Plasma Fetuin-A Responses to a Single Bout of Exercise and Short-term Exercise in Obese and Normal Weight Individuals

by

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Abstract

Fetuin-A, in particular the phosphorylated isoform, is a negative regulator of insulin action, inhibiting insulin receptor tyrosine kinase activity and downstream insulin signaling. In obese individuals, serum fetuin-A concentrations are elevated and are associated with insulin resistance. Recent evidence indicates that fetuin-A is an independent risk factor for type 2 diabetes. However, our understanding of fetuin-A responses to a glucose load or exercise is currently limited. Our goal was to characterize the effects of an oral glucose challenge, single and repeated bouts of exercise on serum fetuin-A concentrations in normal weight and obese individuals.

To examine the temporal changes in serum fetuin-A concentrations in response to an oral glucose challenge and a single bout of exercise, we recruited ten obese and seven age-matched, normal weight men, from the Auburn-Opelika area for this study. All participants underwent a single bout of treadmill walking at 60-70% VO_{2max}, expending 500 kcals. Oral glucose tolerance tests (OGTT) were conducted 4 days prior to and 24-hours after the exercise session. Obese individuals were insulin resistant as evidenced by serum insulin concentrations, glucose-to-insulin ratio, and Homeostasis Model Assessment (HOMA) index. Following an oral glucose challenge, total fetuin-A concentrations were significantly decreased at 30, 60, and 120-min time points in normal weight individuals, but not in obese individuals. After a single bout of exercise, the

glucose/insulin ratio increased 92%, 24 hr after exercise ($p = 0.0067$) and the insulin area under the curve (AUC) was reduced significantly by 16% ($p < 0.05$) post-exercise in obese individuals. Serum Ser-312 phosphorylated fetuin-A (phosphofetuin-A) AUC was significantly lower, 24 hours after a single bout of exercise, in obese men, consistent with improvements in surrogate markers of insulin sensitivity.

Next, we wished to investigate the influence of repeated bouts of exercise on four consecutive days and in the post-exercise period, on plasma fetuin-A concentrations as well as markers of insulin sensitivity. Fifteen obese men, that met the National Cholesterol Education Adult Treatment Panel III (NCEP ATP III) criteria for MetS, were recruited from the Auburn-Opelika area. Participants underwent treadmill walking for four consecutive days, at 70% of their VO_{2max}, expending 350 kcal/exercise session. Fasted blood samples were obtained prior to exercise on days 1 through 4, and 24h and 72h post-exercise. After adjustment for plasma volume, insulin concentrations and HOMA values were decreased significantly on day 2, 3 and 24h post-exercise, compared to baseline, suggesting improved insulin sensitivity. Plasma total fetuin-A concentrations were not altered with repeated bouts of exercise in the post-exercise period. However, a significant decrease in phosphofetuin-A concentrations was observed in the 24h post-exercise period, suggesting that the decreased phosphofetuin-A may contribute to the sustained effects of improved insulin sensitivity following repeated bouts of exercise.

Taken together, these data show for the first time that fetuin-A concentrations are decreased following a glucose load in normal weight, but not obese individuals, suggestive of a physiological role in insulin action. Additionally, the reduced Ser-312 phosphorylated fetuin-A levels observed 24 hours, after a single exercise session

(phosphofetuin-A_{AUC}) and after repeated bouts of exercise, are consistent with improvements in surrogate markers of insulin sensitivity. Given phosphofetuin-A's role of inhibiting insulin action, exercise-induced lowering of phosphofetuin-A, may be one mechanism by which exercise improves insulin action.

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List of Abbreviations

MetS	Metabolic syndrome
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
AMPK	AMP activated protein kinase
HOMA	Homeostasis model assessment
GIR	Glucose insulin ratio
NEFA	Non-esterified fatty acid
MAPK	Mitogen-activated protein kinase
GLUT4	Glucose transporter 4
PI3K	Phosphatidylinositol 3-kinase
RBP4	Retinol-binding protein 4
ER	Endoplasmic reticulum
AHSG	Alpha-2-HS-glycoprotein
NCEP-ATP III	National cholesterol education program-adult treatment panel III

Chapter 1: Introduction

According to World Health Organization, approximately 1.6 billion people worldwide are overweight or obese. Nearly 18 million children under age five are estimated to be overweight (World Health Organization, 2005). Based on the 1999-2008 National Health and Nutrition Examination Survey (NHANES), the age-adjusted obesity prevalence was 32.2% among adult men and 35.5% among adult women in the United States (Flegal et al., 2010). According to Centers for Disease Control and Prevention's Behavioral Risk Factor Surveillance System (BRFSS), Alabama ranked second in obesity prevalence for 2008 (Centers for Disease Control and Prevention, 2010).

Obesity increases the risk for type 2 diabetes, hypertension, dyslipidemia, cardiovascular disease, respiratory problems, certain cancers, gallstones, osteoarthritis, and lowered life expectancy (National Heart Lung and Blood Institute, 1998; Rippe et al., 1998; Solomon and Manson, 1997). The estimated annual cost attributable to obesity-related diseases is approximately $100 billion (National Heart Lung and Blood Institute, 1998). Nearly 280,000 deaths each year may be attributed to obesity related disorders (Allison et al., 1999).

Like obesity, diabetes, especially type 2 diabetes, represents a major global public health threat and together with obesity, constitutes an important contributor to the predicted decline in life expectancy (Campbell, 2009). In the United States, it is currently estimated that over 23 million people or 7.8% of the population have

diabetes. It is estimated that nearly 57 million people are currently living with pre-diabetes (American Diabetes Association, 2007). By 2030, the number of people with type 2 diabetes is predicted to increase to 366 million (Rathmann and Giani, 2004). Diabetes is characterized by elevated blood glucose concentrations that results from an impairment of insulin action, insulin secretion, or both (American Diabetes Association, 2010).

The global epidemic of obesity and diabetes is also associated with the prevalence of metabolic syndrome (Ginsberg and MacCallum, 2009; Ritchie and Connell, 2007). Metabolic syndrome is a complex condition characterized by obesity, hyperglycemia, insulin resistance, hypertension, and dyslipidaemia (Ginsberg and MacCallum, 2009). Metabolic syndrome is associated with an increased risk of cardiovascular disease, type 2 diabetes, stroke, and heart attack.

The hallmark of obesity, diabetes and metabolic syndrome is a condition called insulin resistance, in which the body fails to respond properly to insulin. Insulin resistance impairs the ability of insulin to metabolize glucose and is characterized by glucose intolerance and hyperglycemia followed by an increase in plasma concentrations of insulin; dyslipidemia, characterized by elevated concentrations of triglycerides and diminished HDL cholesterol; elevation of blood pressure, abdominal obesity, and an elevated tendency for thrombosis (Lazar, 2006). At the early stages of insulin resistance, as the body becomes unresponsive to insulin, the pancreas tries to compensate by producing more insulin. As this process goes on, the body gradually becomes more resistant to insulin (Taubes, 2009).

While genetics and environmental factors play a key role in the pathogenesis of insulin resistance, the molecular mechanisms are not clearly understood. Current

evidence regarding the molecular mechanisms of insulin resistance point to an impairment of insulin-stimulated insulin-receptor substrate (IRS)–1 tyrosine phosphorylation resulting in reduced IRS-1–associated phosphatidyl inositol 3 kinase activity, leading to a decrease in insulin-stimulated glucose transport activity (Petersen and Shulman, 2006). In addition, elevated plasma free fatty acids and circulating humoral factors including TNF-α, leptin, resistin, adiponectin, and retinol binding protein-4 have been shown to modulate insulin action (Cefalu, 2009; Wolf, 2008). In this regard, recent studies have implicated a novel hepatokine, fetuin-A, in the pathogenesis of insulin resistance and type 2 diabetes (Ix et al., 2008; Mori et al., 2006; Stefan et al., 2008; Stefan et al., 2006).

Fetuin-A, also known as alpha2-Heremans Schmid glycoprotein, is a liver-secreted plasma glycoprotein. First identified as phosphorylated protein 63 (pp63), fetuin-A is an inhibitor of insulin-stimulated insulin receptor autophosphorylation and tyrosine kinase activity (Auberger et al., 1989; Mathews et al., 2000; Srinivas et al., 1993). Mice null for fetuin-A gene demonstrate improved insulin sensitivity and resistance to diet-induced obesity, compared to their wild-type littermates (Mathews et al., 2002). Further, fetuin-A null mice were protected against obesity and insulin resistance associated with aging (Mathews et al., 2006). In humans, increased fetuin-A concentrations were associated with insulin resistance, metabolic syndrome and a fatty liver (Ix et al., 2006; Kalabay et al., 2002b; Mori et al., 2006; Stefan et al., 2006). Recent studies show that elevated plasma fetuin-A concentrations predicts the incidence of type 2 diabetes (Ix et al., 2008; Stefan et al., 2008). A recent study has shown that weight-loss by lifestyle intervention decreases fetuin-A concentrations and significantly reduces the prevalence of nonalcoholic fatty liver

disease (NAFLD) in children (Reinehr and Roth, 2008). While these studies suggest that fetuin-A may be associated with insulin resistance and diabetes, several questions, including the response of fetuin-A to an oral glucose load, a single bout or repeated bouts of physical activity, or the role of fetuin-A phosphorylation in these conditions, remain unanswered.

There is now strong evidence that regular physical exercise confers protection against the development of type 2 diabetes in high risk populations, supporting the recommendation that increased physical activity, along with the prevention or treatment of obesity by dietary restriction, is an important component of lifestyle modification for people at risk for developing diabetes. Regular physical activity leads to a number of beneficial physiological changes, namely an increase in glucose transporter 4 (GLUT4) expression, that favorably affect muscle glucose uptake and utilization, and an improvement of muscle and liver insulin sensitivity (Hawley and Lessard, 2008; Schenk and Horowitz, 2007; Treebak et al., 2009). These beneficial effects are partly explained by the activation of AMP activated protein kinase (AMPK) during physical activity in skeletal muscle, liver, and adipose tissue (Sriwijitkamol et al., 2007). Stimulation of AMPK activity by muscle contraction increases glucose uptake in an insulin-independent manner, thus bypassing defective insulin signaling, such as observed in type 2 diabetes patients (Frosig et al., 2007).

Several elegant studies in both rodent and humans indicate that a single bout of physical activity can significantly lower blood glucose and insulin concentrations (Gao et al., 1994; Larsen et al., 1997). Following a single bout of exercise, whole body glucose disposal was shown to be increased in obese rodents using the euglycemic-hyperinsulinemic clamp technique, with an associated increase in

insulin-stimulated glucose uptake in skeletal muscle (Musi et al., 2001). This enhancement of insulin action was shown to be associated with an upregulation of specific components of the glucose transport system and includes increased protein expression of GLUT4 and insulin receptor substrate-1 (IRS1) (Henriksen, 2002).

In this study we have examined temporal changes in circulating concentrations of the novel hepatokine, fetuin-A, following a single bout of physical activity and also repeated bouts of physical activity, in obese and lean individuals. Since a single bout of exercise is known to improve insulin sensitivity, we have analyzed changes in total and phosphorylated serum fetuin-A concentrations relative to surrogate markers of insulin sensitivity, such as HOMA and glucose-insulin ratio. To determine if fetuin-A responses resulting from the single bout or repeated bouts of exercise persisted into the immediate post-exercise period and were associated with enhanced insulin sensitivity, we analyzed blood samples obtained 24h and 72h post-exercise.

Our findings indicate that serum fetuin-A concentrations (total fetuin-A and phosphorylated fetuin-A) are responsive to a glucose load and physical activity, suggesting that lowering fetuin-A concentrations by lifestyle modifications can potentially contribute to a lower risk of insulin resistance and diabetes.

Chapter 2: Review of Literature

2.1 Obesity

Obesity is a leading cause of preventable death worldwide. The increasing prevalence of obesity in adults and children make it one of the most serious public health problems of the 21st century (Ogden et al., 2006). Obesity increases the likelihood of various diseases, particularly cardiovascular disease, type 2 diabetes, certain types of cancer, and osteoarthritis (Allison et al., 1999; Kahn and Flier, 2000; Van Gaal et al., 2006). Obesity, defined as body mass index (BMI) greater than or equal to 30 kg/m^2, is most commonly caused by a combination of excessive dietary energy, inadequate energy expenditure, and genetic susceptibility, although a few cases are caused solely by genes, endocrine disorders, medications or psychiatric illness (Aggoun, 2007). While the primary treatment for obesity is dieting and increased physical activity, pharmaceutical interventions (to reduce appetite, or inhibit fat absorption) and surgical options are available (Boney et al., 2005; Hills et al., 2007; Khaodhiar et al., 2009; Priebe et al., 2008; Venables and Jeukendrup, 2009).

2.2 Diabetes

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2010). Type 1 diabetes results from beta cell destruction, usually leading to absolute insulin deficiency. Type 2 diabetes, which accounts for at least 90% of all cases of diabetes, is characterized by defective insulin action, ranging from predominantly insulin resistance with relative insulin deficiency, to predominantly an

insulin secretory defect with insulin resistance. Many people destined to develop type 2 diabetes spend several years in a state of pre-diabetes—a condition where blood glucose concentrations are higher than normal but not yet high enough to be diagnosed as diabetes (Aggoun, 2007). Gestational diabetes affects about 4% of all pregnant women. It may precede development of type 2 diabetes (or rarely type 1 diabetes). Many other forms of diabetes are categorized separately (Mitri and Hamdy, 2009).

Current criteria for the diagnosis of diabetes include: (a) Fasting plasma glucose level ≥ 126 mg/dL (7.0 mmol/L), or (b) plasma glucose ≥200 mg/dL (11.1 mmol/L) two hours after a 75g oral glucose load as in a glucose tolerance test, or (c) symptoms of hyperglycemia and casual (random) plasma glucose ≥200 mg/dL **(**11.1 mmol/L) (American Diabetes Association, 2010). Patients with fasting glucose concentrations from 100 to 125 mg/dL (6.1 and 7.0 mmol/L) are considered to have impaired fasting glucose. Patients with plasma glucose at or above 140 mg/dL (7.8 mmol/L), but not over 200, two hours after a 75g oral glucose load are considered to have impaired glucose tolerance. Of these two pre-diabetic states, the latter in particular is a major risk factor for progression to full-blown diabetes mellitus, as well as cardiovascular disease.

2.3 Insulin action

Insulin, secreted by the beta cells of the pancreas, regulates fat, carbohydrate, and protein metabolism (Ginsberg and MacCallum, 2009). Insulin secretion decreases in response to hypoglycemia, hyperinsulinemia, and increased catecholamine concentrations. It increases in response to hyperglycemia, and elevated plasma concentrations of amino acids, and nonesterified fatty acids (NEFA) (Defronzo, 2009; Ginsberg and MacCallum, 2009; Kelley et al., 1993; Khaodhiar et al., 2009).

Insulin enhances glucose uptake by skeletal muscle and fat tissues. Skeletal muscle accounts for nearly 90% of insulin-stimulated glucose uptake. Insulin increases glycogen synthesis (predominant) and glucose oxidation in muscle. Compared to glucose utilization, the effect of insulin on inhibitory glucose production is greater, but the capacity of the glucose utilization response is much greater than that of glucose production (Fig.2.1) (Rizza et al., 1981).

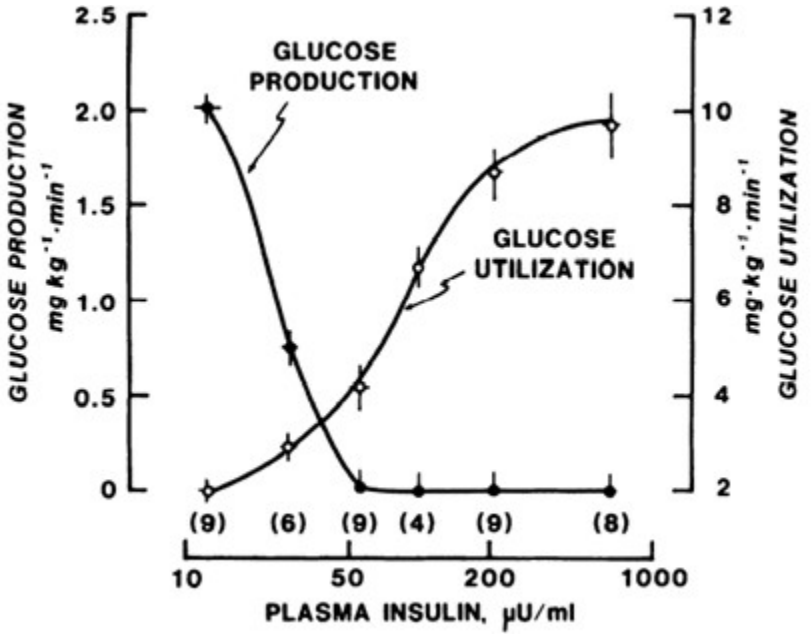

Fig 2.1: Glucose production vs. glucose utilization. Insulin dose-response curve for glucose production and utilization. Compared to glucose utilization, the effect of insulin on glucose production is greater, but the capacity of the glucose utilization response is much greater than that of glucose production. Number of subjects studied is given in parentheses (Rizza et al., 1981).

Insulin inhibits hepatic glucose production (HGP) and this is attributable both to the inhibition of breakdown of glycogen and inhibition of gluconeogenesis. Basal amounts of insulin exert an inhibitory effect on gluconeogenesis, glycogenolysis, lipolysis, and glucagon secretion in the pancreas. Small to moderate changes in plasma insulin concentrations modify HGP primarily by altering hepatic glycogen breakdown (Campbell, 2009; Campbell et al., 2009; DiPenta et al., 2007). The direct

effects of insulin include inhibition of glycogenolysis by glycogen phosphorylase. In the liver, insulin acts to make glycogen phosphorylase less active and slow glycogen breakdown. Insulin also triggers activation of glycogen synthase by blocking the activity of glycogen synthase kinase 3 (GSK3). The indirect effects include inhibition of glucagon secretion, reduction in plasma NEFA concentrations, reduction of the amount of gluconeogenic precursor supplied to the liver, and change in neural input to the liver. Insulin inhibits glucose production through both direct and indirect effects on the liver. It is believed that direct effects of insulin dominate the acute regulation of HGP (Edgerton et al., 2005).

Insulin stimulates the synthesis and storage of triglycerides in adipose tissue and in the liver, and it inhibits the release of triglycerides (Campbell et al., 2009). Insulin also stimulates the synthesis of proteins and of molecules, such as c-Jun N-terminal kinases (JNKs), involved in the function, repair, and growth of cells. Insulin also functions as a signaling molecule conveying information on fuel availability from the periphery to the brain and central nervous system (Koch et al., 2008).

2.4 Insulin signal transduction

2.4.1 Insulin receptor and its substrates

The insulin receptor is a transmembrane receptor, belonging to the class of tyrosine kinase receptors (Hubbard et al., 1994) (Barbetti et al., 1992). Two α subunits and two β subunits, encoded by a single gene, make up the insulin receptor (Barbetti et al., 1992; Ottensmeyer et al., 2000) (Fig.2.2). Insulin binding results in autophosphorylation of the tyrosine residues, which is permitted by the joint participation of two subunits in the insulin binding and by kinase domains in the two beta-subunits situated at juxtaposition (Ottensmeyer et al., 2000). The kinase domain

undergoes a conformational change, which acts as an origin for activation of the kinase and binding of some downstream molecules that participate in the signaling cascades. This intrinsic tyrosine kinase activity phosphorylates various substrates of the insulin receptor, including the insulin receptor substrate-1 (IRS-1) protein (Hubbard et al., 1994).

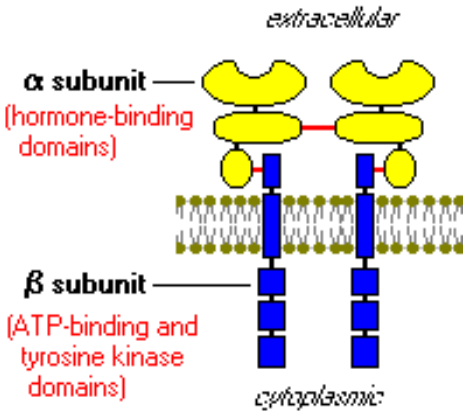

Fig 2.2 Structure of insulin receptor. Insulin receptor has two α subunits (yellow) and two β subunits (blue). The red lines are bonds, showing the sulfur bond between α and β subunits and a disulfide bond between the two complexes (Bowen, 2004).

IRS proteins contain a conserved pleckstrin homology (PH) domain, located at their N terminus, which serves to anchor the IRS proteins to membrane phosphoinositide and helps to localize the IRS proteins to the receptor. The PH domain of IRS proteins is flanked by a phosphotyrosine binding (PTB) domain. It functions as a binding site to the NPXY motif of the IR (Fig.2.3). In addition to phosphorylating IRS 1-4, IR also trans-phosphorylates other substrates on Tyr residues including Shc, Gab1, Cb1, APS, and P60dok (Ottensmeyer et al., 2000). Each of these and the IRS proteins provide specific docking sites for other signaling protein containing Src homology 2 (SH2) domains. These events lead to activation of three main pathways: the IRS/phosphatidylinositol 3 (PI3) kinase pathway;

(RAS)/mitogen-activated protein kinase (MAPK) pathway; and the Cbl-associated protein (CAP)/Cbl pathway (Birnbaum, 2001) (Fig.2.4).

