and cardiovascular disease (Dhingra et al., 2007; Fung et al., 2009).

1.8. Effect of fructose on the sympathetic nervous system

The activating effect of dietary carbohydrates on SNS activity was originally documented more than 25 years ago (Young & Landsberg, 1977). Results showed that high fructose consumption produces hypertension and insulin resistance in rodents (Dai & McNeill, 1995). One of the possible mechanisms was via increases in sympathetic neural outflow which increases plasma insulin and blood pressure (Hwang, Ho, Hoffman, & Reaven, 1987; Reaven, 1993). It was demonstrated in humans that fructose infusion amplified the release of EPI (Gabriely, Hawkins, Vilcu, Rossetti, & Shamoon, 2002). The ingestion of fructose in rats increased plasma and urinary NE (Jansen, Penterman, van Lier, & Hoefnagels, 1987) and increased adrenergic receptor expression in the heart (Kamide et al., 2002). Moreover, fructose feeding in mice produced nocturnal hypertension, sympathetic activation, and CNS catecholaminergic changes (Farah et al., 2006).
1.9. Circadian rhythms and fructose consumption

Circadian rhythms should also be considered in the etiology of metabolic and cardiovascular pathologies associated with diabetes. Organisms adapt to their changing environment through tapping into a central clock, i.e., the suprachiasmatic nucleus (SCN). There are also independent peripheral clocks located in organs such as the liver and fat. Moreover, alterations in the pattern of eating, activity and sleeping may play a role in the development of metabolic syndrome (body weight, blood pressure, and insulin resistance). In humans, this is seen in pathologies associated with shift work, such as diabetes, weight gain, hypertension, coronary artery disease and other maladies (Morikawa et al., 2005; Esquirol et al., 2009). Even a short term alteration in day/night rhythms, such as from jet lag, was sufficient to produce an increase in blood pressure as well as metabolic derangements (Scheer, Hilton, Mantzoros, & Shea, 2009). In animals or humans, alteration of the feeding schedule resulted in significant changes in metabolic and endocrine parameters (Arble, Bass, Laposky, Vitaterna, & Turek, 2009; Kudo et al., 2004; Bodosi et al., 2004; Zvonic et al., 2006; Colles, Dixon, & O'Brien, 2007). People with “night time eating” syndrome have a total caloric intake which is not different from healthy subjects. However, people with this syndrome gain more weight and have depression (Allison et al., 2005; Colles et al., 2007). Experimentally, feeding a group of mice a high fat diet in the inactive time (light period) also gave similar results, that is, greater weight gain without differences in caloric intake or activity (Arble et al., 2009). Similarly, changing the light cycle by exposing the animal to low light during the dark time, resulted in a syndrome of obesity and insulin resistance (Fonken et al., 2010). When animals were forced to eat during the light, leptin levels were also increased (Bodosi et al., 2004).
1.10. Summary

Alterations in the pattern of eating, activity, and sleeping may play a role in the development of the metabolic syndrome (increased body weight, blood pressure, and development of insulin resistance). In humans, this is seen in pathologies associated with shift work, such as, diabetes, obesity, hypertension, and coronary artery disease. Moreover, reversal of day/night cycles has effects on organs such as adipose tissue through the production of leptin and adiponectin. In addition to the function of adipose tissue as a reservoir for fat and an energy source, it is considered to be the body’s largest endocrine organ. In view of previous experiments which demonstrated that the day/night pattern of intake of sugar influences the development of metabolic pathologies, a protocol was designed in which the risk of both was combined. It was hypothesized that fructose provided in the light sleeping period in mice would exacerbate the sympathetic neural and metabolic responses. Consumption of fructose may result in obesity, diabetes, and autonomic dysfunction. Recently, studies have shown that the consumption of fructose enhances the diabetic state by causing elevation in insulin levels. The rise in obesity with fructose consumption may occur because of its role in the lipogenic process. Increasing adiposity and metabolic disturbances are also seen following alterations in day/night feeding schedule. The aim of this study combined the effect of fructose and alteration of day time to test whether light-limited access to fructose alters metabolic function and adipose tissue catecholaminergic activity in mice.
2. HYPOTHESIS AND SPECIFIC AIMS

2.1. Hypothesis

The timing of the access to dietary fructose intake will influence the metabolic and catecholaminergic response to fructose consumption. The hypothesis predicts that fructose given in light period in mice will increase catecholamine use in adipose tissue and catecholaminergic activity in the brainstem. In addition, it is predicted that limiting the time access of fructose to the light period will increase adiposity and influence metabolic parameters.

