INFLUENCE OF DIETARY STARCHES DIFFERING IN GLYCEMIC INDEX ON PRO-OXIDANT AND ANTI-OXIDANT GENE EXPRESSION AND INSULIN SENSITIVITY IN A MOUSE MODEL

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INFLUENCE OF DIETARY STARCHES DIFFERING IN GLYCEMIC INDEX ON PRO-OXIDANT AND ANTI-OXIDANT GENE EXPRESSION AND INSULIN SENSITIVITY IN A MOUSE MODEL

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VITA

Katie E. Colbert, daughter of Tim and Carol Colbert, was born on August 12, 1981, in Winter Park, FL. Upon graduating from Oviedo High School in Oviedo, FL, Katie chose to attend her parents' alma mater, Auburn University. Katie graduated from Auburn University on May 13, 2005 with a Bachelor of Science Degree in Nutrition and Food Science, and an emphasis in Nutrition Science. Encouraged by her previous experience as an undergraduate research scholar, Katie applied to, and was accepted, as a graduate student in the Department of Nutrition and Food Science at Auburn University in August, 2005. Here, she studied as a graduate research assistant under the direction of Dr. Kevin Huggins in order to gain a Master of Science Degree in Nutrition and Food Science.

THESIS ABSTRACT

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The prevalence of obesity and diabetes has reached pandemic proportions worldwide. In the U.S., increased intakes of refined carbohydrates have been reported to parallel this trend. In general, refined carbohydrates (e.g. highly processed grains, sweeteners, and sugar-sweetened beverages) cause more rapid and larger increases in postprandial glycemia and insulinemia than do complex carbohydrates (e.g. whole-grains, fiber-containing products, legumes, fruits and vegetables). Thus, the concept of glycemic index (GI) was developed as a way of ranking carbohydrates according to how they affect postprandial glycemia. In human and animal studies, high-GI diets have been correlated with an increased risk of obesity, insulin resistance, type 2 diabetes, and coronary heart disease; however, the exact molecular mechanism(s) by which high-GI diets elicit adverse effects is unknown. Oxidative stress has been implicated in the pathogenesis of coronary heart disease, insulin resistance, and type 2 diabetes through an overproduction

of reactive oxygen species. Thus, the objectives of this study were to assess the chronic effects of a high-GI diet on 1) pro-oxidant and anti-oxidant gene expression in the adipose tissue, and 2) insulin sensitivity in male, C57Bl/6J mice.

Mice were fed either a high fat (HF) or a low fat (LF) diet, ad libitum, containing either a high-GI starch (100% amylopectin, Amioca®) or a low-GI starch (60% amylose/40% amylopectin, Hi-Maize® or 55% amylose/ 45% amylopectin, HylonV®) for 15 weeks. The expression levels of the pro-oxidant gene, NADPH oxidase, and the anti-oxidant genes, superoxide dismutase-2 (SOD-2), glutathione peroxidase-1 (GPx-1), and catalase were quantified in the epididymal adipose tissue by real-time, reverse transcriptase polymerase chain reaction (RT-PCR) methods. In addition, plasma glucose, insulin, total triacylglycerol, adiponectin, and leptin concentrations were assayed in plasma samples collected at the time of sacrifice. The main treatment effects, the interaction between fat and starch, and differences among the means between groups were assessed using one-way and two-way ANOVA, MANOVA with repeated measures tests, unpaired Student's *t* tests, and orthogonal contrast procedures. The results are reported as LSmeans ± SE, and were considered significant when $P < 0.05$.

In comparison to the LF groups, the high dietary fat resulted in increased weight gain, absolute and relative adipose and liver weight, fasting plasma glucose and insulin, insulin resistance, and NADPH oxidase gene expression. Conversely, increased dietary fat decreased plasma adiponectin concentration, and GPx-1 and catalase gene expression. The high-GI diet, compared to both low-GI diets, increased the post-prandial glycemic response during a meal tolerance test, as well as non-fasting blood glucose concentrations at 4 and 8 weeks. However, the type of dietary starch consumed (whether high-GI or low-

GI) did not influence the expression of pro-oxidant (NADPH oxidase) or antioxidant (Catalase, GPx-1, SOD-2) genes in the adipose tissue of male, C57Bl/6 mice. Similarly, with the exception of the HF/HylonV® group, the type of dietary starch consumed had no influence on insulin sensitivity. Thus, generation of oxidative stress in the adipose tissue of male, C57Bl/6 mice does not appear to be a mechanism by which high-GI diets elicit adverse effects.

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CHAPTER 1. INTRODUCTION

The endemic increase in the incidence of obesity and diabetes in recent decades has prompted researchers to investigate potential mechanisms by which dietary factors may influence the development and/or progression of these conditions (1). In the United States (U.S.), increased intakes of refined carbohydrates have been reported to parallel the upward trend in the prevalence of obesity and type 2 diabetes (2). Examples of refined carbohydrates include sugar-sweetened beverages (sodas), sweeteners, corn syrup, and processed grain products such as white bread and rice (3,4). Complex carbohydrates, on the other hand, consist of whole-grain, fiber-containing bread products (coarse rye bread), oats, barley, bulgar, beans, lentils, legumes, vegetables, and fruit (3-5). In general, refined carbohydrates cause more rapid and larger increases in postprandial (after a meal) blood glucose and insulin than do whole-grain products. Thus, the concept of glycemic index (GI) was developed by Jenkins et al (6) as a physiologic, rather than a structural approach, for classifying carbohydrates according to how much they raise postprandial blood glucose levels (4,7).

Foods with a high-GI (refined carbohydrates) are characterized by rapid digestion and absorption, resulting in large postprandial spikes in blood glucose (postprandial hyperglycemia). This results in an increased insulin demand, triggering hypersecretion of insulin from the pancreatic β-cells (4,7). Foods with a low-GI, on the other hand, are digested and absorbed more slowly, resulting in prolonged and continued absorption of

glucose from the gastrointestinal tract (4,7). Several human studies have correlated high-GI diets with an increased risk of coronary heart disease (CHD) (3,8), the metabolic syndrome (9), insulin resistance, and type 2 diabetes (10-12). Similarly, studies in rodent models have linked high-GI diets with increased adiposity (13-17), decreased lean body mass (16), enhanced lipogenic gene expression (18), increased plasma triglyceride concentration (13), decreased plasma adiponectin concentration (16), hypersecretion of insulin, and insulin resistance (15-17,19).

One observation shared among the animal studies was that altering dietary carbohydrate type, and thus, the GI of the diet, had specific effects on adipose tissue. Adipose tissue is now recognized as an active endocrine organ because it synthesizes and releases a group of biologically active molecules known as adipocytokines (e.g. leptin and adiponectin) (20-22). Interestingly, the normal balance of these adipocytokines is perturbed in obesity, and this alteration may influence the development of insulin resistance, diabetes, metabolic syndrome, and cardiovascular disease (20,23-25). Attempts to identify a link between metabolic disease and altered adipose tissue metabolism have pointed to the potential role of oxidative stress in mediating this complex process. Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses, in favor of the oxidants, potentially leading to tissue damage (26-28). ROS production has been shown to increase in parallel with fat accumulation *in vitro* (3T3-L1 cells) and *in vivo* (various mouse models of obesity) (20). Also, cultured adipocytes (3T3-L1 cells) exposed to hyperglycemic conditions displayed oxidative stress (29), and a direct link between postprandial hyperglycemia and oxidative stress production was identified by Ceriello et

al (30,31). These findings suggest that adipocytes may be susceptible to oxidative stress generated postprandially, or in accumulated fat, through chronic consumption of a high-GI diet.

Although previous studies have indicated that consumption of a high-GI diet may increase the risk of developing certain metabolic diseases, the exact molecular mechanism(s) by which high-GI diets mediate these effects is unknown. Furthermore, no studies to date have investigated the role of oxidative stress in chronic feeding of high-GI diets in experimental animals. Thus, the goal of this study is to examine the effects of a high-GI diet on the expression of genes involved in oxidative stress in the adipose tissue, as well as insulin sensitivity, in male, C57Bl/6 mice. The working hypothesis of this thesis is that mice consuming a high-GI diet will have reduced insulin sensitivity, increased expression of the pro-oxidant gene, NADPH oxidase, and decreased expression of the antioxidant genes, superoxide dismutase- 2, glutathione peroxidase- 1, and catalase, in the adipose tissue, compared to mice consuming the low-GI diets. Furthermore, the low-GI diets will exert protective effects against the generation of oxidative stress in the adipose tissue of mice consuming those diets.

CHAPTER 2. REVIEW OF LITERATURE

Obesity and Diabetes Trends

On a global scale, the magnitude of individuals classified as overweight (body mass index [BMI] of 25-29.9 kg/m^2) or obese (BMI $\geq$ 30 kg/m^2) has reached pandemic proportions (1). In the United States (U.S.) alone, the prevalence of obesity has doubled in the last two decades, with an estimated 35 and 30% of U.S. adults classified as overweight or obese, respectively (1). In the state of Alabama, obesity among adults has increased from less than 10% of the population, in 1986, to greater than 25% of the population, in 2005, according to data provided by the CDC's Behavioral Risk Factor Surveillance System (http://apps.nccd.cdc.gov/brfss/Trends/TrendData.asp). Although the underlying reasons for this global pandemic are multi-factorial, it is believed that the obesity surge can be predominantly attributed to the combined effects of excessive food intake and inadequate physical activity, or the adoption of a "westernized lifestyle" (1,32). Interestingly, a concomitant rise in the incidence and prevalence of obesity-related metabolic diseases, such as type 2 diabetes mellitus, has become evident as well, and closely parallels the obesity pandemic (1,33). In the year 2025, it is estimated that the number of adults with diabetes will reach 300 million worldwide (34). Similar increases in obesity and type 2 diabetes are also occurring in U.S. children, illustrating that these elevations are not due to the aging population alone (1). Due to the adverse health

consequences associated with obesity and diabetes, these diseases have become major public health concerns, and have placed enormous economic burdens on the Western world (1,33,35). Thus, the need to elucidate the main contributory causes of obesity and diabetes is warranted.

At first, public health initiatives targeted dietary factors, namely excessive consumption of high-fat (HF) diets, in an attempt to combat the alarming rise in obesity (32). Of all the macronutrients (fat, protein and carbohydrate), fat has the largest caloric density, providing 9 kilocalories of energy per gram consumed. Thus, excessive consumption of dietary fat was blamed as the nutritional culprit for the obesity phenomenon (3). This idea was further supported by evidence which revealed the adverse effects of HF diets on metabolic disease risk, including the development of obesity, type 2 diabetes, coronary vascular disease (CVD), and cancer (36,37). Taken together, these findings led to the development of dietary recommendations favoring decreased daily consumption of total dietary fat. However, as consumption of one macronutrient decreased, a reciprocal increase in the consumption of another macronutrient took place. In other words, as the prevalence of overweight and obesity increased in the U.S., a significant reduction in fat intake was observed, with a concomitant increase in carbohydrate intake (4,38,39). The quality and composition of dietary carbohydrates in the U.S. food supply changed significantly during the 20th century as well due to industrial and technological advances. Specifically, there was an increase in the production and availability of readily digestible, rapidly absorbed, refined carbohydrates (3). In fact, Gross and colleagues (2) reported that increased intakes of refined carbohydrates (in the form of corn syrup) paralleled the upward trend in the prevalence of

obesity and type 2 diabetes observed in the U.S. during this time (2). Thus, the paradoxical observation of a marked increase in overweight and obese individuals in the U.S., despite reduced dietary fat intake, suggested that dietary carbohydrate amount and type needed consideration in metabolic disease risk profiles as well.

Refined Carbohydrates

To understand better the nature of a refined carbohydrate, a brief mention of the components of a whole grain is needed. There are three parts to a whole grain, the germ, bran, and endosperm, which comprise 5, 15, and 80% of the whole grain, respectively (40). The refining (milling) process of grains involves removal of most of the bran, and much of the germ, resulting in a highly modified and easily digested starch (3). Examples of refined carbohydrates include sugar-sweetened beverages (sodas), sweeteners, corn syrup, potatoes, and processed grain products such as white bread and rice (3,4). On the other hand, complex carbohydrates consist of whole-grain, fiber-containing bread products (coarse rye bread), oats, barley, bulgar, beans, lentils, legumes, vegetables, and fruit (4,5). The main simple sugars in the human diet are glucose, fructose, and lactose, whereas the main complex carbohydrate is starch (41).

In their intact, physical form, whole grains are low in saturated fat, high in fiber, and high in other beneficial micronutrients and phytochemicals; however, as a consequence of the refining process, the remaining grain has a higher starch content, a lower micronutrient and fiber content, and is more susceptible to rapid enzymatic degradation in the digestive tract (3). In fact, a > 10% increase in caloric density, and an 80% and 30% decrease in dietary fiber and protein, respectively, have been reported from processing whole grains into white flour (42). Therefore, different types of carbohydrates

(refined or complex) exert different metabolic and physiologic effects, such that both the quantity and quality of carbohydrate consumed synergistically affect postprandial (following a meal) glycemic and insulinemic responses (7,32,37). In general, refined carbohydrates cause more rapid and larger increases in postprandial blood glucose and insulin than do whole-grain products. This is due to the fact that whole-grain carbohydrates are consumed in their intact physical form, and contain large quantities of fiber and enzymatic inhibitors. These features allow for a decrease in the gastric emptying rate, and also render the starch somewhat resistant to digestion (43,44).

Although dietary recommendations exist regarding the quantity of daily carbohydrate that should be consumed, less-detailed recommendations have been issued in relation to the quality of dietary carbohydrate that should be consumed (37). Currently, the official carbohydrate-related recommendations are high intake of fiber-rich carbohydrates (>55 E% [energy percent] carbohydrate, 25-35 g fiber), low consumption of sugar (< 10 E% sugar), and five to six servings of fruit and vegetables per day (45). To address the need for an alternative classification system which incorporated both the quality of carbohydrate consumed, as well as the resultant physiological response in the body, Jenkins and colleagues (6,7) proposed a concept known as the glycemic index (GI) of carbohydrate-containing foods, in 1981. Currently, no official recommendations exist regarding the GI of the diet (45).

Glycemic Index: Definition and Classification

The classification of carbohydrates according to their chemical and physical properties, such as ‘simple’ (mono- and disaccharides) or ‘complex’ (polysaccharides), arose from the idea that the degree of polymerization, or saccharide chain length, was the

only determinant in the rate of carbohydrate digestion (4,6,7,37) In recent decades, however, the importance of chain length in carbohydrate digestion rate has been questioned, given that this assumption does not incorporate the wide variation in the physiological effect, or blood-glucose-raising potential, of different carbohydrates (4,7,37). Thus, the GI is a physiologic, rather than a structural approach for classifying carbohydrate-containing foods according to glycemic response (4,46). Specifically, GI is defined as the area under the 2-h postprandial blood glucose concentration curve per 50 g available carbohydrate consumed from a test food, relative to a reference food (either white bread or pure glucose) (6,47). A high-GI food is defined as having a GI of ≥ 70, whereas a low-GI food is defined as having a GI of ≤ 55 (37).

Glycemic Load: Definition and Classification

Overall, the GI is useful in that it provides a quantitative assessment of carbohydrate-containing foods based on postprandial blood glucose response (4). However, both the quality and quantity of carbohydrates affect postprandial glycemia and insulinemia. Thus, the utility of GI in accurately predicting these physiologic responses was incomplete because it incorporated only the quality of carbohydrate into the equation. As a result, the term glycemic load (GL) was developed in order to represent both variables (quality and quantity) contributing to the glucose-raising potential of a carbohydrate, and increase the reliability of predicting the glycemic and insulinemic responses of a given diet (10,37). The GL of a particular food is defined as the product of the GI value of that food and the amount of available carbohydrate (g) (that without fiber) in a serving, and is intended to represent the overall glycemic effect of a diet. The total GL of a meal may then be determined by summing the GL contributed by each individual

food (10,48). In fact, with respect to predicting the glycemic response to mixed meals, GL is thought to be superior to either total carbohydrate intake or GI alone (47,48). Foods with a GL value ≤ 10 are considered low GL foods, whereas those with a GL value ≥ 20 are classified as having a high GL (49,50). The American Diabetes Association recommended the use of GI and GL in the management of diabetes in that they may provide a modest additional benefit over that observed when total carbohydrate is considered alone (5).

Factors Affecting the GI of Foods

The rate of digestion and absorption of a carbohydrate, and ultimately, its blood-glucose-raising potential (GI), depends on certain chemical and physical properties of the carbohydrate, as well as the presence of other food components (37). Chemical properties influencing the GI of a carbohydrate include the type of constituent monosaccharides (glucose [highest GI], fructose, galactose), the ratio of amylose to amylopectin present in the raw food (amylose has a lower GI), and the presence of viscous soluble fibers, which can lower GI by delaying gastric emptying and glucose absorption (46,51-53). With respect to the physical properties of carbohydrates, those with an intact botanical structure (course outer layer [wheat bran]) are protected from enzymatic degradation and thus, have a lower GI (54,55). In addition, the extent of ripeness, cooking, processing and refining, which increases the digestibility of the carbohydrate, also increases the GI (56-58). Lastly, the macronutrient distribution of a meal may influence the glycemic response. For example, co-ingestion of fat and/or protein may modify the glycemic response to a carbohydrate-containing food by slowing gastric emptying, and altering the glycemic response (4,5,37). In fact, Henry and colleagues (59) found that the addition of

high fat, high protein toppings to baked potatoes, pasta, and toast (high-GI foods) had a consistent lowering effect on the GI value of that meal.

Effects of GI on Metabolism

The metabolic effect of a carbohydrate refers to how quickly and efficiently the carbohydrate-digesting enzymes, such as pancreatic amylase and numerous other disacchararidases, can break down the carbohydrate into its constituent monosacharrides for absorption along the intestinal brush border. The physiologic effect of a carbohydrate refers to the rate and magnitude with which glucose enters the bloodstream following digestion and absorption, as well as the subsequent demand placed on the pancreas to secrete sufficient amounts of insulin to normalize blood glucose levels. Thus, the resulting hormonal milieu of the body following consumption of a carbohydrate-containing food is highly governed by the quality and quantity of carbohydrate consumed (4,7,37).

In general, foods with a high-GI are characterized by rapid digestion and absorption, resulting in large postprandial spikes in blood glucose. This results in an increased insulin demand, triggering hypersecretion of insulin from the pancreatic beta cells, while strongly inhibiting glucagon release from the pancreatic alpha cells (4,37). The high insulin-to-glucagon ratio in the bloodstream inhibits nutrient mobilization from peripheral tissues, and stimulates rapid uptake of nutrients by insulin-sensitive tissues (muscle, liver, adipose) (37). Overall, the increased ratio of insulin-to-glucagon in the blood exerts a powerful anabolic stimulus favoring nutrient storage, and inhibiting nutrient oxidation (7,60). In the hours following consumption of a high-GI meal, nutrient absorption from the gastrointestinal tract declines; however, the high insulin-to-glucagon

ratio persists. This hormonal environment causes blood glucose to fall rapidly, often into the hypoglycemic range (reactive hypoglycemia), and triggers the release of counterregulatory hormones (cortisol, glucagon, growth hormone)(7,37,60). Counterregulatory hormones function to replenish the bloodstream with the appropriate level of fuels by increasing hepatic gluconeogenesis, decreasing glucose uptake by the skeletal muscle, and increasing lipolysis and non-esterified (free) fatty acid release by the adipose tissue (7,37).

Foods with a low-GI, on the other hand, are digested and absorbed more slowly, resulting in prolonged and continued absorption of glucose from the gastrointestinal tract. Lowering the GI reduces postprandial hyperglycemia and hyperinsulinemia, and stabilizes the hormonal environment following a meal such that reactive hypoglycemia and postprandial rebounds in plasma free fatty acids do not occur (4,37). Thus, due to the absence of a sustained increase in the insulin-to-glucagon ratio in the blood, consumption of low-GI foods are believed to have beneficial effects on rates of lipid oxidation and preservation of lean body tissue (60). This is important given that some of the purported beneficial effects of consuming a low-GI diet, including reduced insulin demand, improved glycemic control, and reduced plasma lipid levels, all relate to a reduction in the rate of carbohydrate absorption, and thus, reduced postprandial glycemia (4).

GI and Postprandial Hyperglycemia

Certain extrinsic variables may influence one's glycemic response to a carbohydrate-containing food, including fasting or pre-prandial (before a meal) blood glucose level, available insulin, and degree of insulin resistance (5). Postprandial hyperglycemia occurs following a meal, when the homeostatic mechanisms aimed at

minimizing glucose fluctuations and restoring normal glucose levels, are blunted (61). In fact, it has been suggested that the increase in the postprandial glycemia and insulinemia following consumption of a high-GI meal may contribute to β-cell exhaustion and development of type 2 diabetes in insulin resistant individuals (10,11,32). Thus, the extrinsic factors mentioned above are highly pertinent in individuals suffering from pre-diabetes or overt diabetes mellitus, given that fasting hyperglycemia and impaired insulin secretion/action may all exacerbate the postprandial glycemic response to a given meal. In these individuals, insulin-mediated suppression of hepatic glucose production, insulin-stimulated glucose uptake in peripheral tissues, and insulin-mediated suppression of fatty acid release from the adipose tissue are all likely impaired (61,62).

One of the primary goals in the management of diabetes is to maintain tight glycemic control, so as to reduce the risk of developing complications over time (63,64). Consideration of postprandial hyperglycemia is relevant in this respect because it contributes substantially to overall glycemia, and has been shown to impact hemoglobin A_{1c} values (HbA_{1c}; currently the gold standard for assessing and monitoring glycemic control), the risk of microvascular complications (e.g. nephropathy and retinopathy), and to a greater extent, the risk of macrovascular disease (CVD), the chief cause of morbidity and mortality in patients with type 2 diabetes (65-68). In fact, Monnier et al (69) reported that the contribution of postprandial glucose to total glycemic load in type 2 diabetic patients varied according to the degree of glycemic control, as assessed by HbA_{1c} values. For example, in patients with $HbA_{1c} > 10.2\%$ (poor glycemic control), postprandial glucose contributed only 30% of the 24-hour area under the blood glucose curve; however, in patients with HbA_{1c} values $< 7.3\%$ (better glycemic control), postprandial

glucose contributed ≥70% of the 24-hour area under the blood glucose curve (69). This study was one of the first of its kind demonstrating that postprandial glucose may be a clinically relevant guide to glycemic control (65,69). Furthermore, high-GI foods may have an additive effect on the degree of postprandial hyperglycemia experienced, such that chronic and frequent postprandial excursions in blood glucose may contribute to the pathophysiology of diabetic complications (70). Therefore, recommending consumption of foods with complex, fiber-containing carbohydrates, or low-GI foods, may aid in reducing the magnitude and frequency of glucose fluctuations, and serve as an attractive dietary strategy for the management of postprandial hyperglycemia.

Effects of GI in Humans

Several cross-sectional, intervention, and prospective cohort studies in humans have shed light on the potential usefulness of dietary GI/GL in the determination and modification of metabolic diseases. For example, a positive association was found between dietary GL and risk of coronary heart disease (CHD) in a large cohort of US women (n = 75,000) after a 10 year follow up (8). Furthermore, a reduced risk in the development of type 2 diabetes in men and women was associated with lower dietary GL in two studies conducted by Salmeron and colleagues (10,11,37). High cereal fiber intakes also paralleled this trend towards decreased risk of diabetes (10,11,37). These findings suggest that decreased consumption of refined carbohydrates may reduce the incidence of diabetes in healthy individuals. Consistent with this assumption, diets high in rapidly absorbed carbohydrates, and low in cereal fiber, were associated with an increased risk of type 2 diabetes in young and middle-aged women, in a prospective examination of the association between GI/GL and dietary fiber (12). Furthermore, a

positive association between high dietary GI and prevalence of metabolic syndrome and insulin resistance was reported in subjects obtained from the Framingham Offspring cohort (9). Similarly, high intakes of fiber and whole grains, both characteristics of low GI foods, were related to lower insulin resistance in this study (9). Liu and colleagues (71,72) reported associations between GI/GL, unfavorable lipid profiles, and elevated inflammatory status by demonstrating a positive relationship between dietary GI/GL and plasma levels of C-reactive protein (a hepatocyte-derived acute-phase reactant protein and sensitive marker of systemic inflammation) and fasting triglycerides in cohorts of women obtained from the Nurses' Health Study. Taken together, these studies provided evidence for the potential role of high GI, low fiber diets in the manifestation of metabolic diseases and their associated complications.

Several intervention studies have also reported beneficial effects of consuming low GI diets on health outcomes. For example, a study conducted in men with advanced CHD, and in women at an increased risk of CHD, revealed that consumption of low GI diets for 3-4 weeks resulted in improved insulin sensitivity in these subjects compared to those consuming a high GI diet (73,74) Furthermore, enhanced insulin secretion was reported not only in subjects with impaired glucose tolerance, following consumption of a low GI diet for 4 months, but also in post-menopausal women who consumed high-fiber rye bread (low GI) for 8 weeks, relative to those that consumed white wheat bread (higher GI) (75,76). However, significant improvements in peripheral insulin sensitivity were not found in these two studies, suggesting that those individuals experienced improved β-cell function as a result of low dietary GI (75,76).

Despite the purported beneficial effects of low-GI diets on various health outcomes, other studies investigating the effects of GI on disease risk, insulin resistance/sensitivity, β-cell function, and lipid profiles have disputed the legitimacy of these findings (37). For example, no relationship between GI and diabetes risk was found in a 6-year follow-up study of post-menopausal women from the Iowa Women's Health Study (77). Also, although associations were observed between GI/GL and CHD risk in U.S. women (8), no relationship was found between GI and CHD risk in men from the Zutphen Elderly Study (78). In addition, no association was found between dietary GI, GL, total carbohydrate intake (including simple sugars), and probability of having insulin resistance from the Danish Inter99 study (79). Consistent with this finding, no relationship was found between GI, GL, carbohydrate intake, and measures of insulin sensitivity, insulin secretion, and adiposity in a cohort of subjects with normal and impaired glucose tolerance from the Insulin Resistance Atherosclerosis Study (80).