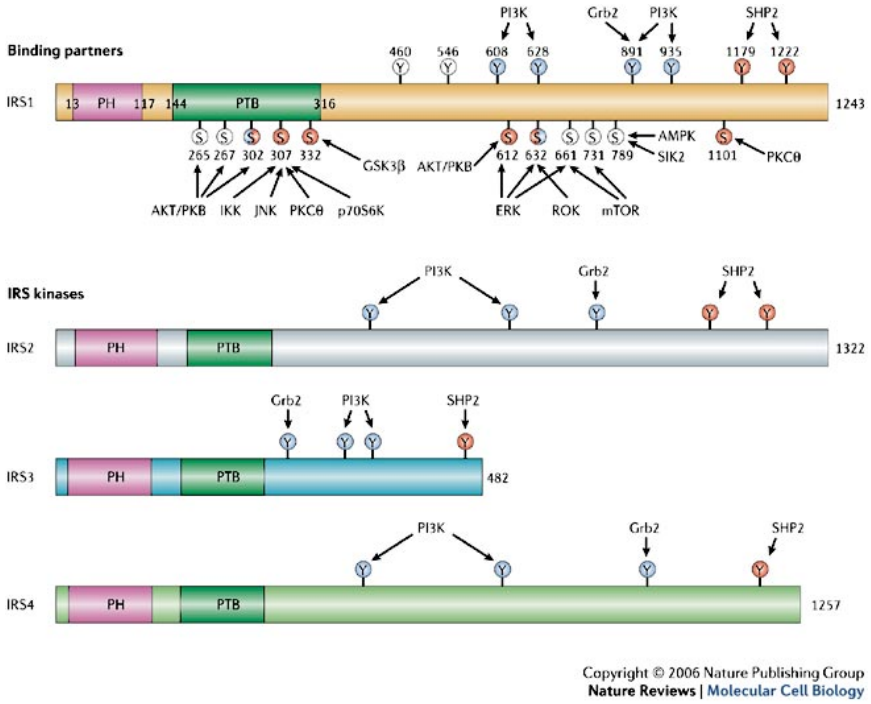

Fig 2.3 Structure of Insulin Receptor Substrate: The four insulin-receptor substrate (IRS) isoforms, IRS1, IRS2, IRS3 and IRS4, share a pleckstrin-homology (PH) domain (magenta), a phosphotyrosine-binding (PTB) domain (dark green) and several sites of phosphorylation on tyrosine and serine residues. The positions of the tyrosine residues (Y) that are phosphorylated by the IR and the downstream-signaling proteins that bind to these sites are shown. The positions of the serine residues (S) and the kinases responsible for their phosphorylation are also shown. Blue circles represent sites of positive regulation, whereas red circles represent sites of negative regulation. White circles represent sites in which the effect of phosphorylation is currently unknown. (Taniguchi et al., 2006).

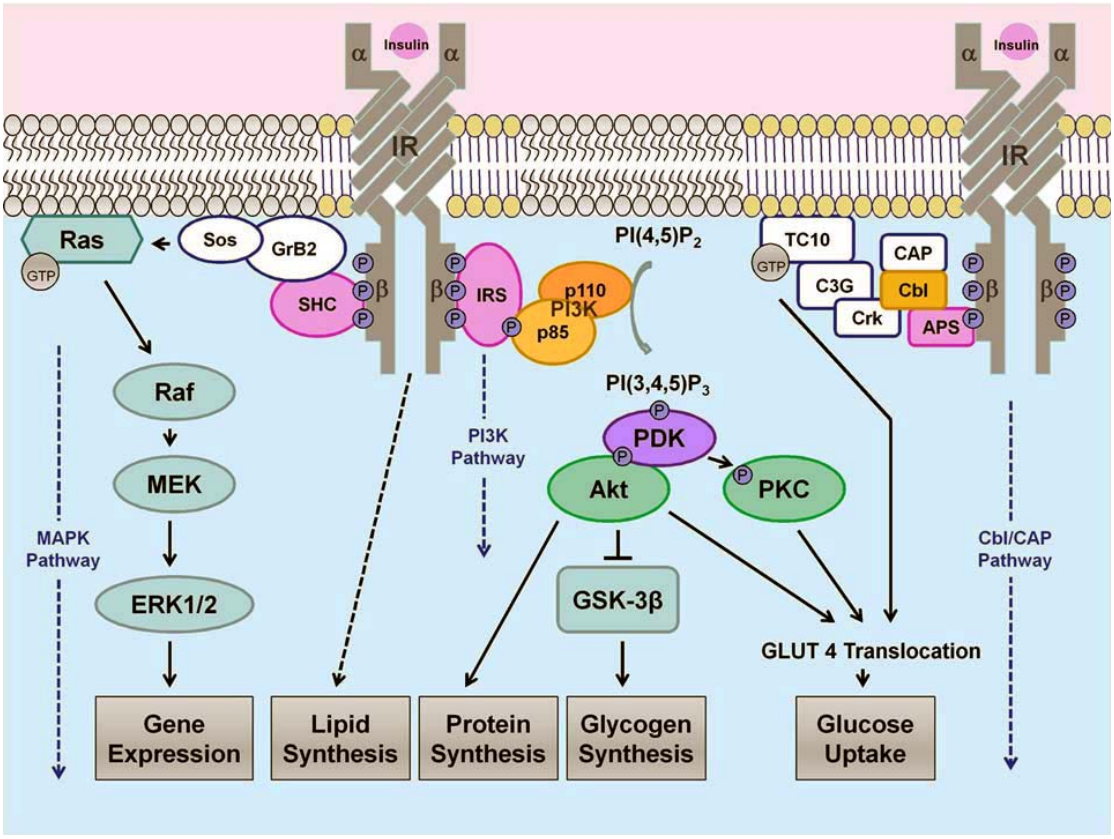

Fig 2.4 Overview of Insulin Signaling Pathway: Insulin binding to its receptor leads to activation of three main pathways: the IRS/phosphatidylinositol 3 (PI3) kinase pathway; (RAS)/mitogen-activated protein kinase (MAPK) pathway; and the Cbl-associated protein (CAP)/Cbl pathway. (Yang, 2009)

2.4.2 PI3 kinase pathway

IR activation leads to the phosphorylation of key tyrosine residues on IRS proteins, some of which are recognized by the SH2 domain of the p85 regulatory subunit of PI3 kinase, a lipid kinase. The catalytic subunit of PI3 kinase, p110, then phosphorylates phosphatidylinositol (4, 5) bisphosphate (PtdIns(4,5)P_2) leading to the formation of Ptd(3,4,5)P_3 (Cusi et al., 2000). A key downstream effector of Ptd(3,4,5)P_3 is AKT (otherwise known as PKB), which is recruited to the plasma membrane. Activation of AKT also requires the protein kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1), which in combination with an as yet unidentified kinase leads to the phosphorylation of AKT (Figure 2.4). Once

active, AKT enters the cytoplasm where it leads to the phosphorylation and inactivation of GSK3 (Figure 2.6). A major substrate of GSK3 is glycogen synthase, an enzyme that catalyses the final step in glycogen synthesis. Phosphorylation of glycogen synthase by GSK3 inhibits glycogen synthesis; therefore the inactivation of GSK3 by AKT promotes glucose storage as glycogen. Also, recent evidence suggests that AKT phosphorylation leads to the exclusion of forkhead transcription factors, which play a role in regulation of expression of genes encoding hepatic enzymes involved in gluconeogenesis. In addition, activation of the PI3 kinase-AKT pathway leads to the stimulation of glucose uptake into cells by inducing translocation of the glucose transporter, GLUT4, from intracellular storage to the plasma membrane (Czech and Corvera, 1999).

2.4.3 The p42/p44 MAPK cascade

The adaptor molecules Shc and Grb2 bind either singly or in combination to the IRS's through their SH2 or PTB domains. Grb2 is complexed to the Ras guanine exchange factor mSOS (son of sevenless). Recruitment of mSOS from the cytosol to the plasma membrane activates Ras (a 21kDa GTPase). In its active GTP bound form, Ras associates with the N-terminal region of the serine/threonine kinase Raf, bringing it to the plasma membrane to become activated. Activated Raf forms a stable complex with another protein kinase termed MKK1 (mitogen activated protein kinase kinase1, also known as MEK1). Phosphorylation of MKK1 by Raf increases MKK1 activity. MKK1 in turn, phosphorylates and activates p42/p44 mitogen activated protein kinase (MAPK). p42/44 MAPKs are members of the MAPK superfamily that also include the p38 MAPK and c-jun N-terminal protein kinase (JNK) isoforms. These latter MAPKs are activated predominantly by cellular stresses such as osmotic stress, oxidative stress, UV irradiation, heat stress and cytokines (Cusi et al., 2000).

Once activated, p42/p44 MAPK phosphorylates many downstream substrates that are involved in numerous cellular processes such as proliferation, differentiation, cell survival, and gene transcription (Cusi et al., 2000). In addition, MAPK is involved in the activation of several downstream serine/threonine protein kinases, such as the p90Rsk isoforms (RSKs 1-3), MSK1/MSK2 (mitogen and stress activated protein kinases) and MNK1/MNK2 (MAPK interacting kinases). Once activated, p90Rsk phosphorylates downstream targets that are involved in gene transcription, cell cycle regulation, and cellular metabolism. The p42/p44 MAPK is key in the mitogenic actions of many hormones and growth factors. Indeed, this molecule appears crucial in the regulation of immediate early genes, such as c-Fos, c-Jun, etc., by insulin in a variety of tissues.

2.4.4 Cbl/CAP pathway

In metabolically responsive cells, Cbl/CAP complex constitutes another signaling pathway required for insulin-stimulated glucose transport. Cbl is recruited to the insulin receptor by interaction with the adapter protein CAP (C-Cbl-associated protein), through one of three adjacent SH3 domains in the carboxy terminus of CAP. When Cbl is phosphorylated, the Cbl/CAP complex translocates to the plasma membrane-domain enriched in lipid rafts. Expression of dominant negative CAP was shown to completely block insulin-stimulated glucose uptake and GLUT4 translocation (Langfort et al., 2003). This suggests that Cbl/CAP complex and PI3 kinase/Akt pathway are two compartmentalized parallel pathways that lead to GLUT4 translocation.

2.5 Insulin resistance

Insulin resistance (IR) is a physiological condition in which insulin becomes less effective in lowering blood glucose. It is characterized by glucose intolerance and

hyperglycemia followed by an increase in plasma concentrations of insulin, dyslipidemia (elevated plasma triglycerides and diminished HDL cholesterol concentrations), elevation of blood pressure, abdominal obesity, and an elevated tendency for thrombosis (Monzillo and Hamdy, 2003; Young-Hyman et al., 2001). Insulin resistance reduces glucose uptake in muscle cells, impairs hepatic glycogen synthesis and increases HGP, and impairs suppression of lipolysis in adipose tissue. Elevated blood fatty-acid concentrations, reduced muscle glucose uptake, and increased liver glucose production all contribute to elevated blood glucose concentration (Kahn and Flier, 2000; Young-Hyman et al., 2001). As tissues become unresponsive to insulin, the pancreas compensates by secreting even more insulin, and gradually the tissues grow more resistant.

While several studies have demonstrated alterations of insulin receptor synthesis, degradation, and function through mutations of the insulin receptor gene in patients with severe insulin resistance (Hubbard et al., 1994), these are not representative of the typical insulin resistance observed in an obese individual. In obese individuals, adipose tissue releases increased amounts of non-esterified fatty acids and glycerol, altered concentrations of hormones such as leptin, adiponectin, resistin, retinol binding protein-4 (RBP-4), and proinflammatory cytokines such as TNF-α, and IL-6 that are involved in the development of insulin resistance (Funaki, 2009).

2.5.2 Role of free fatty acids and insulin resistance

A hallmark of insulin resistance is the excessive release of free fatty acids due to increased resistance of the adipose tissue to the anti-lipolytic effect of insulin (Lago et al., 2009; Lazar, 2005; Rosen and Spiegelman, 2006; Venables and Jeukendrup, 2009). Increased mobilization of stored lipids in these cells elevates free fatty acids in

the blood plasma. Evidence suggests that, compared with their lean counterparts, obese subjects have elevated basal FFA turnover, and impaired basal FFA oxidation, thus raising circulating FFA concentrations (Solomon et al., 2009b). Increased FFA delivery or decreased intracellular metabolism of fatty acids has been shown to increase intracellular content of fatty acid metabolites such as diacylglycerol (DAG), fatty acyl-coenzyme A (fatty acyl-CoA), and ceramides, which, in turn, activate a serine/threonine kinase cascade leading to serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), and a reduced ability of these molecules to activate PI3 kinase (Long et al., 2008), resulting in a diminished insulin signal transduction (Hajduch et al., 2001).

2.5.3 Role of adipocytokines and insulin resistance

Cytokines such as TNF-α and IL-6 are produced by macrophages as well as by adipocytes, and a link between chronic inflammation and obesity and diabetes. TNF-α and IL-6 act through classical receptor-mediated processes to stimulate both the c-Jun amino-terminal kinase (JNK) and the IκB kinase-β (IKK-β)/nuclear factor-κB (NF-κB) pathways, resulting in upregulation of potential mediators of inflammation that can lead to insulin resistance. In obese mouse models a lack of TNF-α function results in improved insulin sensitivity and glucose homeostasis, suggesting that inflammatory response has a critical role in regulation of insulin action in obesity (Defronzo, 2009; Hotamisligil, 2006).

Adiponectin, secreted by adipocytes, has important anti-atherogenic, antidiabetic and anti-inflammatory properties (Lau et al., 2005; Matsuzawa, 2006). In humans, serum adiponectin concentrations are decreased in obesity, diabetes and other insulin resistant states and increased with weight loss or with the use of thiazolidinediones, which enhance sensitivity to insulin (Maeda et al., 2001). It

increases fatty acid oxidation and reduces glucose synthesis in the liver. Ablation of the adiponectin gene in mice leads to severe insulin resistance and lipid accumulation in skeletal muscle (Whitehead et al., 2006).

Resistin, unlike adiponectin, is elevated in many murine models of obesity. It is hypothesized that resistin plays a role in increasing hepatic glucose output (Banerjee et al., 2004; Steppan and Lazar, 2004). Similarly, elevated serum concentrations of retinol-binding protein 4 (RBP4) are shown to be associated with insulin resistance in obese humans and those with type 2 diabetes as well as in lean, nondiabetic people with a family history of diabetes (Graham et al., 2006). RBP4 induces insulin resistance through reduced PI3 kinase signaling in muscle and enhanced expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver through a retinol-dependent mechanism. Consistent with these findings, Rbp4-/- mice demonstrate enhanced insulin sensitivity (Yang et al., 2005).

2.5.4 ER stress and insulin resistance

Recent evidence indicate that in both dietary and genetic obesity, endoplasmic reticulum stress (ER stress) is increased in adipose and liver tissue (Hotamisligil, 2006). The ER is a vast network of membranes in which all the secretary and membrane proteins are assembled (Hotamisligil, 2006). Accumulation of unfolded proteins, energy and nutrient fluctuations, and viral infections gives rise to perturbations in the ER lumen and create stress. The ER activates a complex response system known as the unfolded response to restore the functional integrity of the organelle. During ER stress, two main principal inflammatory pathways that disrupt insulin action, JNK-AP1 and IKK-NFκB, are activated (Colca, 2006; Lazar, 2006). It is hypothesized that insulin resistance develops when excessive fat is shunted into

existing adipocytes that are overstuffed, stressing the ER and attracting macrophages, releasing inflammatory mediators, such as tumor necrosis factor-α (TNF-α), or leaking fatty acids out into the circulation-or any combination of these (Taubes, 2009).

2.5.5 Fatty liver and insulin resistance

Fat accumulation in the liver makes the liver insulin resistant (Rosen and Spiegelman, 2006), overproducing glucose and VLDL, leading to hyperglycemia and hypertriglyceridemia. Shoelson et al. recently demonstrated that obesity-associated hepatic steatosis is associated with increased activity of NF-κB pathway in the liver (Shoelson et al., 2003). The inflamed liver can also be the primary source of systemic factors, including TNF-α, IL-6, and IL-1β, that lead to the development of insulin resistance. Among the liver-secreted proteins shown to be associated with insulin resistance, is the 63 kD phosphorylated glycoprotein initially called pp63 in rats and alpha2-HS glycoprotein (AHSG) in humans, and now known as fetuin-A.

2.6 Fetuin-A

Fetuin-A, a phosphorylated glycoprotein, is synthesized by hepatocytes and secreted into circulation. Fetuin-A has been identified as a major protein during fetal life and is also involved in important functions such as inhibition of the insulin receptor tyrosine kinase activity, protease inhibitory activities and regulation of calcium metabolism and osteogenesis (Colclasure et al., 1988; Triffitt et al., 1976; Weikert et al., 2008). Fetuin-A is also shown to be a key partner in the recovery phase of an acute inflammatory response (Olivier et al., 2000; van Oss et al., 1975).

A second protein of the fetuin family, fetuin-B, was identified in humans and rodents. Based on domain homology, overall conservation of cysteine residues, and chromosomal assignments of the corresponding genes in these species, fetuin-B is

unambiguously a paralog of fetuin-A. Yet, fetuin-A and fetuin-B exhibit significant differences at the amino acid sequence level. Differences and similarities in terms of gene regulation were also observed (Olivier et al., 2000).

2.6.1 Structure of fetuin-A

Fetuin-A consists of two polypeptide chains, a predominant A-chain and a smaller B-chain. These two chains have been shown to be connected via a 'connecting peptide'. Fetuins have both N-linked and O-linked sugar moieties. Two N-glycans are attached to asparagine residues 138 and 158, and two O-glycans are attached to threonine residues at 238 and 252.

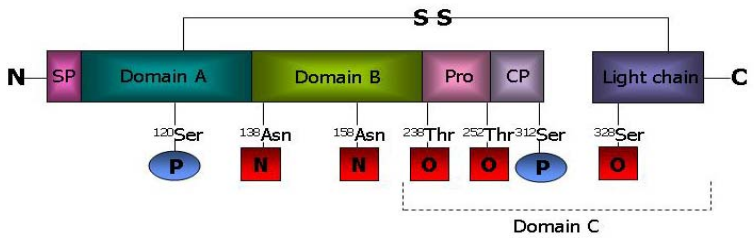

Fig 2.5 Schematic view of the proteolytically processed human fetuin-A structure: Fetuin-A proteolysis occurs at either of the two cleavage sites as indicated by the arrowheads. The resulting fragments (heavy chain + lc-1, top; heavy chain + lc-2, bottom) are interlinked by a disulfide-bond (Cys14–Cys332, by homology). The fetuin-A structure contains *in vivo* phosphorylation (all circles) and *in vitro* CK2 phosphorylation sites. The shaded areas indicate the currently undefined C-termini of the long chain after *in vivo* proteolysis (Kubler et al., 2007).

Fetuin-A is phosphorylated on Ser120 of the A-chain and Ser312 (Fig. 2.5) of the connecting peptide (Haglund et al., 2001). Mass spectrometry analyses indicated that up to 77% of the phosphorylation occurred at Ser312. Further, Haglund et al demonstrated that up to 20% of circulating fetuin-A was phosphorylated (Haglund et al., 2001).

2.6.2 Inhibition of insulin receptor tyrosine kinase activity

The initial investigation of fetuin-A's ability to inhibit tyrosine action revealed

that fetuin-A was a specific inhibitor of insulin-stimulated IR autophosphorylation and receptor tyrosine kinase activity, both *in vitro* and *in vivo* (Srinivas et al., 1996) (Kalabay et al., 1998; Mathews et al., 2000; Srinivas and Grunberger, 1994). Fetuin-A repressed insulin-stimulated tyrosine phosphorylation of IRS-1 and the association of IRS-1 with the p85 subunit of PI3K in H-35 hepatoma cells without competing with insulin for binding to IR (Srinivas et al., 1993).

2.6.3 Fetuin-A and insulin resistance

Using animal models, Grunberger and Mathews demonstrated that fetuin-A gene expression was upregulated in insulin resistant rats and down-regulated in starved conditions (Mathews et al., 2002). Mathews et al. showed that acute injection of recombinant human fetuin-A through the portal vein of rats inhibited insulin-stimulated IR autophosphorylation and IRS-1 phosphorylation in liver and hindlimb muscle (Mathews et al., 2000). Mathews and colleagues showed that mice lacking fetuin-A were more insulin sensitive than their wild-type littermates. Additionally, these mice were resistant to diet-induced obesity (Mathews et al., 2002). They also showed that fetuin-null mice were protected against obesity and insulin resistance associated with aging (Mathews et al., 2006). Recent studies by Papizan and Mathews show increased plasma fetuin-A phosphorylation (Ser312) in insulin resistant, leptin signaling-impaired Zucker diabetic fatty (ZDF) rats and leptin-deficient ob/ob mice, compared to lean controls, suggesting roles for leptin and insulin in the regulation of the Ser312-fetuin-A phosphorylation (Papizan, 2007).

In human, elevated fetuin-A concentrations were observed to be associated with insulin resistance, leading to an increased risk of diabetes, cardiovascular disease, and metabolic syndrome. In humans, fetuin-A gene is located at chromosome 3q27, a susceptibility locus for type 2 diabetes and the metabolic syndrome (Kissebah et al.,

2000). Kalabay and colleagues have shown that fetuin-A concentrations have been reported to be significantly higher in patients with gestational diabetes than in healthy pregnant women and to be correlated with indirect measures of insulin resistance (Kalabay et al., 2002a). Furthermore, Mori et al. show a strong independent contribution of fetuin-A to HOMA, BMI and triglycerides in non-diabetic individuals (Mori et al., 2006). Siddiq et al. also showed that a major allele of a synonymous coding polymorphism in the AHSG gene was associated with type 2 diabetes in French Caucasians (Siddiq et al., 2005). In addition, Stefan and colleagues have shown that fetuin-A is associated with insulin resistance and fat accumulation in the liver by cross-sectional analysis of 106 healthy non-diabetic Caucasians (Stefan et al., 2006).

2.6.4 Fetuin-A and risk of diabetes

Stefan and colleagues have shown that increased plasma fetuin-A concentrations were positively associated with diabetes risk in a 7-year longitudinal study with subjects who were non-diabetic at baseline (Stefan et al., 2008). Similarly, Ix and colleagues demonstrate that higher fetuin-A concentrations predict the incidence of diabetes after adjustment for common insulin resistance measures (age, sex, race, weight, waist circumference, physical activity, blood pressure concentrations, fasting glucose concentrations, HDL cholesterol concentrations, triglyceride concentrations, and C-reactive protein level) (Ix et al., 2008). These findings support the hypothesis that elevated fetuin-A concentrations may play a role in the development of type 2 diabetes.