2.2. Specific aims

The specific aims of this study are:

1. To test the hypothesis that timing (light vs. dark) of fructose consumption will influence body weight, body fat, fat cell size, and metabolic parameters.

2. To test the hypothesis that timing (light vs. dark) of fructose consumption will influence catecholaminergic activity in adipose tissue and brainstem.
3. MATERIALS AND METHODS

3.1. Animals
C57BL/6 male mice with initial body weights (BW) of 22-24 g (Harlan Inc., Indianapolis, IN), were housed individually (22°C) with 12 hr light (270 Lux) and 12 hr dark. Mice were given *ad libitum* access to standard mouse chow (58% CHO, 30% PRO, 12% FAT) and timed access to water or fructose (10% in tap water).

3.2. Animals and treatments:
C57BL/6 male mice were assigned randomly to groups: 1) Control: 24 hr water (n=10); 2) Fructose Light (FL): 12 hr fructose during the light period and 12 hr water during the dark period (n=11); and 3) Fructose Dark (FD): 12 hr fructose solution during the dark period and 12 hr water during the light period (n=11). Total fluid intake, chow consumed, body weight, and body fat was measured at weeks 2, 4 and 6. A glucose tolerance test was performed at week 8. Mice were sacrificed by decapitation at week 9. Experimental protocols were approved by the Wright State University Laboratory Animal Care and Use Committee.

3.3. Histology and cell Measurement of white adipose tissue
Epididymal adipose tissue was fixed in 4% paraformaldehyde. Paraffin embedded tissues were sectioned (5 um) and stained with hematoxylin and eosin (H&E) (AML Laboratories Inc., Rosedale, MD). Tissues were examined using light microscopy and digital images were taken. Images were analyzed using a computerized imaging system, MetaMorph® version 7.6 (Molecular Devices Inc., Sunnydale, CA). Images were saved as 24-bit TIF files for computer processing. Specifically, four tissue sections from each animal were used for analysis with two digital images made of each section. For each image, the number and area of the cells were measured in a defined region. The tissue was manually checked so that broken cells or damaged areas were removed from consideration.
3.4. Immunohistochemical staining for tyrosine hydroxylase (TH) in epididymal fat

To determine the level of tyrosine hydroxylase in fat tissue, fixed sections of epididymal fat were deparaffinized with xylene and hydrated. Sections were incubated in Na citrate (0.01M), boiled at 95°C for 20 min, and cooled for one hour at room temperature. Sections were then washed four times for 5 min each with 0.1 M. phosphate buffered saline (PBS) and washed with 0.5% bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO) plus 0.4% Triton X-100 (Fisher Scientific, Pittsburgh, PA) and 0.3% H₂O₂ (Sigma) for 15 min at room temperature to block endogenous peroxidase. Tissue was then washed with PBS (7.5 pH) four times for 5 min and finally washed with 0.5% BSA for 10 min. A hydrophobic pen was used to draw a circle around the tissue and allowed to dry before adding the blocking solution (normal goat serum in buffer 1% BSA). After incubating with primary TH antirabbit antibody (Pel-Freeze Biologicals, Rogers, AR) [1:500] for 1 hr at room temperature, the tissue was refrigerated overnight.

After 24 hours incubation, the tissue was washed with 0.1M PBS (7.4 pH) four times for 5 min each and then washed with 0.5% BSA for 10 min. A secondary antibody (goat/antirabbit) was added [1:500] to the tissue and incubated for two hours at room temperature. During this two-hour incubation period, ABC solution (Vector Laboratories, Burlingame, CA) was prepared at least 30 min prior to use. Again, the tissue was washed with 0.01M PBS (7.4 pH) four times for 5 min each.