Some investigators have suggested that the beneficial effects of a low GI diet may pertain only to those individuals at an increased risk of, or already diagnosed with, certain metabolic diseases. For example, no effects of consuming a low GI diet on degree of insulin resistance/sensitivity or β-cell function were observed in lean subjects, or those with no parental history of CHD (74,81,82). In fact, the relationship between GI/GL and fasting plasma triglycerides, plasma CRP concentration, risk of type 2 diabetes, and risk of CHD are all stronger among individuals with higher BMIs (8,10,11,71,72,77,83). Perhaps the most inconsistent findings, however, have been with the ability of a low-GI diet to modify lipid profiles in individuals with dyslipidemia. In fact, a Cochrane meta-analysis was conducted in regards to the role of low-GI diets in CHD risk, in which

fifteen randomized controlled trials in subjects with pre-existing CHD risk factors were reviewed (84). This meta-analysis concluded that low-GI diets may provide minor reductions in total cholesterol; however, there was no evidence for the beneficial effects of low-GI diets on other lipids.

One reason for the disparities in these findings may be that food frequency questionnaires (FFQs) were used to assess the GI of diets; however, many of the FFQs were not originally designed measure this concept and thus, may have lack content validity to some extent (37). Also, under-reporting of dietary intake often occurs in FFQs, and may have contributed to inconsistencies in the findings. With respect to intervention studies, several factors may have contributed to the discrepancies observed, including small sample sizes, lack of sufficient statistical power, short duration of the study, subject type, and/or intervention diet composition (37). Due to the high degree of variation in the findings from human studies, health and government agencies in the U.S. have declined to endorse the concept of GI for nutritional intervention (48).

Another area of research fueling intense debate among the scientific community is the role of GI in body weight regulation and obesity. Consumption of a low-GI diet is believed to exert beneficial effects on weight control by promoting satiety and fat oxidation, at the expense of carbohydrate oxidation (60). These assumptions arise from the postprandial reduction in glycemia and insulinemia observed following consumption of a low-GI meal; however, a recent review (85) failed to demonstrate that meals or diets with contrasting glycemic indices had significant effects on carbohydrate or fat oxidation and body composition in humans (32,85). Furthermore, results from a systematic review

of human intervention studies comparing the effects of high-GI vs. low-GI meals on appetite, food intake, energy expenditure, and body weight were inconclusive (86). With respect to short-term studies (<1 day), 15 reported an association between low-GI meals and reduced hunger (greater satiety), whereas 14 found no difference in appetite sensations following consumption of a low-GI meal. Similarly, 14 longer-term intervention studies (<6 months) found no difference in weight loss on a low-GI diet, whereas 4 studies reported an average weight loss of 1.5 kg, and 2 studies actually reported weight loss on the high-GI diet (86). However, many of these studies have been criticized for lacking sufficient statistical power to pick up clinically relevant differences in weight loss (86).

Effects of GI in Animals

In an attempt to circumvent some of the inconsistencies observed in human studies, researchers have also investigated the effects of dietary GI using animal models. Experimental studies using animal models are excellent ways with which to examine both the mechanisms and the effects of dietary components *in vivo*, largely because food intake can be closely monitored and controlled in this setting. However, animal models are also useful in that large, uniform sample sizes can be generated, sufficient controls can be established, and tighter control over confounding variables can be established.

Lerer-Metzger and colleagues (13) investigated the effects of feeding normal and diabetic (streptozotocin [STZ]-injected), male Sprague-Dawley rats, high- (wheat starch) or low-GI diets (mung-bean starch), ad libitum, for 5 weeks. They observed decreases in fed (non-fasting) blood glucose and free fatty acid levels in normal rats on the low-GI

diet, as well as decreases in plasma triacylglycerol levels and adipocyte volume in both normal and diabetic rats fed the low-GI diet (13). In a similar study, in which normal and STZ-diabetic, male Sprague-Dawley rats were fed high-GI (waxy cornstarch) or low-GI (mung bean starch) diets for 3weeks, an increase in insulin-stimulated glucose oxidation, and a decrease in adipocyte diameter and glucose incorporation into total lipids was observed in both normal and diabetic rats fed the mung-bean starch (low-GI) diet (14). In addition, Kabir and colleagues (18) reported increased adipose glucose transporter 4 (GLUT4) and fatty acid synthase (FAS) gene expression, as well as increased FAS activity in the adipose tissue of normal rats fed the high-GI (waxy corn starch) diet.

Given the reported alterations in glucose metabolism, lipid profiles, and adipose tissue characteristics in rats on high-GI diets, it was plausible to assume that GI may also affect insulin sensitivity. In fact, Pawlak and colleagues (15) set out to determine the effects of feeding (for 7 weeks) high-GI (amylopectin) or low-GI (amylose) starches on insulin sensitivity and insulin secretion in Wistar rats. Despite higher peak plasma insulin during an intravenous glucose tolerance test (IVGTT), and greater epididymal fat accumulation in the high-GI rats compared to low-GI rats (with similar body weights), no difference in hepatic or peripheral insulin sensitivity was observed after 7 weeks of feeding. These findings suggest that hypersecretion of insulin, in the absence of insulin resistance, may contribute to enhanced lipogenesis in rats fed high-GI diets (15). However, 7 weeks might not have been sufficient to reveal the development of insulin resistance in these animals. For example, one study fed high amylopectin (high-GI) or high amylose (low-GI) diets to Wistar rats in order to observe differences in the insulin response to an IVGTT (19). Although not apparent at 4 weeks, differences in the insulin

response to an IVGTT began to appear at 8 weeks, and by 12 weeks, rats on the high amylopectin diet displayed a 100% increase in the insulin response relative to those fed the high amylose diet. Furthermore, basal plasma insulin concentration was greater in rats fed the high amylopectin diet (19). These factors were indicative of an insulin resistant, pre-diabetic state.

To mimic the decrease in β-cell mass observed in humans with pre-diabetes, Pawlak and colleagues (16) conducted a long-term study in which partial pancreatectomies were performed on Sprague-Dawley rats (16,87). The animals were fed high-GI (100% amylopectin) or low-GI (60% amylose/40% amylopectin) diets for 18 weeks, during which food intake was restricted in the high-GI group to maintain similar body weights among the groups. Consistent with previous findings, rats fed the high-GI diet had significantly greater adipose mass (differences emerged at 9 weeks) and plasma triglyceride concentrations, and decreased lean body mass, relative to rats fed the low-GI diet (16). Also, areas under the blood glucose and plasma insulin curves following oral glucose tolerance tests (OGTT) were greater in the high-GI group, suggesting impaired glucose tolerance in these animals (16). Lastly, a decrease in plasma adiponectin levels, an anti-inflammatory protein secreted from the adipose tissue and inversely associated with insulin resistance, was observed in the high-GI group (16,88,89). Thus, to investigate whether or not GI could similarly promote changes in adiposity in an obesity-prone mouse strain, Pawlak and colleagues (16) fed C57Bl/6 mice, ad libitum, high-GI or low-GI diets for 9 weeks. Consistent with the changes observed in body composition in rats, mice fed the high-GI diet displayed a significant increase in adipose mass, coupled with a decrease in lean body mass, relative to mice on the low-GI diet (16). These

findings suggested that consumption of a high-GI diet may adversely affect risk factors associated with diabetes and cardiovascular disease in two different rodent models (7,16,90).

Morris and Zemel (17) extended these findings in a study using a transgenic mouse model of diet-induced obesity (adipocyte fatty acid binding protein [aP2]-*agouti* mice), such that the metabolic response to four high-fat (HF, 24% of energy as fat) test diets, varying only in carbohydrate source, could be investigated. In the energy-restricted phase of the study, animals fed the HF/low-GI diets (carbohydrate source obtained from rolled oats or mung bean noodles) displayed reductions in subcutaneous and visceral adipose mass, as well as a decreased plasma insulin-to-glucagon ratio relative to animals fed the HF/high-GI diets (carbohydrate source obtained from instant oatmeal with added sugar or flavored instant oatmeal) (17). In the ad libitum phase of the study, mice given the HF/low-GI diets displayed significant decreases in hepatic fatty acid synthase (FAS) gene expression compared to mice fed the HF/high-GI diets (17). Overall, this study demonstrated that qualitative changes in dietary carbohydrate source influence body composition and weight in aP2-*agouti* transgenic mice (17).

In contrast to the high degree of variation in the findings from human studies, the aforementioned animal studies have provided more consistent and reliable data with respect to the role of dietary GI in metabolic outcomes. Animal models also comprise a unique system in which to investigate mechanisms of dietary components due to the availability of transgenic rodent models, the ability to use surgical techniques as experimental tools, and the ability to control for confounding variables and food intake.

Taken together, these animal studies have provided insight into the potential influence of high-GI diets on body composition, gene expression, and glucose metabolism; however, longer-term intervention studies are warranted to elucidate the exact molecular mechanism(s) through which high-GI diets elicit their effects.

Adipose Tissue as an Endocrine Organ

One observation shared among the animal studies was that altering dietary carbohydrate type, and thus, the GI of the diet, had specific effects on adipose tissue. This was an interesting finding, given that adipose tissue is recognized not only as an energy storage depot, but also an active endocrine organ, and integrator of various physiological pathways (21,22). In addition to the more traditional roles of regulating fat mass and nutrient homeostasis (through lipolysis and release of free fatty acids into the bloodstream when energy is low), adipose tissue is also involved in the control of blood pressure, haemostasis, bone mass, and thyroid and reproductive function (22,91). Moreover, the adipose tissue is unique in that it coordinates many of these processes through the synthesis and release of specific adipocyte-derived secretory proteins, such as leptin (92), adiponectin (93-96), resistin (97,98), visfatin (99), and retinol-binding protein-4 (100), to name a few. Adipocytes are also capable of secreting pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα; 24,25), interleukin-6 (IL-6; 101), monocyte chemoattractant protein-1 (MCP-1; 102), and plasminogen activator inhibitor-1 (PAI-1; 103). Collectively, these adipocyte-derived secretory proteins and pro-inflammatory cytokines comprise a group of biologically active molecules known as adipocytokines (20,21,104). Interestingly, the normal balance of

these adipocytokines is perturbed in obesity, but the exact mechanism by which adipose tissue accumulation leads to dysregulation of adipocytokines remains to be elucidated (20,23). Furthermore, alterations in the synthesis and release of adipocytokines may influence the pathogenesis of certain disease states, including insulin resistance, diabetes, metabolic syndrome, and CVD (20,23,24,25). Attempts to identify a link between metabolic disease and altered adipose tissue metabolism have pointed to the potential role of oxidative stress in mediating this complex process. In fact, reactive oxygen species (ROS) production has been shown to increase in parallel with fat accumulation both *in vitro* (3T3-L1 cells) and *in vivo* (various mouse models of obesity) (20).

Obesity and Oxidative Stress

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses, in favor of the oxidants, potentially leading to tissue damage (26-28). ROS include damaging free radicals such as superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^{\cdot-}$), and hydroxyl ($OH^{\cdot}$), and non-radicals such as hydrogen peroxide (H_2O_2) (105). It is believed that oxidative components, such as ROS, are generated locally within tissues and cells, and then released into systemic circulation, rendering DNA, proteins, and lipids vulnerable to their damaging effects (106). Thus, an individual's susceptibility to oxidative stress is dependent upon the overall balance between factors that exert oxidative stress, and those that exhibit antioxidant capabilities. Oxidative stress has been implicated in the pathogenesis of numerous diseases, and has also been shown to induce inflammatory pathways (107). In fact, it is believed that the macrovascular and microvascular complications associated

with diabetes are all related to an overproduction of superoxide by the mitochondrial electron-transport chain (108-110). Hyperglycemia has been shown to stimulate ROS production in adipocytes, which leads to an increased production of proinflammatory cytokines (29). Given that inflammation is present in obesity, insulin resistance, type 2 diabetes, and CVD, it has been hypothesized that oxidative stress is a common factor underlying each of these conditions (107,111).

In normal individuals, the body utilizes several antioxidant enzymes in order to quench free radicals, such as superoxide, that are generated naturally as a result of oxidative metabolism. However, in obese or diabetic individuals, the body is in a state of metabolic overload, with an increased energy flux through glycolysis, the tricarboxylic acid (TCA) cycle, and the electron-transport chain (110,112). Consequently, an overproduction of electron donors by the TCA cycle occurs, overwhelming the electron-transport chain. This process results in the reduction of molecular oxygen to superoxide within the mitochondria of cells (108-110). In the mitochondria, manganese superoxide dismutase (MnSOD or SOD-2), a key intracellular antioxidant enzyme, catalyzes the rapid conversion of two superoxide anions to hydrogen peroxide and molecular oxygen (110). Following this reaction, two other intracellular antioxidant enzymes, glutathione peroxidase (GPx-1) and catalase, rid the cell of excess hydrogen peroxide. For example, GPx-1 is responsible for the conversion of hydrogen peroxide into two water molecules in a reaction that also requires two molecules of reduced glutathione. In addition, catalase is responsible for the conversion of two hydrogen peroxide molecules into two water molecules and molecular oxygen (113).

Aside from mitochondrial production of superoxide, another major source of ROS within the body comes from the multi-subunit enzyme, NADPH oxidase. In fact, increased NADPH oxidase activity has been implicated in the pathogenesis of hypertension and atherosclerosis by increasing oxidative stress (114). Recently, Furukawa et al (20) investigated the contribution of oxidative stress in obesity and obesity-associated metabolic syndrome. They demonstrated an increase in local oxidative stress production *in vivo* in three different mouse models of obesity (KKA_y, *db/db*, and diet-induced obese mice). This was evidenced by increased lipid peroxidation (represented by thiobarbituric acid reactive substance [TBARS]) and hydrogen peroxide (H_2O_2) production in the white adipose tissue (WAT) of obese mice; however, no such increase occurred in the skeletal muscle, liver, or aorta of these animals (20). A similar increase in plasma TBARS and H_2O_2 was also observed, suggesting that white adipose tissue is the predominant source of increased systemic ROS in obese animals (20). Moreover, the mRNA expression of adiponectin, as well as the key antioxidant enzymes, copper,zinc SOD (Cu,Zn-SOD), GPx-1, and catalase, were all decreased in the WAT of obese mice, whereas the mRNA expression of the pro-oxidant enzyme, NADPH oxidase, was increased (20). These findings suggest that the effects of postprandial nutrient metabolism are first realized in the adipose tissue through changes in redox status.

Postprandial Oxidative Stress

In Western societies, a large part of the day is spent in the postprandial state (106). This is problematic given that metabolic processes following a lipid and/or carbohydrate-rich meal may amplify postprandial oxidative stress, a condition

characterized by increased susceptibility toward oxidative damage following consumption of a meal (31,106,115). Interestingly, postprandial hyperglycemia, a phenomenon that occurs following consumption of a high-GI meal, has been shown to contribute to the development of metabolic disease through generation of oxidative stress (7,116). Hyperglycemia induces an overproduction of ROS by the mitochondrial electron-transport chain, which in turn, is capable of activating the proinflammatory cascade (110,117). In fact, acute hyperglycemia in subjects with normal and impaired glucose tolerance was shown to induce an increase in plasma IL-6 and TNFα concentrations, whereas treatment with the antioxidant, glutathione, completely prevented this rise in systemic inflammatory cytokines (118). Moreover, Ceriello and colleagues (30,31) reported a direct link between postprandial glycemia and the induction of oxidative stress in that a significant depletion of plasma antioxidant capacity (assessed via total radical-trapping antioxidant parameter; TRAP) was observed during an oral glucose tolerance test in both normal, and type 2 diabetic individuals (30). Consistent with this finding, Ceriello et al (116) also reported an increase in plasma malondialdehyde (MDA; marker of lipid peroxidation), and a concomitant decrease in plasma TRAP in type 2 diabetic subjects following consumption of a standard meal, suggesting that postprandial metabolic processes favor oxidative stress production in diabetic individuals (116). These findings were extended in a separate study (31) in which increased postprandial low-density lipoprotein (LDL) oxidation was shown to be directly related to the degree of hyperglycemia in diabetic individuals (31). Furthermore, antioxidant treatment may alleviate postprandial oxidative stress, given that 4 weeks of Vitamin E supplementation in individuals with controlled diabetes resulted in a decrease

in plasma TBARS, and an increase in reduced glutathione, a powerful antioxidant (119). Recently, Monnier et al (120) demonstrated that postprandial glucose excursions in type 2 diabetic subjects exhibit a more specific triggering effect on oxidative stress (assessed by 24-h urinary 8-iso prostaglandin $F_{2\alpha}$) than chronic sustained hyperglycemia alone. Similarly, Hu et al (121) found a positive association between dietary GI/GL, and 2 markers of lipid peroxidation (MDA and F_2-isoprostane) in the plasma of healthy adults. Thus, by reducing postprandial glycemic excursions, low-GI/GL diets may prove beneficial in ameliorating postprandial hyperglycemia-induced overproduction of ROS.

Whole Grains, Fiber, and Inflammation in Humans

In addition to ameliorating postprandial hyperglycemia-induced oxidative stress production, low-GI/GL foods may also be beneficial in augmenting hyperglycemia-induced activation of inflammatory responses. For example, in healthy women, high dietary GL was associated with increased levels of CRP (72), a clinical indicator of inflammation, whereas treatment with a low-GL diet resulted in a 50% decrease in CRP levels (122). Moreover, in a cohort of 902 diabetic women from the Nurses' Health Study, a significant dose-response effect was observed between dietary GI and levels of CRP and tumor necrosis factor receptor 2 (TNF-R2) concentrations (123). Whole grain and bran (good sources of fiber) intakes were associated with significantly lower CRP and TNF-R2 levels as well (123). Also, significant associations between GI/GL, cereal fiber, and plasma adiponectin levels (an anti-inflammatory cytokine) were reported in men (124) and women (125) with type 2 diabetes. These findings suggest that low-GI/GL

diets may protect against inflammation, and that this protection might be mediated through the synergistic effects of fiber and whole grains (40,126).

Rationale, Objectives, and Hypotheses

The overall objective of this study is to elucidate a potential mechanism by which high-GI diets may act to induce obesity, insulin resistance, and diabetes. The overall hypothesis of this study is that chronic consumption of a high-GI diet will result in 1) postprandial hyperglycemia, 2) generation of oxidative stress in the adipose tissue of C57Bl/6 mice, and 3) altered whole-body glucose metabolism, leading to the development of insulin resistance and obesity. Numerous human studies have provided evidence for the beneficial effects of consuming low-GI/GL, high fiber diets, on metabolic disease risk profiles, obesity, inflammation, oxidative stress, insulin resistance, type 2 diabetes, and CHD; however, disparities in these reports have prevented the adoption of nutrition recommendations with respect to GI or GL (48). Furthermore, several investigators have extended the observations from human studies *in vivo*, in obesity-prone animal models. A common observation among the dietary intervention studies in animals was that altering dietary carbohydrate type, and thus, the GI of the diet, had specific effects on adipose tissue. Interestingly, oxidative stress has been shown to increase in parallel with fat accumulation both *in vitro* (3T3-L1 cells) and *in vivo* (obese mouse models) (20). Also, cultured adipocytes (3T3-L1) exposed to hyperglycemic conditions displayed oxidative stress (29). Thus, it is plausible to suspect that high-GI diets result in accumulation of adipose tissue and generation of oxidative stress, in addition to postprandial oxidative stress as a result of frequent glycemic excursions.

However, there have been no studies to date investigating the role of oxidative stress in chronic feeding of high-GI diets in obesity-prone, C57Bl/6 mice.

The specific objective of this thesis is to determine the chronic effects of high-GI diets, under LF and HF conditions, on insulin sensitivity, and pro-oxidant and antioxidant gene expression in the adipose tissue of C57Bl/6 mice. The working hypothesis of this thesis is that 1) high-GI diets will induce oxidative stress in the adipose tissue of male, C57Bl/6 mice by increasing the expression of the pro-oxidant gene, NADPH oxidase, and decreasing the expression of the antioxidant genes, SOD-2, GPx-1 and catalase, and 2) mice on the high-GI diet will have reduced insulin sensitivity compared to mice on the low-GI diets. A long-term feeding study in an obesity-prone animal model (e.g. C57Bl/6 mice) would significantly contribute to our understanding of the mechanism(s) by which high-GI diets elicit adverse effects.

The decision to examine the effects of a high-GI diet under LF and HF conditions was based on several factors. First, there have been no long term animal studies to date investigating the effects of a HF, high-GI diet on oxidative stress and insulin sensitivity. Second, the HF, high-GI diet closely mimicked the typical Western diet, rich in fat and refined carbohydrates, and provided a physiologically relevant setting with which to investigate mechanisms of dietary components. Lastly, it has been argued that the beneficial effects of a low-GI diet pertain only to individuals that are obese, or already diagnosed with certain metabolic diseases. Thus, use of a HF diet in order to induce obesity and insulin resistance in C57Bl/6 mice allowed for the discrimination of any beneficial effects of a low-GI diet in a metabolically challenged rodent.

Selection of the starches used in this study was also based on several factors. To begin with, a commonly used high-GI starch in previously published animal studies has been a waxy cornstarch, comprised of 100% amylopectin (14-16,18,19). Waxy cornstarch was previously determined to have a GI value of 107 ± 7 (14). The high-GI starch used in the present study (Amioca®) was also a waxy cornstarch, comprised of 100% amylopectin; however, Amioca® is a purified starch that has not been used in previously published animal studies. It was suspected that Amioca® would similarly have a high-GI value based on its intrinsic properties. Second, a commonly used low-GI starch in previously published animal studies has been a high amylose cornstarch (Hi-Maize®), comprised of approximately 60% amylose / 40% amylopectin (15,16,19, 138). Although the exact GI value of this starch has not been published, Hi-Maize® has been consistently shown to induce a significantly lower postprandial glycemic and insulinemic response compared to waxy cornstarch (15,16,19, 138). Thus, in the present study, a purified, resistant, high amylose cornstarch (Hi-Maize 260 ®) was utilized as a low-GI starch based on its reported effects on postprandial blood glucose and insulin concentrations. In addition, a second low-GI starch, HylonV®, was used in this study. HylonV® is a purified, high amylose cornstarch, comprised of approximately 55% amylose / 45% amylopectin; however, there have been no published studies to date utilizing this starch. In the present study, HylonV® was used as an additional low-GI starch based on its high amylose content.

CHAPTER 3. MATERIALS AND METHODS

Animals, Diets and Experimental Design:

All animal care, experimental procedures, and euthanasia protocols were reviewed and performed with approval from, and according to the regulations of the Institutional Animal Care and Usage Committee of Auburn University. Six to eight-week-old male, C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and Harlan (Indianapolis, IN), and adapted to the environment for one week prior to the study. All mice were housed in colony cages (4 mice per cage) on a 12-h light/dark cycle in a temperature-controlled environment. The experimental diets used in this study (Harlan-Teklad, Madison, WI) contained one of three different types of starches (National Starch, Bridgewater, NJ), in low-fat (LF) and high-fat (HF) combinations, for a total of six experimental diets (n = 8-10 mice/group). The first starch was a waxy maize cornstarch comprised of 100% amylopectin (Amioca® or AM); the second was a high amylose resistant cornstarch, comprised of 60% amylose / 40% amylopectin (Hi-Maize® or HM); the third was a high amylose cornstarch comprised of 55% amylose / 45% amylopectin (Hylon V® or HY). The macronutrient compositions of the LF diets, expressed as a percentage by weight (g/kg), are as follows: high-GI (H-GI, AM, TD.06136) and low-GI (L-GI, HY, TD.06137; L-GI, HM, TD.06138), 17.9% protein,

6.3% fat, 62.1% carbohydrate. The macronutrient compositions of the LF diets, expressed as a percentage of total energy, are as follows: H-GI, AM and L-GI, HY, 19% protein, 15% fat, 66% carbohydrate; L-GI, HM, 21% protein, 17% fat, 62% carbohydrate. The macronutrient compositions of the HF diets, expressed as a percentage by weight (g/kg), are as follows: H-GI, AM (TD.06536), L-GI, HY and L-GI, HM (TD.06537 and TD.06538), 17.7% protein, 18.2% fat, 50.9% carbohydrate. The macronutrient compositions of the HF diets, expressed as a percentage of total energy, are as follows: H-GI, AM and L-GI, HY, 16.2% protein, 37.4% fat, 46.5% carbohydrate; L-GI, HM, 17.4% protein, 40.2% fat, 42% carbohydrate. The H-GI and L-GI diets differed only in the nature of the component starch. The amount of starch (carbohydrate) in the HF diet was reduced in order to add fat in the form of lard.

Mice were fed the experimental diets (Harlan-Teklad, Madison, WI) ad libitum for 15 weeks, and given free access to water. Body weights were recorded weekly, with the exception of week 9 in the LF diet groups. Blood samples were collected from the tail vein after 4, 8 and 15 weeks of experimental feeding for blood glucose analysis using a handheld glucometer (OneTouch® Ultra® Meter, OneTouch® Ultra® Test Strips with FastDraw™ Design, Lifescan). The compositions of the LF and HF diets are listed in Tables 1 and 2, respectively.

This study utilized a 2X3 factorial design, given that two factors, fat and starch, were present, and the type of starch existed at three different levels (AM, HM, HY). As a result of injuries experienced due to fighting in colony cages, nine mice had to be sacrificed prior to the completion of the study. Consequently, certain analyses contained unequal sample sizes.