2.6.5 Fetuin-A and weight loss

Reinehr and colleagues found that high fetuin-A concentrations were related to insulin resistance and metabolic syndrome in a study with obese children with

nonalcoholic fatty liver disease (NAFLD) (Reinehr and Roth, 2008). At baseline, fetuin-A concentrations were higher in children with NAFLD than those without NAFLD. Fetuin-A concentrations decreased significantly in obese children with substantial weight loss, whereas fetuin-A concentrations did not change significantly in patients without change of weight status. The prevalence of NAFLD decreased significantly in the obese children with substantial weight loss in contrast to the children without substantial weight loss.

2.6.6 Fetuin-A and metabolic syndrome

Data from the Heart and Soul study also suggest that higher fetuin-A concentrations are strongly associated with metabolic syndrome (Ix et al., 2006). The highest fetuin-A quartile was associated with metabolic syndrome in unadjusted and adjusted analyses. Compared with 24% of participants in the lowest quartile, a total of 45% of participants in the highest quartiles of fetuin-A had metabolic syndrome.

2.7 Metabolic syndrome

Metabolic syndrome (MetS), an insulin resistant condition (Després and Lemieux, 2006) that afflicts approximately 50 million Americans (Aggoun, 2007), is associated with abdominal obesity, altered blood lipids, inflammation, cardiovascular disease, and full-blown diabetes (Ginsberg and MacCallum, 2009; Ritchie and Connell, 2007). Although there is a debate surrounding the concept of metabolic syndrome, it is recognized as a major risk factor for diabetes and cardiovascular disease by NCEP-ATP III, World Health Organization, and the International Diabetes Federation (IDF) (Després and Lemieux, 2006).

The criteria for the diagnosis of MetS, as defined by NCEP-ATP III are shown in Table 1. These criteria take into consideration the contribution of abdominal obesity, insulin resistance and related metabolic markers to cardiometabolic risks (Després

and Lemieux, 2006). A combination of two to three of the characteristics significantly increases the risks for cardiovascular disease and diabetes. When any three of the five listed characteristics are present, a diagnosis of MetS is made.

Table 2.1: NCEP-ATP III clinical identification of metabolic syndrome (any 3 of the following)

Risk Factor	Defining Level
Abdominal Obesity(Waist circumference)	
Men	>102 cm (> 40 in)
Women	> 88 cm (>35 in)
Triglycerides	≥150 mg/dL
HDL cholesterol	
Men	<40 mg/dL
Women	<50 mg/dL
Blood pressure	≥130/≥85 mmHg
Fasting glucose	≥110 mg/dL

2.8 Strategies in the prevention and control of type 2 diabetes

Since diabetes is a chronic disease with no currently available cure (Campbell et al., 2009), diligent ongoing treatment and careful monitoring are required to reduce the risk of long term complications (Bailey, 2009; Crandall et al., 2008; Delahanty and Nathan, 2008; Hawley and Lessard, 2008; Iqbal, 2007; James et al., 1985; Kim and Lee, 2009; Kivelèa et al., 2007; Nield et al., 2008; Priebe et al., 2008). Effective management of type 2 diabetes requires the combination of diet and exercise to promote weight loss (Campbell et al., 2009; Davis et al., 2009; Hawley and Lessard, 2008; Huffman et al., 2008; Khaodhiar et al., 2009; Mitri and Hamdy, 2009; Schenk and Horowitz, 2007; Villareal et al., 2008), various oral diabetic drugs (type 2 only), and insulin use (type 1 and for type 2 not responding to oral medications) (Nield et al., 2008) (Priebe et al., 2008) (Iqbal, 2007). Whereas exercise in healthy people has little impact on blood glucose concentrations, moderate-intensity exercise in patients with type 2 diabetes is usually associated with a decrease in blood glucose concentrations towards normal (Delahanty and Nathan, 2008; DiPenta et al., 2007; Kirwan et al.,

2009; Mitri and Hamdy, 2009).

2.8.1 Lifestyle intervention studies

With a goal to prevent diabetes, several large, randomized clinical trials involving adults at high risk for the development of type 2 diabetes have been conducted in the United States, Europe, and around the world (Table 2.2). These studies, as outlined hereafter, unequivocally show that a lifestyle modification program with energy restriction and increased physical activity effectively decreased the risk for the development of diabetes.

The Diabetes Prevention Program conducted in the United States, which included lifestyle-modification that combined a low-fat diet and at least 150 minutes of physical activity per week, with the goal of at least a 7 percent weight loss, showed a reduction in the incidence of diabetes by 58% (Knowler et al., 2002). Lifestyle intervention was significantly more effective at preventing diabetes than a second part of the study examining prevention with the use of metformin.

In the Finnish Diabetes Prevention Study, 533 overweight adults with impaired glucose tolerance were randomly assigned to either an intensive lifestyle intervention group with energy restriction and increased physical activity aimed at reducing weight or into a nonintervention control group (Tuomilehto et al., 2001). Subjects were monitored for an average of 3.2 years with an OGTT performed each year. The risk for diabetes was reduced by 58% in the intervention group.

Table 2.2 Summary of selected lifestyle intervention studies

Clinical Study	Location	Participants	Duration	Study Design	Major Outcome
Diabetes Prevention Program (Knowler et al., 2002)	United States	3,234 nondiabetic persons with elevated fasting or post-load plasma glucose concentrations	4 years	1. Metformin (MET) or placebo 2.Lifestyle modification (LSM) (low fat diet and physical activity of moderate intensity)	A 58% reduction in the incidence rate of diabetes in life style modification group
Da Qing Study (Pan et al, 1997)	China	577 people with impaired glucose tolerance	6 years	1.diet 2.exercise 3.diet + exercise.	diet, exercise, and diet + exercise interventions were associated with 31%, 46%, and 42% reductions in risk of developing diabetes.
Finnish Diabetes Prevention Program (Tuomilehto et al., 2001)	Finland	522 middle-aged overweight subjects with impaired glucose tolerance	3.2 years	1.Intervention: Diet and exercise 2. Control	58% diabetes risk reduction in the intervention group
Nurses' study (Hu et al., 2001)	United States	84941 healthy female nurses	16 years	A low-risk group was defined according to dietary and lifestyle variables: BMI under 25, an average of at least one half-hour per day of vigorous or moderate activity, no smoking, an average of 5g or more alcohol per day, a diet high in fiber and polyunsaturated fat and low in trans fat and glycemic load.	Women who were in the low-risk groups had a relative risk of diabetes of 0.09 as compared with all other women
Indian Diabetes Prevention Program (Ramachandran et al., 2006)	India	531 subjects with impaired glucose tolerance	30 moths	1. Control 2. LSM 3. MET 4. LSM + MET	Accumulative incidences of diabetes were 55.0%, 39.3%, 40.5%, and 39.5% in group 1-4. The relative risk reduction was 28.5% with LSM, 26.4% with MET, and 28.2% with LSM + MET.

In the Indian Diabetes Prevention Program, 531 adults with impaired glucose tolerance were randomized into four groups (Ramachandran et al., 2006). Group1 was control, Group 2 was given advice on lifestyle modification, Group 3 was treated with metformin, and Group 4 was given lifestyle modification advice plus metformin. Accumulative incidences of diabetes were 55.0%, 39.3%, 40.5%, and 39.5% in Group 1-4 respectively. The relative risk reduction was 28.5% with Lifestyle Modification (LSM), 26.4% with Metformin (MET), and 28.2% with LSM + MET. The authors speculate that the difference in the scale of risk reduction compared to the Diabetes Prevention Program, may be due to distinctions in ethnicity of study population (Asian Indians vs Caucasions).

The Da Qing study in China divided 577 people with impaired glucose tolerance into a control group and three active treatment groups including diet alone, exercise alone, or diet plus exercise (Pan et al, 1997). Participants were monitored over 6 years with an OGTT every 2 years. All treatment groups had a significant decrease in the incidence of diabetes compared to the control group, and this effect was independent of the initial BMI. The highest overall reduction in the incidence of diabetes when compared with the control group in a proportional hazards model was in the exercise only group (46%) when adjusted for baseline plasma glucose concentrations and BMI.

The Nurses' Health Study recruited 84,941 healthy female nurses (Hu et al., 2001) . During the following 16 years, information on their diets and lifestyles were recorded. During the follow-up period, 3,300 new cases of type 2 diabetes were documented. The most important risk factor for type 2 diabetes was BMI. Lack of exercise, a poor diet, current smoking, and abstinence from alcohol were all associated with a significantly increased risk of diabetes even after adjustment for

BMI (Hu et al., 2001). Women who were in the low-risk groups had a relative risk of diabetes of 0.09 as compared with all other women.

2.8.2 Mechanism of exercise-induced improvement of insulin sensitivity

Although the mechanism of the protective effect of regular exercise is not addressed in the above clinical trials, a large body of literature demonstrates that physical training is associated with lower plasma insulin concentrations, improved muscle and liver insulin sensitivity, increased muscle glucose uptake and utilization and overall glycemic control (Frosig et al., 2007; Hawley and Lessard, 2008; Huffman et al., 2008; Kivelèa et al., 2007; Mikines et al., 1988; Park et al., 2008; Schenk and Horowitz, 2007; Venables and Jeukendrup, 2009; Weiss and Holloszy, 2007; Wojtaszewski and Richter, 2006). Although there are differences in related studies (Clark et al., 2004), these beneficial effects are partly explained by AMP-activated protein kinase (AMPK) activation during physical activity not only in skeletal muscle but also in liver and adipose tissue.

2.8.3 Muscle contraction and activation of AMPK

AMPK plays an important role in the regulation of cellular and whole-body energy homeostasis (Viollet et al., 2009). AMPK is composed of three different subunits α, β, and γ. The α-subunit contains a serine/threonine protein kinase catalytic domain in the N-terminal end. The β subunit appears to stabilize the interaction between alpha and γ subunits through its binding domain in the C-terminal end (Viollet et al., 2009). Regulation of AMPK activity involves both direct allosteric activation by AMP and reversible phosphorylation of AMPK α-subunit on Thr172 by upstream kinases. (Fig.2.6). Under conditions of high cellular energy demands, intracellular ATP is reduced, AMP concentrations rise and the AMP/ATP ratio forms a very sensitive indicator of cellular energy status. Binding of AMP to the regulatory

γ-subunit of AMPK promotes allosteric activation. AMPK is activated by phosphorylation of Thr172 in response to increase in the AMP/ATP ratio and Ca^{2+}. Thr172 is dephosphorylated by PP2C protein phosphatase switching active AMPK to the inactive form. There are at least two protein kinases capable of phosphorylating Thr172 in vivo, LKB1 and Ca^{2+}/calmodulin-dependent kinase kinase, especially the beta isoform (CAMKKβ). The LKB1 complex is supposed to be constitutively active and to promote activation by the AMP-dependent pathway. CaMKKβ responds to changes in cytoplasmic Ca^{2+} concentrations, indicating that AMPK may be activated in the absence of increase concentrations of AMP (Langfort et al., 2003).

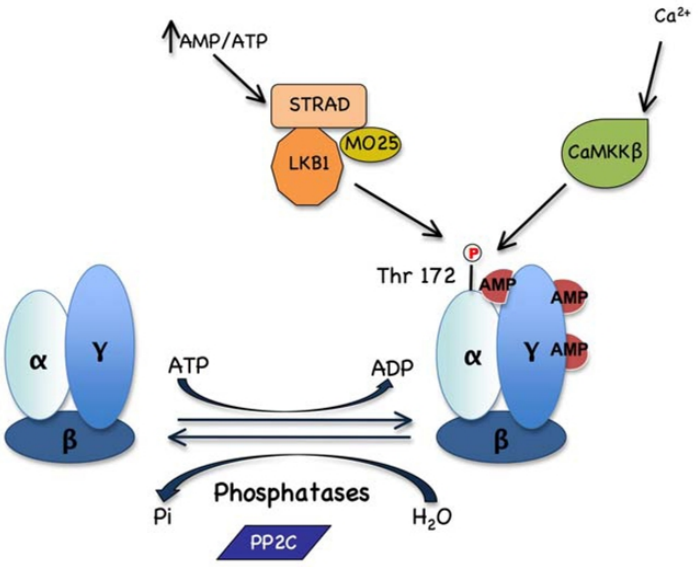

Fig 2.6 Regulation of AMPK activation: AMPK is activated by phosphorylation of Thr 172 catalysed by LKB1:STRAD:MO25 complex in response to increase in the AMP/ATP ratio and by CaMKKβ in response to elevated Ca^{2+} concentrations. Thr172 is dephosphorylated by PP2C protein phosphatase switching active AMPK to the inactive form (Treebak et al., 2009).

During muscle contraction, ATP is hydrolyzed, forming ADP. ADP then helps to replenish cellular ATP by donating a phosphate group to another ADP, forming an ATP and an AMP. As more AMP is produced during muscle contraction, the AMP: ATP ratio dramatically increases, leading to the allosteric activation of AMPK.

Stimulation of AMPK in the muscle is an efficient method to increase glucose uptake in an insulin-dependent manner, thus bypassing defective insulin signaling, as observed in type 2 diabetes patients. It has been recently discovered that the downstream target of Akt, Akt substrate of 160kDa (AS160/TBC1D4), plays a major role in regulating insulin-stimulated glucose uptake (Frosig et al., 2007). Another possible downstream effect of AMPK in the regulation of muscle glucose transport is an AS160/TBC1D4 homolog, TBC1D1 (Treebak et al., 2009). AS160/TBC1D4 is a Rab-GTPase activating protein that regulates the translocation of GLUT4 from intracellular vesicles to the plasma membrane (Langfort et al., 2003; Wojtaszewski and Richter, 2006).

2.8.4 AMPK and regulation of hepatic metabolism

Also, activation of AMPK leads to the inhibition of cholesterol synthesis by the phopshorylation of HMG-CoA reductase (Viollet et al., 2009). By inhibiting ACC and activating malonyl CoA decarboxylase (Ogden et al.), AMPK increases fatty acid oxidation via the regulation of malonyl CoA concentrations, which is both a critical precursor for biosynthesis of fatty acids and a potent inhibitor of CPT-1, the shuttle that controls the transfer of Long Chain acyl CoAs (LCACoA) into the mitochondria. AMPK inhibits hepatic glucose production via the phosphorylation of TORC2 and inhibition gene expression for key gluconeogenic enzymes, G6Pase and PEPCK, and for the transcriptional co-activator PGC-1.

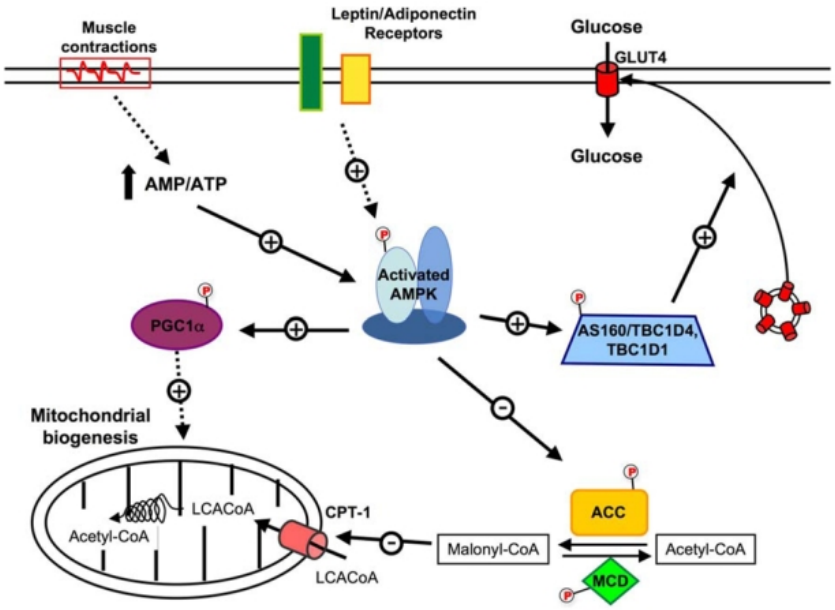

Fig 2.7 AMPK and the regulation of hepatic metabolism: Activation of AMPK leads to the inhibition of cholesterol synthesis by the phopshorylation of HMG-CoA reductase. By inhibiting ACC and activating MCD, AMPK increases fatty acid oxidation via the regulation of malonyl CoA concentrations, which is both a critical precursor for biosynthesis of fatty acids and a potent inhibitor of CPT-1, the shuttle that controls the transfer of LCACoA into the mitochondria. AMPK inhibits hepatic glucose production via the phosphorylation of TORC2 and inhibition gene expression for key gluconeogenic enzymes, G6Pase and PEPCK, and for the transcriptional co-activator PGC-1α. ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CPT1-α, carnitine palmitoyl transferase-1; G6Pase, glucose-6-phosphatase; LCACoA, Long Chain acyl CoAs; MCD, malonyl-CoA decarboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, PPARγ co-activator 1α; TORC2, transducer of regulated CREB activity 2 (Treebak et al., 2009).

2.8.5 Single bout of exercise and insulin sensitivity

Several elegant studies in both rodent and humans indicate that a single bout of physical activity can significantly lower glucose and insulin concentrations (Gao et al., 1994; Larsen et al., 1997). Following a single bout of exercise, whole body glucose disposal was shown to be increased in obese rodents using the euglycemic-hyperinsulinemic clamp technique, with an associated increase in insulin-stimulated glucose uptake in skeletal muscle (Musi et al., 2001). This enhancement of insulin action was shown to be associated with upregulation of

specific components of the glucose transport system and includes increased protein expression of GLUT4 and insulin receptor substrate-1 (IRS1) (Henriksen, 2002). Consistent with an increase in glucose uptake, AS160 phosphorylation (substrate of Akt) was shown to be increased in exercised legs in 12 healthy trained men, 4h after a one-legged exercise (Treebak et al., 2009).

2.8.6 Short-term exercise training

Short term exercise training is also shown to improve insulin action in humans. Improved insulin sensitivity and responsiveness as well as enhanced suppression of hepatic glucose production were observed in fourteen obese patients with type 2 diabetes after seven days of exercise training (Kirwan et al., 2009). Also, fasting glucose, insulin, triglycerides, and cholesterol decreased significantly in older obese individuals after short-term exercise training (Solomon et al., 2009a). Decreased glucose, insulin, triglycerides, C-reactive protein concentrations were reported in sixteen obese men after six days of exercise training (Black et al., 2005).

2.9 Exercise-induced plasma volume changes

Early cross-sectional comparisons between endurance-trained and sedentary male and female subjects revealed that blood volume was higher in trained individuals compared with untrained individuals. This relationship was independent of body size and existed similarly in both males and females (Cannon and Kluger, 1983). Further comparisons demonstrated that larger blood volume in athletes was contributed by higher plasma volume and erythrocyte volume (Carroll et al., 1995). Several longitudinal investigations demonstrated a blood volume expansion with endurance exercise training. In these investigations, both plasma volume and erythrocyte volume were increased (Carroll et al., 1995; Convertino, 1993; Gillen et al., 1991).

However, moderate-to-intense exercise results in a decrease in blood and plasma volume as water moves from the plasma compartment into both the interstitial and intracellular fluid compartments of contracting muscle. Changes in plasma volume changes during and after exercise are thought to result from transient fluid shifts into (hemodilution) and out of (hemoconcentration) the intravascular space (Kargotich et al., 1998). Firstly, the magnitude of plasma volume changes has been shown to be influenced by capillary fluid pressure. Cohn showed that the loss of plasma volume was related to hydrostatic pressure (Cohn, 1966). Secondly, according to Kargotich, plasma volume shifts are also influenced by osmotic gradients (Kargotich et al., 1997). It may therefore be critical to consider the effect of plasma volume changes on blood-borne parameters when conducting exercise studies.

2.10 Study goals

Fetuin-A, particularly the phosphorylated form (phosphofetuin-A), inhibits insulin receptor autophosphorylation and tyrosine kinase activity *in vitro* and *in vivo*. Elevated levels of fetuin-A have been shown to be associated with insulin resistance, metabolic syndrome, and an increased risk for type 2 diabetes. It is well known that increased physical activity contributes to lower fasting and postprandial insulin concentrations and improved insulin sensitivity. However, there are few reports on the effects of acute or short-term exercise training on fetuin-A levels and its phosphorylation status. The goal of this study was to examine daily variations in fetuin-A, the effect of an oral glucose challenge, and the effects of single and repeated bouts of exercise on total fetuin-A and phosphofetuin-A levels in obese individuals with MetS, and correlate these with changes in insulin sensitivity.

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Chapter 3: Temporal changes in plasma fetuin-A concentrations in response to an oral glucose challenge and a single bout of exercise in normal weight versus obese men

3.1 Abstract

Fetuin-A, in particular the phosphorylated isoform, is a negative regulator of insulin action, inhibiting insulin receptor tyrosine kinase activity and downstream insulin signaling. In obese individuals, serum fetuin-A levels are elevated and are associated with insulin resistance. Recent evidence indicates that fetuin-A is an independent risk factor for type 2 diabetes. However, our understanding of fetuin-A responses to a glucose load and exercise, is currently limited. Our goal was to characterize the effect of an oral glucose challenge and a single bout of exercise on serum fetuin-A levels in normal weight and obese individuals. Ten obese and seven age-matched normal weight men underwent a single bout of treadmill walking at 60-70% VO_{2max}, expending 500 kcals. Oral glucose tolerance tests were conducted 4 days prior to and 24-hours after the exercise session. Obese individuals were insulin resistant as evidenced by serum insulin concentrations, glucose-to-insulin ratio, and Homeostasis model assessment (HOMA) index. Following an oral glucose challenge, total fetuin-A concentrations were significantly decreased at 30, 60 and 120-min time points in normal weight individuals, but not in obese individuals. Serum Ser312-phosphorylated fetuin-A levels (phosphofetuin-A) also decreased significantly at 2 hour time point compared to 0-min time point in lean but not obese individuals. After a single bout of exercise, the glucose/insulin ratio increased 92%, 24 hr after exercise ($p = 0.0067$) and the insulin AUC was reduced 16% post-exercise ($p < 0.05$)

in obese and 20% post-exercise ($p < 0.05$) in normal weight individuals. Phosphofetuin-A area under the curve was significantly lower 24 hours after a single bout of exercise, while total fetuin-A concentrations remained unchanged with exercise in obese men. Additionally, the reduced phosphofetuin-A AUC observed after a single exercise session, in obese individuals, is consistent with improvements in surrogate markers of insulin sensitivity. Taken together, we demonstrate for the first time, that total fetuin-A and phosphofetuin-A concentrations are decreased following a glucose load in normal weight individuals, suggesting that fetuin-A levels are responsive to physiological cues with potential implications in insulin action. Further, lowering of phosphofetuin-A levels in obese individuals, following a single bout of exercise, may be one mechanism by which exercise improves insulin action.