The next step involved tissue washing with 0.5% BSA + 0.4% Triton -X100 in 0.01M PBS (7.4 pH) for 10 min. Following this, 1 ml of 0.01 M PBS + 2 µl of reagent A + 2 µl of reagent B were combined and allowed to sit on ice for 30 min. This solution was then added to the slides, which sat for 1 hour at room temperature. The tissue was washed with 50 mM buffer A (8.505 g NaCl + 0.3405 g Midazol dissolved in 500 ml dH₂O) two times for 5 min each,
washed with diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA) solution (40 ml buffer A + 10 mg tablet DAB 3 + 3, 3'-diaminobenzidine tetrahydrochloride + 200 µl of H2O2 + 10 µl of 30% H2O2 + 1 ml dH2O) and placed on a shaker for 10 min or until the tissue took on a brown color. Slides were again rinsed with 0.01M PBS two times for 3 min each. Finally, the slides were dipped in H&E for 5 sec and dehydrated by going through increasing concentrations of ethanol (70%, 95%, and 100%) for 1 min each, ending in a 5 min xylene wash. Cover slips were placed over the tissue using mounting media (Fisher Scientific, Pittsburgh, PA) and dried. Slides were photographed at 20X magnification.

3.5. Immunohistochemistry of TH in brainstem

The method was the same for TH staining in brainstem. Frozen (-80°C) brainstem sections (25 um) from each group were processed for immunohistochemistry. The brainstem sections were rinsed in 0.01 M PBS (7.4 pH) solution. Slides were placed on a tray and allowed to sit at room temperature for one hour. This step was followed by washing with 0.01 M PBS (7.4 pH) two times for 2 min each. The slides were next inserted into a container with 15% H2O2 ((2 µl of H2O2 + 10 ml of 0.1 M PBS (7.4 pH)) and placed on a shaker for 15 min. After 15 min, the tissue was washed with 0.01 M PBS (7.4 pH) three times for 5 min each. A hydrophobic pen was used to draw a circle around the tissue and allowed to dry for few seconds before adding the blocking solution ((10 ml 0.01 M PBS (7.4 pH) + 150 ul of normal goat serum + 10 µl of 0.4% Trion-100)). Primary antibody (Pel-Freeze Biologicals, Rogers, AR) rabbit/antirabbit [1:1000] was added. The tissue then sat at room temperature for one hour followed by refrigeration overnight.

The next day slides were again washed with 0.1M PBS (7.4 pH) two times for 5 min each. Again, the tissues on slides were circled with a hydrophobic pen. The secondary antibody,
goat/antirabbit (Vector Laboratories, Burlingame, CA), was added [1:1000] to the tissue and incubated for one hour. While the tissue was incubating, an avidin-biotin peroxidase complex ((2 µl of reagent A + 2 µl of reagent B + 1 ml of 0.01 M PBS (7.4 pH)) was prepared at least 30 min prior to use and kept on ice. After incubation, tissues were washed with buffer B. This step was followed by washing the tissue with 50 mM of buffer A two times for 5 min each and lastly washed with DAB solution ((20 ml buffer B + 10 mg tablet DAB + 200 ul of H2O2 +10 µl of 30% H2O2 + 1 ml dH2O)). Containers with the slides were then set on the shaker with the DAB solution for 10 min (or until the tissue turned black).

Tissues were rinsed to remove the DAB solution with 0.01 M PBS two times for 5 min each. Slides were then dried for 30 min to 1 hour and dehydrated using increasing percentages of ethanol 70%, 95%, 100% solutions for 1 min each. Slides were then placed in xylene for 5 min. Cover slips were placed on the slide with mounting media (Fisher Scientific, Pittsburgh, PA) and dried. Slides were photographed at 20X magnification. The TH was measured quantitatively by comparing one slide per animal from each group.

3.5. High-performance liquid chromatography coupled with electrochemical detection (HPLC-ED)

To determine the concentrations of norepinephrine (NE), epinephrine (EPI), and dopamine (DA) in fat, 60 mg of epididymal tissue was removed from the -80⁰ C freezer and placed in a tube with 500 µl of cold 0.4 N perchloric acid and homogenized using a sonicator three time at 20 sec each. Samples were then centrifuged at 4⁰ C and 13,000 rpm for 10 min. Supernatant (400 µl) was transferred to a 2 ml tube. To this tube, 50 mg of alumina (aluminium oxide) (Sigma-Aldrich, St. Louis, MO) and 45 µl of dihydroxybenzylamine hydrobromide (DHBA - 50 ng/ml) were added. DHBA was used as the internal standard and 0.5 M Tris buffer (9.0 pH) was
added to adjust the pH to 8.5. The mixture was vortexed for 30 sec and centrifuged for 1 min at 1000 rpm so that the alumina precipitated to the bottom.