Meal Tolerance Tests (MTT):

Meal tolerance tests were performed in the LF and HF diet groups at weeks 8 and 12, respectively. The blood glucose response to experimental diets was determined in fasted mice. Mice were transferred into individual cages and fasted overnight (~15 h) before testing. At 8:00 AM the following morning, blood glucose concentrations were obtained from each mouse via the tail vein (time 0). Next, the animals were given access to a previously weighed amount of food from one of five experimental diets: LF/AM, LF/HM, LF/HY, HF/AM or HF/HM. After a 20-min feeding period, the remaining pellet, as well as any food that spilled into the bottom of the cage during that time, was weighed and recorded. Blood glucose concentrations were obtained from the tail vein at 20, 45, 60, and 120 min after food ingestion for calculation of the incremental area under the blood glucose curve ($AUC_{glucose}$) (127). The incremental $AUC_{glucose}$ over 120 min was calculated according to the trapezoidal rule using SigmaPlot 8.0. In addition, blood samples were obtained from the tail vein of the HF/AM and HF/HM groups at 0, 20, 60, 120, and 240 min after food ingestion for measurement of plasma insulin. Blood samples were not collected in the LF diet groups during the meal tolerance test for analysis of plasma insulin. At the completion of the test, animals were placed back into their colony cages and given the appropriate experimental diet.

Food Intake:

Twenty-four hour food intake was measured by cage, in duplicate, at weeks 4 and 12. Animals were transferred into fresh cages, and food intake measurements were recorded by weighing pellets before and after 24 h. Attempts were made to record any food that may have spilled into the bottom of the cage during feeding. Average food

intake values from weeks 4 and 12 were combined for statistical analyses. Data is expressed as food intake in g/day/mouse due to unequal numbers of mice per cage. In addition, 24 h food intake values from each diet group at weeks 4 and 12 were multiplied by the caloric density of the respective diet for calculation of energy intake. Average energy intake values for each diet group from weeks 4 and 12 were combined for statistical analyses.

Glucose Tolerance Tests (GTT):

Glucose tolerance tests were performed at the beginning of week 15. Mice were transferred into individual cages and fasted overnight (~15 h) prior to the test. At 8:00 AM the following morning, blood glucose concentrations were obtained from each mouse via the tail vein (time 0), followed by an intraperitoneal injection of a filter-sterilized glucose solution (1.5 g/kg body weight). Blood glucose concentrations were obtained from the tail vein at 15, 30, 60, and 120 min after glucose injection for calculation of the incremental $AUC_{glucose}$ (127). The incremental $AUC_{glucose}$ over 120 min was calculated according to the trapezoidal rule using SigmaPlot 8.0. At the completion of the test, animals were placed back into their colony cages and given the appropriate experimental diet. At 30, 60, and 120 min after glucose injection, 2 mice, 6 mice, and 1 mouse, respectively, had blood glucose concentrations that exceeded the maximum detection limit of the glucometer (600 mg/dl). Thus, the true $AUC_{glucose}$ for the HF/HY group may have actually exceeded the calculated $AUC_{glucose}$ reported in this thesis.

Tissue Collection:

At the end of week 15, following a 5 h fast, mice were anesthetized with an intraperitoneal injection of ketamine-xylazine (70 mg/kg and 5 mg/kg, respectively), upon which a terminal blood sample was collected from the retro-orbital venous plexus using heparinized capillary collection tubes. In addition, the liver and epididymal adipose tissue depots were rapidly excised and weighed. A portion of each tissue was placed in RNA later stabilization reagent (Qiagen, Valencia, CA) for gene expression analysis.

RNA Isolation and real-time RT-PCR analysis:

Total RNA was isolated according to the manufacturer's protocol from 50-100 mg of mouse epididymal adipose tissue, with on-column DNase digestion, using an RNeasy® Lipid Tissue Mini Kit (Qiagen, Valencia, CA). Adipose tissue samples were removed from a -80°C freezer, placed on dry ice, cut with a sterile razor blade, and weighed using an analytical scale. The weighed tissue sample was placed in 1.0 ml of QIAzol Lysis Reagent (Qiagen, Valencia, CA), and homogenized for 45 seconds using a tissue homogenizer (PRO 200, PRO Scientific Inc., Oxford, CT). For RNA quantification, 2 µl of the RNA sample was diluted with 48 µl of Tris-EDTA (TE) buffer (pH 7.5, 1:25 dilution). The absorbencies of the diluted RNA sample were obtained at 260 nm and 280 nm using a spectrophotometer (BioRad SmartSpec™ Plus, Hercules, CA). The concentration of the RNA sample was calculated as (44 x A_{260} x dilution factor) / 1000 (RNeasy® Lipid Tissue Handbook, Qiagen, Valencia, CA). RNA purity was assessed by calculating the ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}). RNA was considered pure with an A_{260}/A_{280} ratio of 1.9–2.1 (RNeasy® Lipid Tissue Handbook, Qiagen, Valencia, CA).

Complementary DNA (cDNA) was synthesized according to the manufacturer's protocol from 1 µg of total adipose RNA using an iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA). A subset of animals from each diet group (n = 5) was used for gene expression analysis. Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed on an iCycler real-time PCR detection system (BioRad, Hercules, CA) using 0.5 µl cDNA, 3.0 µl of a 1.25 µM (final concentration) primer mix, and 12.5 µl of iQ SYBR Green Supermix (BioRad, Hercules, CA) per reaction. Mouse gene specific primers were selected from Primer Bank (http://pga.mgh.harvard.edu/primerbank/citation.html), and purchased from Integrated DNA Technologies, Inc. (IDT, Inc., Coralville, IA). The following primers (5'-3') were used: mouse catalase, NM_009804, AAGCGACCAGATGAGCAGTG (forward) and TCCGCTCTCTGTCAAAGTGTG (reverse); mouse *gp91^{phox}*(NADPH oxidase), NM_007807, TGAATGCCAGAGTCGGGATTT (forward) and CCCCCTTCAGGGTTCTTGATTT (reverse); mouse glutathione peroxidase (GPx-1), NM_008160, AGTCCACCGTGTATGCCTTCT (forward) and GAGACGCGACATTCTCAATGA (reverse); mouse superoxide dismutase 2 (SOD-2), NM_013671, CAGACCTGCCTTACGACTATGG (forward) and CTCGGTGGCGTTGAGATTGTT (reverse). The following PCR amplification cycle was used: 95.0°C, 3:0 min; 95.0°C, 0:15 min, 60.0°C, 0:30 min, 72.0°C, 0:30 min, repeated 40x; 55.0 - 95.0°C, 0:10 min, repeated 80x (Appendix A and B). In real-time RT-PCR, the Ct value is a measure of how many cycles it takes for each sample to generate sufficient product to provide a signal that crosses a pre-determined threshold, and provides a measure of how much starting material is present in a given sample. Thus,

expression levels were first normalized to β-actin by calculating the ΔCt (ΔCt = β-actin Ct – Target gene Ct). Next, relative mRNA expression was calculated as $2^{\Delta Ct}$; this value was used for statistical analyses (Real-Time PCR Applications Guide, BioRad, Hercules, CA) (Appendix C).

Plasma Measurements:

Plasma glucose concentrations were determined with a handheld glucometer (OneTouch® Ultra® Meter, OneTouch® Ultra® Test Strips with FastDraw™ Design, Lifescan); plasma immunoreactive insulin, leptin, and adiponectin concentrations were measured by ELISA using mouse insulin, leptin, or adiponectin as a standard (Linco Research Inc, St. Charles, MO); plasma IL-6 concentrations were determined by ELISA using mouse IL-6 as a standard (R&D Systems, Minneapolis, MN); and plasma total triacylglycerol concentrations were determined using a colorimetric assay (Sigma®, St. Louis, MO). The homeostasis model assessment (HOMA) index was used to assess insulin sensitivity (128). HOMA index was calculated as fasting glucose (mmol/L) x fasting insulin (μU/mL) / 22.5.

Statistical analyses:

The main treatment effects of fat and starch, and the interaction between fat and starch among the treatment groups were obtained with a two-way analysis of variance (ANOVA) procedure. In the LF diet groups, one-way ANOVA procedures were performed to test for differences among the means in relative plasma leptin levels, blood glucose concentrations at each time point during the meal tolerance test, incremental $AUC_{glucose}$ over 120 min during the meal tolerance test, and energy intake during the meal tolerance test. In the HF diet groups, unpaired Student's *t* tests were performed to test for

differences between the means in blood glucose concentrations at each time point during the meal tolerance test, incremental $AUC_{glucose}$ over 120 min during the meal tolerance test, and energy intake during the meal tolerance test. When one-way and two-way ANOVA tests were significant, follow-up unpaired Student's *t* tests and orthogonal contrasts were performed to test for differences among the means between groups for the following: plasma glucose, insulin, adiponectin, IL-6, total triacylglycerol, relative gene expression values (as $2^{\Delta Ct}$), incremental $AUC_{glucose}$ over 120 min during the glucose tolerance test, tissue weights, and 24 h food and energy intake. For analysis of changes in body weight over time, a multivariate ANOVA (MANOVA) with repeated measures test was used to determine if the main effects of fat and starch on body weight varied with time. Data are presented as LSmeans ± SE. Statistical significance was declared for *P* values < 0.05. Statistical analysis was performed using JMP 6™ from SAS (Cary, NC).

TABLE 1
Nutrient composition of LF experimental diets

	H-GI AM	L-GI HM	L-GI HY
Components (g/kg)			
Casein	200	200	200
L-cysteine	3	3	3
Experimental Starch	500	500	500
Maltodextrin	109.49	109.49	109.49
Sucrose	50	50	50
Cellulose (Fiber)	30	30	30
Soybean oil	60	60	60
Mineral Mix*	35	35	35
Vitamin Mix†	10	10	10
Choline Bitartrate	2.5	2.5	2.5
TBHQ (antioxidant)	0.01	0.01	0.01

*AIN-93-G-MX (TD 94046)
†AIN-93-VX (TD 94047)

TABLE 2
Nutrient composition of HF experimental diets

	H-GI AM	L-GI HM	L-GI HY
Components (g/kg)			
Casein	200	200	200
L-cysteine	3	3	3
Experimental Starch	380	380	380
Maltodextrin	109.49	109.49	109.49
Sucrose	42.4	42.4	42.4
Cellulose (Fiber)	30	30	30
Soybean oil	60	60	60
Mineral Mix*	41	41	41
Vitamin Mix†	11.6	11.6	11.6
Choline Bitartrate	2.5	2.5	2.5
TBHQ (antioxidant)	0.01	0.01	0.01
Lard	120	120	120

*AIN-93-G-MX (TD 94046)
†AIN-93-VX (TD 94047)

CHAPTER 4. RESULTS

Meal Tolerance Tests:

To assess the effects of the experimental diets on postprandial glycemic response, meal tolerance tests were performed. FIG. 1 shows the glycemic response to each LF diet during the meal tolerance test. Blood glucose concentrations at time 0 (basal) were significantly greater in the LF/HM, L-GI group compared to the LF/HY, L-GI and LF/AM, H-GI groups (118 ± 4.3 vs. 85 ± 2.5 and 99 ± 5.1 mg/dl, $P = 0.0001$). However, blood glucose concentrations were significantly greater in the LF/AM, H-GI group compared to the LF/HM and LF/HY, L-GI groups at 20, 45, and 60 min following food consumption (t = 20, 214 ± 8.3 vs. 171 ± 6.1 and 165 ± 11.8 mg/dl, $P = 0.0003$; t = 45, 219 ± 12.5 vs. 186 ± 9.2 and 186 ± 9.2 mg/dl, $P = 0.019$; t = 60, 202 ± 13 vs. 166 ± 9.1 and 177 ± 12.1 mg/dl, $P = 0.045$, FIG. 1). The incremental $AUC_{glucose}$ over 120 min was significantly greater in the LF/AM, H-GI diet compared to the LF/HM or LF/HY, L-GI diets (21996 ± 732 vs. 18725 ± 782 or 18771 ± 845 mg/dl x 120 min, $P = 0.009$, FIG. 2). Energy intake (kcal consumed in 20 min) during the meal tolerance test was significantly lower in the LF/AM, H-GI group compared to the LF/HM or LF/HY L-GI groups (0.65 ± 0.09 vs. 0.98 ± 0.12 or 1.28 ± 0.17 kcal / 20 min, $P = 0.006$, FIG. 3). FIG. 4 shows the glycemic response to the HF/AM, H-GI and HF/HM, L-GI diets during the meal tolerance test. This test was not performed in the HF/HY, L-GI group. Blood glucose

concentrations at time 20 and 45 were significantly greater in the HF/AM, H-GI group compared to the HF/HM, L-GI group (t = 20, 193 ± 13.2 vs. 153 ± 5.3 mg/dl, $P = 0.0095$; t = 45, 215 ± 7.6 vs. 181 ± 7.3 mg/dl, $P = 0.011$). A similar trend was observed in the HF groups in that the incremental $AUC_{glucose}$ over 120 min was significantly greater in the HF/AM, H-GI diet compared to the HF/HM, L-GI diet (21489 ± 593 vs. 18988 ± 550 mg/dl x 120 min, $P = 0.012$, FIG. 5). There was no significant difference in energy intake (kcal / 20 min) between the HF/AM and HF/HM group during the meal tolerance test (FIG. 6). There were also no significant differences in plasma insulin levels at any time point during the meal tolerance test in the HF/AM and HF/HM groups (FIG. 7). Plasma insulin levels during the meal tolerance tests were not measured in the LF groups.

24-h Food Intake:

Food intake was measured by cage, in duplicate, at weeks 4 and 12. The combined averages at 4 and 12 weeks were used for statistical analyses. When expressed as g / 24 h / mouse, there was a significant main effect of fat ($P = 0.002$), with animals in the HF groups consuming less food per 24 h than animals in the LF groups (FIG. 8). However, when expressed in terms of energy intake (kcal/ 24 h/ mouse), there were no significant differences among any of the groups (FIG. 9).

Body Weight:

Figure 10 shows weekly changes in body weight over 15 weeks of experimental feeding. All diets increased body weight over 15 weeks ($P < 0.0001$). A significant interaction between time and fat ($P < 0.0001$) was observed, demonstrating that the effect of fat on body weight varied with time. Weight gain in the HF diet groups was significantly greater than weight gain in the LF diet groups over 15 weeks of feeding

(FIG. 11). Although there was a trend towards an interaction between time and starch ($P = 0.0539$), the effect of starch on body weight did not vary with time. With respect to final body weight, a significant main effect of fat ($P = 0.0001$) was observed, in which the HF diet groups weighed more than the LF diet groups at the conclusion of the study; however, there were no differences in body weight between groups within the LF and HF diet groups (FIG. 12).

Epididymal Adipose and Liver Weight:

Epididymal adipose and liver weights at the time of sacrifice (end of week 15) are shown in figures 13 and 14, respectively. There was a significant main effect of fat for epididymal adipose and liver weight ($P < 0.0001$), with animals in the HF groups having increased tissue weights (FIG. 13 and 14). However, there were no significant differences in epididymal adipose or liver weight between groups within the LF or HF diet groups. When expressed per gram of body weight, there was also a significant main effect of fat for epididymal adipose ($P = 0.0005$) and liver ($P < 0.0001$). Animals in the HF groups had increased relative tissue weights (FIG. 15 and 16), indicating that the high-fat-fed mice were not only growing, but also getting fatter. There were no significant differences in relative epididymal adipose weight between groups within the LF or HF diet groups. Similarly, there were no significant differences in relative liver weight between groups consuming the LF diets. There was a significant difference in relative liver weight between groups consuming the HF diets, with animals in the HF/HY, L-GI group having significantly increased relative liver weight compared to those in the HF/HM, L-GI group (0.045 ± 0.002 vs. 0.038 ± 0.002 g / g body weight, $P = 0.008$; FIG. 16). In addition,

there was a trend towards a main effect of starch ($P = 0.089$), and an interaction between starch and fat ($P = 0.078$) with respect to relative liver weight.

Plasma Metabolites:

Non-fasting blood glucose concentrations were measured at week 4 and 8 of experimental feeding. There was a significant main effect of starch ($P = 0.0062$) at 4 weeks, with diets containing the AM, H-GI starch having the greatest blood glucose concentrations (HF/AM, 159 ± 7.4 mg/dl; LF/AM, 156 ± 6.1 mg/dl; FIG. 17). Within the LF diet group, there was a significant increase in non-fasting blood glucose concentration in the LF/AM, H-GI group compared to the LF/HM, L-GI group (156 ± 6.1 vs. 132 ± 6.1 mg/dl, $P = 0.009$; FIG. 17).Within the HF diet group, there was a significant increase in non-fasting blood glucose concentration in the HF/AM, H-GI group compared to the HF/HY, L-GI group (159 ± 7.4 vs 136 ± 6.4 mg/dl, $P = 0.025$; FIG. 17). At 8 weeks, no differences in non-fasting blood glucose concentrations were detected; however, a trend towards a significant main effect of fat ($P = 0.0629$), and an interaction between fat and starch ($P = 0.0508$) was observed. Interestingly, the LF/AM group had a significantly greater non-fasting blood glucose concentration at 8 weeks compared to all other experimental groups ($P = 0.001$; FIG. 18).

Table 3 shows the plasma measurements obtained from 15-week-old mice at time of sacrifice. There was a significant main effect of fat ($P = 0.0001$) in fasting blood glucose levels, with the HF diet groups having greater blood glucose concentrations than the LF diet groups; however, there were no significant differences between groups within the LF and HF diet groups. There were significant main effects of fat ($P < 0.0001$), starch ($P = 0.0001$), and an interaction with fat and starch ($P < 0.0001$) in fasting plasma insulin

levels, with HF diet groups having greater insulin levels than the LF diet groups. There were no significant differences between LF diet groups. Unexpectedly, insulin levels were greater in both HF/L-GI groups compared to the HF/H-GI group. For example, the HF/HY, L-GI diet group had a significant increase in plasma insulin levels compared to the HF/HM, L-GI and HF/AM, H-GI groups ($P < 0.001$). Furthermore, the HF/HM, L-GI group had a significant increase in plasma insulin levels compared to the HF/AM, H-GI group ($P = 0.03$). No significant differences in plasma total triacylglycerol concentrations were detected; however, a trend towards a main effect of fat ($P = 0.0536$) and starch ($P = 0.0504$) was observed.

The concentrations of two different adipocytokines, adiponectin and leptin, were also measured in the plasma. With respect to adiponectin, a significant main effect of fat ($P = 0.0002$) was observed, with HF diet groups having decreased plasma adiponectin concentrations at the time of sacrifice, as expected. There were no significant differences between HF diet groups, but the LF/HY, L-GI group had significantly decreased plasma adiponectin concentration compared to LF/AM, H-GI and LF/HM, L-GI groups ($P = 0.007$). With respect to relative plasma leptin levels (leptin [ng/ml] /g body weight), one way ANOVA revealed a significant difference among LF diet groups ($P = 0.0325$), with the LF/AM, H-GI group having a significant increase in relative plasma leptin concentrations compared to the LF/HM, L-GI group ($P = 0.0098$). Plasma leptin concentrations in the HF diet groups were beyond the detection limit of the assay, and were not included in the statistical analysis.

Concentrations of the inflammatory cytokine, interleukin-6 (IL-6), were measured in the plasma as well. A significant interaction between fat and starch ($P = 0.013$) was

observed. Although there were no significant differences between HF diet groups, the LF/HY, L-GI group had significantly increased plasma IL-6 concentrations compared to the LF/AM, H-GI and LF/HM, L-GI groups ($P = 0.005$). Furthermore, plasma IL-6 concentrations were significantly greater in the LF/HY, L-GI group compared to the HF/HY, L-GI group ($P = 0.038$).

Glucose Tolerance Tests:

Intraperitoneal glucose tolerance tests (IPGTTs) were performed at the beginning of week 15 following an overnight fast (~15 h). Blood glucose concentrations were obtained from the tail vein at time 0 (before glucose injection [1.5 mg glucose/ kg body weight]), 15, 30, 60, and 120 min after glucose injection for calculation of the incremental $AUC_{glucose}$. FIG. 19 shows the glycemic response during the IPGTT in the LF diet groups. Blood glucose concentrations at time 0 (basal) were significantly greater in the LF/HM, L-GI group compared to the LF/HY, L-GI and LF/AM, H-GI groups (127 ± 6.6 vs. 100 ± 6.6 and 92 ± 7.8 mg/dl, $P < 0.0001$). Blood glucose concentrations at time 15 were also significantly greater in the LF/HM, L-GI group compared to the LF/AM, H-GI group (435 ± 26.6 vs. 354 ± 24.7 mg/dl, $P = 0.0329$). FIG. 20 shows the glycemic response during the IPGTT in the HF diet groups. Blood glucose concentrations at time 15 were significantly greater in the HF/HY, L-GI group compared to the HF/AM, H-GI group (452 ± 23.1 vs. 365 ± 29.2 mg/dl, $P = 0.0254$). In addition, blood glucose concentrations at time 30, 60, and120 were significantly greater in the HF/HY, L-GI group compared to the HF/HM, L-GI and HF/AM, H-GI groups (t = 30, 541 ± 19.6 vs. 405 ± 21 and 449 ± 25 mg/dl, $P = 0.0005$; t = 60, 551 ± 27 vs. 416 ± 28.8 and 435 ± 34.1 mg/dl, $P = 0.0037$; t = 120, 471 ± 30 vs. 322 ± 31.9 and 306 ± 37.8 mg/dl, $P = 0.0025$).

FIG. 21 shows the incremental $AUC_{glucose}$ during the IPGTT in both the LF and HF diet groups. Significant main effects of fat ($P < 0.001$), starch ($P = 0.0203$), and an interaction between fat and starch ($P = 0.0314$) were observed. The $AUC_{glucose}$ was increased in the HF diet groups, although the majority of this fat effect may be attributed to the HF/HY, L-GI group in that the $AUC_{glucose}$ for the HF/HY group was significantly greater than all other experimental groups ($P = 0.001$; FIG. 21). In addition, there was a significant increase in the $AUC_{glucose}$ in the HF/AM, H-GI group compared to the LF/AM, H-GI group (58936 ± 2382 vs. 37224 ± 3369 mg/ dl x 120 min, $P = 0.034$; FIG. 21). There were no significant differences in $AUC_{glucose}$ between LF diet groups.

HOMA Index:

The HOMA index, a measure of insulin resistance, was calculated as fasting blood glucose concentration at the time of sacrifice (mmol/L) x fasting insulin concentration at the time of sacrifice (μU/mL) / 22.5. Significant main effects of fat ($P < 0.0001$), starch ($P = 0.0005$), and an interaction between fat and starch ($P = 0.0002$) was observed. HOMA indices were increased in the HF groups; however, similar to results obtained from the IPGTTs, the majority of this fat effect may be attributed to the HF/HY, L-GI group in that the HOMA index for the HF/HY group was significantly greater than all other experimental groups ($P < 0.001$; FIG. 22). In addition, there was a significant increase in the HOMA index in the HF/HM, L-GI group compared to the LF/ HM, L-GI group (9.2 ± 1.0 vs. 3.3 ± 0.7, $P < 0.0001$), and a trend towards a significant increase in the HF/AM, H-GI group compared to the LF/AM, H-GI group (6.5 ± 1.2 vs. 3.8 ± 0.7, $P = 0.0515$; FIG. 22). There were no significant differences in HOMA indices between LF diet groups.

Relative Expression of the Pro-oxidant enzyme, NADPH oxidase, in Epididymal Adipose Tissue:

In epididymal adipose tissue, expression of murine NADPH oxidase (pro-oxidant enzyme), was quantitated by real-time RT-PCR, and normalized to β-actin expression. After 15 weeks of experimental feeding, there was a significant main effect of fat ($P < 0.0001$) with respect to NADPH oxidase expression, in which mRNA levels were significantly increased in each HF diet group compared to the comparable LF diet group (FIG. 23). For example, there was a significant increase in NADPH oxidase expression in the HF/AM, H-GI group compared to the LF/AM, H-GI group ($P = 0.002$), in the HF/HM, L-GI group compared to the LF/HM, L-GI group ($P = 0.008$), and in the HF/HY, L-GI group compared to the LF/HY, L-GI group ($P = 0.036$). However, there were no significant differences in NADPH oxidase expression between groups within the LF and HF diet groups.

Relative Expression of the Anti-oxidant enzymes, GPx-1, Catalase, and SOD-2 in Epididymal Adipose Tissue:

In epididymal adipose tissue, expression of murine catalase, glutathione peroxidase-1 (GPx-1), and superoxide dismutase-2 (SOD-2) was quantitated by real-time RT-PCR, and normalized to β-actin expression. After 15 weeks of experimental feeding, there was a significant main effect of fat ($P < 0.0001$) with respect to GPx-1 expression, in which mRNA levels were decreased in the HF diet groups compared to the LF diet groups (FIG. 24). For example, there was a significant decrease in GPx-1 expression in the HF/AM, H-GI group compared to the LF/AM, L-GI group ($P = 0.014$), and in the HF/HM, L-GI group compared to the LF/HM, L-GI group ($P = 0.026$; FIG. 24).

However, there were no significant differences in GPx-1 expression between groups within the LF and HF diet groups. Similarly, there was a significant main effect of fat ($P = 0.0037$) with respect to catalase expression, in which mRNA levels were also decreased in the HF diet groups compared to the LF diet groups. (FIG. 25). There was a significant decrease in catalase expression in the HF/AM, H-GI and HF/HY, L-GI group compared to the LF/AM, H-GI group ($P = 0.006$); however, there were no significant differences in catalase expression between groups within the LF and HF diet groups (FIG. 25). Lastly, although there was a trend toward a main effect of fat with respect to SOD-2 expression ($P = 0.0567$), there were no significant differences in SOD-2 expression among any of the groups (FIG. 26).

CHAPTER 5. DISCUSSION

The prevalence of obesity and diabetes has reached pandemic proportions in recent decades, prompting researchers to investigate potential mechanisms by which dietary factors may influence the development and/or progression of these conditions. The glycemic index (GI) was proposed as a way of classifying carbohydrates according to how they affect post-prandial glycemia (6). It is believed that consumption of high-GI (H-GI) carbohydrates may render one susceptible to adverse metabolic consequences (4,7,37). In fact, several human studies have correlated H-GI diets with an increased risk of coronary heart disease (CHD) (3,8), the metabolic syndrome (9), insulin resistance and type 2 diabetes (10-12). Similarly, studies in rodent models have linked H-GI diets with increased adiposity (13-17), decreased lean body mass (16), enhanced lipogenic gene expression (18), increased plasma triglyceride concentrations (13), decreased plasma adiponectin concentrations (16), hypersecretion of insulin and insulin resistance (15-17,19); however, the exact mechanism(s) by which H-GI diets elicit adverse effects is unknown.