3.2 Introduction

Obesity represents a major global public health threat, and together with diabetes, it constitutes an important contributor to the predicted decline in life expectancy (Baskin et al., 2005). Metabolic syndrome is a complex condition, closely linked to obesity, insulin resistance, hypertension, and dyslipidemia, and is associated with an increased risk of cardiovascular disease, type 2 diabetes, stroke and heart attack (Ginsberg and MacCallum, 2009). Insulin resistance, a primary underlying abnormality leading to MetS, is a physiological condition in which insulin becomes less effective in lowering blood glucose. It is characterized by glucose intolerance and hyperglycemia followed by increase in plasma concentrations of insulin, dyslipidemia with elevated levels of triglycerides and diminished HDL cholesterol, elevation of blood pressure, abdominal obesity and with the elevated tendency for thrombosis (Lazar, 2006).

Fetuin-A, also known as alpha 2-HS glycoprotein, a liver-secreted humoral factor, is a physiological inhibitor of insulin-stimulated insulin receptor autophosphorylation and tyrosine kinase activity both *in vitro* and *in vivo* (Auberger et al., 1989; Cintron et al., 2001; Mathews et al., 1997; Olivier et al., 2000; Srinivas et al., 1993). Phosphorylation status of fetuin-A has been shown to be critical for its inhibition of insulin receptor tyrosine kinase activity. In humans, circulating plasma fetuin is partially phosphorylated (about 20% of total) on Ser120 and Ser312 (Haglund et al., 2001). In animal models of diet-induced obesity, plasma fetuin-A concentrations are elevated and mice that lack fetuin-A demonstrate improved insulin sensitivity and resistance to diet-induced obesity (Hennige et al., 2008; Lin et al., 1998; Mathews et al., 2006; Mathews et al., 2002). In humans, plasma fetuin-A concentrations are positively associated with body mass index (BMI), visceral adiposity, insulin resistance, metabolic syndrome, fatty liver, and an increased risk for type 2 diabetes (Ix et al., 2006; Ix et al., 2009; Ix et al., 2008; Kalabay et al., 2002; Mori et al., 2006; Stefan et al., 2008; Stefan et al., 2006) . Recent studies by Reinehr and colleagues demonstrate that elevated fetuin-A levels were related to insulin resistance and MetS in a study with obese children with nonalcoholic fatty liver disease and substantial weight loss following a 1-year lifestyle intervention decreased fetuin-A concentrations significantly (Reinehr and Roth, 2008).

Several elegant studies in both rodent and humans indicate that a single bout of physical activity can significantly lower glucose and insulin concentrations (Gao et al., 1994;Larsen et al., 1997;Richter et al., 1985). Following a single bout of exercise, whole body glucose disposal was shown to be increased in obese rodents and humans using the euglycemic-hyperinsulinemic clamp technique, with an associated increase in insulin-stimulated glucose uptake in skeletal muscle (Cusi et al., 2000) (Hoene et

al., 2009; Musi et al., 2001), which was associated with increased expression and translocation of more GLUT4 to the cell surface (Holloszy, 2005).

The goal of this study was to examine the effect of an oral glucose challenge and a single bout of exercise on serum total fetuin-A and phosphorylated fetuin-A concentrations in obese individuals. Surrogate markers of insulin sensitivity, including plasma glucose concentrations, insulin concentrations, HOMA values and glucose to insulin ratio were also assessed.

3.3 Methods

3.3.1 Recruitment of subjects:

This study used a sample cohort of subjects initially recruited for participation in a larger investigation. We analyzed the data from ten obese (BMI $\geq$ 30 kg/m^2 or % body fat $\geq$ 30, waist circumference $\geq$ 102 cm) and seven normal weight (BMI $\leq$ 25 kg/m^2) men. Volunteers were recruited from the local community by distributing recruitment fliers as well as through Auburn University's daily faculty/staff announcement email. All volunteers met the following criteria: between 30 and 65 years of age, non-smokers, no reported cardiovascular or metabolic disease, not currently taking medication known to alter lipid or glucose metabolism, weight stable for the past six months and did not engage in regular physical activity for the past six months. This study was approved by the Auburn University Institutional Review Board (IRB) for research involving human subjects.

3.3.2 Study protocol:

Participants were asked to record their diet and physical activity for three days. These records were analyzed using The Food Processor SQL nutrition and fitness software (Esha Research, Salem, OR). Participants were counseled to maintain a similar energy and nutrient intake and to refrain from any kind of exercise, during the

blood sampling timeline (Fig.3.1). Blood samples (10 mL) were obtained after an overnight fast.

Participants then underwent an oral glucose tolerance test (OGTT) consuming 75 g of standard carbohydrate syrup. Blood samples were drawn at 30 min intervals for 2 hours following consumption of the glucose solution. Fasting blood samples were obtained again on days 5 and 7, and blood samples were assayed for total fetuin-A and phosphofetuin-A to analyze daily variation. On day 7, participants underwent a single bout of treadmill walking expending 500 kcal at 60-70% VO_{2max}. Blood samples were taken prior to and after exercise. To examine the sustained effects of the single bout of exercise, participants were given a second OGTT 24 hours post-exercise, and blood samples were collected at 30 min intervals for 2 hours. Serum samples were stored in a -80˚C freezer until further analyses.

3.3.3 Metabolic assays:

Serum glucose concentrations, based on the glucose oxidase method, were assayed according to the manufacturer's protocol (Raichem, San Diego, CA). Serum insulin concentrations were measured by a human insulin-specific radioimmunoassay (Millipore Corporation, Billerica, MA). HOMA values were calculated by the following formula: Insulin (μU/mL) x [glucose (mmol/L)/22.5]. Glucose to insulin ratio was calculated as glucose (mg/dL)/insulin (μU/mL). Serum non-esterified fatty acid concentrations were assayed, in duplicate, using a 96-well NEFA assay kit (Wako, Richmond, VA).

3.3.4 Total fetuin-A assay:

Plasma concentrations of total fetuin-A were assayed by a sandwich ELISA, according to the manufacturer's protocol (BioVendor, LLC, Candler, NC). Plasma samples were assayed in duplicate, using human fetuin-A standards ranging from 2 to

100 ng/mL. Briefly, standards, quality controls, and diluted serum samples were added to anti-human fetuin-A antibody coated microtiter strips. After incubation, washing, and addition of conjugate solution, the substrate was added, and the absorbance was read in a microplate reader, at 450 nm.

3.3.5 Phosphofetuin-A Western blot:

Plasma samples were assayed for phosphofetuin-A by Western blotting. Proteins were separated on a 4 ~ 20% gradient SDS-PAGE gel (NuSep iGel, Austell, GA), transferred to nitrocellulose, and detected using a Ser312-phospho-fetuin-A antibody. Chemoluminescence was imaged and quantitated with UVP Bioimaging system (UVP, Upland, CA).

3.3.6 Statistical analysis:

Student's t-test, repeated measures analysis of variance (ANOVAs) and Duncan's multiple range tests were used to determine significant findings. $P < 0.05$ was considered to be significant. Data was analyzed using Statistical Analysis System (SAS, Cary, NC).

3.4 Results

3.4.1 Baseline anthropometric and metabolic indices

Obese men, recruited for this study, demonstrated significant differences in body weight, BMI, waist circumference, fat (% body weight), absolute $VO_{2max,}$ blood glucose, serum insulin, glucose to insulin ratio, and HOMA values compared to normal weight individuals (Table 3.1). After exclusion of individuals that did not meet the selection criteria, all individuals in the normal weight group had a BMI less than 25 kg/m^2.

3.4.2 Daily variation of fetuin-A in normal weight and obese individuals

Since there were no earlier reports on daily variation of total fetuin-A or

phosphorylated fetuin-A levels in humans, it was of interest to determine daily variation of fetuin-A in normal weight and obese individuals. However, no significant daily variations were observed in fasting concentrations of total fetuin-A, phosphofetuin-A, or in any of the other assayed metabolites, including blood glucose, serum insulin, NEFA, glucose to insulin ratio, HOMA values, in either the normal weight or obese individuals. (Table 3.2)

3.4.3 Fetuin-A response to an oral glucose load in normal weight and obese individuals

To analyze alterations of fetuin-A in response to a glucose load, total fetuin-A and phosphofetuin-A levels were assayed following an oral glucose load. Obese men demonstrated significantly higher glucose levels AUC compared to normal weight individuals, during the oral glucose tolerance test (Fig.3.2A; Table 3.5). However, average blood glucose concentrations in obese men were below 140 mg/dl at the 2h time point, and therefore, did not meet the criteria for impaired glucose tolerance. Insulin levels tended to be higher in obese individuals, during the OGTT, but these were not significantly different, compared to normal weight group (Fig.3.3B; Table 3.5). Total fetuin-A and phosphofetuin-A levels and AUC were significantly higher for the obese group at all time points (Fig.3.3C, D; Table 3.5) than the normal weight group. Interestingly, we observed a dramatic decrease in total fetuin-A levels 30 minutes after consumption of an oral glucose load, in normal weight individuals (Fig.3.3C). This decrease, in total fetuin-A, in normal weight individuals, was sustained for the duration of the OGTT. Phosphofetuin-A levels tended to be lower ($p = 0.15$), at the 30 minute time-point, following an oral glucose load. A significant decrease in phosphofetuin-A levels were observed at the 2h time-point in normal weight individuals (Fig.3.3D). However, in contrast to normal weight individuals,

total fetuin-A and phosphofetuin-A levels were unaltered in obese individuals after an oral glucose load.

3.4.4 Effects of a single bout of exercise on fetuin-A and surrogate markers of insulin sensitivity

After an overnight fast, all participants underwent a single bout of exercise on day 7, in the fasted condition, and blood samples were obtained prior to and immediately after exercise. Blood samples were also obtained 24 hours after exercise (day 8) to characterize latent effects of the single bout of exercise. No significant changes in blood glucose, serum insulin, glucose to insulin ratio, or HOMA levels were observed in both normal weight and obese individuals immediately after exercise (Tables 3.3 and 3.4). As expected, NEFA levels increased significantly, immediately after a single bout of exercise in both normal weight and obese individuals. Obese individuals demonstrated an improvement in glucose to insulin ratio ($p < 0.0067$), and a tendency to decrease HOMA values ($p < 0.061$), suggestive of an improvement of insulin sensitivity (Table 3.4). To further analyze improvement of insulin sensitivity, an oral glucose challenge was administered 24 hours after the single bout of exercise. While the areas under the curve for glucose (Fig.3.3A) were not significantly different before and after exercise, in both normal weight and obese groups, the AUC for insulin, during the OGTT, were significantly lower after exercise, in both normal weight and obese groups (Table 3.5 and Fig.3.3B), indicative of improved insulin sensitivity.

Serum total fetuin-A concentrations were not altered immediately after a single bout of exercise, in either normal weight or obese individuals (Tables 3.3 and 3.4, Figs.3.3C and E). Interestingly, phosphofetuin-A levels were significantly decreased following a single bout of exercise in both normal weight and obese

individuals. In normal weight individuals, phosphofetuin-A levels were decreased immediately after exercise; and 24h after the single bout of exercise ($p < 0.05$; Table 3.3). However, administration of an OGTT after the single bout of exercise did not significantly alter the AUC for phosphofetuin-A for normal weight individuals (Table 3.5 and Fig. 3.3F). On the contrary, in obese individuals, after a single bout of exercise, phosphofetuin-A_{AUC} during the OGTT was significantly lower, compared to normal weight individuals (Table 3.5 and Fig.3.3D).

3.5 Discussion

This is the first report analyzing serum total fetuin-A and phosphorylated fetuin-A responses to a single bout of exercise and an oral glucose load. It is well established that a single bout of physical activity leads to significant blood glucose lowering effects in obese rodent models and individuals with type 2 diabetes (Hubinger et al., 1987, Goodyear and Kahn, 1998; Hayashi et al., 1997). A decrease in plasma insulin has also been observed in type 2 diabetic individuals during an exercise bout (Larsen et al., 1997; Musi et al., 2001; Wallberg-Henriksson, 1992). Further, the enhanced glucose transport activity has been shown to persist into the immediate post-exercise period. This was shown to be associated with an improvement of insulin sensitivity, both immediately after and 20 h after exercise (Devlin et al., 1987; Minuk et al., 1981).

Fetuin-A, a physiological inhibitor of insulin-stimulated insulin receptor autophosphorylation and tyrosine kinase activity, has been shown to be associated with insulin resistant conditions such as obesity, metabolic syndrome, and type 2 diabetes (Ix et al., 2006; Ix et al., 2008; Mori et al., 2006; Stefan et al., 2008; Stefan et al., 2006). In this study we demonstrate that, in obese men, phosphorylated fetuin-A levels were decreased 24 hours after a single bout of exercise. This is consistent with

the improvement in insulin sensitivity observed 24 hours after the single bout of exercise. Thus, it is possible that the decreased phosphofetuin-A may relieve the inhibitory effects on the insulin receptor, and contribute to the latent effects of exercise-induced improvement of insulin sensitivity.

While the the primary mechanism for this glucose-lowering effect of acute exercise was shown to be due to an increase in GLUT4 translocation (Wallberg-Henriksson and Holloszy, 1984; Kennedy et al., 1999), the mechanisms for the long-term effect of improvement of insulin sensitivity following a single bout of exercise, in insulin-resistant skeletal muscle are not clear. Insulin resistant individuals demonstrate decreased GLUT4 protein translocation, significantly less insulin-stimulated insulin receptor and IRS-1 tyrosine phosphorylation, reduced PI3-kinase and Akt activity in skeletal muscle (Bjornholm et al., 1997; Goodyear et al., 1995; Krook et al., 1998; Ryder et al., 2001; Zierath et al., 1996).

Cusi et al demonstrated that, 24 hours after a single exercise session, insulin-stimulated skeletal muscle tyrosine phosphorylation of the insulin receptor and of IRS-1 were significantly enhanced in type 2 diabetic subjects (Cusi et al., 2000). Improvements in whole-body insulin-mediated glucose uptake have been attributed to enhaced intracellular signaling through activation of PI3-kinase in humans (Cusi et al., 2000; Houmard et al., 1999; Kirwan et al., 2000). An alternate mechanism attributed to the improvement in insulin sensitivity, is the activation of contraction-induced 5'-AMP-activated protein kinase (AMPK). AMPK activation is not only found in skeletal muscle but also found in liver and adipose tissue (Musi et al., 2001; Ruderman et al., 2003). Exercise-induced AMPK activation has been shown to induce AS160 phosphorylation in skeletal muscle (Birk and Wojtaszewski, 2006). AS160, a downstream target of Akt, plays a major role in regulating GLUT4 transport. Also,

studies showed that AMPK activation down-regulates gluconeogenic gene expression, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Lochhead et al., 2000). Recent studies showed that a single bout of exercise resulted in a very rapid and pronounced induction of hepatic metabolic enzymes and regulators of metabolism or transcription: G6Pase, pyruvate dehydrogenase kinase-4 (PDK4), IRS-2, peroxisome proliferator activated receptor coactivator 1α (PGC-1α) (Hoene et al., 2009). This may lead to improved insulin signal transduction in the liver. This suggests that the markedly increased protein amounts of insulin pathway intermediates induced by the acute exercise could be responsible for enhanced action of insulin.

It was surprising to find that total fetuin-A and phosphorylated fetuin-A levels decrease following an oral glucose load in normal weight individuals, but not in obese individuals. Whether this decrease is because of alterations in $glucose_{AUC}$ or due to alterations in insulin sensitivity between normal weight versus obese individuals is not clear. Also, whether the decrease in fetuin-A was because of a decrease of its secretion by the liver, or due to an increased intake into target cells, is not known. Fetuin-A has been shown to be rapidly internalized into cells and secreted from the cells into which it was internalized, suggestive of a receptor-mediated internalization (Wajih et al., 2004). In this context, annexin II and annexin VI have been reported as putative receptors for fetuin-A (Chen et al., 2007). Additional experiments are required to understand the role of fetuin-A in insulin action and to clarify the mechanisms involved in its rapid clearance from circulation.

Our study has several limitations. The sample size of our studied population was small. Our studied population may not provide proper representation of the obese, insulin-resistant population. Additionally, the long-term effects of exercise should be

taken into consideration to gain a critical insight into the role of fetuin-A play in insulin resistance condition. Future research examining the response of phosphorylated fetuin-A to a 5-8% weight-loss, as demonstrated in the Diabetes Prevention trials, can enhance our understanding of fetuin-A in insulin action and address some of the limitations of this study.

In summary, in conjunction with existing animal and human data, our data showing the lowering of total fetuin-A, following an oral glucose load suggest that fetuin-A is responsive to physiological cues in normal weight individuals with normal insulin action. However, in obese insulin-resistant individuals this action is blunted. Further, lowering of phosphofetuin-A levels in obese individuals, following a single bout of exercise, may be one mechanism by which exercise improves insulin action.

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Table 3.1: Baseline physiological and metabolic characteristics in normal weight (n=7) and obese (n=10) individuals

Variables	Normal weight	Obese
Age (yrs)	42.3 ± 8.5	39.4 ± 8.2
Height (in)	68.3 ± 3.0	71.5 ± 3.6
Weight (lbs)	164.1 ± 11.3	260.4 ± 55.5***
BMI (kg/m^2)	24.8 ± 1.4	35.6 ± 6.7***
Waist Circumference (inches)	34.9 ± 2.5	45.9 ± 6.3***
Fat (% body weight)	25.2 ± 5.5	37.6 ± 5.2***
$VO_{2\ abs}$ (L/min)	2.6 ± 0.47	3.3 ± 0.6*
$VO2_{\ rel}$ (ml/kg/min)	35.2 ± 7.3	28.8 ± 6.2
Systolic BP (mm Hg)	118 ± 12	126 ± 8
Diastolic BP(mm Hg)	76 ± 6	83 ± 6
Total Cholesterol (mg/dL)	191 ± 31	172 ± 44
LDL Cholesterol (mg/dL)	116 ± 29	104 ± 33
HDL Cholesterol (mg/dL)	43 ± 7	35 ± 6
Triglycerides (mg/dL)	157 ± 66	163 ± 99
NEFA (mEq/L)	0.37 ± 0.2	0.56 ± 0.26
Glucose (mg/dL)	96 ± 5	113 ± 22*
Insulin (µU/mL)	7.5 ± 7.1	31.5 ± 18.1**
Glucose/insulin	22.3 ± 13.9	4.6 ± 2.3**
HOMA	1.8 ± 1.7	9.2 ± 6.3**

Data are expressed as Means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 3.2: Analysis of daily variation in fasted normal weight (n=7) and obese (n=10) individuals

	Normal weight		Obese	
Variables	Day 5	Day 7	Day 5	Day 7
Glucose (mg/dL)	99 ± 11	99 ± 6	120 ± 22	106 ± 11
Insulin (µU/mL)	22.7 ± 11.0	25.5 ± 12.2	31.5 ± 18.1	28.8 ± 8.7
Glucose/insulin	5.3 ± 2.5	4.9 ± 2.8	4.6 ± 2.3	4.0 ± 1.2
HOMA	5.5 ± 2.6	6.5 ± 3.2	9.2 ± 6.3	7.6 ± 2.7
NEFA (mEq/L)	0.29 ± 0.15	0.36 ± 0.24	0.56 ± 0.26	0.54 ± 0.22
Total Fetuin-A (µg/ml)	274 ± 41	282 ± 34	346 ± 23	341 ± 11
Phosphofetuin-A (arbitrary scan units)	323 ± 412	321 ± 422	271 ± 160	332 ± 230

Data are expressed as Means ± SD.