After removing the buffer, the remaining alumina was washed with 1 ml of dH2O, vortexed, and centrifuged three times. Elution was performed by adding 200 µl of 0.1 N perchloric acid (0.1 N perchloric acid with 1.0 mM Na metabisulfate). Following a 30 sec vortex and spin for 1 min, the acid was removed. The remaining homogenates were centrifuged at 13,000 rpm at 4°C for 10 min. Two aliquots (20 µl) of supernatant were injected into the HPLC. Chromatographic separation was conducted using an ESA 80 × 4.6 mm column packed with 3 µm of C18 resin and a LC-4B amperometric detector (Bioanalytical Systems Inc. BASi) optimized to 0.75 V. The mobile phase consisted of a stock of 8.204 sodium citrate, 0.0584 g EDTA disodium salt (C_{10}H_{14}O_{8}Na_{2}N_{2}), 11.516 g citric acid, and 110 ml ProClin® solution. For running urinary catecholamines, 500 ml of stock solution + 0.12884 g 1-octanesulfonic acid (C_{8}H_{17}O_{3}SNa: sodium ethyl hexyl sulfonate) and 35 ml MeOH (methanol) was made. The pH was adjusted to 8.5 using Tris buffer (9.0 pH). The HPLC flow rate was set at 0.6 ml/min. A standard, containing a known amount of the monoamines - norepinephrine (NE), epinephrine (EPI), dihydroxybenzylamine hydrobromide (DHBA), and dopamine (DA), was injected into the chromatography system. The concentrations of monoamines in the samples were determined by comparing the peak amplitude from the samples to that obtained from known standard concentrations. The concentrations of NE, EPI, and DA were determined and expressed as pg/ng of tissue.

3.6. Statistical analysis

Male C57BL mice were given standard chow and assigned to one of three groups: Control (n=10, water 24h); FL (n=11, 10% fructose solution during 12h light period); and FD (n=10,
10% fructose solution during 12h dark period). Statistical analysis was performed using GraphPad Prism® (v. 5.01) (GraphPad Software, La Jolla, CA) and STATISTICA® software (v.10) (StatSoft, Tulsa, OK). All data are expressed as mean ± SEM. Statistical significance was assessed by one-way, two-way, or repeated-measures analysis of variance (ANOVA) followed by Tukey’s HSD post hoc test. The significance level was set at two-tailed $p<0.05$. 

4. RESULTS

4.1. Metabolic parameters

4.1.1 Change in body weight: BW increased significantly for FL group over the 6 wk time course (Fig 2). There was a significant difference in BW (\(p<0.05\)) observed for FL as compared to FD.

4.1.2 Change in body fat: There was a significant increase in BF in FL group (Fig 2) (2.6 and 1.5 fold higher than Control and FD, respectively). FL animals had significantly more body fat (FL>FD>C, \(p<0.05\)).

4.1.3 Adipocyte size: Fructose consumption induced a change in adipose cell size (Group effect, \(p<0.03\)) (Fig 3). Adipocytes were significantly larger in the FL group when compared to Control (\(p<0.05\)).

4.1.5 Plasma insulin: At the experiment’s 8-week termination, insulin was measured in non-fasted mice in the morning (Figure 4). Mice fed fructose during the light period (FL) had significantly higher insulin levels compared to mice fed fructose during the dark (FD, \(p<0.05\)). There was no difference as compared to controls (Fig 4).

4.1.6 Plasma leptin: There was a significant elevation in plasma leptin for mice fed fructose in the light period (FL) compared to those fed fructose during the dark period and controls. (Fig 4, \(p<0.05\))

4.1.7 Glucose tolerance test GTT: There was no significant difference among treatment groups for GTT data (FL: 192 ± 7.4 AUC/min, FD: 187 ± 7.5 AUC/min, Control: 204 ± 8.6 AUC/min) (Table 1).
4.1.8 Plasma glucose: There was no significant difference in plasma glucose level among the groups, although there was significant increasing plasma levels of insulin and leptin hormones (FL: 139 ± 22.0 mg/dL, FD: 154.4 ± 21 mg/dL, Control: 157 ± 23.6 mg/dL) (Table 1).