The objective of this study was to determine the chronic effects of a H-GI diet on local oxidative stress production in the adipose tissue, and insulin sensitivity in male, C57Bl/6 mice. We hypothesized that generation of oxidative stress in the adipose tissue was a mechanism by which H-GI diets elicit adverse effects. Specifically, we

hypothesized that mice consuming the H-GI diet would have reduced insulin sensitivity, increased expression of the pro-oxidant gene, NADPH oxidase, and decreased expression of the antioxidant genes, superoxide dismutase- 2, glutathione peroxidase- 1, and catalase, in the adipose tissue, compared to mice consuming the low-GI (L-GI) diets. We also hypothesized that the L-GI diets would exert protective effects against the generation of oxidative stress in the adipose tissue of mice consuming those diets. An additional level of complexity was incorporated into our experimental design by including two different levels of fat - low fat (LF) and high fat (HF). First, we wanted to explore the main effects and interactions of fat and starch with respect to oxidative stress and other metabolic characteristics in an obesity-prone mouse model (C57Bl/6). Second, the composition of the HF diets, in which 37.4% of kcal were derived from fat (compared to 15% of kcal in the LF diets), closely mimicked the amount of fat found in the typical Western diet, and provided a physiologically relevant setting with which to investigate mechanisms of dietary components (129,130). Furthermore, some have argued that the beneficial effects of a L-GI diet may only pertain to individuals that are obese, and at an increased risk of, or already diagnosed with certain metabolic diseases (74,81-83). With this in mind, the C57Bl/6 mouse was an appropriate animal model to use in our study for the following reasons: 1) when allowed ad libitum access to a HF diet, C57Bl/6 mice have been shown to develop obesity, hyperinsulinemia, hyperglycemia, and hypertension, 2) this process is believed to be similar to the progression of metabolic disease observed in humans, 3) when restricted to a LF diet, C57Bl/6 mice remain lean and physically normal (131-133). Thus, the C57Bl/6 mouse was a particularly good model to use in order to investigate the effects of H-GI or L-GI starches in LF and HF diets.

In the present study, we reported that increasing the amount of dietary fat had influences on weight gain, absolute and relative adipose and liver weight, glucose tolerance, insulin sensitivity (HOMA index), fasting plasma glucose, insulin and adiponectin concentrations, and NADPH oxidase, GPx-1 and catalase gene expression. In addition, we reported that a qualitative change in dietary carbohydrate composition (e.g. H-GI starch vs. L-GI starch) had influences on the post-prandial glycemic response during a meal tolerance test, non-fasting blood glucose concentrations (4 weeks), glucose tolerance, fasting plasma insulin levels, and insulin sensitivity (HOMA index). However, the type of dietary starch consumed, whether H-GI (AM) or L-GI (HM or HY) did not influence the expression of pro-oxidant (NADPH oxidase) or antioxidant genes (Catalase, GPx-1, SOD-2) in the adipose tissue of male, C57Bl/6 mice.

The acute effects of our experimental diets were analyzed by performing meal-tolerance tests in our mice. Data from these tests verified that the AM starch (100% amylopectin, H-GI) produced a greater post-prandial blood glucose response ($AUC_{glucose}$) compared to the HM (60% amylose/ 40% amylopectin, L-GI) and HY (55% amylose/ 45% amylopectin, L-GI) starches. These observations were consistent with those of Pawlak and colleagues (16) and Byrnes and colleagues (19), in which significant differences in the post-prandial glycemic response to test diets were observed upon feeding equal amounts of high-amylopectin (H-GI) or high-amylose (L-GI) foods. Although equal amounts of food were not administered in our meal tolerance tests, our data showed that the increased glycemic response to the H-GI diet was significant, despite reduced energy intake in these animals compared to those consuming the L-GI diets (HM and/or HY).

As expected, animals consuming the HF diets experienced greater increases in body weight over time compared to those consuming the LF diets. This was evidenced by a significant interaction with time and fat, as well as a significant main effect of fat on weight gain and final body weight. The increased body weight in the HF groups compared to the LF groups was partially attributed to increases in the weights of both the liver and epididymal adipose tissue depots. Although the type of dietary starch consumed (whether H-GI or L-GI) had no effect on body weight, relative liver weight, or relative epididymal adipose weight among the groups, there was a trend towards increased relative epididymal adipose weight in the H-GI, AM groups compared to the L-GI, HM and HY groups in both the LF and HF diets, despite comparable body weights. In addition, an increase in relative leptin concentration (leptin [ng/ml] / g body weight) was observed in the LF/AM, H-GI group compared to the LF/HM, L-GI group. This finding was consistent with previous studies (15-17), and suggested that the H-GI starch promoted increases in adiposity despite comparable body weights. It is important to note that only epididymal adipose tissue weights (a subcutaneous depot) were used as a measure of adiposity in our mice; however, the type of dietary starch consumed might influence the abundance of other adipose tissue depots as well (e.g. visceral adipose depots), a phenomenon observed by Morris and Zemel (17).

Previous studies have also reported no differences in body weight among rodents fed either a H-GI or a L-GI diet anywhere from 3 to 9 weeks (13-15,18,19). In other rodent studies, food restrictive measures were implemented when body weights differed among high-GI and low-GI groups under ad libitum feeding conditions (16,17). Although there were no differences in food intake between groups within the LF or HF diet groups,

there was an effect of fat on food intake, in which animals consuming the HF diets ate significantly less (grams of food) than those consuming the LF diets. However, when expressed in terms of 24 h energy intake, there were no differences among any of the groups. Interestingly, C57Bl/6 mice have been shown to exhibit increased feed efficiency (weight gained / kcal consumed) when fed a HF diet (131,134-136). So, increased body weight in the HF diet groups despite similar energy intake compared to the LF diet groups supports the notion of increased feed efficiency in the HF-fed mice. Some additional issues concerning food intake measurements and the compositions of our experimental diets warrant further consideration as well.

The diets used in this study had identical macronutrient and micronutrient compositions, differing only in the nature of the starch (100% amylopectin [AM] vs 60% amylose/ 40% amylopectin [HM] and 55% amylose/ 45% amylopectin [HY]). Amylopectin, the major component of most starches, is a large glucose homopolysaccharide containing both α-1,4 (linear) glucose linkages, and α-1,6 branching points (137,138). Amylose, on the other hand, is a straight chain polymer of D-glucose units (137,138). In addition, amylose contains various amounts of resistant starch, or non-digestible fibers, which resist α-amylase digestion in the small intestine, and are fermented to short-chain fatty acids by the microflora in the large intestine (139-142). It has been suggested that adding resistant starch to the diet dilutes dietary energy density (139). Interestingly, the HM starch used in the present study is a fermentable, high amylose-resistant corn starch, and has been shown to consist of 39.9% fiber, and 33% resistant starch (139). Due to the resistant starch component, HM was calculated to provide a metabolizable energy value of 2.8 kcal/g, as opposed to the traditional 4.0

kcal/g provided by digestible starch (139). HY, the other low-GI starch used in the present study, is a high amylose corn starch comprised of 55% amylose and 45% amylopectin; however, the amount of resistant starch in HY has not been experimentally determined. For the purposes of this study, we assumed that the metabolizable energy value of the HY starch was intermediate between that of the HM (2.8 kcal/g) and AM (3.6 kcal/g) starches. Nonetheless, both L-GI diets had varying degrees of energy dilution compared to the H-GI diet. Previous studies have shown that mature rats will compensate for energy dilution of the diet (e.g. addition of resistant starch) by consuming more of the diet (139,143), and that food spillage in rats is significantly greater for energy-diluted diets (139). Thus, additional food intake studies, using metabolic cages, need to be performed to accurately account for spillage in our experimental animals. Given that the body weights did not significantly differ between groups within the LF and HF diets, we suspect that increased consumption of food in the HM and HY groups compensated for the dilution in dietary energy densities in these diets.

Next, we wanted to investigate the effects that a H-GI diet may have on various measures of glucose metabolism. A study conducted by Lerer-Metzger and colleagues (13) reported significantly decreased non-fasting plasma glucose concentrations in rats fed a L-GI starch (mung-bean starch) for 5 weeks, compared to those fed a H-GI starch (ground French toast). Similarly, we reported that mice fed the LF and HF, L-GI diets (HM and HY) had decreased non-fasting blood glucose concentrations at 4 weeks compared to those fed the LF and HF, H-GI diets (AM). By 8 weeks, however, only the LF/AM group remained significantly elevated. Although it is possible that mice consuming the AM starch had just eaten prior to our blood glucose measurements at 4

and 8 weeks, it appeared that the H-GI starch had acute influences on fed blood glucose levels early-on in the study. However, increases in fed blood glucose levels in the AM groups early-on did not translate into impaired glucose tolerance or insulin resistance in these animals at 15 weeks (discussed below).

At the completion of the study, the HF diet groups had significantly elevated fasting blood glucose concentrations compared to the LF diet groups, although the type of dietary starch consumed had no influence on the magnitude of fasting blood glucose in any of the groups. Consistent with increased fasting blood glucose concentrations, the HF diet groups also had significantly elevated fasting plasma insulin levels compared to the LF diet groups at week 15. In addition, there was an interaction between fat and starch with respect to fasting plasma insulin levels, indicating that the effect of fat on insulin levels was dependent upon the type of starch in the diet; however, there were no differences in fasting plasma insulin concentration between the LF diet groups. This was inconsistent with the finding of Byrnes et al. (138), in which rats fed a high amylopectin diet displayed higher basal plasma insulin concentration after 12 weeks of feeding compared to rats fed a high-amylose diet. This disparity may have been due to diet and/or species differences. Surprisingly, the interaction between fat and starch was manifested in both HF/L-GI groups (HF/HM and HF/HY), in which fasting insulin concentrations were significantly greater than that of the HF/AM, H-GI group. Increased basal insulin concentrations, despite similar fasting blood glucose concentrations, were suggestive of a hyperinsulinemic, pre-diabetic state in the HF/HM and HF/HY groups. It is unclear why the L-GI starches were acting in this manner.

To further investigate the effects of dietary starch and/or fat on glucose tolerance, we performed intraperitoneal glucose tolerance tests (IPGTTs) in our mice at the beginning of week 15. The HF-fed mice showed a reduced ability to clear the glucose load as indicated by elevated blood glucose concentrations at each time point compared to the LF diet groups. As expected, the areas under the blood glucose concentration curves ($AUC_{glucose}$) during IPGTTs were greater in the HF groups compared to the LF groups, indicating some degree of impaired glucose tolerance in the HF-fed animals. Similarly, Pawlak and colleagues (15) observed impaired glucose tolerance during an intravenous glucose tolerance test (IVGTT) in rats fed a HF diet, whereas no difference in glucose tolerance was observed in rats fed a H-GI (100% amylopectin) diet (15). In the current study, the HF/HY, L-GI group displayed a marked increase in the $AUC_{glucose}$ compared to all other groups, indicating significantly impaired glucose tolerance in these animals. In fact, several animals in the HF/HY group had blood glucose concentrations above the maximal detection limit of the glucometer (600 mg/dl) at 30, 60, and 120 minutes after glucose injection, a phenomenon that did not occur in any other group. Excluding the HF/HY group, the $AUC_{glucose}$ in the H-GI groups (HF/AM or LF/AM) was not greater than the $AUC_{glucose}$ in the L-GI groups (HF/HM or LF/HM, LF/HY). This data suggested that a qualitative change in dietary carbohydrate composition did not affect glucose tolerance in C57Bl/6 mice after 15 weeks of experimental feeding. Although previous studies have found significant increases in the insulin response to an IVGTT in rats fed a high-amylopectin (H-GI) diet compared to those fed a high-amylose (L-GI) diet, no differences in glucose tolerance were ever observed among any of the experimental groups in these studies (15,19,137). One study did report increases over

time in the $AUC_{glucose}$ during an oral GTT in rats fed a H-GI (100% amylopectin) diet compared to those fed a L-GI diet (60% amylose/40% amylopectin); however, partial pancreatectomies were carried out in these animals in order to resemble a pre-diabetic condition (16). Thus, the applicability of their findings with respect to the current study is difficult to define.

The impaired glucose tolerance observed in the HF/HY, L-GI group may have been related to body weight, given that this group displayed the greatest increase in weight gain throughout the study, and had the highest final body weight. In addition, HOMA indices were significantly greater, and plasma adiponectin concentrations significantly lower, in the HF diet groups compared to the LF diet groups. In fact, the HF/HY group had a HOMA index significantly greater than all other groups at the end of the study. The HOMA index is a practical and valid approach for assessing insulin sensitivity because it incorporates both fasting plasma insulin and fasting plasma glucose values into the equation. Thus, consistent with significantly impaired glucose tolerance, the HF/HY, L-GI group was also the least insulin sensitive according to their HOMA index. On the other hand, HOMA indices were not significantly different between the HF/AM, H-GI and HF/HM, L-GI groups, or between the LF/AM, H-GI and LF/HM or LF/HY, L-GI groups. Therefore, excluding the HF/HY, L-GI group, insulin sensitivity was unaffected by the type of starch in the diet.

Following characterization of the metabolic phenotypes among our experimental groups, we set out to determine if the GI of the diet altered the expression of genes involved in oxidative stress in the adipose tissue of our animals. Oxidative stress is defined as an imbalance between the production of reactive oxygen species and

antioxidant defenses, in favor of the oxidants (26-28). We hypothesized that generation of oxidative stress within the epididymal adipose tissue of C57Bl/6 mice was a potential mechanism by which H-GI diets elicit adverse effects.

Adipose tissue is now recognized as an active endocrine organ, and integrator of numerous physiological pathways (21-23). In fact, studies in both humans and animals have demonstrated that the adipose tissue is particularly sensitive to changes in the GI of the diet. Recently, Kallio and colleagues (144) analyzed global changes in gene expression in subcutaneous adipose tissue samples from subjects with the metabolic syndrome, following intervention with 2 test diets. One diet contained a rye-pasta modification, analogous to a L-GI starch, whereas the other diet contained an oat-wheat-potato modification, analogous to a H-GI starch. In the rye-pasta group, a coordinate down-regulation of genes involved in insulin signaling and apoptosis was observed, whereas a coordinate up-regulation of genes related to stress, cytokine-chemokine-mediated immunity, and the interleukin pathway was observed in the oat-wheat-potato group (144). These findings suggested that a qualitative change in dietary carbohydrate composition differentially modulates gene expression in the adipose tissue of humans with metabolic disease.

In the present study, increasing the amount of fat in the diet significantly increased the expression of the pro-oxidant gene, NADPH oxidase, and significantly decreased the expression of the anti-oxidant genes, GPx-1 and catalase. The effects of fat on the expression of genes involved in oxidative stress were consistent with those of Furukawa et al. (20), in which the expression of NADPH oxidase increased, and the expression of GPx and catalase both decreased in the adipose tissue of obese mice.

Similarly, Suh et al. (145) reported a decrease in the expression of antioxidant defense genes in the adipose tissue of Zucker diabetic fatty rats (145). These findings suggest that accumulated adipose tissue in obese animal models is intimately involved in the generation of oxidative stress; however, we showed that a qualitative change in dietary carbohydrate composition (whether H-GI or L-GI) had no effect on the expression of pro-oxidant and anti-oxidant genes in the adipose tissue of C57Bl/6 mice. Thus, on the basis of gene expression data, there was not enough evidence to support the hypothesis that dietary GI influences the expression of genes involved in oxidative stress in the epididymal adipose tissue of LF- and HF-fed C57Bl/6 mice.

Chronic consumption of a H-GI diet has also been suggested to influence the pathogenesis of diabetic complications and cardiovascular disease (CVD) through frequent postprandial glycemic excursions (70). Endothelial cells are particularly vulnerable to changes in the metabolic environment early on in the development of CVD, and acute hyperglycemic spikes have been shown to induce endothelial dysfunction (146-148). This may be due to the fact that glucose uptake occurs via facilitative diffusion in endothelial cells, such that in times of metabolic overload, they are incapable of downregulating nutrient influx (107). In addition, acute glycemic variations influence a series of events involved in coagulation, thrombosis, and inflammation, including an increase in circulating levels of the pro-adhesive molecule, intercellular adhesion molecule (ICAM)-1, and increases in plasma cytokines, such as IL-6 and TNF-α (118,146,149-151). Attempts to identify a link between postprandial hyperglycemia and metabolic disease have pointed to the role of oxidative stress in mediating this complex process (107,146). For example, Soriano et al. (152) demonstrated that acute increases in

glucose stimulated superoxide production in the mitochondria of endothelial cells, which in turn may lead to endothelial dysfunction and atherosclerosis.

In the context of obesity and diabetes, the body is in a state of metabolic overload, with an increased energy flux through glycolysis, the tricarboxylic acid (TCA) cycle, and the electron-transport chain (110,112). Consequently, an overproduction of electron donors by the TCA cycle occurs, overwhelming the electron-transport chain, and resulting in the reduction of molecular oxygen to superoxide within the mitochondria of cells (108-110). In fact, it is believed that the macrovascular and microvascular complications associated with diabetes are all related to an overproduction of superoxide by the mitochondrial electron-transport chain (108-110). Thus, in the present study, it is possible that postprandial hyperglycemia favored oxidative stress generation through direct effects on mitochondrial ROS production in other cell types, such as endothelial cells (107, 110).

Postprandial hyperglycemia may also influence markers of oxidative stress in the plasma. For example, Marfella et al. (153) showed that acute hyperglycemia in normal subjects raised circulating levels of nitrotyrosine. Similarly, Hu et al. (121) found a positive association between dietary GI/GL, and 2 markers of lipid peroxidation (MDA and F_2-isoprostane) in the plasma of healthy adults. In fact, Monnier et al. (120) demonstrated that postprandial glucose excursions in type 2 diabetic subjects exhibited a more specific triggering effect on oxidative stress (assessed by 24-h urinary 8-iso prostaglandin $F_{2\alpha}$) than chronic sustained hyperglycemia alone. Taken together, these studies and others (118,146,149-151) suggest that metabolic processes following acute glycemic excursions favor the production of oxidative stress, coagulation, and

inflammation. Based on the glycemic response to our H-GI diet during the meal tolerance test, it is plausible to suspect that similar processes occurred in the plasma of our animals in the postprandial state as well.

In the current study, chronic consumption of a H-GI diet had no significant effect on body weight, relative liver and epididymal adipose weight, expression of genes involved in oxidative stress in the adipose tissue, or fasting plasma glucose, triglyceride and adiponectin concentrations. In the LF diet group, chronic consumption of a H-GI diet increased relative leptin concentration compared to animals consuming a L-GI diet, indicating increased adiposity in the H-GI group. The H-GI diet also increased non-fasting blood glucose concentrations at 4 (LF/AM and HF/AM) and 8 (LF/AM) weeks of feeding, but these differences did not translate into impaired glucose tolerance or insulin resistance at the end of the study in the H-GI groups. In fact, the HF/HY, L-GI group was more insulin resistant than the HF/AM, H-GI group at the end of the study. Thus, the effect of starch on glucose tolerance, fasting plasma insulin concentration, and insulin sensitivity were predominantly attributed to differences in the HF/HY, L-GI group compared to all other groups. These findings merit further investigation on how the quality of dietary carbohydrate consumed affects whole-body metabolism and metabolic disease risk profiles.

CHAPTER 6. CONCLUSIONS

In comparison to the LF groups, the high dietary fat resulted in increased weight gain, absolute and relative adipose and liver weight, fasting plasma glucose and insulin, insulin resistance, and NADPH oxidase gene expression. Conversely, increased dietary fat decreased plasma adiponectin concentration, and GPx-1 and catalase gene expression. The H-GI diet, compared to both L-GI diets, increased the post-prandial glycemic response during a meal tolerance test, as well as non-fasting blood glucose concentrations at 4 and 8 weeks. However, the type of dietary starch consumed (whether H-GI or L-GI) did not influence the expression of pro-oxidant (NADPH oxidase) or antioxidant (Catalase, GPx-1, SOD-2) genes in the adipose tissue of male, C57Bl/6 mice. Similarly, with the exception of the HF/HY, L-GI group, the type of dietary starch consumed had no influence on insulin sensitivity. Thus, on the basis of gene expression data, there was little evidence that consumption of a high-GI diet influenced the expression of genes involved in oxidative stress in the adipose tissue of male, C57Bl/6 mice.

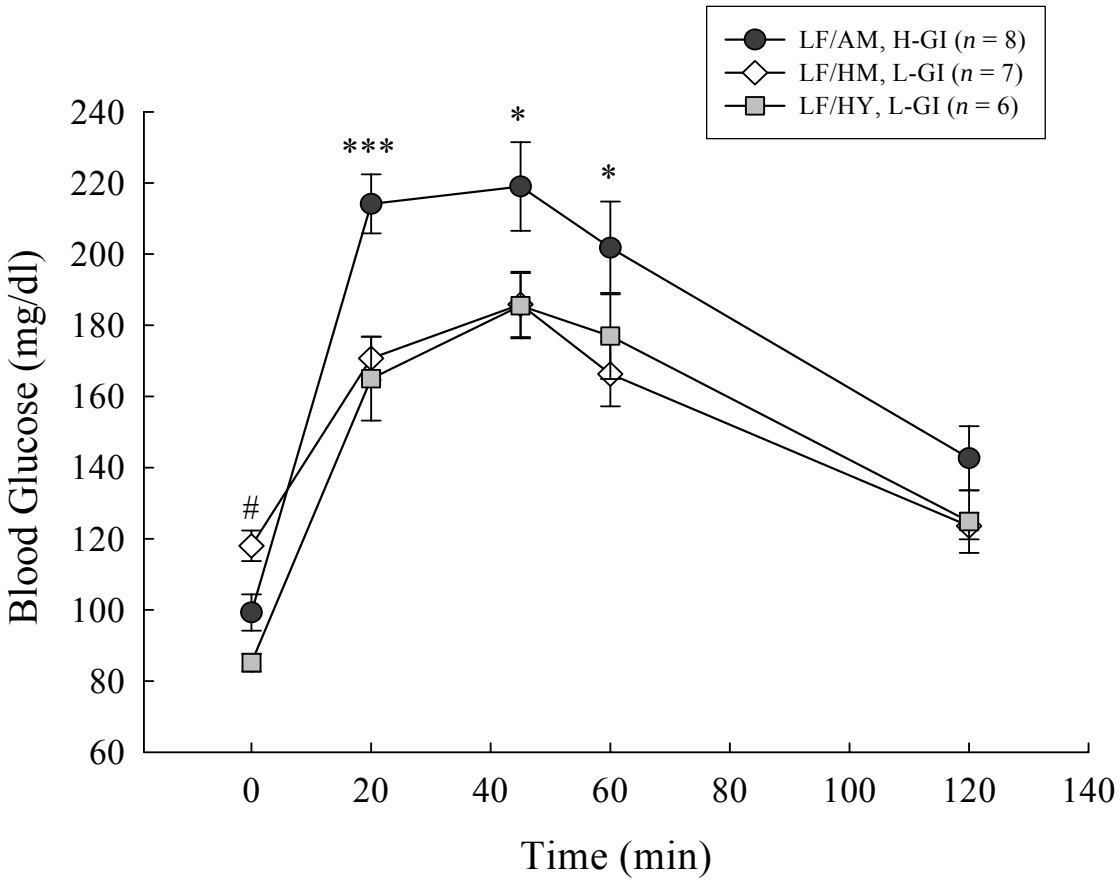

FIG. 1. Blood glucose response during a meal tolerance test in the low fat diet groups. Meal tolerance tests were performed in the low fat diet groups at week 8 following an overnight fast. Blood glucose concentrations were obtained from each mouse via the tail vein (time 0) using a handheld glucometer. Animals were then given access to a weighed portion of food from one of three experimental diets: LF/AM, H-GI (dark gray circles), LF/HM, L-GI (white diamonds) or LF/HY, L-GI (light gray squares). After a 20-min feeding period, the remaining pellet was removed and weighed. Blood glucose concentrations were obtained from the tail vein at 20, 45, 60, and 120 min after food ingestion for calculation of the incremental area under the blood glucose curve ($AUC_{glucose}$) (127). Blood glucose concentrations shown are LSmean (mg/dl) ± SE (n = 6 – 8 mice per group). #P = 0.0001 (LF/HM, L-GI vs. all other groups; one-way ANOVA). ***P = 0.0003 (LF/AM, H-GI vs. all other groups; one-way ANOVA). *P = 0.019 at 45 min, and *P = 0.045 at 60 min (LF/AM, H-GI vs. all other groups; one-way ANOVA).