Table 3.3: Effects of a single bout of exercise (expending 500 kcal) on serum glucose, insulin, total fetuin-A and phosphofetuin-A concentrations in normal weight (n=7) individuals

Variables	Day 7		Day 8 (% change)	P value
	Before Exercise	After Exercise (% change)		
Glucose (mg/dL)	98.7 ± 5.6	94.3 ± 7.2 (-4.45%)	97.9 ± 10.5 (-0.81%)	0.074
Insulin (µU/mL)	25.5 ± 12.2	20.9 ± 10.8 (-18.0%)	15.4 ± 6.4 (-39.6%)	0.17
Glucose/insulin	4.9 ± 2.8	5.6 ± 2.7 (14.3%)	9.2 ± 8.6 (87.8%)	0.39
HOMA	6.5 ± 3.2	5.0 ± 3.0 (-23%)	3.7 ± 1.5 (-43%)	0.17
NEFA (mEq/L)	0.37 ± 0.24	0.74 ± 0.51 (100%)$^{\#}$	0.33 ± 0.08 (-10.8%)	0.015
Total Fetuin-A (µg/ml)	282 ± 33.9	293 ± 35.4 (3.9%)	309 ± 89.5 (9.6%)	0.69
Phosphofetuin-A (arbitrary scan units)	743 ± 486	500 ± 195 (-32.7%)	324 ± 200 (-56.4%)*	0.044

Data are expressed as Means ± SD. *$^{\#}$ $p < 0.05$, * compared to before exercise, $^{\#}$ compared to Day 8

Table 3.4: Effects of a single bout of exercise (expending 500 kcal) on serum glucose, insulin, total fetuin-A and phospho fetuin-A concentrations in obese (n=10) individuals

Variables	Day 7		Day 8 (% change)	P value
	Before exercise	After exercise (% change)		
Glucose (mg/dL)	106 ± 10.8	103 ± 11.4 (-2.83%)	105 ± 12.0 (-0.94%)	0.78
Insulin (µU/mL)	28.8 ± 8.7	21.9 ± 8.0 (-24.0%)	19.5 ± 17.2 (-32.3%)	0.11
Glucose/insulin	4.0 ± 1.2$^{\#\#}$	4.0 ± 1.2 (0%) $^{\#\#}$	7.7 ± 3.5 (92.5%)	0.0067
HOMA	7.6 ± 2.7	7.6 ± 2.7 (0%)	5.1 ± 4.4 (-32.9%)	0.061
NEFA (mEq/L)	0.54 ± 0.22	1.08 ± 0.36 (100%) ***	0.54 ± 0.24 (0%)	<0.001
Total fetuin-A (µg/ml)	341 ± 230	343 ± 12.9 (0.59%)	336 ± 60.9 (-1.5%)	0.99
Phosphofetuin-A (arbitrary scan units)	332 ± 230	328 ± 179 (-1.2%)	256 ± 88.8 (-22.9%)	0.64

Data are expressed as Means ± SD. *** $p < 0.001$, compared to before exercise, $^{\#\#}$ $p < 0.01$, compared to Day 8

Table 3.5: Alterations in metabolic indices, fetuin-A, and phosphofetuin-A during an oral glucose tolerance test (OGTT), before and 24-hours after a single bout of exercise in normal weight (n=7) and obese (n=10) individuals

Variables	Normal weight		Obese	
	OGTT before Ex.	OGTT 24h after Ex.	OGTT before Ex.	OGTT 24h after Ex.
$Glucose_{AUC}$	1410 ± 175	1323 ± 136	1813 ± 405$^{\#}$	1571 ± 627
$Insulin_{AUC}$	739 ± 144	598 ± 230*	937 ± 277	789 ± 300*
Total fetuin-A_{AUC}	2991 ± 432	2993 ± 614.5	3955 ± 565$^{\#\#}$	4050 ± 619
Phosphofetuin-A_{AUC}	1719 ± 1519	1703 ± 1342	3853 ± 1364$^{\#\#}$	2996 ± 745*
$NEFA_{AUC}$	31.1 ± 23.3	23.1 ± 5.8	40.3 ± 13.1	39.7 ± 12.8

AUC: Area under the curve; Data are expressed as Means ± SD.
*$p < 0.05$, **$p < 0.01$ (comparing before and after exercise)
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (comparing normal weight vs. obese groups)

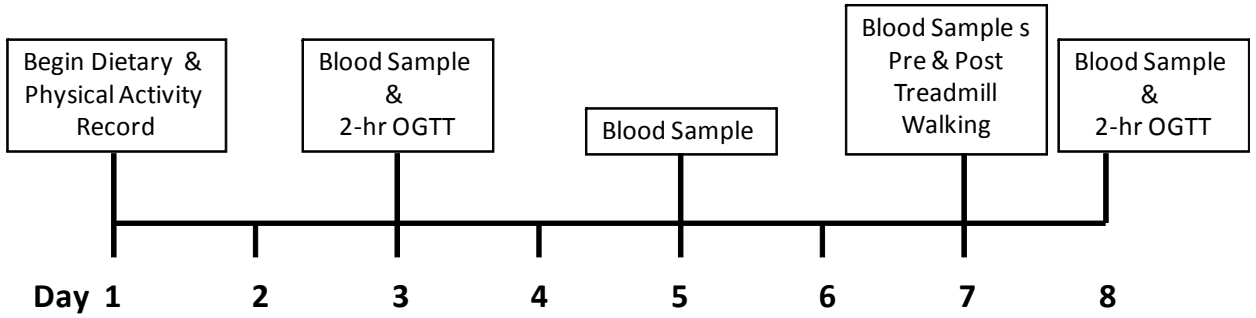

Fig 3.1 Baseline procedure and blood sampling

Fig.3.2

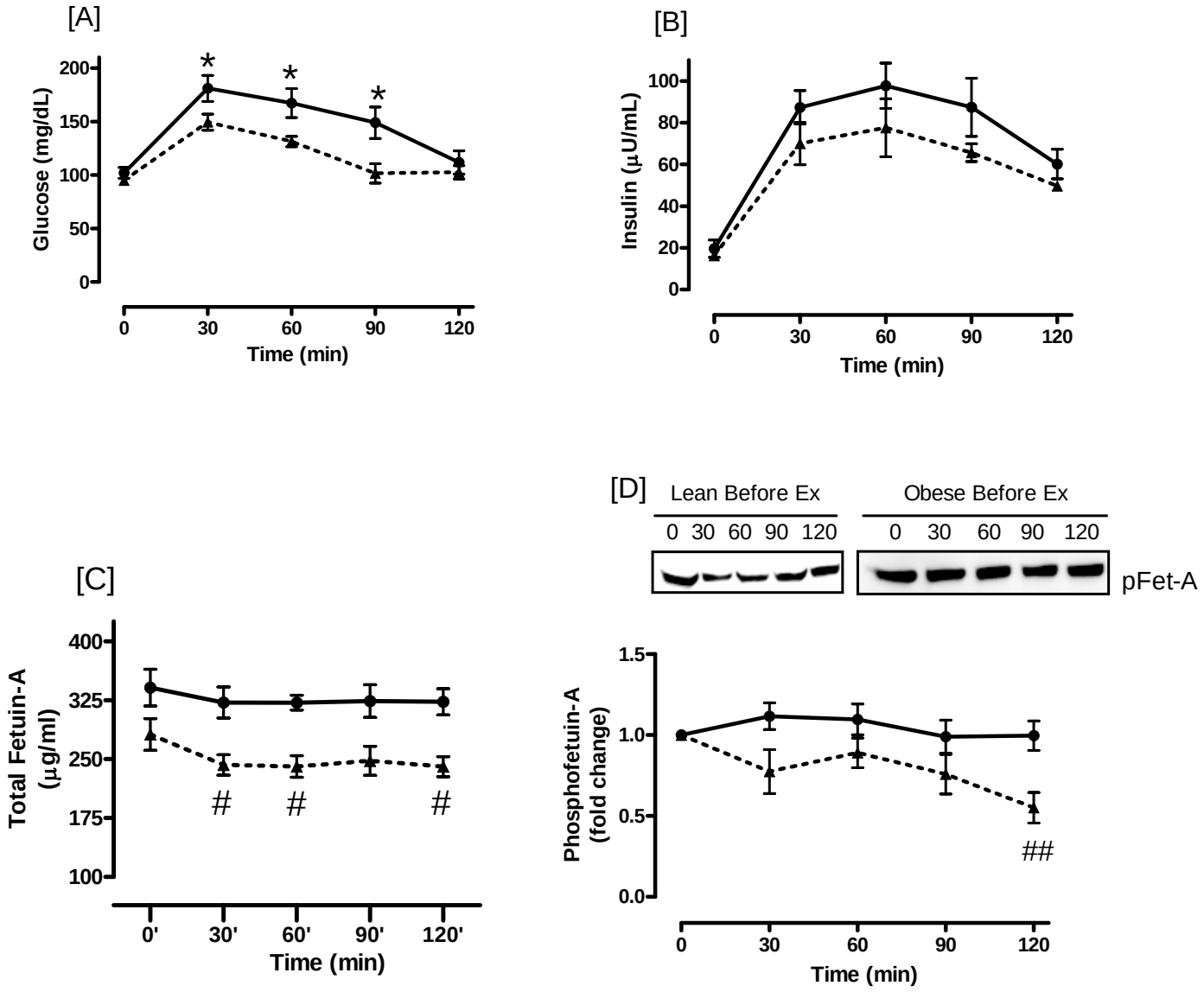

Fig.3.2: Effects of an oral glucose challenge. All participants were subjected to an oral glucose tolerance test before exercise. Blood samples were taken at 30 min intervals over a 2-hour period and assayed for glucose (A), insulin (B), total fetuin-A (C), and phosphofetuin-A (D). —●— obese group before exercise. --▲-- normal weight group before exercise.*$p < 0.05$, comparing normal weight vs. obese groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to 0' time point within each group.

Fig.3.3

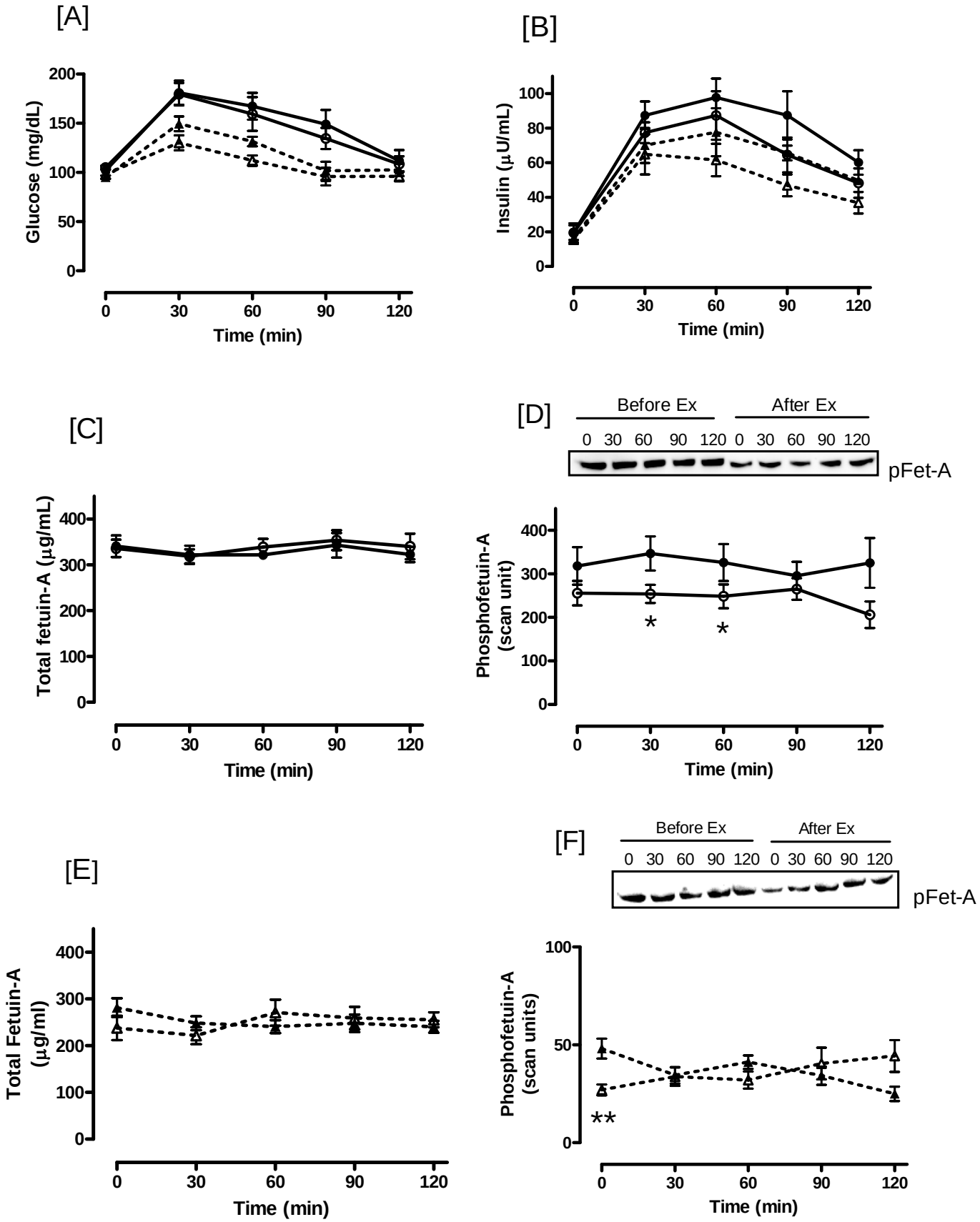
[A]
Glucose (mg/dL)
200
150
100
50
0
0 30 60 90 120
Time (min)
[B]
Insulin (μU/mL)
100
80
60
40
20
0
0 30 60 90 120
Time (min)
[C]
Total fetuin-A (μg/mL)
400
300
200
100
0
0 30 60 90 120
Time (min)
[D]
Before Ex
After Ex
0 30 60 90 120 0 30 60 90 120
pFet-A
Phosphofetuin-A (scan unit)
400
300
200
100
0
*
*
0 30 60 90 120
Time (min)
[E]
Total Fetuin-A (μg/ml)
400
300
200
100
0
0 30 60 90 120
Time (min)
[F]
Before Ex
After Ex
0 30 60 90 120 0 30 60 90 120
pFet-A
Phosphofetuin-A (scan units)
100
50
0
**
0 30 60 90 120
Time (min)

Fig.3.3: Effects of a single bout of exercise. All participants were subjected to a single bout of exercise (treadmill walking at 60-70% VO_{2max} to achieve a 500 kcal expenditure). OGTT was administered before exercise and 24 hours after exercise. Blood samples were taken at 30 min intervals over a 2-hour period and were assayed for glucose (A), insulin (B), total fetuin-A (C,E) and phosphofetuin-A (D,F). —●— obese group before exercise, –O— obese group after exercise, --▲-- normal weight group before exercise, --Δ--indicates normal weight group after exercise. * $p < 0.05$, ** $p < 0.01$, comparing before and after exercise.

Chapter 4: Effects of Short Term Exercise on Plasma Fetuin-A Concentrations in Men with Metabolic Syndrome

4.1 Abstract:

Fetuin-A, particularly the phosphorylated form (phosphofetuin-A), inhibits insulin receptor autophosphorylation and tyrosine kinase activity *in vitro* and *in vivo*. Elevated concentrations of fetuin-A have been shown to be associated with insulin resistance, metabolic syndrome, and an increased risk for type 2 diabetes. It is well known that increased physical activity contributes to lower fasting and postprandial insulin concentrations and improved insulin sensitivity. However, the fetuin-A response to exercise in men with metabolic syndrome is unknown. The goal of this study was to investigate the influence of repeated bouts of exercise on four consecutive days and in the post-exercise period on plasma fetuin-A concentrations as well as markers of insulin action in fifteen obese men with metabolic syndrome. Participants underwent treadmill walking at 70% of their VO_{2max} to expend 350 kcal daily for four days. Fasting blood samples were obtained prior to exercise on days 1 through 4, and 24h and 72h post-exercise. Plasma insulin concentrations and HOMA values were decreased significantly on day 2, 3 and 24h post-exercise compared to baseline, suggesting improved insulin sensitivity. While plasma concentrations of total fetuin-A were not altered with repeated bouts of exercise and in the post-exercise period, a significant decrease in phosphofetuin-A concentrations was observed in the 24h post-exercise period, suggesting that the decreased phosphofetuin-A may contribute to the improved insulin sensitivity following sustained effects of the repeated bouts of exercise.

4.2 Introduction

Metabolic syndrome, which affects one in five people in the world, is a clustering of metabolic abnormalities, including obesity, insulin resistance, glucose intolerance, dyslipidemia, cardiovascular disease, hypertension, and diabetes (Aggoun, 2007). Although there's a debate surrounding the concept of MetS (Kahn et al., 2005; Simmons et al., 2010), it is recognized as a major risk factor for diabetes and cardiovascular disease. Insulin resistance is thought to be one of the primary underlying abnormalities leading to MetS. In insulin-resistant individuals, humoral factors secreted by adipose tissue and liver, including non-esterified fatty acids, leptin, adiponectin, TNF-α, IL-6, resistin, and RBP4, have been implicated in the development of MetS and diabetes (Ginsberg and MacCallum, 2009; Rosen and Spiegelman, 2006).

Fetuin-A, a liver-secreted humoral factor, is a physiological inhibitor of insulin-stimulated insulin receptor autophosphorylation and tyrosine kinase activity both *in vitro* and *in vivo* (Auberger et al., 1989; Cintron et al., 2001; Mathews et al., 1997; Olivier et al., 2000; Srinivas et al., 1993). Phosphorylation status of fetuin-A has been shown to be critical for its insulin receptor tyrosine kinase activity. Recent studies indicate that in humans, circulating plasma fetuin is partially phosphorylated (about 20% of total) on Ser120 and Ser312 (Haglund et al., 2001). In animal models of diet-induced obesity, plasma fetuin-A concentrations are elevated and mice that lack fetuin-A demonstrate improved insulin sensitivity and resistance to diet-induced obesity (Hennige et al., 2008; Lin et al., 1998; Mathews et al., 2006; Mathews et al., 2002). In humans, plasma fetuin-A concentrations are positively associated with BMI, visceral adiposity, insulin resistance, metabolic syndrome, fatty liver, and an increased risk for type 2 diabetes (Ix et al., 2006; Ix et al., 2009; Ix et al., 2008; Kalabay et al., 2002; Mori et al., 2006; Stefan et al., 2008; Stefan et al., 2006) . Recent studies by

Reinehr and Roth demonstrate that elevated fetuin-A concentrations were related to insulin resistance and MetS in obese children with nonalcoholic fatty liver disease (NAFLD) and substantial weight loss following a 1-year lifestyle intervention decreased fetuin-A concentrations significantly (Reinehr and Roth, 2008).

Exercise training increases insulin action in humans. Acutely, it increases muscle glucose transport, and in a sustained manner, exercise improves insulin sensitivity (Dela et al., 1992; Dengel et al., 1996; Frosig et al., 2007; Houmard et al., 1999; Kirwan et al., 2009). Since fetuin-A is a regulator of insulin action, it was of significant interest to understand the effects of short-term exercise training on circulating concentrations of total fetuin-A and its phosphorylated form. Therefore, in this study, we examined the effects of repeated bouts of exercise on fetuin-A and phosphofetuin-A concentrations in fifteen obese male individuals with MetS. Surrogate markers of insulin sensitivity, including plasma glucose concentrations, insulin concentrations, HOMA values and glucose to insulin ratio were also assessed.

4.3 Methods

4.3.1 Participants:

Fifteen middle-aged obese males, with BMI $\geq$ 30 kg/m^2 and who met the National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) criteria for metabolic syndrome, and who did not engage in regular physical activity (< 3 sessions/week and < 30 min/session) were recruited from the local community by distributing fliers. All volunteers were screened for contraindications to exercise, cardiovascular and metabolic disease, and drugs known to alter lipid metabolism, prior to entry into the study. This study was approved by the Auburn University Institutional Review Board (IRB) for research involving human subjects.

4.3.2 Experimental Protocol:

Following initial anthropometric measurements, participants underwent a standardized graded exercise test on a treadmill to assess heart rate, blood pressure, respiratory gas responses to exercise, and cardiovascular fitness.

Participants were asked to record their diet and physical activity for three days. These records were analyzed using The Food Processor SQL nutrition and fitness software (Esha Research, Salem, OR). Participants were counseled to maintain a similar energy and nutrient intake and to refrain from any kind of exercise, during the blood sampling timeline. Blood sampling and exercise protocols were initiated after one week of stable dietary and nutrition intake (Fig.4.1). Blood samples were obtained after an overnight fast and prior to exercise on days 1 through 4. Exercise was completed by expending 350 kcal of energy via treadmill walking at 60-70% of VO_{2max}. Post-exercise blood samples (10 mL) were obtained at the same time of day, and under the same conditions on days 5 and 7.

4.3.3 Metabolic assays:

Serum glucose concentrations, based on a glucose oxidase method, were assayed according to the manufacturer's protocol (Raichem, San Diego, CA). Serum insulin concentrations were measured by a human insulin-specific radioimmunoassay, according to the manufacturer's protocol (Millipore Corporation, Billerica, MA). HOMA values were calculated by the following formula: Insulin (μU/mL) x [glucose (mmol/L)/22.5]. Glucose to insulin ratio was calculated as glucose (mg/dL)/insulin (μU/mL).

4.3.4 Total fetuin-A assay:

Plasma concentrations of total fetuin-A were assayed by a sandwich ELISA, according to the manufacturer's protocol (BioVendor, LLC, Candler, NC). Plasma

samples were assayed in duplicate, using human fetuin-A standards ranging from 2 to 100 ng/mL. Briefly, standards, quality controls, and diluted serum samples were added to anti-human fetuin-A antibody coated microtiter strips. After incubation, washing, and addition of conjugate solution, the substrate was added, and the absorbance was read in a microplate reader at 450 nm.

4.3.5 Phosphofetuin-A Western blot:

Plasma samples were assayed for phosphofetuin-A by Western blotting. Proteins were separated on a 4 ~ 20% gradient SDS-PAGE gel (NuSep iGel, Austell, GA), transferred to nitrocellulose, and detected using a Ser312-phospho-fetuin-A antibody. Chemoluminescence was imaged and quantitated with UVP Bioimaging system (UVP, Upland, CA).

4.3.6 Statistical analysis:

Repeated measures analysis of variance (ANOVAs) and Duncan's multiple range tests were used to determine significant findings. $P < 0.05$ was considered to be significant. Data were analyzed using Statistical Analysis System (SAS, Cary, NC).

4.4 Results

4.4.1 Baseline anthropometric measurements and metabolic indices in participants

Study participants (n=15) were hypertensive, dyslipidemic, and met the NCEP-ATP III criteria for metabolic syndrome. Fasting blood glucose and insulin concentrations were within the normal range (Table 4.1).

4.4.2 Changes in serum glucose, insulin concentrations with repeated bouts of exercise during and in the post- exercise period

Since cross-sectional comparisons between endurance-trained and sedentary male and female subjects revealed that plasma volume and erythrocyte volume were higher in trained individuals compared with untrained individuals (Cannon and Kluger,

1983; Carroll et al., 1995), adjustments were made for plasma volume changes following exercise. Blood glucose concentrations in study participants were not significantly altered with repeated bouts of exercise or in the 24h or 72h post-exercise period. However, insulin concentrations and HOMA concentrations were significantly decreased after a first bout of exercise and remained decreased with repeated bouts of exercise. Further, insulin and HOMA concentrations were significantly lower 24h after the last bout of exercise, compared to concentrations at baseline, suggesting a sustained effect of improved insulin sensitivity. However, this sustained effect failed to last for 72h, with plasma insulin and HOMA demonstrating values comparable to baseline concentrations. Fasting glucose to insulin ratio, a measure of insulin sensitivity, tended to increase; but this was not statistically significant, either with repeated bouts or in the post-exercise period (Table 4.2).