4.1.9 Plasma triglyceride and cholesterol levels: After 8 weeks, cholesterol and triglyceride levels of the fructose fed mice and the control group were measured using specific colorimetric assays. There was no significant difference among groups for these variables: cholesterol (FL 92.6 ± 8.0 mg/dL, FD: 90 ± 5.5 mg/dL, Control: 82.7 ± 6.3 mg/dL); triglycerides (FL: 89 ± 12.0 mg/dL, FD: 89 ± 8.0 mg/dL, Control: 82.7 ± 6.3 mg/dL) (Table 1).

4.2. Measurement of Catecholamine Activity

4.2.1 Immunohistochemistry of TH in white adipose tissue: Immunohistochemistry was conducted and followed by HPLC analyses, which quantitatively measured the catecholamine level. Immunostaining of TH for FL on adipose tissue and brainstem was used since it was difficult to measure catecholaminergic activity accurately using TH staining. Electrochemical methods for direct measurement of NE and EPI levels (Fig 6) were employed.

4.2.2 Catecholamine level in white adipose tissue: To investigate the effect of fructose limited access time on catecholamine activity, HPLC method was performed to measure NE and EPI quantitatively in epididymal adipose tissue. There was a trend for higher levels of catecholamine in the FL group, which indicated that the time of response is critical (Fig 8).

4.2.3 Immunohistochemistry of TH in brainstem: There was robust staining for TH in the brainstem, specifically in the locus coeruleus (LC). Staining was qualitatively greater in the FL group (Fig 9).
Table 1. Results of the glucose tolerance test (GTT) and plasma levels of glucose, adiponectin, triglycerides and cholesterol (after 8 weeks)

<table>
<thead>
<tr>
<th>Group</th>
<th>GTT (AUC/min)</th>
<th>Glucose (mg/dL)</th>
<th>Adiponectin (µg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>Cholesterol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>204±8.6</td>
<td>157.1±23.6</td>
<td>12.±0 1.8</td>
<td>77± 9.3</td>
<td>82.7± 6.3</td>
</tr>
<tr>
<td>F12L</td>
<td>192± 7.4</td>
<td>139 ±22.0</td>
<td>10.7± 2.4</td>
<td>89± 12.0</td>
<td>92.6± 8.0</td>
</tr>
<tr>
<td>F12D</td>
<td>187± 7.5</td>
<td>154.5± 21.0</td>
<td>11.5± 2.1</td>
<td>92.6± 8.0</td>
<td>90.2± 5.5</td>
</tr>
</tbody>
</table>