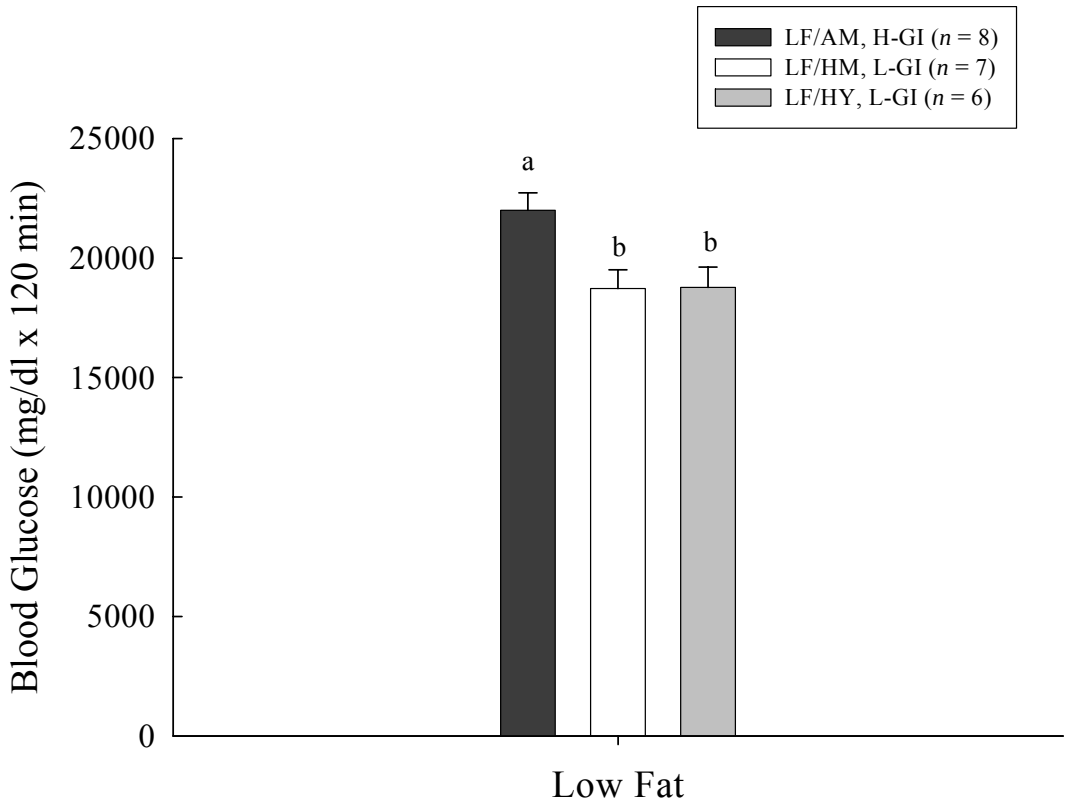

FIG. 2. Area under the blood glucose response curve ($AUC_{glucose}$) during a meal tolerance test the low fat diet groups. Meal tolerance tests were performed in the low fat diet groups at week 8 following an overnight fast. Blood glucose concentrations were obtained from each mouse via the tail vein (time 0) using a handheld glucometer. Animals were then given access to a weighed portion of food from one of three experimental diets: LF/AM, H-GI (dark gray bar), LF/HM, L-GI (white bar) or LF/HY, L-GI (light gray bar). After a 20-min feeding period, the remaining pellet was removed and weighed. Blood glucose concentrations were obtained from the tail vein at 20, 45, 60, and 120 min after food ingestion for calculation of the incremental area under the blood glucose curve ($AUC_{glucose}$) (127). The incremental $AUC_{glucose}$ over 120 min was calculated according to the trapezoidal rule using SigmaPlot 8.0. Values shown are LSmean (mg/dl x 120 min) ± SE (n = 6 - 8 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (LF/AM, H-GI vs. all other groups, one-way ANOVA).

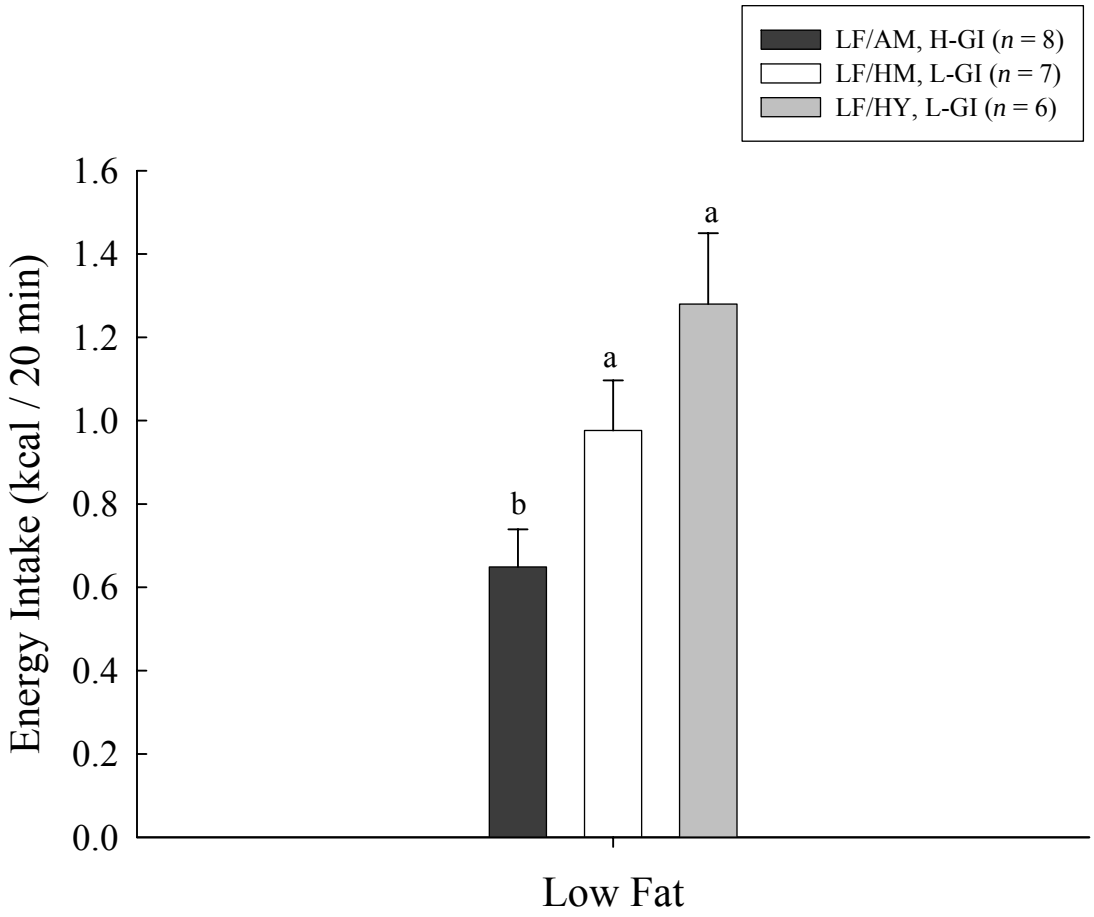

FIG. 3. Energy intake during a meal tolerance test in low fat diet groups. Meal tolerance tests were performed in the low fat diet groups at week 8 following an overnight fast. Animals were administered a weighed portion of food from one of three experimental diets: LF/AM, H-GI (dark gray bar), LF/HM, L-GI (white bar) or LF/HY, L-GI (light gray bar). After a 20-min feeding period, the remaining pellet, as well as any food that spilled into the bottom of the cage, was removed and weighed. The weight of the remaining pellet (g) was multiplied by the caloric density of the respective diet for calculation of energy intake (kcal / 20 min) from each mouse. Average energy intake from each low fat diet group during the twenty-minute feeding period is reported. Values shown are LSmean (kcal/20 min) ± SE (n = 4 – 6 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (LF/AM, H-GI vs. all other groups, one-way ANOVA).

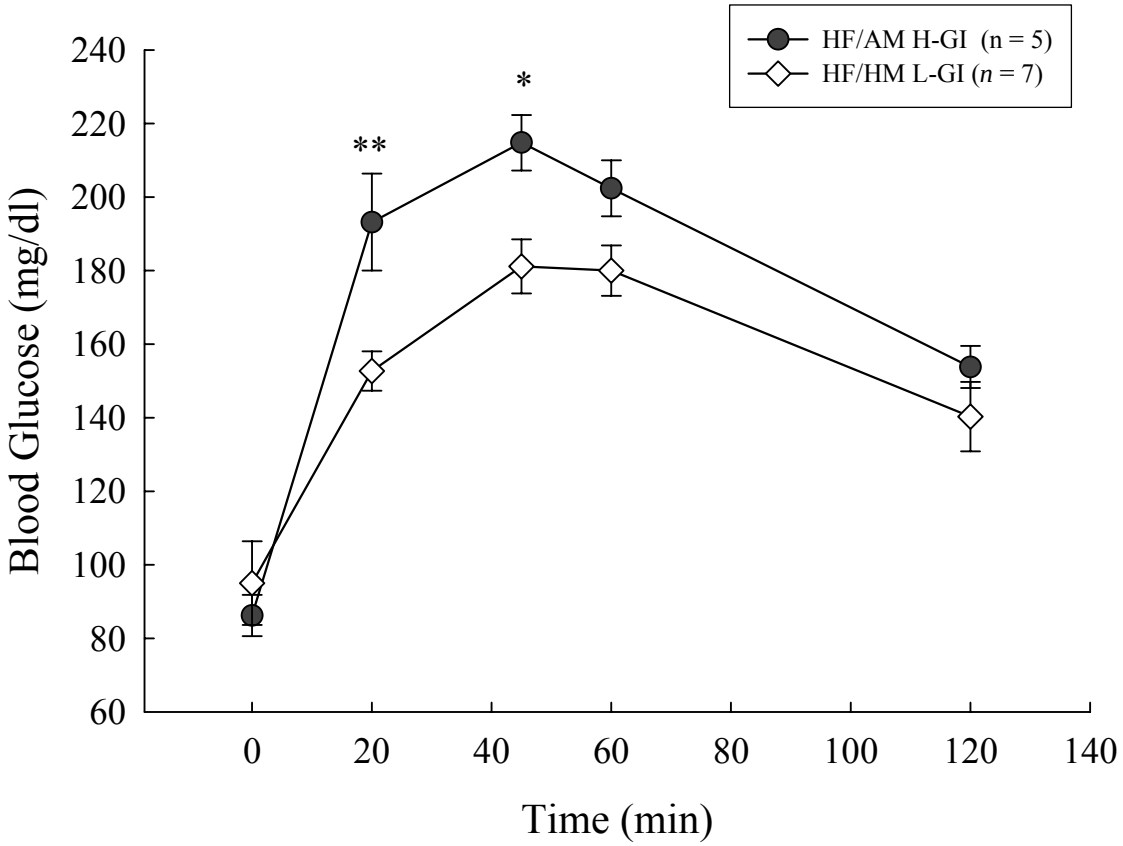

FIG. 4. Blood glucose response during a meal tolerance test in the high fat diet groups. Meal tolerance tests were performed in the high fat diet groups at week 12 following an overnight fast. Blood glucose concentrations were obtained from each mouse via the tail vein (time 0) using a handheld glucometer. Animals were then given access to a weighed portion of food from one of two experimental diets: HF/AM, H-GI (dark gray circles) or HF/HM, L-GI (white diamonds). After a 20-min feeding period, the remaining pellet was removed and weighed. Blood glucose concentrations were obtained from the tail vein at 20, 45, 60, and 120 min after food ingestion for calculation of the incremental area under the blood glucose curve ($AUC_{glucose}$) (127). Blood glucose concentrations shown are LSmean (mg/dl) ± SE (n = 5 – 7 mice per group). **P = 0.0095, *P = 0.011 (HF/AM, H-GI vs. HF/HM, L-GI; unpaired Student's t test).

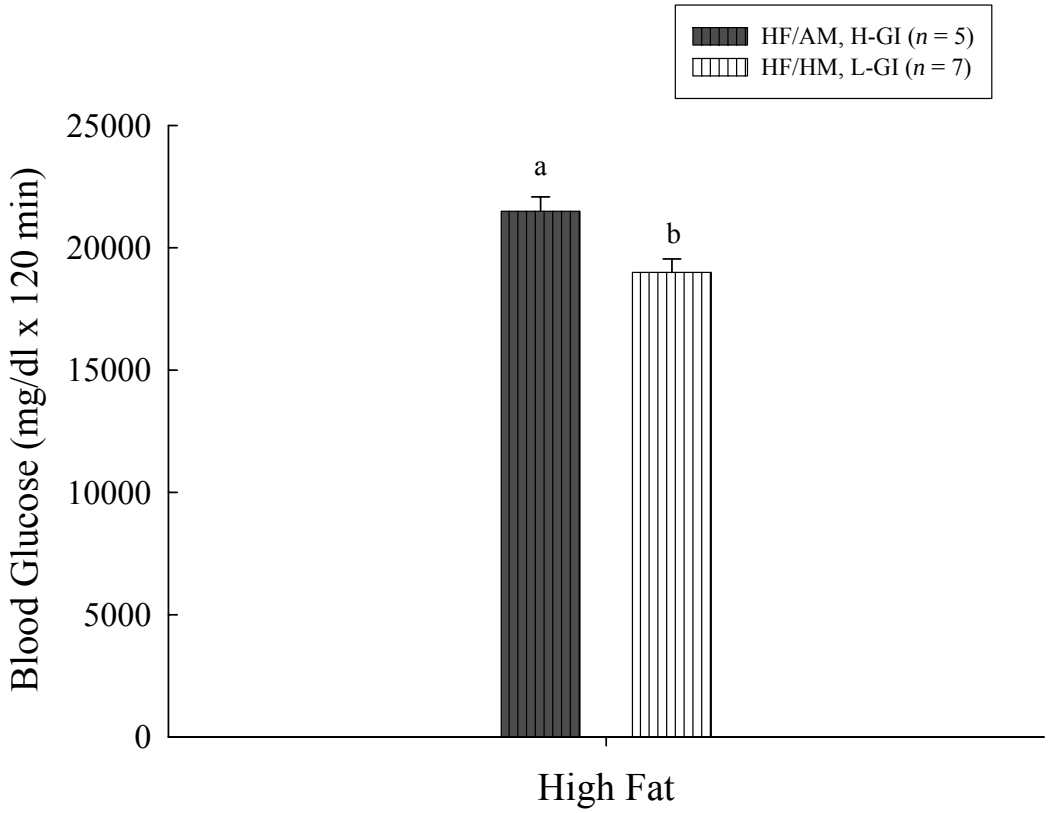

FIG. 5. Area under the blood glucose response curve ($AUC_{glucose}$) during a meal tolerance test the high fat diet groups. Meal tolerance tests were performed in the high fat diet groups at week 12 following an overnight fast. Blood glucose concentrations were obtained from each mouse via the tail vein (time 0) using a handheld glucometer. Animals were then given access to a weighed portion of food from one of two experimental diets: HF/AM, H-GI (dark gray bar) or HF/HM, L-GI (white bar; pattern-filled bars denote high fat diet groups). After a 20-min feeding period, the remaining pellet was removed and weighed. Blood glucose concentrations were obtained from the tail vein at 20, 45, 60, and 120 min after food ingestion for calculation of the incremental area under the blood glucose curve ($AUC_{glucose}$) (127). The incremental $AUC_{glucose}$ over 120 min was calculated according to the trapezoidal rule using SigmaPlot 8.0. Values shown are LSmean (mg/dl x 120 min) ± SE (n = 5 - 7 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (HF/AM, H-GI vs. HF/HM, L-GI; unpaired Student's t test).

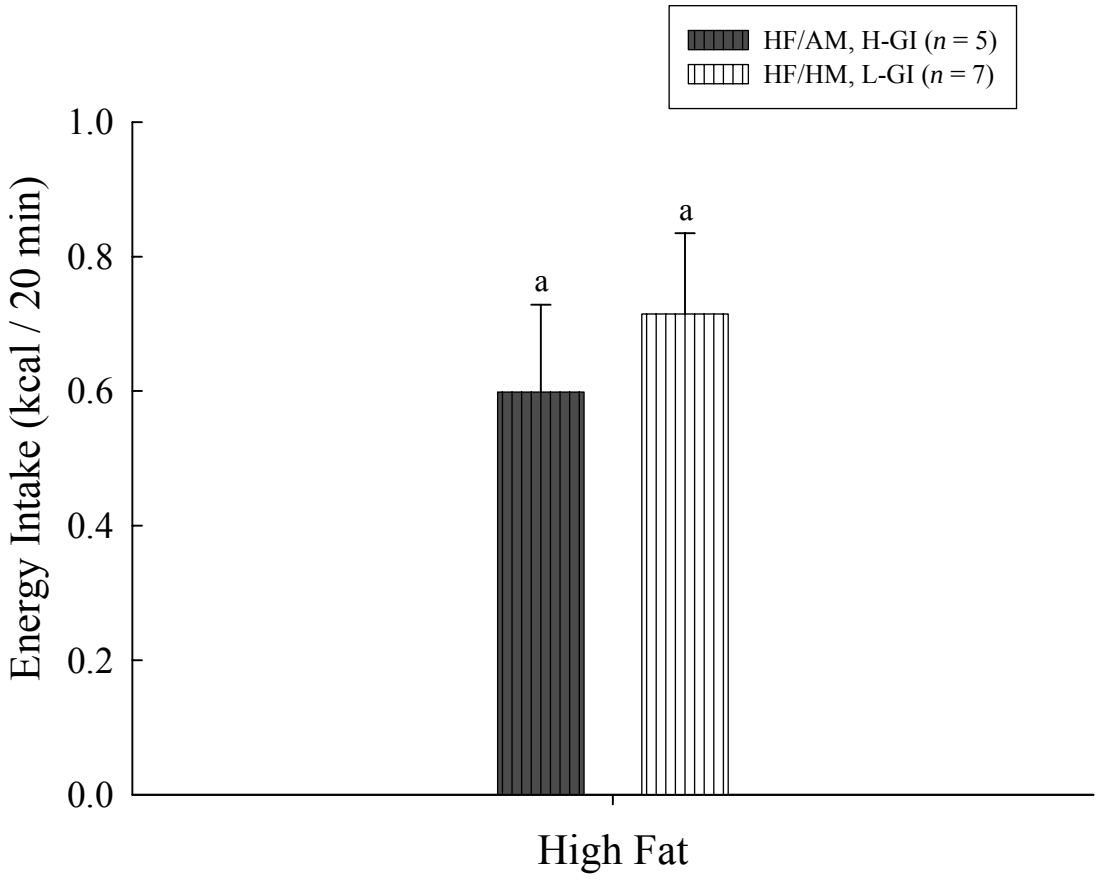

FIG. 6. Energy intake during a meal tolerance test in high fat diet groups. Meal tolerance tests were performed in the high fat diet groups at week 12 following an overnight fast. Animals were administered a weighed portion of food from one of two experimental diets: HF/AM, H-GI (dark gray bar) or HF/HM, L-GI (white bar; pattern-filled bars denote HF diet groups). After a 20-min feeding period, the remaining pellet, as well as any food that spilled into the bottom of the cage, was removed and weighed. The weight of the remaining pellet (g) was multiplied by the caloric density of the respective diet for calculation of energy intake (kcal / 20 min) from each mouse. Average energy intake from each high fat diet group during the twenty-minute feeding period is reported. Values shown are LSmean (kcal/20 min) ± SE ($n = 5 – 7$ mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (HF/AM, H-GI vs. HF/HM, L-GI; unpaired Student's *t* test).

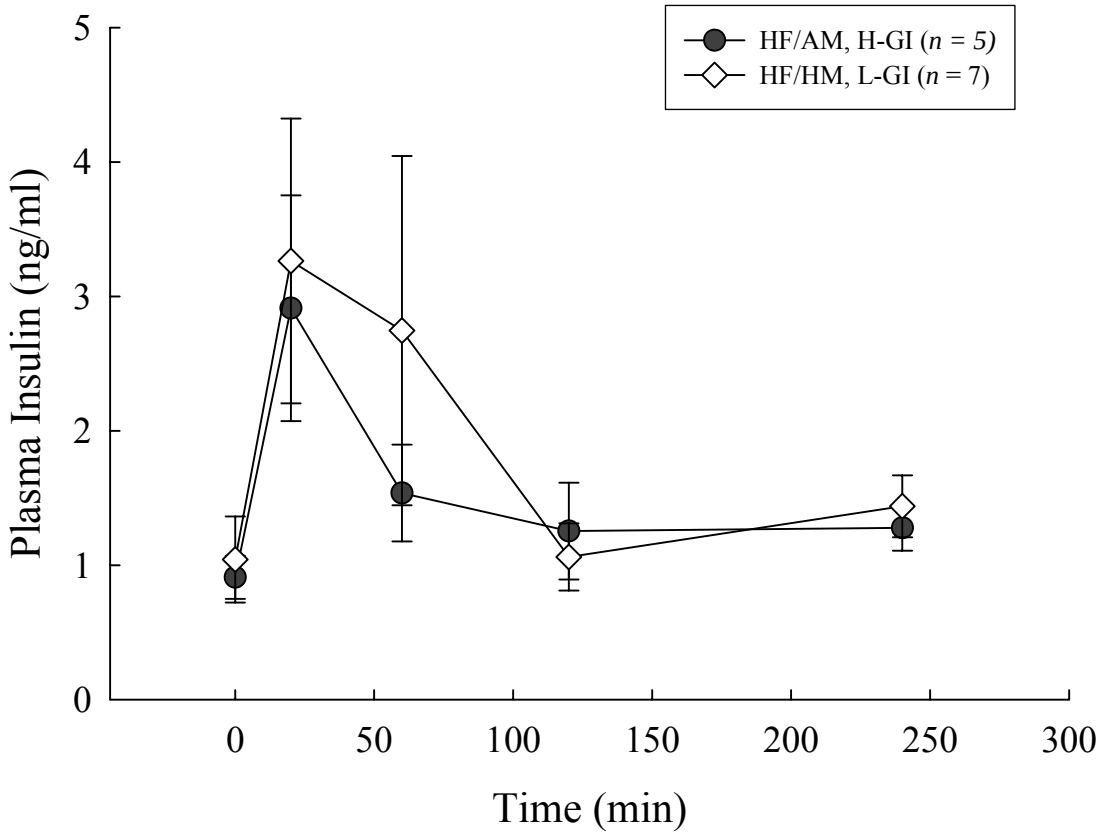

FIG. 7. Plasma insulin response during a meal tolerance test in the high fat diet groups. Meal tolerance tests were performed in the high fat diet groups at week 12 following an overnight fast. Animals were given access to a weighed portion of food from one of two experimental diets: HF/AM, H-GI (dark gray circles) or HF/HM, L-GI (white diamonds). After a 20-min feeding period, the remaining pellet was removed and weighed. Blood samples were collected via the tail vein at 0 (basal), 20, 60, 120 and 240 minutes after food ingestion. Plasma insulin concentrations shown are LSmean (ng/ml) ± SE (n = 5 – 7 mice per group). There were no significant differences in plasma insulin concentrations between groups at any time point.

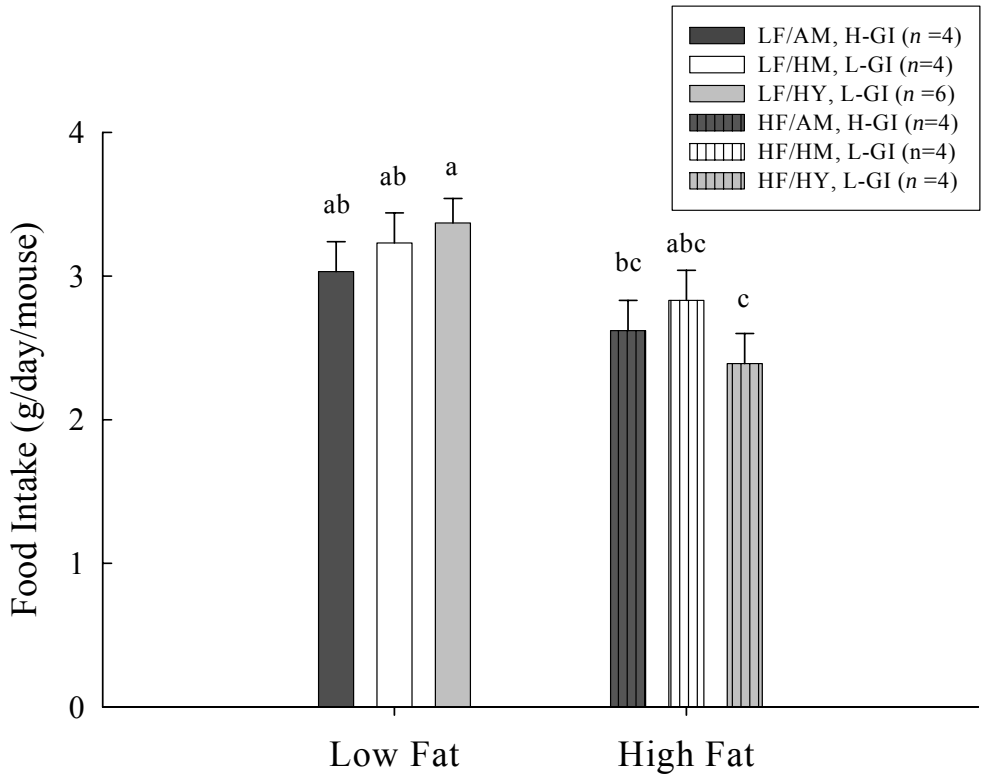

FIG. 8. Twenty-four hour food intake (g). Twenty-four hour food intake was measured by cage, in duplicate, at weeks 4 and 12. Measurements were made by weighing food pellets before and after twenty-four hours, and attempts were made to record any food that may have spilled into the bottom of the cage during feeding. Food intake values for each diet group from weeks 4 and 12 were combined for statistical analyses. Average food intake from each diet group is reported, and data is expressed as food intake in g/day/mouse due to unequal numbers of mice per cage (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]).Values shown are LSmean (g/day/mouse) ± SE (n = 4 – 6 cages per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

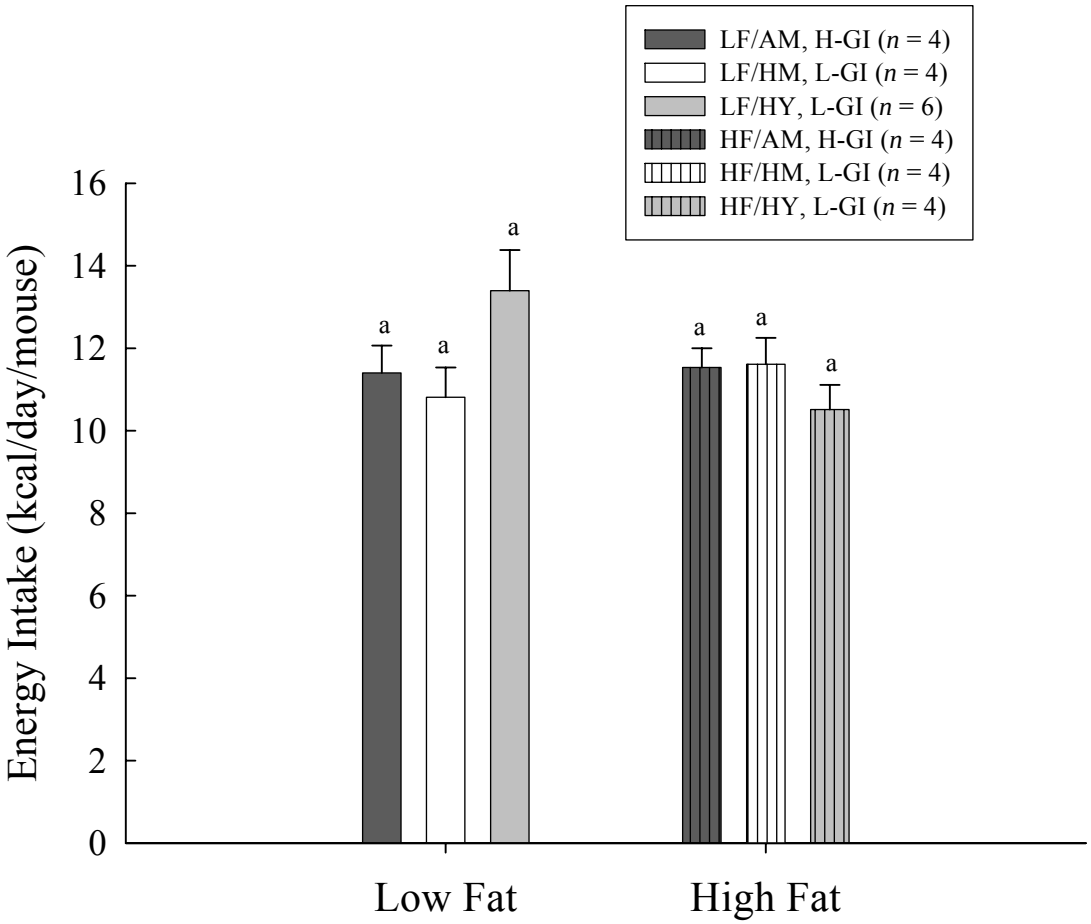

FIG. 9. Twenty-four hour energy intake (kcal/day/mouse). Twenty-four hour food intake values from each diet group at weeks 4 and 12 were multiplied by the caloric density of the respective diet for calculation of energy intake. Energy intake values for each diet group from weeks 4 and 12 were combined for statistical analyses. Average energy intake from each diet group is reported, and data is expressed as energy intake in kcal/day/mouse due to unequal numbers of mice per cage (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (kcal/day/mouse) ± SE ($n = 4 – 6$ cages per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's *t* tests).