4.4.3 Changes in plasma total fetuin-A and phosphofetuin-A with repeated bouts of exercise and in the post-exercise period

In this study, baseline fetuin-A concentrations ranged from 239 μg/ml to 714 μg/ml, in obese individuals with metabolic syndrome. After adjustment for plasma volume changes, total fetuin-A concentrations were not altered with repeated bouts of exercise, or in the post-exercise period (Table 4.2). Since phosphorylation of fetuin-A was critical for its inhibitory action, it was of interest to examine its phosphorylation status. Previously, it was shown that, of the two phosphorylation sites, the majority of the phosphorylation occurred on Ser312 (Haglund et al., 2001). In this study, we demonstrate that Ser312-phosphofetuin-A concentrations were significantly lower in the 24h post-exercise period, compared to baseline (Fig.4.2). Interestingly, consistent with changes in insulin and HOMA concentrations, the observed decrease in phosphofetuin-A, 24h after the last bout of exercise, was not sustained over 72h of the

post-exercise period, and returned to concentrations comparable to baseline (Fig.4.2).

4.5 Discussion

In this study, we demonstrate that, total fetuin-A concentrations are not altered either with repeated bouts of exercise or in the post-exercise period. We observed a sustained effect of exercise with a decrease in the phosphorylated form of fetuin-A, and insulin 24h after the last bout of exercise, but not at the 72h post-exercise time-point. These findings are consistent with the changes we observed with sustained improvements in insulin sensitivity, 24h following the last bout of exercise.

Fetuin-A, synthesized by hepatocytes and secreted into circulation, exists in both the phosphorylated and dephosphorylated forms in plasma. The phosphorylated form of fetuin-A has been shown to be an inhibitor of insulin receptor tyrosine kinase activity. Interestingly, the fetuin-A gene in humans is localized to a site previously linked to the MetS quantitative trait locus (Kissebah et al., 2000). Elevated plasma fetuin-A concentrations correlate with waist circumference, hypertension, decreased HDL-cholesterol concentrations, and insulin resistance; features of MetS, and accumulating evidence implicates fetuin-A in cardiovascular disease, and incident diabetes (Ix et al., 2008; Kalabay et al., 2002; Mori et al., 2006; Reinehr and Roth, 2008; Stefan et al., 2008). Previously, we have shown that phosphorylated fetuin-A levels were correlated with serum triglyceride concentrations in individuals with MetS (Kaushik et al., 2009). Fetuin-A has also been identified as a key partner in the recovery phase of an acute inflammatory response (Olivier et al., 2000; van Oss et al., 1975). Fetuin-A concentrations are positively correlated with C-reactive protein and negatively correlated with adiponectin concentrations (Hennige et al., 2008), indicating a link between fetuin-A and a proinflammatory state. An increase in liver fat has been proposed as the link between obesity and increased fetuin-A concentrations (Stefan et

al., 2006). Recent studies demonstrate that substantial weight loss in children was associated with a significant decrease of fetuin-A (Reinehr and Roth, 2008) while a recent publication (in press, 2010) demonstrates that short-term exercise training for 6 weeks does not alter fetuin-A concentrations in non-diabetic obese women (Schultes et al., 2010). To our knowledge, this is the first report examining the response of phosphorylated fetuin-A, to repeated bouts of exercise, in obese men with MetS.

In our study, we show that plasma insulin concentrations and HOMA values decreased significantly after the first bout of exercise, and this effect was sustained 24h after the last bout of exercise, indicating that short-term exercise improves insulin sensitivity in obese men. This is consistent with several previous reports demonstrating that short-term exercise training, for 6-10 days, increases insulin sensitivity in obese individuals with impaired glucose tolerance, and in patients with type 2 diabetes (Arciero et al., 1999; Black et al., 2005; O'Gorman et al., 2006; Winnick et al., 2008). We demonstrate that such sustained effects on improved insulin sensitivity were observed even with four days of repeated bouts of exercise. While previous studies demonstrate an improvement in fasting glucose concentrations after 7 days of exercise training (O'Gorman et al., 2006), our study did not show significant changes in fasting glucose after 4 days of exercise. Further, in agreement with other studies (Burstein et al., 1985), we show that the increase in insulin sensitivity associated with physical conditioning was lost 72h after exercise was discontinued.

Although the mechanisms of improved insulin sensitivity are not fully delineated, it may be related to enhanced sensitivity and responsiveness of peripheral glucose uptake, presumably by muscle, to insulin, as well as an increased inhibition of hepatic glucose production by insulin. It is well documented that exercise training induces an increase in the expression and translocation of more GLUT4 to the cell

surface, in muscle of humans and laboratory rodents (Dela et al., 1992; Holloszy, 2005; O'Gorman et al., 2006). Contraction-induced 5'-AMP-activated protein kinase (AMPK) may play a role in, at least part of the mechanism by which exercise enhances glucose uptake. AMPK activation is not only found in skeletal muscle but also found in liver and adipose tissue (Ruderman et al., 2003). Also, exercise results in activation of AMPK in the liver (Carlson and Winder, 1999) and AMPK activation down-regulates gluconeogenic gene expression, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Lochhead et al., 2000), leading to an enhanced suppression of hepatic glucose production (Kirwan et al., 2009).

In our study, the decrease in plasma phosphofetuin-A concentrations observed 24h after the last bout of exercise was not sustained for 72h. This is consistent with the changes in insulin concentrations and HOMA concentrations. Earlier studies from our laboratory, and those of others demonstrate that phosphorylated fetuin-A inhibits insulin action at the concentration of the insulin receptor tyrosine kinase (Auberger et al., 1989; Kalabay et al., 1996; Srinivas et al., 1996). Though several studies suggest that the beneficial effects of exercise are independent of improvements in insulin signal transduction (Nesher et al., 1985; Ploug et al., 1984; Richter et al., 1985; Wojtaszewski and Richter, 2006), some rodent and human studies suggest otherwise. In obese and type 2 diabetic subjects, acute exercise was shown to increase insulin receptor and IRS1 phosphorylation (Cusi et al., 2000). Similarly, a single bout of exercise in mice regulated hepatic IRS protein expression, leading to improved cellular insulin signal transduction. The mechanisms related to lowering of phosphofetuin-A following exercise are currently unknown. Since insulin was shown to decrease fetuin-A phosphorylation status (Akhoundi et al., 1994), it may be speculated that the observed decrease in fetuin-A phosphorylation with exercise training, could be associated with

the improvement of insulin sensitivity.

In conclusion, this study demonstrates that while total fetuin-A concentrations are not altered with repeated bouts of exercise, phosphorylated fetuin-A concentrations are decreased 24h following 4 days of exercise training, in conjunction with an improvement in insulin sensitivity. Our study has several limitations. The sample size of our study population was relatively small (n=15). This studied population may not provide accurate representation all obese individuals with MetS. Another limitation is the absence of a normal weight control group. Since the diabetes prevention trials show the benefits of a 5-8% weight loss (Knowler et al., 2002; Ramachandran et al., 2006; Tuomilehto et al., 2001), additional studies to investigate the influence of lifestyle intervention by diet and exercise, on phosphofetuin-A, the biologically active form, can shed light on fetuin-A's contribution to the regulation of insulin sensitivity.

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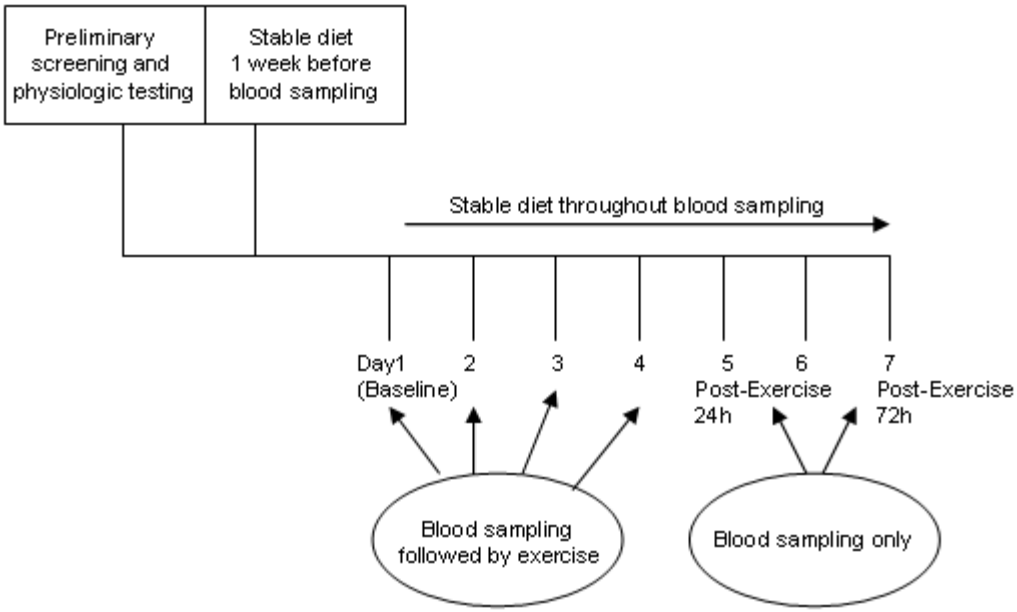

Fig.4.1: Schematic of experimental protocol

Table 4.1: Baseline descriptive measurements in participants

Variables	Mean ± SD (n=15)
Age (yrs)	50.3 ± 8.2
Height (cm)	177.7± 6.1
Weight (kg)	101.5 ± 17.7
BMI (kg/m^2)	32.0 ± 4.6
Fat (% body weight)	28.9 ± 4.1
VO_{2max} (L/min)	2.89 ± 0.43
VO_{2rel} (ml/kg/min)	28.9 ± 4.26
Systolic blood pressure (mm Hg)	140 ± 9
Diastolic blood pressure (mm Hg)	92 ± 6
Glucose (mg/dL)	100 ± 13
Insulin (µU/mL)	15.5 ± 10.0
Total cholestrol (mg/dL)	253 ± 44
LDL choleterol (mg/dL)	168 ± 42
HDL cholesterol (mg/dL)	32 ± 5
Triglycerides (mg/dL)	267 ± 146

Table 4.2: Effect of repeated bouts of exercise (plasma volume adjusted) on metabolic indices and total fetuin-A concentrations in obese individuals (n=15)

Variables	Baseline	Day 2	Day 3	Day 4	24h Post-Ex	72h Post-Ex
Glucose (mg/dL)	99.6 ± 12.8[a,b]	96.1 ± 9.63[b]	101.8 ± 11.4[a,b]	101.7 ± 14.2[a,b]	102.9 ± 10.8[a,b]	104.7 ± 12.1[a]
Insulin (µU/mL)	15.5 ± 10.0[a]	11.5 ± 6.56[b]	10.7 ± 4.42[b]	13.0 ± 8.3[a,b]	9.48 ± 3.68[b]	12.5 ± 7.74[a,b]
Glucose/insulin ratio	9.40 ± 6.96[a]	11.4 ± 7.09[a]	11.1 ± 4.44[a]	11.7 ± 7.76[a]	13.2 ± 7.38[a]	13.0 ± 11.4[a]
HOMA	3.92 ± 2.79[a]	2.79 ± 1.69[b]	2.72 ± 1.28[b]	3.41 ± 2.46[a,b]	2.41 ± 0.96[b]	3.32 ± 2.34[a,b]
Total fetuin-A (µg/mL)	297.2 ± 69.4[a]	300.3 ± 49.8[a]	287.1 ± 52.5[a]	285.7 ± 70.4[a]	289.1 ± 62.7[a]	298.8 ± 51.3[a]

Means with the same letter are not significantly different.
Data are shown in Mean ± SD.

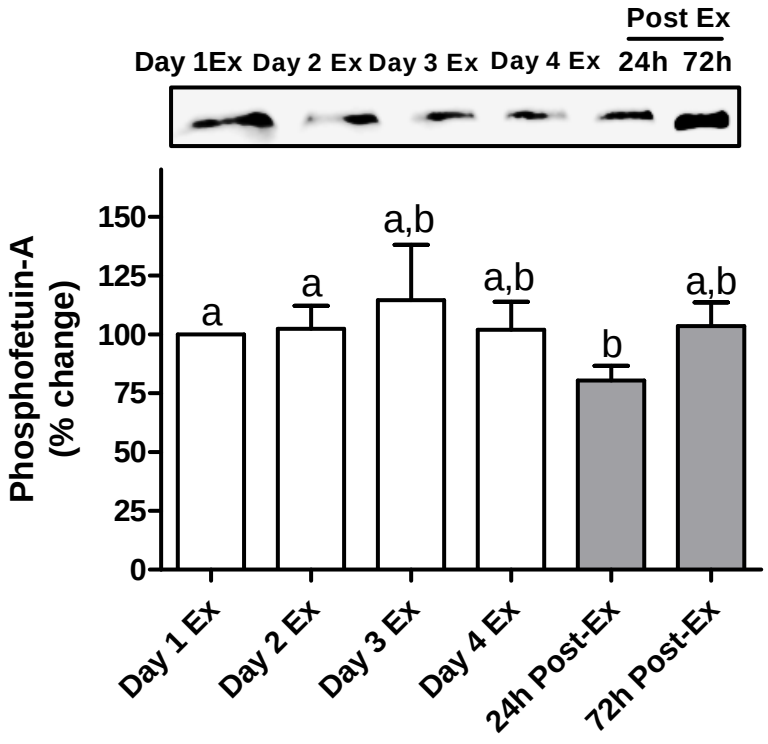

Fig 4.2 Plasma phosphofetuin-A concentrations with repeated bouts of exercise and in the post-exercise period: Blood samples were obtained after an overnight fast and prior to exercise on days 1 through 4. Exercise was completed by expending 350 kcal of energy by treadmill walking at 70% of VO_{2max}. Post-exercise blood samples were obtained at the same time of day, and under the same conditions, on days 5 and 7. Upper panel is a representative phosphofetuin-A Western blot, from one individual, and the bottom panel shows the percent change compared to Day 1 of all study participants. Means with the same letter are not significantly different.

Chapter 5: Summary and Conclusions

The global epidemic of obesity and diabetes is associated with an increased prevalence of MetS and an increased risk of diabetes, cardiovascular disease, stroke and heart attack. Collectively, these diseases constitute a major current threat to global human health and welfare.

The hallmark of obesity, diabetes and metabolic syndrome is a condition called insulin resistance, in which the body fails to respond properly to insulin. Insulin resistance impairs the ability of insulin to metabolize glucose and it is characterized by glucose intolerance and hyperglycemia followed by an increase in plasma concentrations of insulin, dyslipidemia, with elevated levels of triglycerides and diminished HDL cholesterol, elevation of blood pressure, abdominal obesity, and an elevated tendency for thrombosis.

While genetics and environmental factors play a key role in the pathogenesis of insulin resistance, the molecular mechanisms are not clearly understood. More and more scientists have blamed 'low-grade' inflammation, triggered by nutrients ormetabolic surplus, such as elevated plasma free fatty acids and circulating humoral factors including TNF-α, leptin, resistin, adiponectin, and retinol binding protein-4.

Fetuin-A, particularly the phosphorylated form (phosphofetuin-A), inhibits insulin receptor autophosphorylation and tyrosine kinase activity *in vitro* and *in vivo*. Elevated levels of fetuin-A have been shown to be associated with insulin resistance, metabolic syndrome, and an increased risk for type 2 diabetes. It is well known that increased physical activity contributes to lower fasting and postprandial insulin

concentrations and improved insulin sensitivity. However, there are few reports on the effects of acute or short-term exercise training on fetuin-A levels and its phosphorylation status. The goal of this study was to examine daily variations in fetuin-A, the effect of an oral glucose challenge, and the effects of single and repeated bouts of exercise on total fetuin-A and phosphofetuin-A levels in obese individuals with MetS, and correlate these with changes in insulin sensitivity.

We demonstrate, for the first time, that fetuin-A levels are decreased following an oral glucose challenge in lean, but not obese individuals. In obese individuals, a single bout of exercise decreased phosphofetuin-A levels, 24 hours post-exercise, consistent with an improved insulin sensitivity following exercise. Similarly, with repeated bouts of exercise phosphofetuin-A levels are decreased in conjunction with an improvement of insulin sensitivity, assessed using surrogate markers of insulin sensitivity, viz., insulin levels, glucose to insulin ratio, and HOMA levels.

In summary, we conclude that fetuin-A levels are responsive to physiological cues in lean, but not obese individuals. Secondly, our findings suggest that the exercise-induced lowering of phosphofetuin-A may contribute to the improvement in insulin sensitivity. Future studies on the effects of weight loss and long-term lifestyle modification on fetuin-A, particularly phosphofetuin-A levels, are needed to understand the role of fetuin-A in the modulation of insulin sensitivity.

Appendix

Protocol For Research Involving Human Subjects

AUBURN UNIVERSITY

Institutional Review Board

Project Title: Changes in Lipoprotein Lipid Concentrations and Particle Densities with Repeated Bouts of Aerobic Exercise in Hypercholesterolemic Men

1. PURPOSE OF STUDY

Both an increase in high-density lipoprotein cholesterol (HDL-C) concentrations and a shift in HDL particle density towards larger, less dense HDL have been associated with a reduced risk for the development of coronary artery disease (CAD). A single session of aerobic exercise has been shown to increase serum HDL-C and reduce triglyceride (TG) concentrations in sedentary hypercholesterolemic men. These changes are thought to be mediated in part, by an increase in plasma lipoprotein lipase activity (LPLA) after exercise. LPLA facilitates the hydrolysis and clearance of TG-rich lipoproteins and enhances the transfer of cholesterol from these molecules to HDL. Thus, it is thought that

HDL particles may become larger and less dense as cholesterol is taken up by this fraction; yet, post-exercise changes in HDL particle densities of hypercholesterolemic men have not been described previously. In general, the changes in serum lipid concentrations and LPLA after a single exercise session are optimal 24 hours post-exercise and often return to pre-exercise levels 72 hours after the exercise session. These changes, however, are usually attributed to chronic exercise training, since post-training blood samples are frequently obtained within 48 hours of the last exercise bout. Currently, it is not known whether the favorable lipid profiles observed 24 hours after a single exercise session can be maintained by successive bouts of exercise on consecutive days. Likewise, the capacity of successive exercise bouts to prolong lipid and enzyme changes after the last bout of exercise has not been determined in hypercholesterolemic men. Addressing these questions will help to distinguish the influence of a single bout of exercise from what is generally held to be an effect of exercise training on serum lipid concentrations and the distribution of lipids among lipoprotein fractions.

The objectives of this project are to:

1) describe the exercise-induced shifts in particle densities within the HDL fraction (HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, and HDL_{3c} particle densities) and relate these shifts to the lipid and enzyme responses
2) determine if the lipid and enzyme responses after a single exercise session are maintained or enhanced by aerobic exercise on successive days, and;

3) determine if successive bouts of aerobic exercise on consecutive days prolong the influence of the last exercise session on lipid concentrations and enzyme activities in sedentary hypercholesterolemic men.

2. SUBJECT POPULATION

A. Description:

A total of 20 sedentary[1] male volunteers, previously identified as having elevated levels of serum cholesterol (total serum cholesterol levels $\geq$ 240 mg / dl)[2] will be recruited from Auburn University, the Auburn – Opelika, and surrounding communities for this investigation. All subjects will be between 30 and 50 years of age and their body fat will be less than 30% of their total weight.[3] Subjects meeting the above criteria will be further screened to exclude those who show evidence of medical contraindications to exercise, are taking drugs known to affect lipid or lipoprotein levels, use tobacco products, or consume more than 2 oz. of alcohol per day.

[1] *Sedentary individuals are those who have not participated in any regular physical activity in the previous 6 month period. Regular physical activity is defined as moderate or intense physical exertion (50 - 80% of $\dot{V}O_{2max}$) for 20 minutes or more at least two times per week.*

[2] *Previous identification of the volunteers' blood lipid profiles will have been accomplished through a recent (within the previous two months), routine blood analysis carried out by medical personal in clinics, health fairs, insurance screenings, or their personal physician. The subject will voluntarily provide all medical information. No*

attempt will be made to obtain information on potential subjects other than direct contact with the subjects themselves. Potential subjects will not be coerced to participate in this study either directly or through physician - patient relationships. The blood lipid status of the subject will be verified by an initial blood draw during the preliminary screening (A detailed description of the preliminary screening, blood sampling, and lipid analysis is included in section 3 of this IRB).

[3] *Individuals that are obese (> 30% of total body weight) have been shown to respond differently to exercise training than those with an average or below average percent body fat (average body fat is related to age).*

These subjects are not considered a "vulnerable" population. They are, however, at greater risk for the development of CAD than the normal population. Because the subjects will be screened for the physiological factors mentioned above, their increased risk for CAD may be largely attributed to elevated levels of serum cholesterol.

B. Sample Size:

The minimum number of subjects needed to validate the study: **20**

The maximum number of subjects to be recruited: **40**

The maximum number of subjects that will be included in this study: **40**

C. Subject Recruitment:

Methods of recruitment will include; personal contacts, advertisement via mass mailings to each department at Auburn University, flyer postings around the Auburn University campus, advertisement in the university and local newspapers, and public appearances by the principle investigator on local television and radio stations. There will

be no subject coercion, either directly or through physician-patient relationships. All advertisements, flyers, mailings, bulletins, and public appearances will be informational only. There will be no deceptive wording contained in any of the recruiting instruments (*see attached announcement*).

Every effort will be made to recruit minorities and those from diverse backgrounds; however, the nature of our research mandates that our subjects fit certain physiological and demographic criteria. It is anticipated that it will be difficult to find a demographically proportionate representation of sedentary hypercholesterolemic males with respect to ethnicity or minority group association. Anticipated difficulties stem from the fact that many individuals that can be classified as hypercholesterolemic: a) are unaware of their lipid and lipoprotein profile; b) have unrelated metabolic diseases that may affect their lipid profile; c) have contraindications to exercise, and; d) may be using alcohol or tobacco products which are known to alter their lipid concentrations, particle densities, or the enzymes that are involved with blood lipid metabolism. Therefore, the majority of our efforts will be directed toward getting an adequate number of subjects to satisfy statistical power concerns. In this regard, stratification of subjects based on race or ethnicity would be prohibitive.

D. Group Assignment and Other Pertinent Information:

Twenty subjects will be randomly selected and assigned to either an experimental (n=10) or control (n=10) group from the pool of eligible volunteers. Experimental and control subjects will undergo the same procedures, with the exception that the control subjects will not go through the exercise interventions during the experimental protocol.