Data are means ± SEM. There were no significant differences among the groups for any of the variables at 8 weeks. Control (n=10, water 24h); FL (n=11, 10% fructose solution during 12h light period); and FD (n=10, 10% fructose solution during 12h dark period).
Figure 2. Data are means ± SEM. Changes in (A) bodyweight and (B) body fat at 2 (▪) and 6 weeks (■) after the initiation of dietary treatment. Control (n=10, water 24h); FL (n=11, 10% fructose solution during 12h light period); and FD (n=10, 10% fructose solution during 12h dark period). Two-way repeated measures ANOVA showed a significant effect of time on bodyweight ($F_{1,29} = 47.2; \ p < 0.0001$). Two-way repeated measures ANOVA for body fat showed significant effects of Group ($F_{2,29} = 3.85; \ p < 0.03$), Time [(F$_{1,29} = 60.6; \ p < 0.001$) and a Group × Time interaction ($F_{2,29} = 5.04; \ p < 0.013$). **$p < 0.01$ compared with 2 weeks; †$p < 0.01$ compared with control values at 6 weeks; ‡$p < 0.05$ compared with fructose dark group at 6 weeks.
Figure 3. Procedure for MetaMorph® for white adipose tissue (WAT). Images of WAT were processed to measure the adipocyte size on the mice in the three groups Control: water only; FL: water during dark period and fructose during light period; FD: water during light period and fructose during dark period. A and D are original images of WAT. B, E, C, and F are images of result of adipocyte size in certain region was created (800pixelx800 pixel), For each image, the number and area of the cells were measured in a defined region.
Figure 4. Data are means ± SEM. Cell size in white epididymal fat. Fixed, hematoxylin and eosin stained tissue was examined using light microscopy and image analysis. Control (n=10, water 24h); FL (n=11, 10% fructose solution during 12h light period); and FD (n=10, 10% fructose solution during 12h dark period). Analysis using ANOVA showed a group effect (F_{2,18} = 3.80; p < 0.042). *p < 0.05 compared with Control.
Figure 5. Data are means ± SEM. Insulin and leptin levels in the Control (n=10, water 24h); FL (n=11, 10% fructose solution during 12h light period); and FD (n=10, 10% fructose solution during 12h dark period). Analysis using ANOVA showed a significant Group effect for insulin (F\(_{2,17} = 5.62; p< 0.015\)) and leptin (F\(_{2,18} = 3.85; p< 0.042\)). *p< 0.05 compared with the FD group.
Figure 6. Immunostaining of tyrosine hydroxylase (TH) in the white adipose tissue. TH staining showed localization of the brown reaction product in the adipose cells. A) Control: Water only; (B) FL: Water during dark period and fructose during light period; and (C) FD: Water during light period and fructose during dark period.
Figure 7. Standards chart for catecholamine measuring (1nA). A standard, containing a known amount of the monoamines - norepinephrine (NE), epinephrine (EPI), dihydroxybenzylamine hydrobromide (DHBA), and dopamine (DA), was injected into the chromatography system. The concentrations of monoamines in the samples were determined by comparing the peak amplitude from the samples to that obtained from known standard concentrations. The concentrations of NE, EPI, and DA were determined and expressed as pg/ng of tissue.
Figure 8. Data are means ± SEM. Plasma hormone levels (ng/mL). One-way ANOVA showed that there were no significant differences among the groups for catecholamine levels of NE and EPI. Control (n=10, water 24h); FL (n=11, 10% fructose solution during 12h light period); and FD (n=10, 10% fructose solution during 12h dark period).
Figure 9. Immunostaining of tyrosine hydroxylase (TH) on brainstem of C57BL/6 mice in the locus coeruleus (LC). After 8 weeks of 10 % fructose treatment, staining was qualitatively greater in the FL group compared to FD and Control groups. Control (n=10, water 24h); FL (n=11, 10% fructose solution during 12h light period); and FD (n=10, 10% fructose solution during 12h dark period).
5. DISCUSSION

This study tested the hypothesis that limited time access to fructose causes activation of adipose and brain catecholaminergic activity as well as metabolic pathologies in mice. The results show that consumption of fructose during the light or sleep period in male C57BL/6 mice produced altered metabolic and endocrine responses (Morris et al., 2011). This was seen as enhanced insulin and leptin levels with increased adiposity and fat cell size. Immunohistochemistry showed staining for TH in adipose tissue and brainstem. Qualitative examination of the stain suggested increased catecholaminergic activity in both tissues. Electrochemical measurement of catecholamines, NE and EPI, in adipose tissue showed no significant changes. Our results document the importance of the timing of nutrient intake in causing metabolic disease. Questions remain as to the role of catecholamines in mediating the changes.

Epidemiological and experimental studies in humans and animals confirm that high fructose consumption causes metabolic and autonomic disorders including insulin resistance, obesity, autonomic dysfunction, and lipid and renal abnormalities (Bray, Nielsen, & Popkin, 2004; Katovich et al., 2001; Dai & McNeill, 1995; Farah et al., 2006; Hsieh, 2005). Autonomic dysfunction plays a major role in the etiology of such syndromes. The clusters of metabolic and hemodynamic alterations are characterized by an increase in sympathetic drive (Grassi & Seravalle, 2006; Landsberg, 2001; De, Senador, Mostarda, Irigoyen, & Morris, 2012). There is much information on the cardiovascular and metabolic effects of a fructose supplemented diet in animal models. Fructose fed rats and mice show a moderate hypertension and glucose intolerance associated with high levels of plasma cholesterol, and TG (Lindmark, Wiklund,
Bjerle, & Eriksson, 2003; Kamide et al., 2002; Dai & McNeill, 1995; Farah et al., 2006; Hsieh, 2005). There is evidence for a role of the sympathetic nervous and renin angiotensin systems (RAS) in fructose-induced cardiovascular and renal changes (Cunha et al., 2007; Van Gaal, Mertens, & De Block, 2006). In a study done with fructose feeding in rats, fructose induced sympathetic activity and increased urinary catecholamine excretion (Kamide et al., 2002). An association between cardiovascular, autonomic and metabolic dysfunction in humans and animals has been demonstrated (Van Gaal et al., 2006; Hellstrom, 2007).