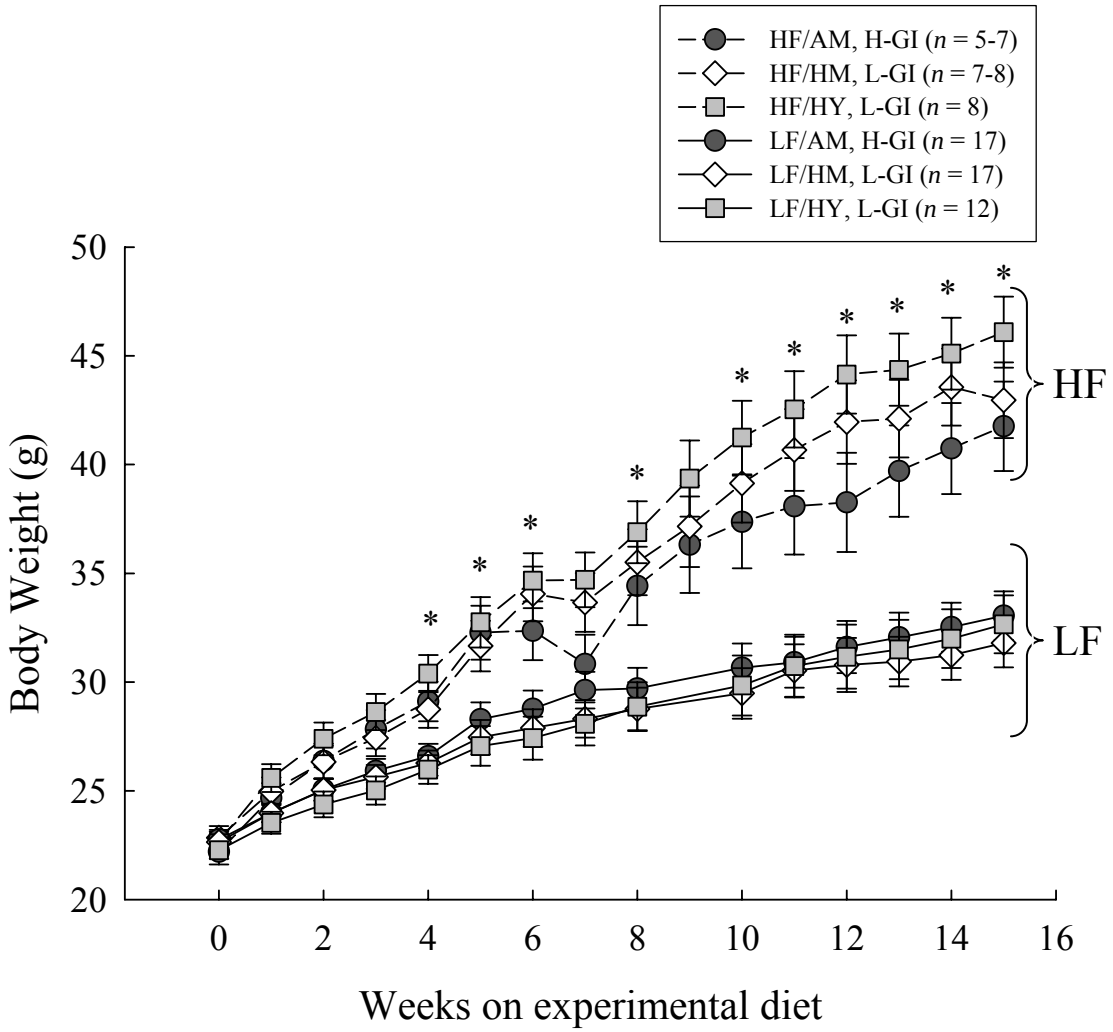

FIG. 10. Body weight over 15 weeks of experimental feeding. C57Bl/6 mice were fed one of six experimental diets, ad libitum, for 15 weeks (LF/AM, H-GI [dark gray circles], LF/HM, L-GI [white diamonds], LF/HY, L-GI [light gray squares], HF/AM, H-GI [dark gray circles, dashed line], HF/HM, L-GI [white diamonds, dashed line], HF/HY, L-GI [light gray squares, dashed line]). Body weights were not recorded at week 9 in the low fat diet groups. Low fat and high fat diet groups are labeled with brackets. Values shown are weekly LSmean body weights (g) ± SE (n = 5-17 mice per group). *$P < 0.05$, all high fat diet groups vs. all low fat diet groups (MANOVA with repeated measures test).

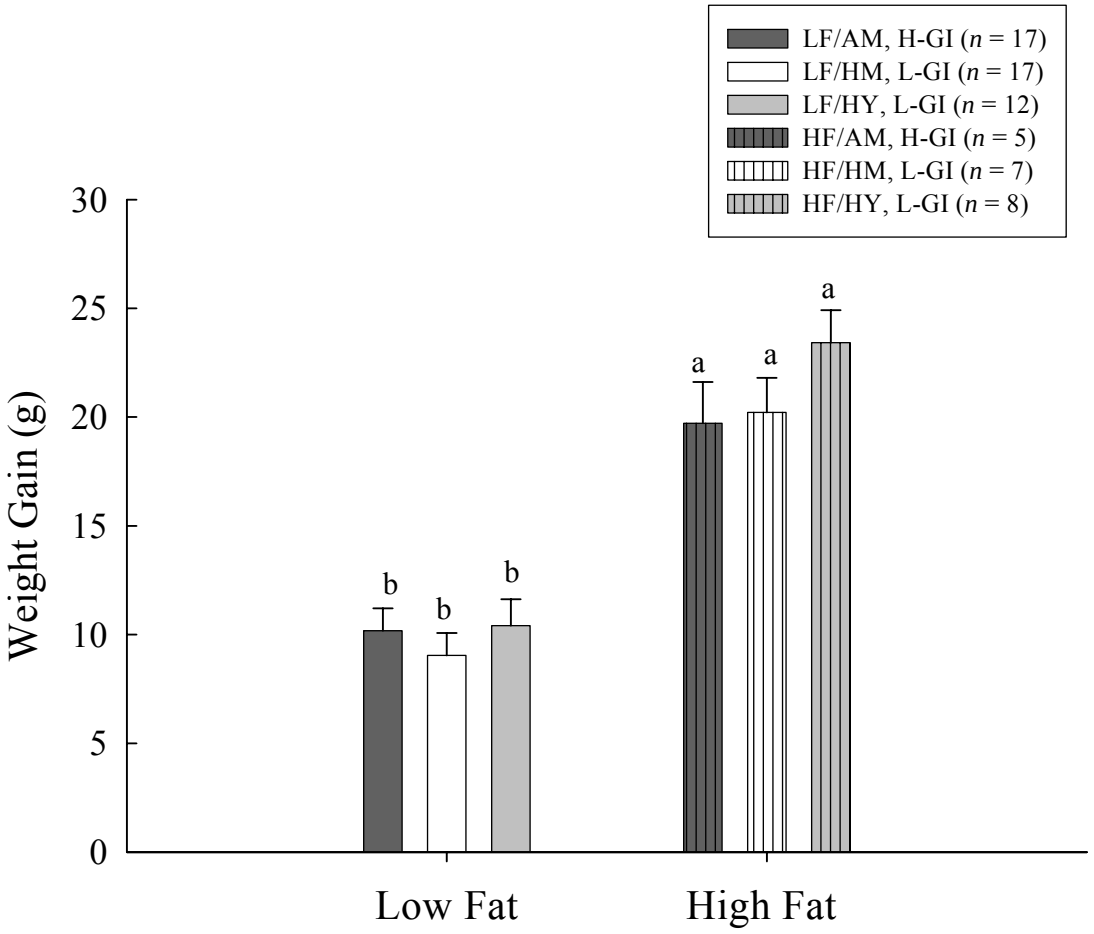

FIG. 11. Weight gain over 15 weeks of experimental feeding. C57Bl/6 mice were fed one of six experimental diets, ad libitum, for 15 weeks. Average weight gain from each diet group is reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (g) ± SE (n = 5-17 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

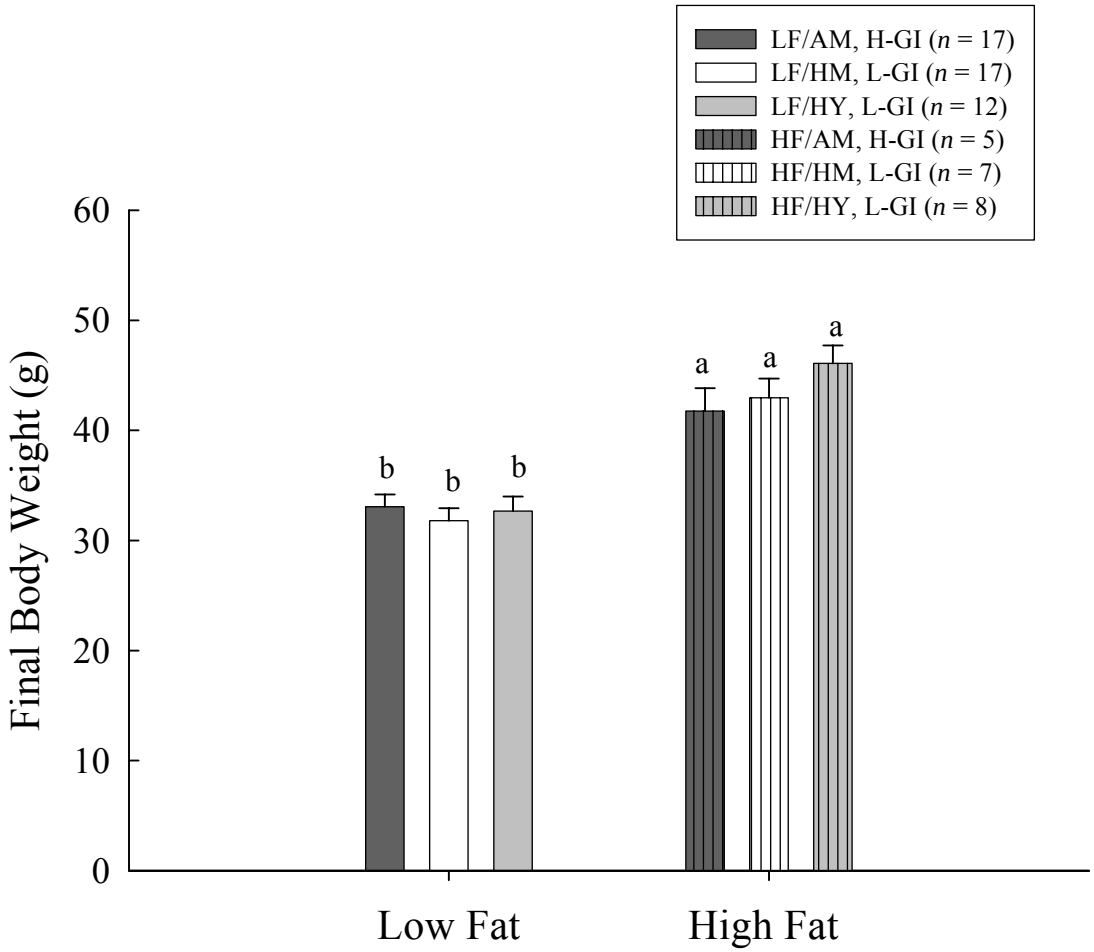

FIG. 12. Final body weight after 15 weeks of experimental feeding. C57Bl/6 mice were fed one of six experimental diets, ad libitum, for 15 weeks. Average final body weight from each diet group is reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (g) ± SE (n = 5-17 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

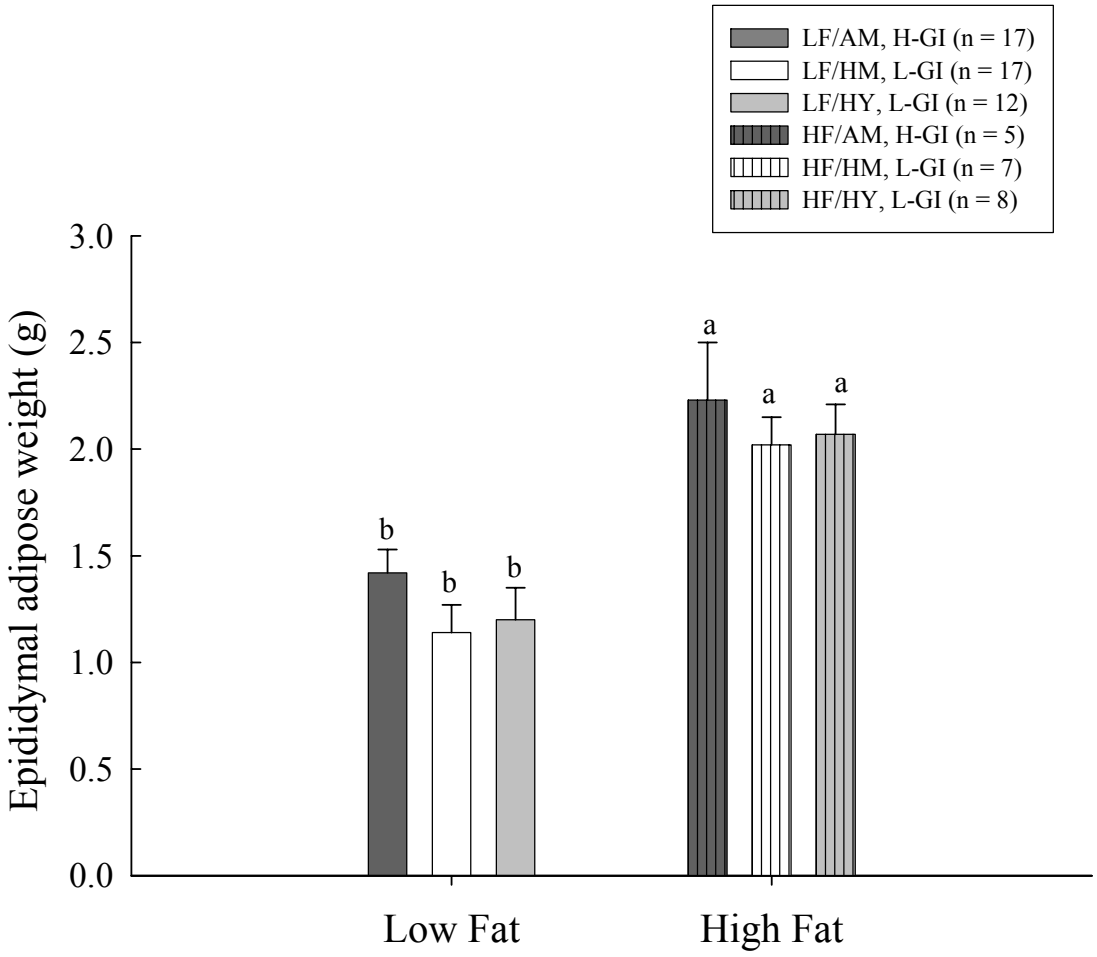

FIG. 13. Epididymal adipose weight. Right and left epididymal adipose depots were excised and weighed at the time of sacrifice (end of week 15). The combined average weight of both the right and the left epididymal adipose depots from each diet group is reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (g) ± SE (n = 5 – 17 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

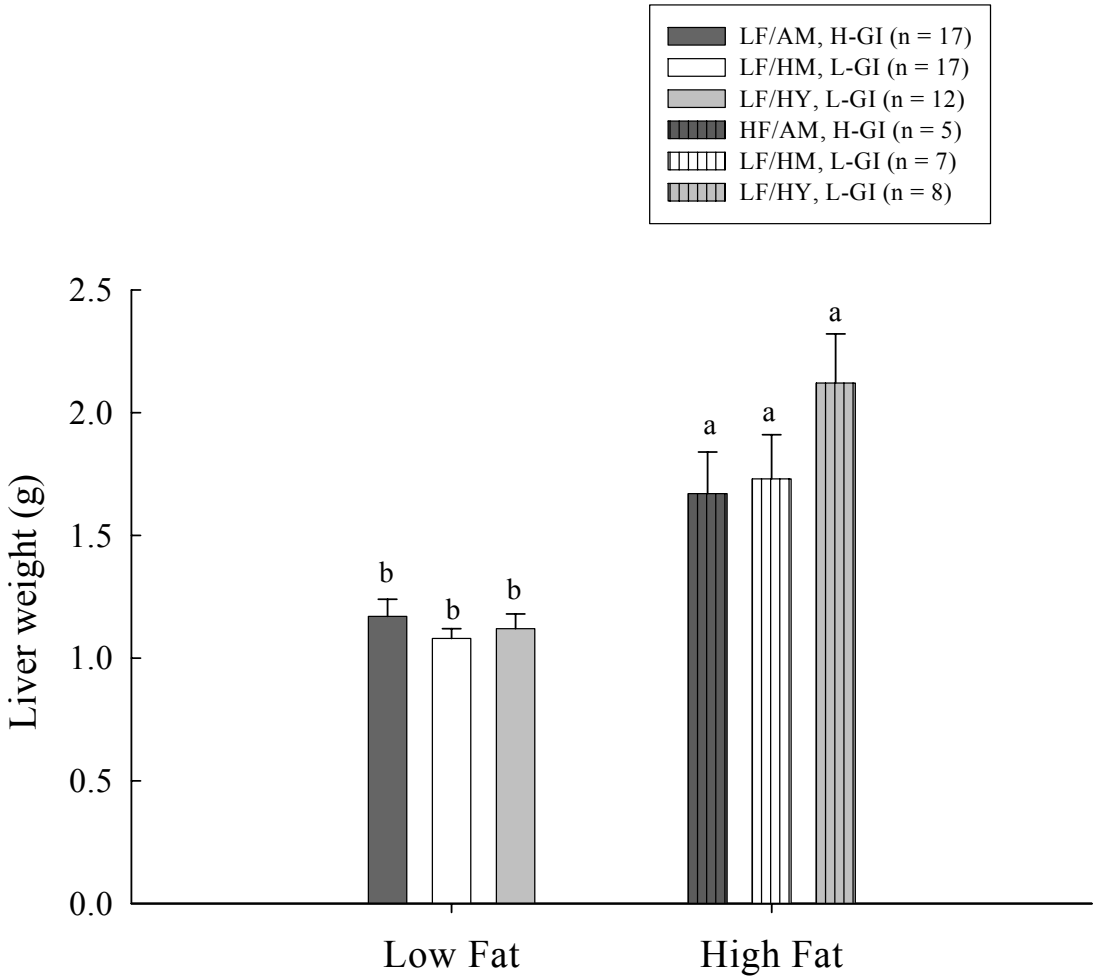

FIG. 14. Liver weight. All lobes of the liver were excised and weighed at the time of sacrifice (end of week 15). The combined average weight of all lobes of the liver from each diet group is reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (g) ± SE (n = 5 – 17 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

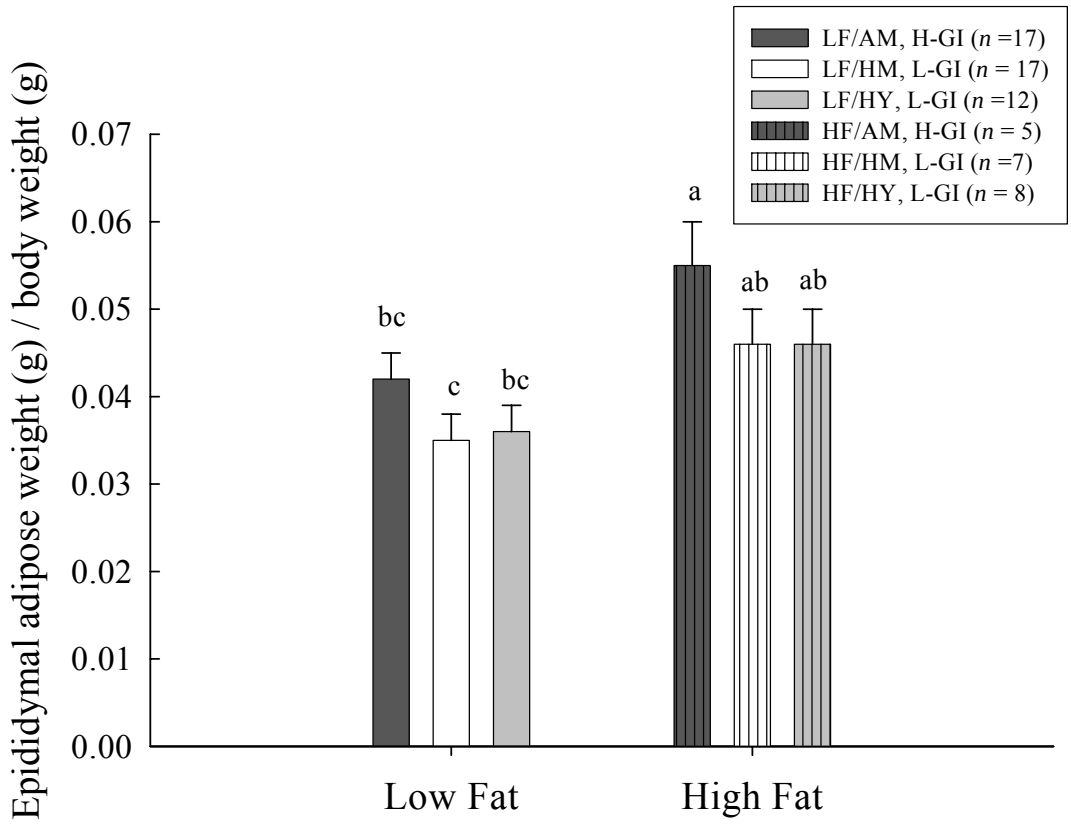

FIG. 15. Relative epididymal adipose weight. Right and left epididymal adipose depots were excised and weighed at the time of sacrifice (end of week 15). Relative epididymal adipose weight for each mouse was calculated by dividing the combined weight of the right and the left epididymal adipose depots (g) by the body weight of each mouse (g). The average relative epididymal adipose weight from each diet group is reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (g) ± SE (n = 5 – 17 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