3. EXPERIMENTAL METHODS AND STUDY DESIGN

A. Preliminary Procedures:

On the first visit to the laboratory and after being fully informed regarding the nature of the study and the risks involved, each subject will sign an informed consent approved by the Auburn University Institutional Review Board for Research Involving Human Subjects. Each subject will then complete a health history questionnaire and a physical activity questionnaire (*see attached*). Subsequently, each subject will be measured for height, weight, waist and hip circumferences, and relative body fat using a seven-site skinfold method. On this same visit, all subjects will undergo a 5 ml blood draw from an antecubital vein (5 ml is equivalent to one teaspoon) in order to verify their lipid and lipoprotein status.

All subjects meeting the criteria outlined in section 2 of this IRB form and randomly chosen from the pool of volunteers will be invited back to the laboratory for a general subject meeting. At this time, the subjects will be notified of their group assignments and the experimental protocol will be reviewed. Subsequently, subjects will be given instructions related to the dietary and physical activity requirements of the study (*a detailed description to follow*). At this time, a three-day diet questionnaire and a seven-day physical activity record will be distributed (*see attached*). Subjects will be asked to record their dietary intake for two week days and one weekend day. The three-day dietary data will be analyzed for total caloric intake and nutritional composition using a commercially available dietary analysis software package. Likewise, subjects will be

asked to record their daily activity for one week in order to estimate their average daily energy expenditure.

On the third visit to the laboratory, each subject will undergo a physical examination conducted by a physician (*The collaborating physician on this project has not yet been determined. The name of the physician and a brief description of his/her credentials will be submitted to the IRB Review Board for approval prior to initiating this study.*) and the following battery of tests: 1) height and weight, 2) lung function and volumes, 3) a continuously graded exercise test (Bruce Protocol) on a motor driven treadmill (Quinton Q-65) in order to assess maximal exercise capacity. For ten minutes prior to the test, subjects will rest in a supine position while heart rate and rhythm and blood pressure will be monitored. Throughout the test and during recovery blood pressure will be obtained manually at the end of each three-minute stage, and heart rate and rhythm will be monitored continuously using a 12-lead electrocardiogram (Medical Graphics CPX/D Integrated Systems). Respiratory gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$) will be measured continuously and averaged over 15 sec intervals via open-circuit spirometry utilizing an automated metabolic cart calibrated with gas mixtures of known composition between each test. The peak oxygen uptake achieved during exercise will be recorded as $\dot{V}O_{2max}$ (L/min). The exercise tests will be conducted under the direct supervision of the collaborating physician and the principle investigator. The test results will be evaluated by the physician for cardiac contraindications to exercise training.

Using this exercise data, heart rate (HR) and ratings of perceived exertion (RPE) will be regressed on $\dot{V}O_2$ for each subject. The regression equations will be used to calculate

the individualized exercise HR and RPE, which corresponds to the desired exercise intensities for each subject. Individual energy costs (kcal/min) of exercise at the various exercise intensities will be estimated as the product of $\dot{V}O_2$ (L/min) and the respiratory exchange ratio energy-oxygen equivalent (kcal/LO_2) measured during the exercise test at each respective intensity of interest. Using this relationship, the exercise duration (min) required to expend 350 kcal of energy will be calculated for each individual.

Following the preliminary testing, each subject will be given their individualized, calorically neutral diet designed to maintain their current body weight and stabilized their lipid profile. (This diet was determined from the subject's 3-day diet record analysis described previously.) All subjects will receive standardized instructions on the dietary guidelines. Each subject will receive an exchange list of foods (American Dietetic Association & American Diabetic Association Food Exchange List) from which choices can be made to attain the prescribed dietary composition. All subjects will maintain their dietary records for one week prior to and throughout the experimental protocol period. All diets will be analyzed to evaluate subject compliance with the dietary recommendations.

B. Experimental Procedures:

Following 7 days on the controlled diet and abstaining from any physical exercise for 72 hours, each subject will return to the laboratory after a 12-hour fast (restricted to water only) for baseline, pre-exercise blood sampling (PRE). (*A detailed description of the blood sampling procedures will follow.*) Next, all subjects in the experimental group will perform a submaximal exercise bout. Specifically, subjects will be asked to walk or jog

on a motorized treadmill at 70% of their $\dot{V}O_{2max}$ for duration needed to expend 350 kcals of energy. The experimental subjects will follow this same procedure on each of the next three days (blood samples will be labeled EX+24, EX+48, EX+72). Fasting blood samples will also be obtained at 24 hours (POST24) and 72 hours (POST72) following the last submaximal exercise session. All experimental blood samples will be obtained at the same time each morning. Subjects in the control group will undergo the same blood sampling protocol without exercise intervention.

Each subject will be asked to come to the lab a total of nine times. The total time commitment will be approximately 10 hours. There will be a total of 7 blood samples taken for each subject. The first blood sample (used to verify the subject's blood lipid status as hypercholesterolemic) will be approximately 5 ml (one tablespoon), and the remaining six blood samples (PRE, EX+24, EX+48, EX+72, POST24, POST72) will be 30 ml each (6 tablespoons). A total of 185 ml of blood (37 tablespoons) will be obtained over a four-week period (Note: Approximately 180 ml of blood of this blood will be obtained over the one-week experimental protocol period.)

The blood sampling protocol and analytical methods are described below. The initial 5 ml blood sample will be handled in the same manner as all other blood sample; however, this sample will only be analyzed for TC. This analysis will only be used to verify the lipid and lipoprotein status of the subjects prior to data collection.

Following a 12-hour fast, pre-heparin blood samples (20 ml) will be obtained through a venous catheter from the antecubital vein of each subject. Immediately following the blood draw, a small portion of the sample (80 l) will be used to determine the

hematocrit and hemoglobin concentrations using the calculations described by Costill and Fink, 1974. The remainder of the sample will be refrigerated at 4 C, and centrifuged at 1500 X g for 30 min at 4 C within three hours of collection. Aliquots of serum will be taken for lipid concentration and particle density analyses and for separating high-density lipoprotein (HDL) and the subfraction HDL_3, then stored at -70 C until analyzed at the conclusion of the study. After the fasting blood sample is obtained, a post-heparin blood sample (10 ml) will be collected for lipoprotein lipase activity (LPLA) and hepatic triglyceride lipase activity (HTGLA) in heparinized tubes 10 min after the intravenous injection of heparin (75 IU/kg). Plasma will be separated and stored as described above for the serum samples.[4]

[4] *The principle investigator will perform all phlebotomy procedures, including heparin injection. My expertise in this area includes obtaining over 2000 blood samples through venous catheters, the administration of over 500 heparin injections, and obtaining 500 post-heparin blood samples. As a doctoral student, I performed these procedures throughout 4 funded research projects at Texas A&M University. The attending cardiologist, Dr. J. James Rohack, and my doctoral mentor, Dr. Stephen F. Crouse, approved the procedures that I used.*

High-density lipoprotein will be separated from 2.5 ml of plasma by the method of Warnick and Albers (1978). This procedure utilizes the heparin-manganese (II) reagent to precipitate apoB-containing lipoproteins. The cholesterol in the supernatant is predominantly HDL-C. The HDL subfraction, HDL_2, will then be precipitated using dextran sulfate (MW 15,000) as described by Gidez et al. (1982); subsequently, this

supernatant will be analyzed for cholesterol, which is predominately HDL_3-C. The cholesterol content of HDL and HDL_3 will be determined enzymatically according to Allain et al. (1974). The concentration of HDL_2-C will be calculated as the difference between HDL-C and HDL_3-C.

Like the HDL-C and HDL_3-C analysis, plasma samples will be analyzed enzymatically for TC and triglycerides (TG) content (Allain et al., 1974 and Bucolo and David, 1973, respectively). Fresh serum samples from each timepoint will be analyzed by gradient polyacrylamide gel electrophoresis (PAGE) as described by Ramjan et al., (1996). One serum aliquot will undergo PAGE to determine the relative density distribution of very low-density (VLDL), low-density (LDL), and HDL lipoprotein fractions. The distribution of particle densities within the HDL fraction (HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, and HDL_{3c}) will be determined by introducing another fresh serum aliquot into a higher resolution PAGE system. LPLA and HTGLA will be assayed in the post-heparin plasma samples using the procedures described by Thompson et al. (1986). To minimize interassay variation, all samples from each Ss will be determined in duplicate in a single assay run. Internal quality control will include commercially prepared serum and plasma lipid samples.

4. BENEFITS

The subjects will receive no monetary compensation for participation; however, all volunteers will receive the results of their blood analysis and preliminary screening. All subjects will receive a complete report of their individual testing results and a summary of our research findings upon completion of the study.

The preliminary screening will provide valuable information to each subject regarding their relative risk for cardiovascular disease (CVD) and present physical fitness status. Blood pressure and ECG will be monitored during the maximal exercise test; this will provide each subject with important information related to the functional status of the cardiovascular system during maximal exertion and may be used diagnostically by the attending physician to detect the presence or absence of significant dysrhythmias and coronary heart disease. Assessment of plasma lipids and lipoproteins will provide further information regarding the risk associated with CVD. The evaluation of lipid-regulating enzymes will provide each subject with information about their individual ability to metabolize fats and cholesterol. The body composition assessment will provide each subject with information regarding ideal body weight and the amount of fat that may be reasonably and safely lost. From the dietary information provided, each subject will learn about the composition of a healthy, low-cholesterol, low-fat diet, and will practice living on such a diet. It is likely that, after the investigation, this healthy diet may be continued. Any individual with abnormalities noted on any of the preliminary screenings or testing procedures will be advised to consult their private physician for further evaluation. This could prove extremely valuable in identifying latent disease, preventing a major coronary event, and possibly preventing sudden death. Finally, it is likely that participation in this study, with the development of healthful lifestyle habits, will contribute to a lower cardiovascular disease risk profile, and will enhance the overall health of each subject.

In the opinion of this investigator, no alternative procedures will provide the information essential to answer the questions posed in this study.

5. & 6. RISKS and PRECAUTIONS

Subjects that participate in this study may encounter physical risks. The reasonable risks that are associated with this protocol are described below. The precautionary measures that will be taken for each possible risk are outlined after each description of risk.

A. Blood Sampling:

Risk(s): A pre-heparin (20 ml) and post-heparin (10 ml) sample of blood will be drawn into vacutainers on six occasions throughout the experimental protocol. The puncture of the skin is accompanied by minor discomfort and may result in the development of a minor contusion adjacent to the puncture site. Additionally, as with any similar procedure that disrupts the skin barrier, there is a risk of infection.

Precaution(s): This risk to the subjects (and to the technician) will be minimized through the use of accepted sterile procedures which include: (1) use of surgical rubber gloves by the technician; (2) antiseptic cleansing (70% alcohol) of the involved site prior to the puncture; (3) use of sterile equipment and instruments for each sample; and (4) proper dressing of the wound with antiseptic and Band-Aid following sample collection. As part of the study entry criteria, subjects will be screened by questionnaire for the HIV or Hepatitis virus: Those reporting positive for either will be excluded from the study.

Risk(s): The injection of heparin sodium may be associated with some risk to the subject. Heparin inhibits reactions that lead to the clotting of blood. Hemorrhage is the

chief complication that may result from heparin. Bleeding time is usually unaffected by heparin. Clotting time is prolonged by full therapeutic doses (150 IU/kg); in most cases it is not measurably affected by low doses, like those used in this study (75 IU/kg). Also, the half-time of heparin elimination is relatively fast ($t_{1/2}$ = 10 min), further minimizing the risk of any adverse effects from a single injection of a sub-therapeutic dose.

Precaution(s): Despite the minimal risk associated with the small amount of heparin used, patients with contraindications to heparin will be screened out of this study. The conditions for which heparin is contraindicated include: hypersensitivity to heparin, severe hypertension, hemophilia, thrombocytopenia, vascular purpuras, ulcerative lesions of the stomach or small intestine, liver disease with impaired hemostasis, and menstruation. Any subject taking oral anticoagulants will be screened out of the study, or, on approval of their physician, will be asked to discontinue use of these drugs prior to heparin treatment.

B. Maximal Exercise Capacity Test:

Risk(s): The risks associated with the $\dot{V}O_{2max}$ test are comparable to those encountered during any strenuous physical activity. In otherwise healthy individuals who are properly screened for contraindications to exercise prior to the test and properly supervised by trained personnel during the test, the risk of potentially harmful or life-threatening events is extremely low. These include the risk of occasional abnormal blood pressure responses, syncope, heart dysrhythmia, severe dyspnea, and, in rare instances, heart attack. In addition, there is a risk of falling on the treadmill with subsequent

contusions or abrasions, or of suffering minor orthopedic injuries, such as ankle, knee, or hip strain.

Precaution(s): Mortality and morbidity rates for maximal exercise testing are reportedly 0.5 and 8.3, respectively, per 10,000 tests. Every effort will be made to minimize the small risk inherent in this procedure through the preliminary screening by health history questionnaire, physical exam conducted by a cardiologist, identification of contraindications to exercise testing, and by observation during testing by a cardiologist and trained personnel. Throughout all testing procedures, the 4th edition of the American College of Sports Medicine's "Guidelines for Exercise Testing and Prescription" (1996) will be closely observed.

C. Diet:

No risks are associated with the consistent diet in this investigation. The diet is based on guidelines by the American Dietetic Association and the American Diabetes Association and is not designed to induce a loss of body weight.

D. Submaximal exercise bout:

Risk(s): Again, as with the maximal exercise test, there exists a risk of abnormal blood pressure response, syncope, heart dysrhythmia, severe dyspnea, and, in rare instances, heart attack during exercising. Also, during exercise there exists a risk of an injury to joints or muscles, such as ankle, knee, or hip sprains or, rarely, fractures, and muscle strains.

Precaution(s): These risks will be minimized by exercise stress testing and examination (by a physician) of all subjects prior to performing the acute bout of

submaximal exercise; those with contraindications to heavy exercise will be screened out of the study. Thus, the health risks to those remaining are considered negligible. However, every reasonable precaution will be taken to insure that the exercise is carried out in a safe manner. Exercise heart rates and blood pressures will be by checked and charted regularly by trained exercise technicians who are certified in CPR. All exercise prescription procedures will conform to the "Guidelines for Exercise Testing and Prescription" published by the American College of Sports Medicine (1996).

7. LOCATION OF EXPERIMENTS

All subject screening, preliminary testing, and experimental protocol will be conducted in the Exercise Physiology Laboratory in the Department of Health and Human Performance (2134 Beard-Eaves Memorial Coliseum).

8. PROTECTION OF DATA

The results of all tests will be classified as medical records (CONFIDENTIAL) and will be kept in a metal filing cabinet in the principle investigator's office in the Department of Health and Human Performance (2070 Beard-Eaves Memorial Coliseum). The door to this office is always shut and locked when the principle investigator is not present.

Each subject's identity will be coded with a number to ensure confidentiality throughout data analysis. Subjects will be coded with a letter, indicating their group affiliation (E for exercise group and C for control group) and a number (1 through 20) according to the order in which the subjects were randomly selected. (*Examples of coded identifications are E12 and C08.*) Except in the case of laboratory personnel (the

principle investigator, collaborating physician, and graduate students directly involved with this project), written subject authorization will be required for the release or viewing of these records. All future references to the data obtained in this investigation will be in collective terms only. Subject names and individual results will not be delineated or discussed outside of the research group directly involved in data collection and analysis. Any information that may link a subject to the data obtained in this project will be removed and destroyed after all manuscripts from this project have been published (2 to 3 years after completing the data collection and analysis).

INFORMED CONSENT

Title of the Study: Changes in Lipoprotein Lipid Concentrations and Particle Densities with Repeated Bouts of Aerobic Exercise in Hypercholesterolemic Men

Principal Investigator: Peter W. Grandjean, Ph.D., C.S.C.S.

Address: Department of Health and Human Performance
2070 Beard-Eaves Memorial Coliseum
Auburn University
Auburn, AL 36849-5323

Phone: (334) 844 – 1462

E-mail: grandpw@mail.auburn.edu

Purpose of this Study:

The purposes of this study are to 1) to compare the effects of a single bout and repeated bouts of aerobic exercise on the lipid and lipoprotein profile in hypercholesterolemic men, 2) to describe the changes in enzymes that alter the lipid and lipoprotein molecules as a result of a single bout and multiple bouts of exercise, and 3) to determine how long exercise-induced changes in blood lipid profiles can last in hypercholesterolemic men.

Date Subject's initials

Impact of this Study:

Addressing these questions will help to distinguish the influence of a single bout of exercise from what is generally held to be an effect of exercise training on serum lipid concentrations and the distribution of lipids among lipoprotein fractions. The results of this study may potentially lead to determining an optimal exercise regimen for therapeutically modifying blood lipids and reducing cardiovascular disease risk in hypercholesterolemic individuals.

Procedures to be Followed:

After you volunteer and give your informed consent to be a subject in this study, you will be given a health history questionnaire and a physical activity questionnaire to answer. You are encouraged to answer these questionnaires to the best of your knowledge so that we can make an accurate decision about the safety of the study for you. Subsequently, we will measure your height, weight, waist and hip circumferences, and estimate your relative body fat by skinfold analysis. We will then draw a 5 ml blood sample (about 1 tablespoon of blood) in order to establish your lipid and lipoprotein status.

If you meet the criteria for entry into the study and are randomly chosen from the pool of eligible volunteers, you will be asked to return to the lab for a general meeting. At this time, you will be given instructions on the dietary and physical activity requirements of the study. We will also review the experimental protocol, the dates and times for preliminary physiologic testing, and the exercise and blood sampling

procedures. You will then be asked to record your dietary habits over a three-day period and your physical activity over a seven-day period. We will ask you to return the three-day diet record and the physical activity record within 10 days of this meeting.

On the third visit to you will be given a physical exam by a physician. Following review of the questionnaire and your physical exam, your lung function will be measured. This test involves you breathing in and out as hard as you can through a mouthpiece connected to a computer. Next, you will be asked to exercise on a treadmill. For this test, ten electrodes will be placed on your skin for recording the activity of your heart at rest and during exercise. This heart recording is called an electrocardiogram (ECG). After resting ECG and blood pressure measurements have been made, you will be asked to complete a maximal graded exercise stress test on a motor-driven treadmill. This type of test is used to determine your fitness level and is sometimes called a $\dot{V}O_{2max}$ test. It is performed hundreds of times each year in our laboratory without any problems. Strict protocols will be followed, and a physician or exercise physiologist will be present throughout the test to insure your safety. For this test the treadmill will maintain a constant walking speed, but the incline will increase every third minute until you can no longer continue walking. Hence, you will be able to maintain a walking pace throughout the test. You will be allowed to stop exercise when you decide you are too exhausted to continue. During the course of this test you will breath through a mouthpiece which is connected to a computer so that exercise variables like your oxygen uptake and carbon dioxide production can be measured. Also, throughout the test, we will monitor your

Date Subject's initials

ECG and blood pressure. If you have a normal ECG and blood pressure response to exercise (as determined by the attending physician) you will be allowed to continue in this study.

If you are selected to continue, you will be given further instructions about the dietary and physical activity requirements of the study. We will determine the caloric intake and nutritional composition of your diet and provide you with an individualized diet to follow throughout the study. (This diet was determined from the information you provided us with on your 3-day diet record.) This diet is designed to maintain your current body weight and to stabilize your lipid and lipoprotein profile throughout the testing period. Briefly, we will provide you with a food exchange list that is designed to assist you with the planning of a consistent diet to be followed for two weeks (One week prior to the data collection and the week of data collection.) This is a healthy, well balanced diet based on suggestions from the American Dietetic Association. During this two-week period you will be asked to fill out daily diet records so we can check the type and amount of foods you are eating. We will also ask you to refrain from any physical exercise.

After 7 days on the diet and an overnight (12-hour) fast, you will be asked to return to the laboratory to have your blood drawn. You will be asked to refrain from any moderate or strenuous physical activity for 72 hours (3 days) prior to the blood sampling. A blood sample, equal to about 4 tablespoons (20 ml), will be obtained by inserting a small plastic tube (catheter) into a vein in your arm. This catheter will remain in your arm for about 15 minutes until all blood samples have been obtained. This is much like an I.V. you

might have in a hospital. This way, you will not need to receive more than one needle stick. While the catheter is in your arm, it does not usually cause pain. The blood collected will be analyzed for blood hemoglobin, hematocrit, and lipid risk factors. After this blood sample is obtained, you will receive an injection of a small amount of a substance called heparin through the catheter in your arm. This substance will cause the release of certain proteins, called enzymes, from your blood vessels. We are interested in measuring these enzymes since they affect the different kinds and amounts of lipid risk factors in your blood. After ten minutes, another 2 tablespoons (10 ml) blood sample will be drawn through the same catheter, and then the catheter will be removed and direct pressure will be held over the puncture site to prevent bleeding.

Immediately after these blood samples have been collected, you will be asked to walk or jog on a treadmill at an intensity equal to 70% of your $\dot{V}O_{2max}$ for a length of time needed to burn 350 kcals of energy. This will involve exercising by walking or jogging for about 30 to 50 minutes. This submaximal exercise bout will be personalized so that you will exercise on a treadmill at a level that is comfortable for you. This same procedure will be repeated on each of the next three days (for a total of 4 exercise sessions). You will also be asked to repeat all the blood sampling procedures 24 hours and at 72 hours after the last exercise bout. (Subjects in the control group will undergo the same blood sampling protocol without the exercise interventions.) Therefore, a total of 6 experimental blood samples will be taken. Your exercise session will be supervised

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by trained exercise technicians, and will be conducted in the Exercise Physiology Laboratory at Auburn University.

Discomforts or Risks to be Reasonably Expected:

The following few paragraphs gives you information about the potential risks and discomforts that you may experience as a result of participating in this study. Additionally, at all times during the course of the study we urge you to voice questions and concerns so we may address these as they arise.

No risks are associated with the diet used in this study. The diet is not designed to induce body weight loss, and will give you a balanced intake of all nutrients.

The assessment of lung function and volume simply requires that you place a clamp on your nose, and take several breaths into a tube connected to an open-loop spirometer. The primary risk of this procedure is contamination of the mouthpiece and tubing between you and the previous subject. This risk will be minimized using disposable mouthpieces and by sterilizing the other equipment with a germ-killing solution called Cydex between each subject use.