As an index of catecholaminergic activity in adipose tissue, immunostaining for TH and electrochemistry for measurement of NE and EPI levels was utilized. Staining showed localization of the brown reaction product in the adipose cells. Given the structure of adipocytes which contain a large central vacuole (Fig 1), it was difficult to quantitatively compare staining in the tissues. This difficulty was also seen in the quantitative measurement of TH in vascular tissue. TH fibers were seen around vessels and in close association with adipocytes, but it was not possible to verify the level of change (Murano, Barbatelli, Giordano, & Cinti, 2009). In epididymal WAT of Siberian hamsters, it was also difficult to detect TH due to the close packing of the adipocytes (Shi, Song, Giordano, Cinti, & Bartness, 2005). However, in the present study it appeared that WAT staining was heavier in the FL group which had more fat and greater metabolic damage. In a quantitative assessment of TH activity in epididymal adipose tissue of fructose-fed rats, there were no significant differences between groups (Baret et al., 2002). It was easier to detect nerve fibers in fat tissue from animals in a fasted state since cell size was smaller and parenchymal area was larger (Slavin & Ballard, 1978). Because it was difficult to measure catecholaminergic activity accurately using TH staining, electrochemical methods for
direct measurement of NE and EPI levels were used (Lucot, Jackson, Bernatova, & Morris, 2005).

For the HPLC/electrochemical method, fat tissue is extracted with alumina, amines are separated by HPLC, and NE and EPI are measured by electrochemistry (Lucot, Crampton, Matson, & Gamache, 1989). This method is useful for measuring catecholamine levels accurately in tissues, urine, and plasma. NE levels in WAT were 0.08 pg/ng in FD vs. 16 pg/ng in FL. EPI levels were 0.14 pg/ng in FD vs. 9.45 pg/ng in FL (Fig. 8). The levels appear higher in the FL group as hypothesized, but because of the large variance, changes were nonsignificant. There are other reports of catecholamines in mice and rats WAT. Short term feeding (6 days) of fructose showed no change in NE levels in fat (Young, Weiss, & Boufath, 2004). Animals fed fructose for 6 weeks showed no significant change in SNS activation in epididymal (Baret et al., 2002). Most experiments using fructose provide 24 hr accesses. The design of the present study is unique since it compares the effect of limited light/dark consumption. Indeed, there was a trend for increase both for TH and catecholamine content.

As a complement to the adipose measurements, TH staining in the brainstem source for sympathetic innervation of adipose tissue was examined. There was robust staining for TH in the brainstem, specifically in the locus coeruleus (LC). Staining was qualitatively greater in the FL group (Fig. 10). Similar results were seen when fructose was given in the food (Farah, Joaquim, & Morris, 2006; Farah et al., 2006). Fructose fed mice showed marked increases in brainstem TH mRNA expression, indicating central actions of the diet. CNS activation may play a role in the cardiovascular and metabolic changes induced by fructose.

With regard to metabolic changes resulting from fructose consumption, body fat, fat cell size, and plasma levels of insulin and leptin were significantly increased. The changes were
specific for the FL group, verifying the important influence of timing (light/dark). In a study performed in people who were fed three meals at different times over 24 hours (morning, afternoon, night), results showed that the group eating at night had higher cholesterol levels than the morning and afternoon groups although these groups ate more food (Morikawa et al., 2007). High levels of TG were seen in shift workers, indicating that alteration of activity patterns is a risk factor for coronary disease (Knutsson, Akerstedt, & Jonsson, 1988). Moreover, individuals who worked at night showed higher risk for diabetes. Glycated hemoglobin was higher in night workers compared to those working on a fixed day time schedule (Morikawa et al., 2005). These studies established that alterations in sleeping or eating times are associated with risk factors related to type 2 diabetes and heart disease. Along with these results, studies also show increases in body weight in shift workers (obesity) (van Amelsvoort, Schouten, & Kok, 1999). Experimentally, increases in blood pressure and plasma insulin and glucose were also seen in individuals when circadian misalignment was induced, even acutely (Scheer et al., 2009). Similarly, in animals, such a misalignment (light-dark cycle reversed) influenced the induction of heart failure (Penev, Kolker, Zee, & Turek, 1998). Interestingly, heart attack and strokes were found to occur more often during the early waking hours in humans (Muller, 1999; Elliott, 1998).