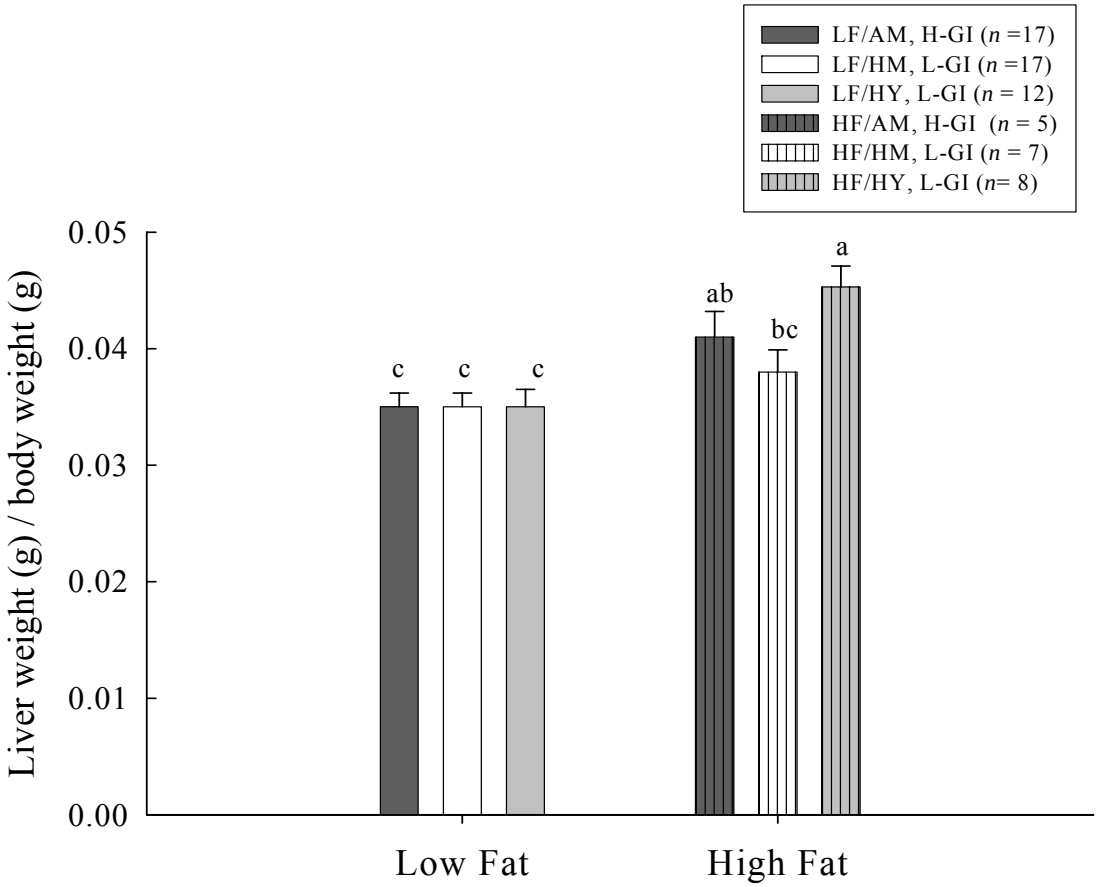

FIG. 16. Relative liver weight. All lobes of the liver were excised and weighed at the time of sacrifice (end of week 15). Relative liver weight for each mouse was calculated by dividing the combined weight of all lobes of the liver (g) by the body weight of each mouse (g). The average relative liver weight from each diet group is reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (g) ± SE (n = 5 – 17 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

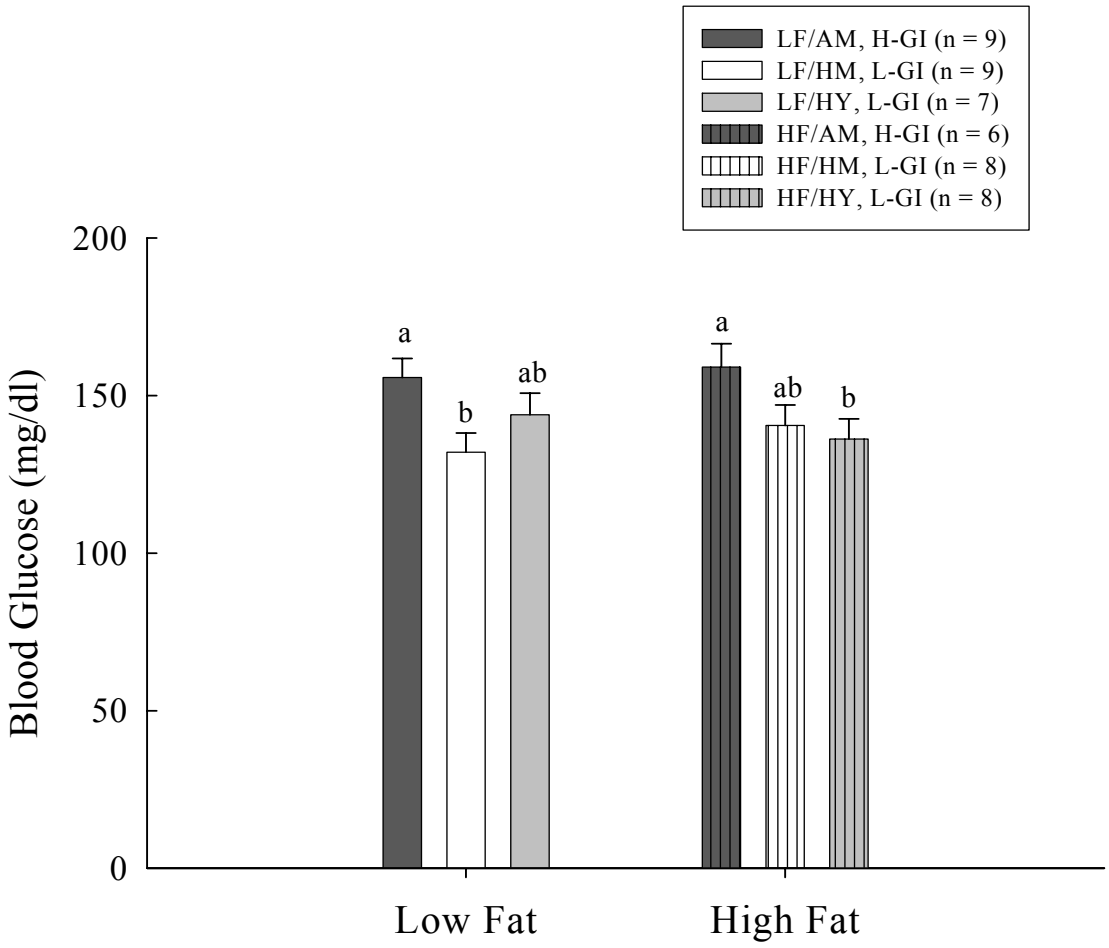

FIG. 17. Non-fasting blood glucose levels at week 4. Non-fasting blood glucose concentrations were measured at 8:00 AM on week 4 using a handheld glucometer. The average non-fasting blood glucose concentration from each diet group is reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (mg/dl) ± SE (n = 6 – 9 mice per group). Bars not sharing the same letter are significantly different, P < 0.05 (two-way ANOVA and follow-up unpaired Student's t tests).

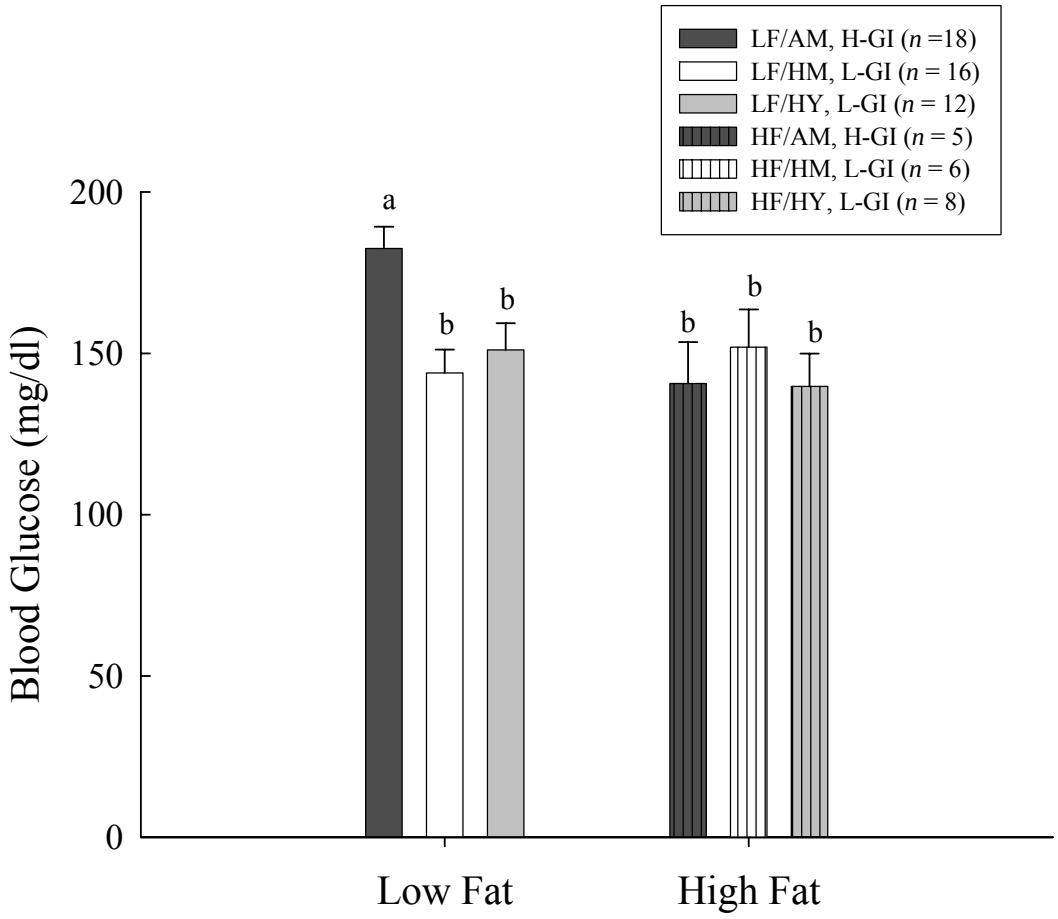

FIG. 18. Non-fasting blood glucose levels at week 8. Non-fasting blood glucose concentrations were measured at 8:00 AM on week 8 using a handheld glucometer. The average non-fasting blood glucose concentration from each diet group is reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean (mg/dl) ± SE (n = 5 – 18 mice per group). Bars not sharing the same letter are significantly different, $P <$ 0.05 (two-way ANOVA and follow-up unpaired Student's t tests).

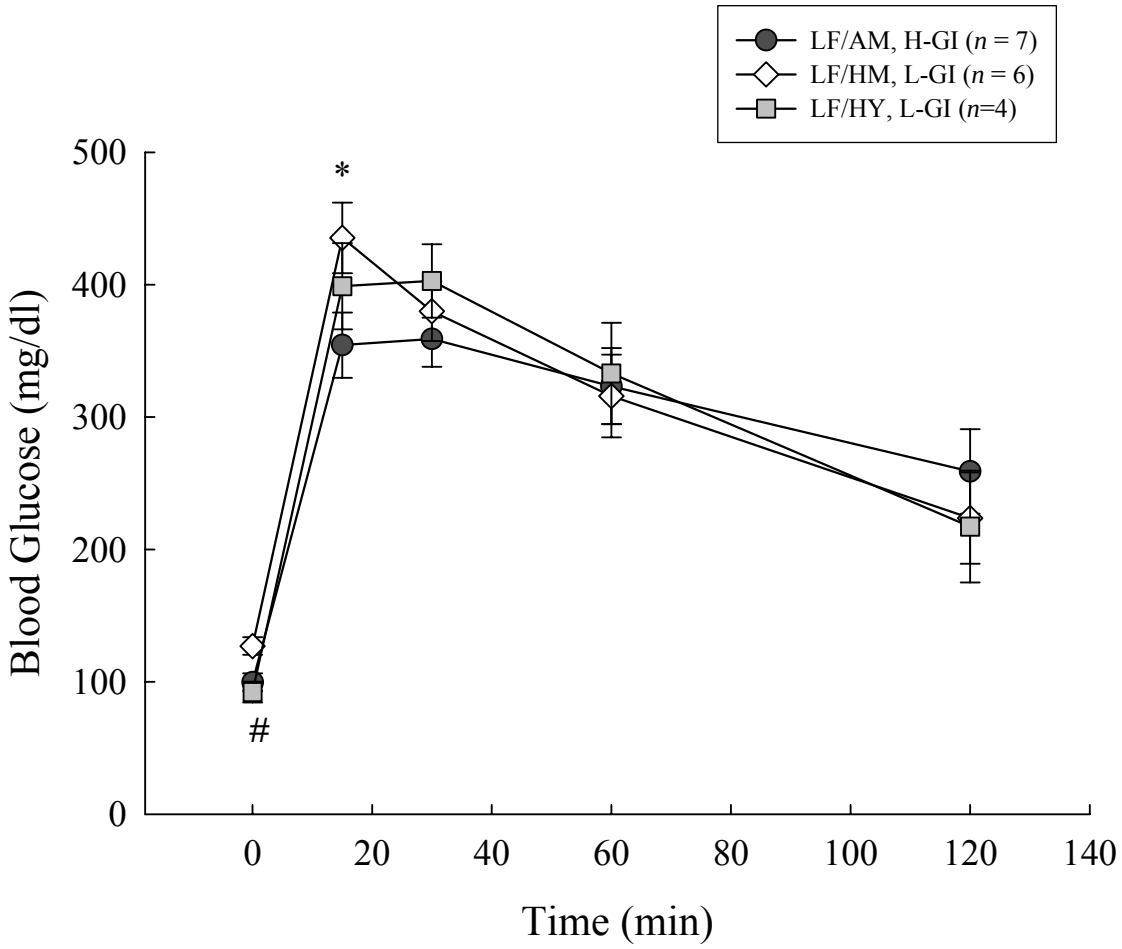

FIG. 19. Intraperitoneal glucose tolerance test in low fat diet groups. Glucose tolerance tests were performed in the low fat diet groups at the beginning of week 15 following an overnight fast (LF/AM, H-GI [dark gray circles], LF/HM, L-GI [white diamonds], LF/HY, L-GI [light gray squares]). Blood glucose concentrations were obtained from each mouse via the tail vein (time 0) using a handheld glucometer. Animals were then administered an intraperitoneal injection of a filter-sterilized glucose solution (1.5 g/kg body weight). Blood glucose concentrations were obtained from the tail vein at 15, 30, 60, and 120 min after glucose injection for calculation of the incremental $AUC_{glucose}$ (127). Blood glucose concentrations shown are LSmean (mg/dl) ± SE (n = 4 – 7 mice per group). # $P < 0.0001$ (LF/HM, L-GI vs. all other groups; one-way ANOVA), *$P = 0.0329$ (LF/HM, L-GI vs. LF/AM, H-GI; one-way ANOVA).

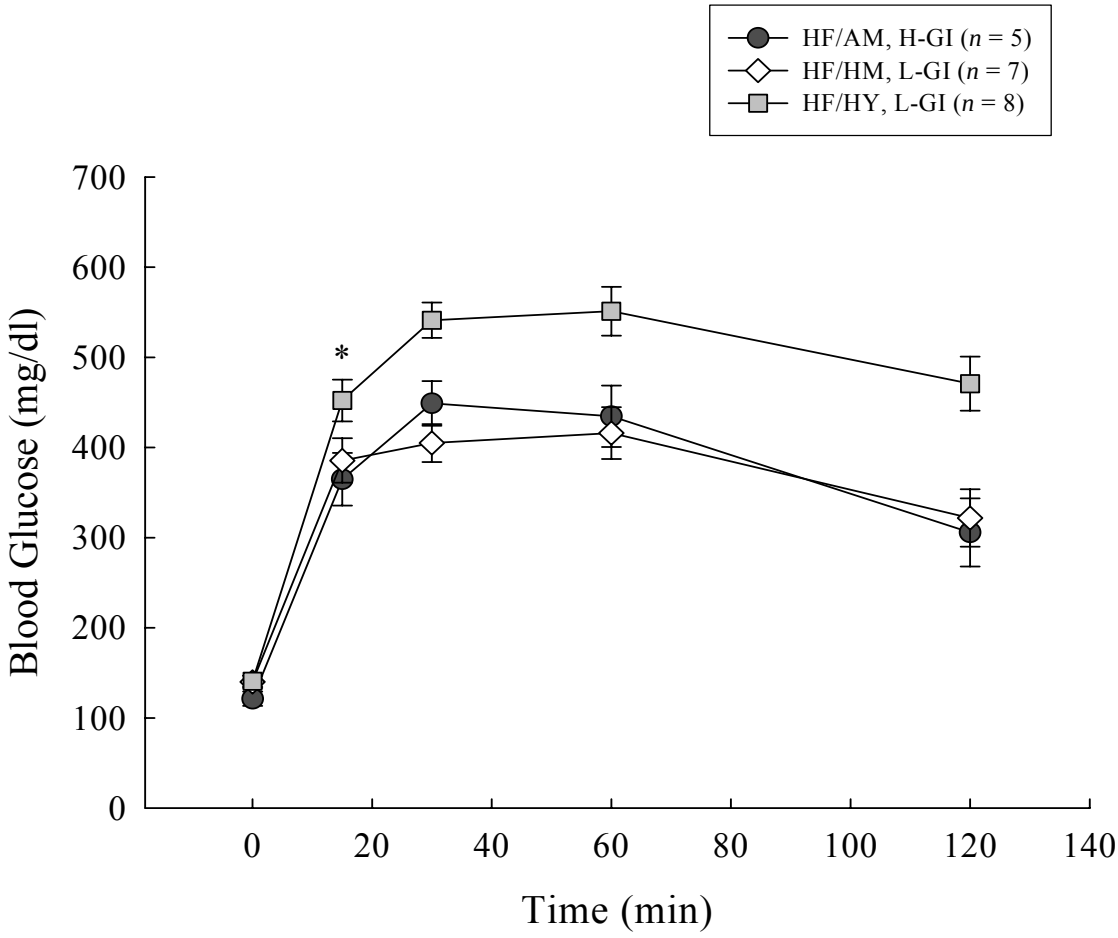

FIG. 20. Intraperitoneal glucose tolerance test in high fat diet groups. Glucose tolerance tests were performed in the high fat diet groups at the beginning of week 15 following an overnight fast (HF/AM, H-GI [dark gray circles], HF/HM, L-GI [white diamonds], HF/HY, L-GI [light gray squares]). Blood glucose concentrations were obtained from each mouse via the tail vein (time 0) using a handheld glucometer. Animals were then administered an intraperitoneal injection of a filter-sterilized glucose solution (1.5 g/kg body weight). Blood glucose concentrations were obtained from the tail vein at 15, 30, 60, and 120 min after glucose injection for calculation of the incremental $AUC_{glucose}$ (127). In the HF/HY, L-GI group at 30, 60, and 120 min, 2 mice, 6 mice, and 1 mouse, respectively, had blood glucose concentrations beyond the maximum detection limit of the glucometer (600 mg/dl). Blood glucose concentrations shown are LSmean (mg/dl) ± SE (n = 5 – 8 mice per group). * P = 0.0254 (HF/HY, L-GI vs. HF/AM, H-GI; one-way ANOVA), ***P = 0.0005 at 30 min, ***P = 0.0037 at 60 min, and ***P = 0.0025 at 120 min (HF/HY, L-GI vs. all other groups; one-way ANOVA).

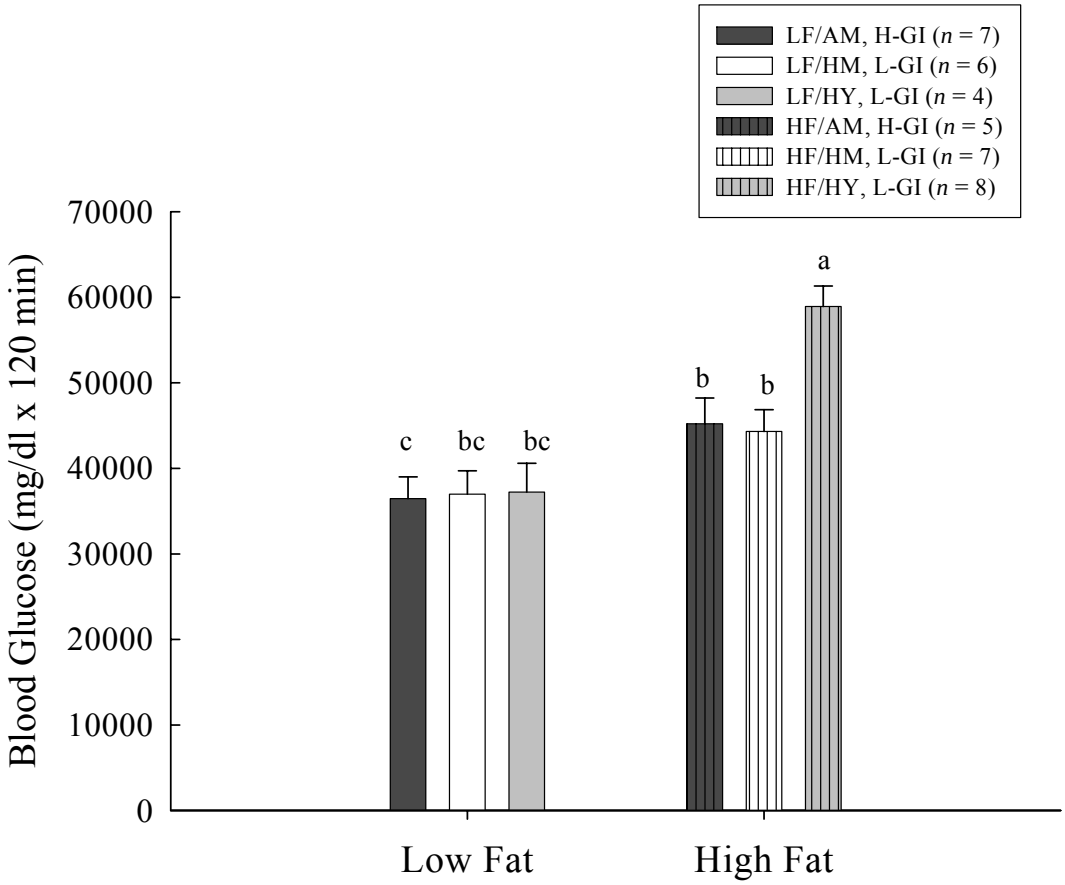

FIG. 21. Area under the blood glucose response curve ($AUC_{glucose}$) during an intraperitoneal glucose tolerance test. Glucose tolerance tests were performed in each diet group at the beginning of week 15 following an overnight fast (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Blood glucose concentrations were obtained from each mouse via the tail vein (time 0) using a handheld glucometer. Animals were then administered an intraperitoneal injection of a filter-sterilized glucose solution (1.5 g/kg body weight). Blood glucose concentrations were obtained from the tail vein at 15, 30, 60, and 120 min after glucose injection for calculation of the incremental $AUC_{glucose}$ (127). The incremental $AUC_{glucose}$ over 120 min was calculated according to the trapezoidal rule using SigmaPlot 8.0. Values shown are LSmean (mg/dl x 120 min) ± SE (n = 4 - 8 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

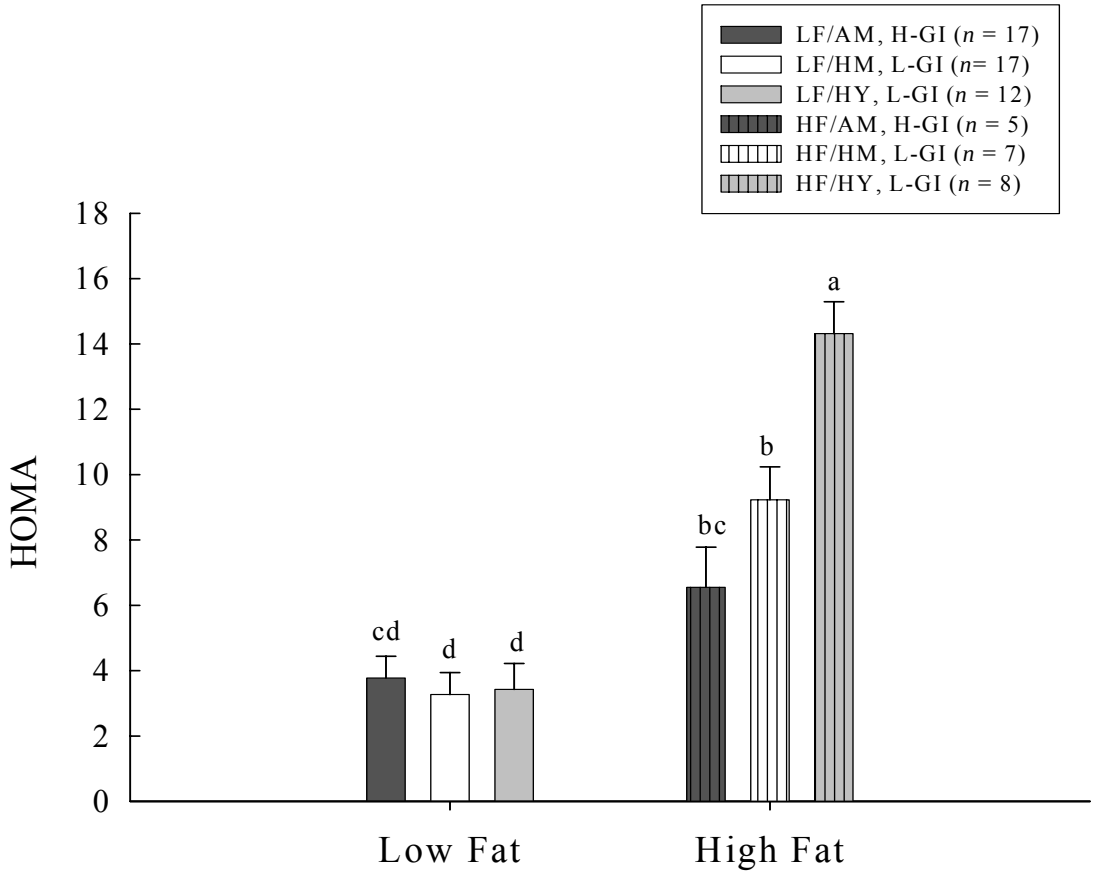

FIG. 22. Homeostasis model assessment (HOMA) index at the end of week 15. The homeostasis model assessment index was used as a measure of insulin resistance (128). HOMA index values were calculated as fasting blood glucose (mmol/L) x fasting plasma insulin (μU/mL) / 22.5. Average HOMA index values from each diet group are reported (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Values shown are LSmean ± SE (n = 5-17 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