In mice exposed to chronic stress for 7 days, there were circadian patterns in the blood pressure responses, higher levels in the light vs. dark (Bernatova, Key, Lucot, & Morris, 2002). The experiments in the present study were designed to test the hypothesis that limited time access to fructose causes activation of adipose and brain catecholamine activity as well as metabolic pathologies.

Unlike glucose, fructose metabolism is not regulated by the energy state. After fructose ingestion, its metabolism occurs independently of insulin because of the limitation of fructose
uptake by the pancreas that lacks GLUT5 fructose transporters (Curry, 1989). In the liver, fructose is rapidly phosphorylated by fructokinase and further converted to glyceraldehyde, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate, intermediates in the glycolytic pathway. These intermediates are metabolized to generate TG through the lipogenic process. The speed of the metabolism is due mainly to the high affinity of fructokinase for fructose and the phosphorylation which occurs independent of insulin. Therefore, fructose consumption has been suggested to increase hepatic TG which can be packed into very-low density lipoproteins by the liver and reduce TG clearance by adipose tissue. Although several studies linked fructose intake with elevation in TG and cholesterol (Dai & McNeill, 1995; Hsieh, 2005), in the present study there were no significant differences in TG or cholesterol among the treatment groups.

Most studies provide continuous access to fructose. The current study is unique since the access of fructose was coupled to a limited daily period (12 hr light vs. dark) demonstrating that there were significant differences among treatment groups depending on whether the sugar was given during the light or dark period. The FL group showed significant increases in adiposity and elevation in leptin and insulin levels. This occurred without differences in caloric intake among the groups. These results establish additional experimental evidence that the time of intake is critical to metabolism and the consequent pathologies. For example, when people are required to work at night, they demonstrate elevated TG, lower HDL cholesterol, and weight gain (Esquirol et al., 2009). People who work at night also have health implications in endocrine responses (Holmback et al., 2003). Alterations in the pattern of metabolic hormone secretion as well as increased body weight were confirmed in humans with nighttime snacking syndrome even though total food intake was not different from controls (Goel et al., 2009; Colles et al., 2007). Obesity and lack of blood glucose rhythms was noted in rats fed only during light time and
exposed to constant slow movement (Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010). In the present study, fructose fed mice had an increase in fat cell size and body fat. There were metabolic changes, but no decrease in activity. Increased epididymal fat and cell size were noted in rats fed fructose (Furuhashi et al., 2004).

The current data showed significant differences in metabolic response in mice fed fructose during the 12 hr light period. FL group had the highest levels of insulin and leptin plasma with enhanced adiposity. Leptin is mainly produced by adipose tissue. This hormone plays an important role in regulation of energy status by binding to specific receptors in the hypothalamus causing decreased food intake and increased energy expenditure (Havel, 2005). This organ is affected by alteration in circadian rhythms like other vital organs such as liver, heart and kidney (Damiola et al., 2000; Zvonic et al., 2006). Additionally, leptin displays a diurnal pattern (Ahren, 2000; Teff et al., 2004) with other adipokines that are released from adipose tissue (Gavrila et al., 2003). Circulating leptin level is directly correlated to adiposity (Maffei et al., 1995). In addition, insulin and leptin have synergistic effects. Insulin increases leptin gene expression and has a role in the diurnal pattern of leptin secretion (Saad et al., 1998; Havel, 2002).
6. CONCLUSION

The present study demonstrated that limited access time to a 10% fructose solution in mice increased adiposity and produced diabetic like symptoms. There was a trend for SNS activation as documented with TH staining (WAT and brainstem) and catecholamine levels in adipose tissue. The changes were specific for the FL group and were dissociated from caloric intake. Changes occurred when mice were given the sugary liquid during the light (inactive) period as compared to dark phase consumption. Hormonal effects included increases in leptin and insulin without changes in glucose or glucose tolerance. In conclusion, the results of the present study document that restricting fructose access to the light inactive period produced pathological effects as related to body fat, adipocyte size, and metabolic hormones with a trend toward SNS changes. These data may have clinical implications, since the timing of intake could be important in the control of adiposity and the development of metabolic syndrome.
7. REFERENCES


Characterization of peripheral circadian clocks in adipose tissues. Diabetes, 55, 962-970.