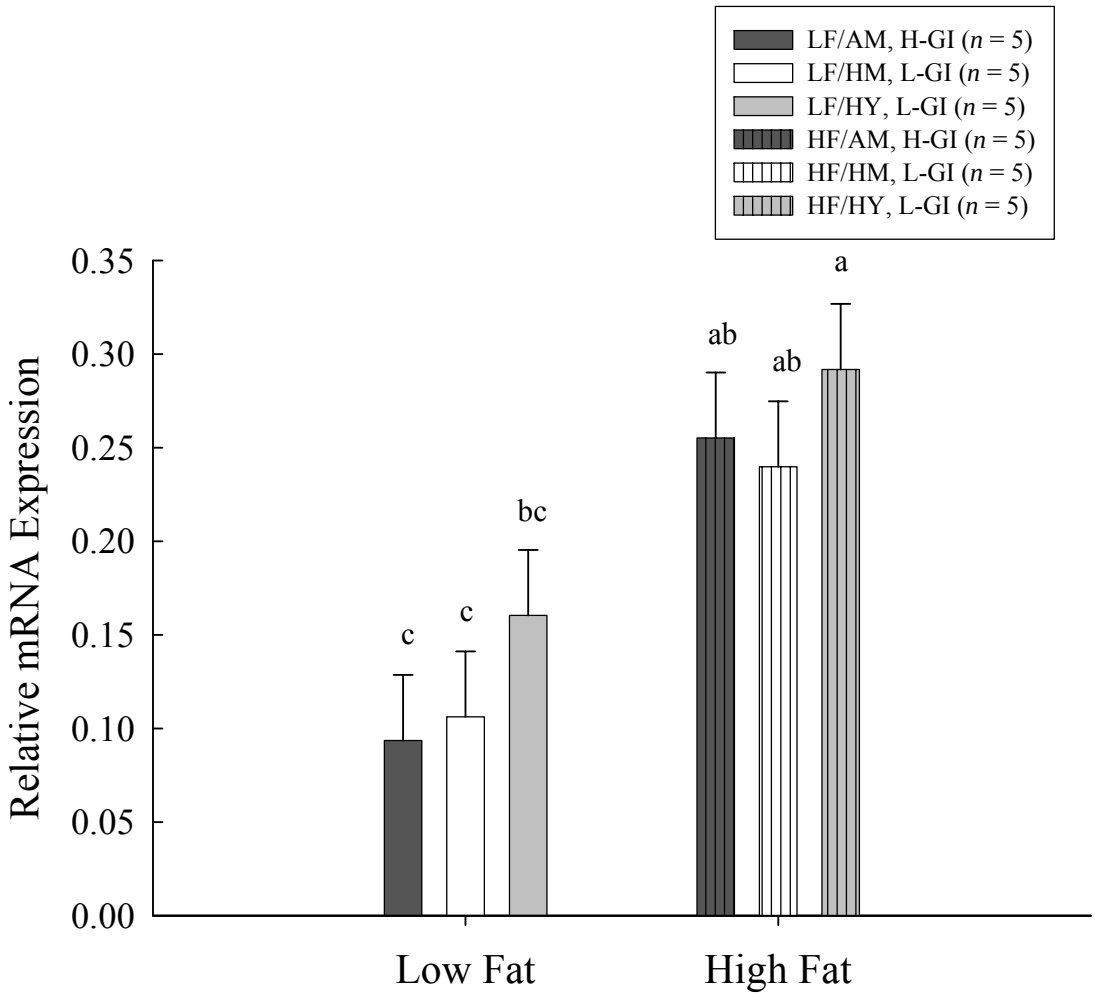

FIG. 23. Relative NADPH oxidase mRNA expression in the epididymal adipose tissue of C57Bl/6 mice. Total RNA was isolated from 50-100 mg of mouse epididymal adipose tissue. Complementary DNA (cDNA) was synthesized from 1μg of total RNA, and used for real-time reverse transcriptase polymerase chain reaction (RT-PCR). A subset of animals from each diet group was used for gene expression analysis (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Expression levels were normalized to β-actin by calculating the ΔCt (ΔCt = β-actin Ct – NADPH oxidase Ct). Relative mRNA expression was calculated as $2^{\Delta Ct}$ (Real-Time PCR Applications Guide, BioRad, Hercules, CA; Appendix C). Values shown are LSmean (x100) ± SE (n = 5 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

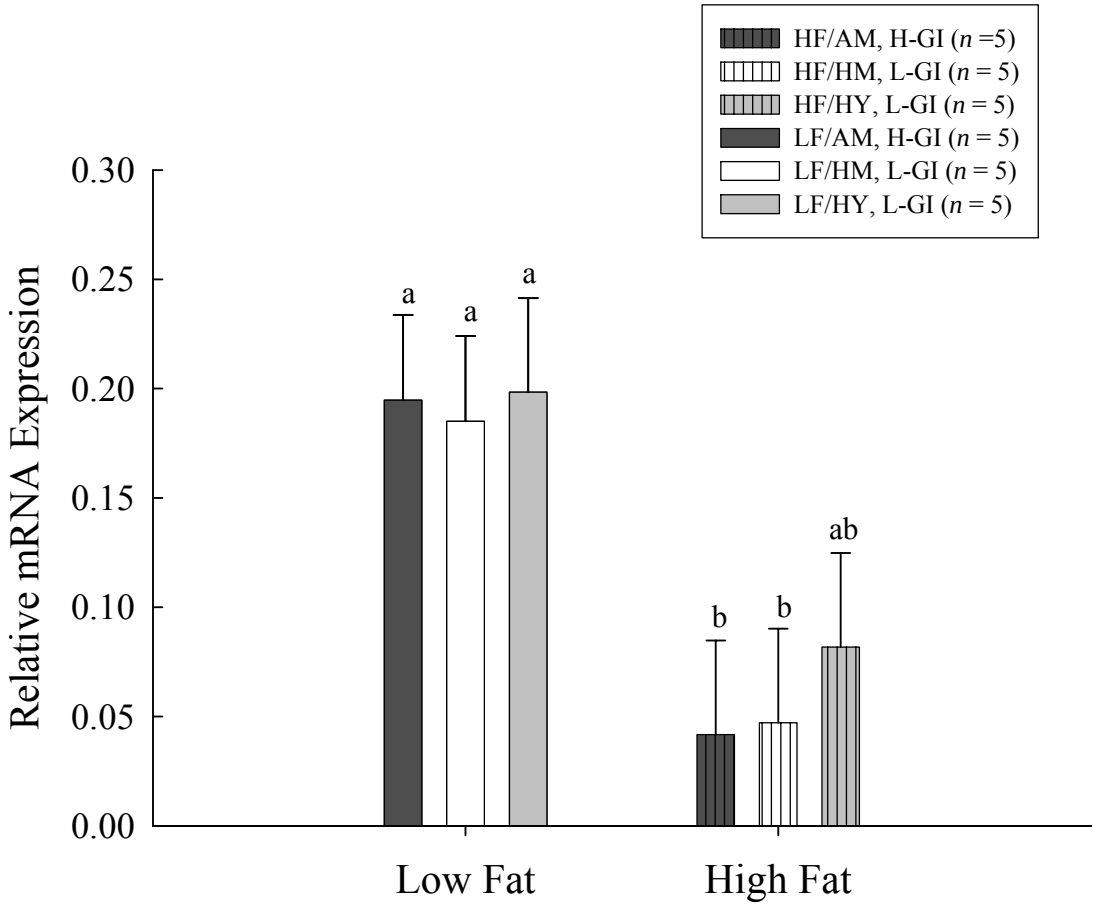

FIG. 24. Relative glutathione peroxidase-1 mRNA expression in the epididymal adipose tissue of C57Bl/6 mice. Total RNA was isolated from 50-100 mg of mouse epididymal adipose tissue. Complementary DNA (cDNA) was synthesized from 1µg of total RNA, and used for real-time reverse transcriptase polymerase chain reaction (RT-PCR). A subset of animals from each diet group was used for gene expression analysis (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Expression levels were normalized to β-actin by calculating the ΔCt (ΔCt = β-actin Ct – glutathione peroxidase-1 Ct). Relative mRNA expression was calculated as $2^{\Delta Ct}$ (Real-Time PCR Applications Guide, BioRad, Hercules, CA; Appendix C). Values shown are LSmean ± SE (n = 5 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

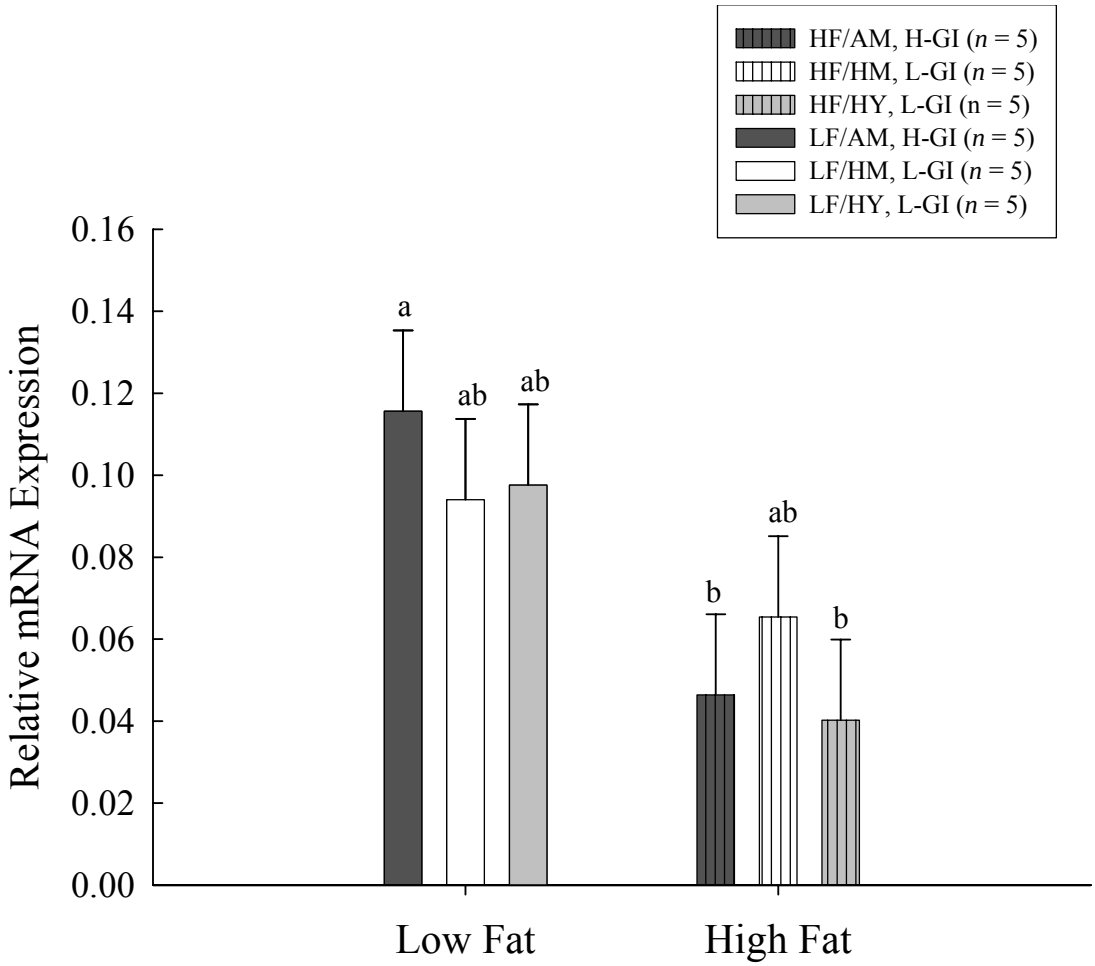

FIG. 25. Relative catalase mRNA expression in the epididymal adipose tissue of C57Bl/6 mice. Total RNA was isolated from 50-100 mg of mouse epididymal adipose tissue. Complementary DNA (cDNA) was synthesized from 1µg of total RNA, and used for real-time reverse transcriptase polymerase chain reaction (RT-PCR). A subset of animals from each diet group was used for gene expression analysis (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Expression levels were normalized to β-actin by calculating the ΔCt (ΔCt = β-actin Ct – catalase Ct). Relative mRNA expression was calculated as $2^{\Delta Ct}$ (Real-Time PCR Applications Guide, BioRad, Hercules, CA; Appendix C). Values shown are LSmean ± SE (n = 5 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's t tests).

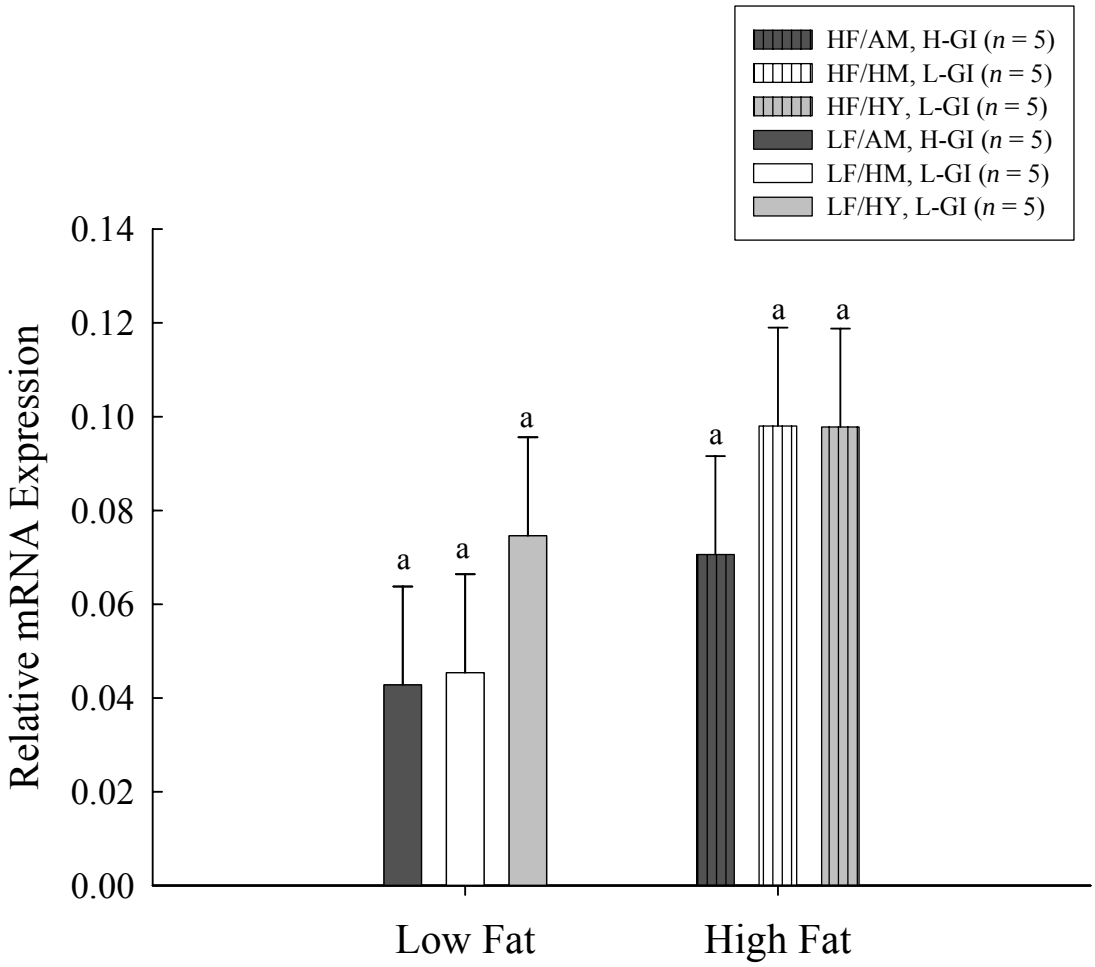

FIG. 26. Relative superoxide dismutase-2 mRNA expression in the epididymal adipose tissue of C57Bl/6 mice. Total RNA was isolated from 50-100 mg of mouse epididymal adipose tissue. Complementary DNA (cDNA) was synthesized from 1µg of total RNA, and used for real-time reverse transcriptase polymerase chain reaction (RT-PCR). A subset of animals from each diet group was used for gene expression analysis (LF/AM, H-GI [dark gray bar], LF/HM, L-GI [white bar], LF/HY, L-GI [light gray bar], HF/AM, H-GI [dark gray pattern-filled bar], HF/HM, L-GI [white pattern-filled bar], HF/HY, L-GI [light gray pattern-filled bar]). Expression levels were normalized to β-actin by calculating the ΔCt (ΔCt = β-actin Ct – superoxide dismutase-2 Ct). Relative mRNA expression was calculated as $2^{\Delta Ct}$ (Real-Time PCR Applications Guide, BioRad, Hercules, CA; Appendix C). Values shown are LSmean ± SE (*n* = 5 mice per group). Bars not sharing the same letter are significantly different, $P < 0.05$ (two-way ANOVA and follow-up unpaired Student's *t* tests).

TABLE 3
Plasma measurements in 15-week-old mice[*, †, ‡]

	Low Fat			High Fat			Main Effects		Interactions
	H-GI AM (*n*)	L-GI HM (*n*)	L-GI HY (*n*)	H-GI AM (*n*)	L-GI HM (*n*)	L-GI HY (*n*)	Fat	Starch	Fat x Starch
Blood Glucose (mg/dl)	153 ± 6.0 (17)[b]	151 ± 6.0 (17)[b]	144 ± 7.1 (12)[b]	186 ± 11.0 (5)[a]	178 ± 9.3 (7)[a]	185 ± 8.7 (8)[a]	*P* < 0.0001	NS	NS
Insulin (ng/ml)	0.44 ± 0.06 (17)[c]	0.37 ± 0.06 (17)[c]	0.40 ± 0.07 (12)[c]	0.57 ± 0.1 (5)[c]	0.88 ± 0.09 (7)[b]	1.3 ± 0.08 (8)[a]	*P* < 0.0001	*P* = 0.0001	*P* < 0.0001
Triglyceride (mg/dl)	43 ± 7.1 (7)[bc]	57 ± 7.1 (7)[abc]	37 ± 8.4 (5)[c]	62 ± 8.4 (5)[ab]	65 ± 7.1 (7)[a]	47 ± 6.6 (8)[abc]	NS	NS	NS
Adiponectin (µg/ml)	16 ± 1.2 (17)[a]	16 ± 1.2 (15)[a]	12 ± 1.4 (12)[b]	10 ± 2.1 (5)[b]	9.0 ± 1.8 (7)[b]	10 ± 1.7 (8)[b]	*P* = 0.0002	NS	NS
Leptin[§‖] (ng/ml/ g body weight)	0.41± 0.05 (15)[a]	0.23± 0.04 (16)[b]	0.30± 0.05 (12)[ab]	ND	ND	ND	ND	*P* = 0.0325	ND
IL-6 (pg/ml)	3.68 ± 0.44 (7)[c]	4.99 ± 0.6 (7)[bc]	9.5 ± 1.4 (5)[a]	7.86 ± 1.8 (5)[ab]	7.01 ± 2.1 (7)[abc]	5.51 ± 0.73 (8)[bc]	NS	NS	*P* = 0.0130

[*]Data reported as LSmean ± SE (*n*).
[†]Means within a row with different letters designate a significant difference at $P < 0.05$ (two-way ANOVA and unpaired Student's *t*-test; NS = not significant, ND = not determined).
[‡]Mice were fasted for 5-6 hours prior to blood collection.
[§]Leptin levels exceeded the detection limit of the assay in the high fat diet groups.
[‖]One-way ANOVA performed for comparison of leptin concentrations in the low fat diet groups.

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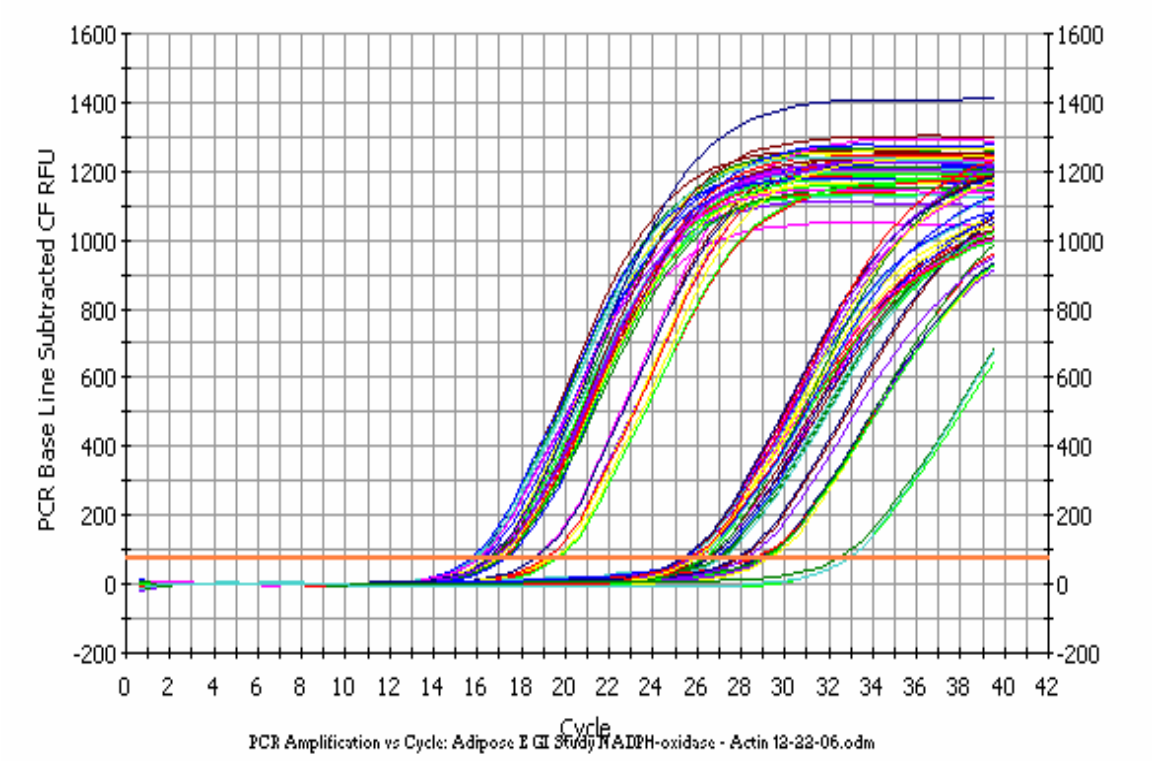
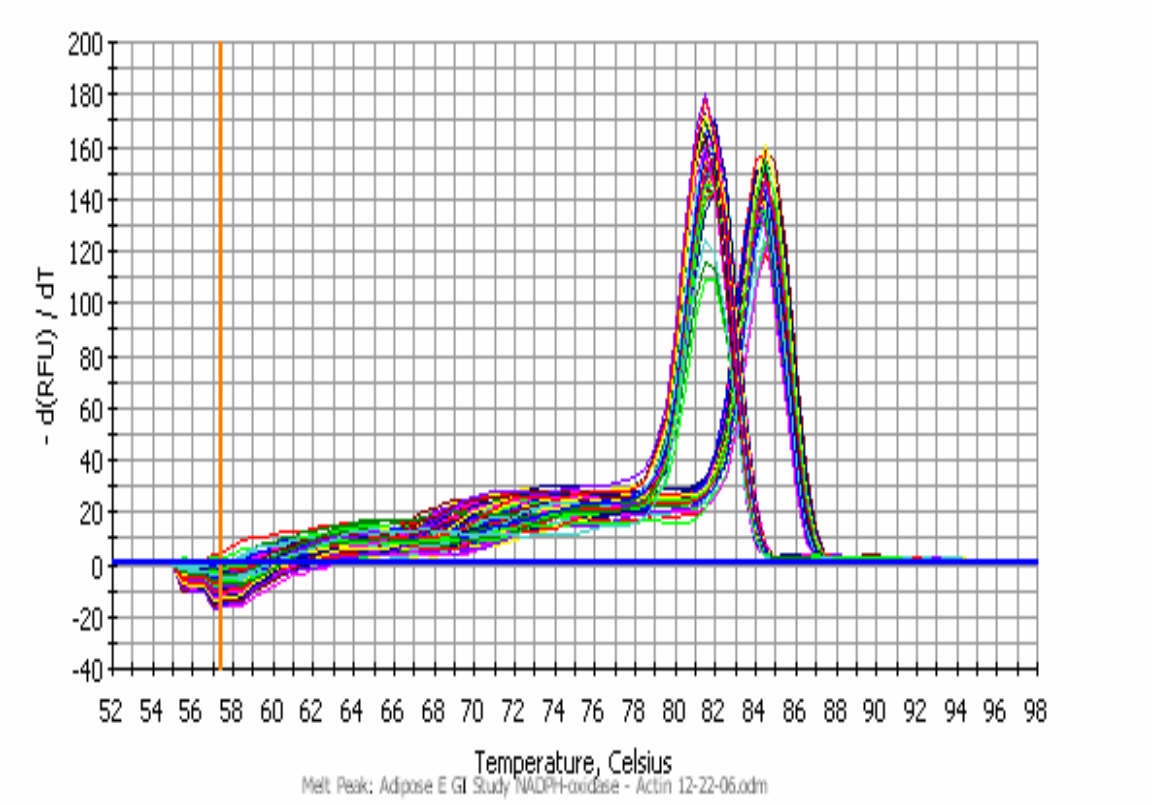
APPENDICES

Appendix A



Appendix A: Real-time PCR amplification cycle graph for NADPH oxidase and β-actin products from epididymal adipose tissue samples of mice on the LF diets (*n* = 5 mice per group).

Appendix B



Appendix B: Real-time PCR melt curve graph for NADPH oxidase and β-actin primers. The formation of a single peak indicates that a single product was formed.

Appendix C

Sample calculation: **The ΔC_T method using a reference gene**

Sample	C_T NADPH oxidase (target)	C_T β-actin (reference)
1. Adipose mRNA (LF/ High-GI A)	26.4	16.3
2. Adipose mRNA (HF/ High-GI A)	22.9	14.2

Equation:

$2^{(C_T(\text{Reference})- C_T(\text{Target}))}$ = Relative expression

$2^{(C_T(\beta\text{-actin})- C_T(\text{NADPH oxidase}))}$ = Relative expression

Solution:

Sample 1. $2^{(16.3 - 26.4)} = 0.0009$

Sample 2. $2^{(14.2 - 22.9)} = 0.0024$