For the ECG, 10 electrodes are put on your skin to measure electrical activity of the heart. The 10 electrode sites will be prepped by cleaning the skin with alcohol and rubbing it lightly with an abrasive material like sandpaper. These procedures may cause some irritation and a mild stinging sensation. In addition, a slight possibility exists that you may be allergic to the gel used in the electrodes. This may cause some itching and redness of the area that might last for a day or two. The equipment used to record your

Date Subject's initials

ECG has been specifically designed to measure the electrical activity of your heart, and meets all safety specifications to minimize any risk of electrical shock.

The risks to you associated with the GXT or $\dot{V}O_{2max}$ test are comparable to those you face whenever you perform hard exercise, which causes you to sweat and breath heavily. These include occasional abnormal blood pressure responses, the possibility of fainting, potentially abnormal heartbeats, heavy and difficult breathing, and, in rare instance, a heart attack. In addition, there is a risk of falling on the treadmill, which could cause cuts, scrapes, or bruises. You could also suffer orthopedic injuries, such as ankle, knee, hip or muscle strains and sprains, or, rarely, fractures of bones. Studies have shown that your risk for death during this type of test is about 0.5 in 10,000, and your risk for harmful affects is about 5 to 8 in 10,000. We will make every effort to minimize these risks by carefully reviewing your health and medical history questionnaire, evaluating your risk factors for disease, and having a cardiologist give you a physical examination. All these procedures will be done before you are allowed to exercise. If we find some physical problems that, in our judgment, make exercise risky, for your own protection we will not allow you to exercise in this study. In addition to these pretest procedures, trained exercise technicians and exercise physiologists will be in charge of conducting the test and observing your ECG and blood pressure during exercise. They are trained to recognize problems in your heart or in your

bodily responses to the exercise test, which could be dangerous, and to stop the test if necessary. In addition to these trained exercise personnel, your first maximal test may

Date Subject's initials

also be supervised by a physician, who will be on hand in case of emergencies, and will diagnose the health of your heart after the test.

Obtaining the blood sample by using a catheter inserted into a vein in your arm is a routine procedure in our laboratory and in many clinical settings. Although adverse effects are rare, the puncture of the skin is accompanied by minor discomfort and may result in the development of a minor bruise next to the puncture site. However, as with any similar procedure disrupting the skin barrier, there is a risk of contracting an infection. This risk to you (and to the technician) will be minimized through the use of accepted sterile procedures which include: (1) use of surgical rubber gloves by the technician; (2) antiseptic cleansing (70% alcohol) of the involved site prior to puncture; (3) use of sterile equipment and instruments for each sample; and (4) proper dressing of the wound with antiseptic and Band-Aid following sample collection.

There is a small risk associated with the injection of heparin. Heparin is a drug that is used clinically to reduce blood clotting. We will be using a very small dose (between 1/4 and 1/2 a teaspoon) to cause small amounts of fat-regulating enzymes to be released from your blood vessels. There is a chance that this small dose of heparin may affect your blood clotting time and could, therefore, cause you to bleed more than you normally would if you were to suffer an injury soon after injection or had a medical condition, such as an ulcer, that can cause bleeding internally. In most people, however, blood clotting time is not measurably affected by a dose of heparin as low as we will be using in this study. Furthermore, any effect caused by such a small dose will not last long, usually

Date Subject's initials

less than a few hours. Despite the minimal risk associated with the small amount of heparin used in healthy people, it would be particularly risky for you to receive heparin if you have any of the following conditions: known hypersensitivity or allergy to heparin, severe high blood pressure, hemophilia, thrombocytopenia, vascular purpuras, ulcers of the stomach or small intestine, and liver disease with impaired hemostasis. If you are taking oral blood-thinning medication, you should not participate in this study, unless, on approval of your physician, you can discontinue use of these drugs prior to heparin treatment. You MUST make us aware of any of these conditions before we draw your blood by truthfully informing us verbally and by answering the questions on the health and medical history questionnaire. The use of heparin will also increase the likelihood that you will have a bruise under your skin at the sight of the needle puncture for blood sampling. Finally, some people report local irritation or hypersensitivity to heparin with chills, fever, nausea, and vomiting.

Benefits of participation and alternative procedures:

The health/medical history and the physical exam by a physician will provide you with valuable information about the health of your heart, your risk of developing cardiovascular disease (CVD), and the safety of hard exercise. Furthermore, as part of the research procedures, blood pressure and ECG will be monitored during a maximal exercise test as well as during all experimental exercise sessions. These measurements will provide you with important information related to the functional status of the cardiovascular system during maximal exertion and will be used diagnostically by the

physician to detect the presence or absence of abnormal heartbeats and coronary heart disease. The blood tests for cholesterol, lipoproteins, and other blood fats and enzymes will provide you with further information about your risk of developing heart disease.

The body composition assessment will provide you with information about the total amount of body fat you have, and what your ideal body weight should be. This will enable you to make educated decisions about how much weight you need to lose to achieve your ideal body weight. Although the diet you will be asked to follow for two-week periods is not designed to promote weight loss, you will gain knowledge about a healthy low-fat, low-cholesterol diet as recommended by the American Diabetes Association/American Dietetic Association.

Compensation:

As a subject in this study, you will receive the previously outlined physical examinations and tests at no cost to you. You will be given your individual results for all screening procedures, the exercise test (GXT), your results from the experiment, and the overall findings from this study. These results will be made available to you upon completion of all data analysis.

Medical treatment, if any, available to the subject during or after the experiment if complications arise.

We will make reasonable and proper efforts to prevent physical injury to you and to insure your safety throughout all phases of this research project. However, as noted above, participation in this study is not without risk. Compensation for physical injuries

or adverse effects incurred as a result of participating in this research is NOT available. The investigators are prepared to advise you about medical treatment in case you experience adverse consequences of any of the study procedures. It will be your responsibility to report any injuries or ill effects to one of the investigators or study supervisors as soon as possible. Phone numbers where the investigators may be reached are listed in the heading of this form.

Questions concerning the research and the procedures involved:

When you volunteer for this study, the procedures will be discussed with you in detail by one of the investigators. If you have any questions about the research or about your rights as a subject, we want you to ask us. If you have any questions later, please contact one of the investigators listed in the heading of this form.

Be instructed that consent to participate in the research may be withdrawn at any time, and that you may discontinue participation without prejudice.

Participation in this research is entirely voluntary. Refusal to participate will involve no penalty of any kind. If you decide to participate, you are free to withdraw your consent and discontinue participation at any time and for any reason. This will be without prejudice and any results that were obtained up to the time of your withdrawal will still be reported to you.

Be informed of the conditions under which your participation may be terminated by the investigator without regard to your consent.

_____

Date Subject's initials

Failure to comply with dietary recommendations prior to blood sampling, failure to report as scheduled for all blood sampling specified in the methods, or to follow instructions with regards to data collection and/or the submaximal exercise bout may result in termination of your participation in this study without your consent.

You have the opportunity to decide to consent or not to consent to participate in research without the intervention of any element of force, fraud, deceit, duress, coercion, or undue influence on your decision.

Your decision whether or not to participate will not jeopardize your future relations with Auburn University or the Department of Health and Human Performance.

Your right to privacy.

You have the right to privacy. All information that is obtained in this study that can be identified with you will remain confidential, and will be stored in the office of the principal investigator (2070 Beard-Eaves Memorial Coliseum). All information that can be identified with you will be known only to the investigators and to those who will be responsible for statistical analysis of the data. It may be released to your private physician upon your request. The results of this study may be published in scientific journals without identifying you by name.

Date Subject's initials

INSTRUMENT TO OBTAIN INFORMED CONSENT

I,_____, have been informed by the investigators that I have been selected to participate in a study entitled: **Changes in Lipoprotein Lipid Concentrations and Particle Densities with Repeated Bouts of Aerobic Exercise in Hypercholesterolemic Men**

1. I have been given and have read an explanation of the procedures to be followed in this study, including an identification of those that are experimental.

2. I have been given and have read a description of the risks and discomforts which may be associated with the experimental procedures used in this study, including those associated with blood sampling, heparin injection, exercise testing and exercise sessions, assessment of body composition, and evaluation of lung function and volume.

3. I have been given and have read a description of the benefits that I may expect from participating in this study.

4. I have been offered an answer to any inquiries concerning the procedures.

_____ Date Subject's initials

5. I have been assured that steps will be taken to insure the confidentiality of my results, which will be housed in the office of the principle investigator. Neither my name nor any other descriptor that can identify me will be associated with the publication of the results of this study.

6. I understand that in the event of physical injury resulting from the research procedures described to me, there will be no financial compensation or free medical treatment offered to me.

7. I have not been requested to waive or release the institution, its agents or sponsors from liability for the negligence of its agents or employees.

8. I have read and understand the explanations provided to me and voluntarily agree to participate in this study. **I understand that I will be given a copy of the entire informed consent document to keep for my own records.**

_____ _____

Date Signature of Subject

_____

____ Subject's initials

Date

Signature of Principal Investigator

This research has been reviewed and approved by the Institutional Review Board for Research Involving Human Subjects, Auburn University. For more information regarding your rights as a participant you may contact Ms. Jeanna Sasser at (334) 844 - 5966 or Dr. Leanne Lamke at (334) 844 - 3231.

I understand that, in case of any further questions, I may contact the following individual:

Peter W. Grandjean, Ph.D., C.S.C.S.

Department of Health and Human Performance, Auburn University

(334) 844 - 1462

Date Subject's initials

AUBURN UNIVERSITY INSTITUTIONAL REVIEW BOARD for RESEARCH INVOLVING HUMAN SUBJECTS

RESEARCH PROTOCOL REVIEW FORM

For information or help completing this form, contact: **THE OFFICE OF HUMAN SUBJECTS RESEARCH**, 307 Samford Hall,
Phone: 334-844-5966 **e-mail:** hsubjec@auburn.edu **Web Address:** http://www.auburn.edu/research/vpr/ohs/index.htm

Complete this form using Adobe Acrobat Writer (versions 5.0 and greater).

1. **PROPOSED DATES OF STUDY:** **FROM:** 10/20/2007 **TO:** 10/19/2008

REVIEW TYPE (Check one): ☑ FULL BOARD ☐ EXPEDITED ☐ EXEMPT

2. **PROJECT TITLE:** Alterations in Phosphorylated Fetuin-A, a Novel Regulator of Insulin Action, in Insulin Resistance and Metabolic Syndrome: Effects of Lifestyle Modification

3.

Peter Grandjean	Assoc Prof	KINE	4-1462	grandpw@auburn.edu
PRINCIPAL INVESTIGATOR	TITLE	DEPT	PHONE	E-MAIL

2050 Memorial Coliseum — ADDRESS FOR CORRESPONDENCE

334-844-1467 — FAX

4. **SOURCE OF FUNDING SUPPORT:** ☐ Not Applicable ☐ Internal ☑ External (External Agency): AAES

5. **STATUS OF FUNDING SUPPORT:** ☐ Not Applicable ☑ Approved ☐ Pending ☐ Received

6. **GENERAL RESEARCH PROJECT CHARACTERISTICS**

A. Research Content Area

Please check all descriptors that best apply to this proposed research project.

- ☐ Anthropology
- ☑ Anthropometry
- ☐ Biological Sciences
- ☐ Behavioral Sciences
- ☐ Education
- ☐ English
- ☐ History
- ☐ Journalism
- ☑ Medical
- ☑ Physiology
- ☑ Other (Please list:) Metabolism

Please list 3 or 4 keywords to identify this research project:

Insulin Resistance, Metabolic Syndrome, Exercise,

Diet, Weight Loss

B. Research Methodology

Please check all descriptors that best apply to the research methodology.

Data collection will be: ☑ Prospective ☐ Retrospective ☐ Both

Data will be recorded so that participants can be directly or indirectly identified: ☐ Yes ☐ No

Data collection will involve the use of:

- ☐ Educational Tests (cognitive, diagnostic, aptitude, achievement)
- ☑ Surveys / Questionnaires
- ☐ Private Records / Files
- ☐ Interview / Observation
- ☐ Audiotaping and / or Videotaping
- ☑ Physical / Physiologic Measurements or Specimens

C. Participant Information

Please check all descriptors that apply to the participant population.

- ☑ Males
- ☐ Females

Vulnerable Populations

- ☐ Pregnant Women
- ☐ Children
- ☐ Prisoners
- ☐ Adolescents
- ☐ Elderly
- ☐ Physically Challenged
- ☐ Economically Challenged
- ☐ Mentally Challenged

Do you plan to recruit Auburn University Students? ☐ Yes ☑ No

Do you plan to compensate your participants? ☑ Yes ☐ No

D. Risks to Participants

Please identify all risks that may reasonably be expected as a result of participating in this research.

- ☑ Breach of Confidentiality
- ☐ Coercion
- ☐ Deception
- ☑ Physical
- ☐ Psychological
- ☐ Social
- ☐ None
- ☐ Other (please list):

For OHSR Office Use Only

DATE RECEIVED IN OHSR: _____ **by** _____ **PROTOCOL #** _____

DATE OF OHSR CONTENT REVIEW: _____ **by** _____ **DATE ASSIGNED IRB REVIEW:** _____ **by** _____

DATE OF IRB REVIEW: _____ **by** _____ **DATE IRB APPROVAL:** _____ **by** _____

INTERVAL FOR CONTINUING REVIEW: _____

7. PROJECT ASSURANCES

PROJECT TITLE: Alterations in Phosphorylated Fetuin-A, a Novel Regulator of Insulin Action, in Insulin Resistance and Metabolic Syndrome: Effects of Lifestyle Modification

A. PRINCIPAL INVESTIGATOR'S ASSSURANCE

1. I certify that all information provided in this application is complete and correct.
2. I understand that, as Principal Investigator, I have ultimate responsibility for the conduct of this study, the ethical performance this project, the protection of the rights and welfare of human subjects, and strict adherence to any stipulations imposed by the Auburn University IRB.
3. I certify that all individuals involved with the conduct of this project are qualified to carry out their specified roles and responsibilities and are in compliance with Auburn University policies regarding the collection and analysis of the research data.
4. I agree to comply with all Auburn policies and procedures, as well as with all applicable federal, state, and local laws regarding the protection of human subjects, including, but not limited to the following:
   a. Conducting the project by qualified personnel according to the approved protocol
   b. Implementing no changes in the approved protocol or consent form without prior approval from the Office of Human Subjects Research (except in an emergency, if necessary to safeguard the well-being of human subjects)
   c. Obtaining the legally effective informed consent from each participant or their legally responsible representative prior to their participation in this project using only the currently approved, stamped consent form
   d. Promptly reporting significant adverse events and/or effects to the Office of Human Subjects Research in writing within 5 working days of the occurrence.
5. If I will be unavailable to direct this research personally, I will arrange for a co-investigator to assume direct responsibility in my absence. This person has been named as co-investigator in this application, or I will advise OHSR, by letter, in advance of such arrangements.
6. I agree to conduct this study only during the period approved by the Auburn University IRB.
7. I will prepare and submit a renewal request and supply all supporting documents to the Office of Human Subjects Research before the approval period has expired if it is necessary to continue the research project beyond the time period approved by the Auburn University IRB.
8. I will prepare and submit a final report upon completion of this research project.

Peter Grandjean		
Principal Investigator (Please Print)	**Principal Investigator's Signature**	**Date**

B. FACULTY SPONSOR'S ASSSURANCE

1. By my signature as sponsor on this research application, I certify that the student or guest investigator is knowledgeable about the regulations and policies governing research with human subjects and has sufficient training and experience to conduct this particular study in accord with the approved protocol.
2. I certify that the project will be performed by qualified personnel according to the approved protocol using conventional or experimental methodology.
3. I agree to meet with the investigator on a regular basis to monitor study progress.
4. Should problems arise during the course of the study, I agree to be available, personally, to supervise the investigator in solving them.
5. I assure that the investigator will promptly report significant adverse events and/or effects to the OHSR in writing within 5 working days of the occurrence.
6. If I will be unavailable, I will arrange for an alternate faculty sponsor to assume responsibility during my absence, and I will advise the OHSR by letter of such arrangements.
7. I have read the protocol submitted for this project for content, clarity, and methodology.

Faculty Sponsor (Please Print)	**Faculty Sponsor's Signature**	**Date**

C. DEPARTMENT HEAD'S ASSSURANCE

By my signature as department head, I certify that every member of my department involved with the conduct of this research project will abide by all Auburn University policies and procedures, as well as with all applicable federal, state, and local laws regarding the protection and ethical treatment of human participants.

Mary Rudisill		
Department Head (Please Print)	**Department Head's Signature**	**Date**

8. **PROJECT ABSTRACT: Prepare an abstract (400-word maximum) that includes: I.) A summary of relevant research findings leading to this research proposal; II.) A concise purpose statement; III.) A brief description of the methodology; IV.) Expected and/or possible outcomes, and V.) A statement regarding the potential significance of this research project. *Please cite relevant sources and include a "Reference List" as Appendix A.***

Metabolic syndrome (MetS), currently affecting over 55 million people in the United States, is a clustering of metabolic abnormalities, associated with an increased risk of cardiovascular disease and type 2 diabetes. The major underlying pathophysiologic determinant of MetS is a condition called insulin resistance (IR), in which tissue responsiveness to normal insulin action is blunted. Current understanding of IR indicates that humoral factors secreted by the fat and liver tissues affect insulin sensitivity. We have identified fetuin-A as a novel physiological regulator of insulin action in vitro, in intact cells and in vivo in animals. We have shown that circulating levels of fetuin-A are increased in several animal models of insulin resistance and diabetes. Additionally, several human investigation studies demonstrate a correlation of fetuin-A levels with body mass index, insulin resistance, and with a fatty liver. Fetuin-A interacts with the activated insulin receptor (IR) and inhibits IR tyrosine kinase activity, a proximal step of insulin signal transduction. Recently, we have elucidated the role of fetuin-A phosphorylation in the regulation of insulin action, demonstrating that phosphorylation is critical for the inhibitory activity of fetuin-A.

There are several objectives of this study: (1) Quantitate phosphorylated fetuin-A levels and the daily variation in these levels in individuals with insulin resistance and metabolic syndrome; (2) Investigate the short-term responses of fetuin-A phosphorylation and changes in insulin resistance that occur with a single session of aerobic exercise; (3) Characterize and compare the total and temporal changes in fetuin-A phosphorylation and insulin resistance that occur with an 8 to 10% weight loss induced by chronic exercise training versus that achieved by dietary modification, and; (4) Investigate the influence of weight loss on the short-term responses of fetuin-A phosphorylation and changes in insulin resistance that occur with a single session of aerobic exercise.

Our hypothesis is that phosphofetuin-A levels are tightly correlated with insulin resistance and that lifestyle modifications will improve insulin sensitivity and decrease phosphorylated fetuin-A levels. We propose to recruit volunteers based on the presence or absence of metabolic syndrome. The first phase of this study will assay daily variation and the effects of acute exercise on fetuin-A levels and phosphorylation status and correlate these to insulin sensitivity as measured by the homeostasis model assessment (HOMA) and the quantitative insulin sensitivity check (QUICKI) indexes, and an oral glucose tolerance test (OGTT). In the second phase, volunteers with and without MetS will be randomized to one of two groups: exercise intervention or diet intervention, to analyze the effect of weight loss through exercise training and dietary modification on phosphofetuin levels and insulin action.

This study could potentially implicate phosphorylated fetuin-A as a critical factor in the pathophysiology of insulin resistance and the metabolic syndrome. Understanding the molecular mechanisms of insulin resistance and MetS may help us identify phosphofetuin-A as a therapeutic target for developing targeted lifestyle intervention, pharmaceutical agents, and identifying genetic links.

9. **PURPOSE & SIGNIFICANCE.**

   a. **Clearly state all of the objectives, goals, or aims of this project.**

The objectives of this study are to:

1. Quantitate phosphorylated fetuin-A levels and the daily variation in these levels in individuals with insulin resistance and metabolic syndrome;

2. Investigate the short-term responses of fetuin-A phosphorylation and changes in insulin resistance that occur with a single session of aerobic exercise;

3. Compare the total and temporal changes in fetuin-A phosphorylation and insulin resistance that occur with an 8 to 10% weight loss induced by chronic exercise training versus that achieved by dietary modification, and;

4. Investigate the influence of weight loss on the short-term responses of fetuin-A phosphorylation and changes in insulin resistance that occur with a single session of aerobic exercise.

   b. **How will the results of this project be used? (e.g., Presentation? Publication? Thesis? Dissertation?)**

The results of this project will be prepared for professional presentation and manuscript submission to peer reviewed scientific journals. Examples of targeted journals are Metabolism, Journal of Lipid Research, Journal of Applied Physiology, and Medicine and Science in Sports and Exercise, Diabetes, Diabetes Care. Since these findings are expected to have broad clinical and scientific impact, the results will be presented at international meetings of the American Heart Association and the American College of Sports Medicine, Experimental Biology. The results of this project will also be used as a dissertation project for Mr. Felipe Araya. Results from this study will be used as preliminary data to attract additional research grant funding from the American Heart Association, American Diabetes Association and the National Institutes of Health.

10. **KEY PERSONNEL INVOLVED WITH DATA COLLECTION. Identify each individual involved with the conduct of this project and describe his or her roles and responsibilities related to this project. Be as specific as possible.**

Individual: Peter Grandjean **Title:** Assoc Prof **Dept/ Affiliation:** KINE
Roles / Responsibilities:

As a Co-PI, Dr. Grandjean will be responsible for overseeing all aspects of human participant data collection, including recruitment, screening volunteers, the informed consent process, participant education, scheduling participant appointments, lab visits and coordinating all physiological assessments, participant record-keeping, blood sampling, handling and storage and statistical analyses.

Individual: Suresh Mathews **Title:** Asst Prof **Dept/ Affiliation:** NUFS
Roles / Responsibilities:

As a Co-PI, Dr. Mathews will be responsible for overseeing the project & standardization of all assays. Dr. Mathews will coordinate diet-analysis and dietary counseling. Dr. Mathews will coordinate the storage and analyses of all blood samples. Drs. Mathews and Grandjean will convene regular meetings with postdoctoral fellow, and the graduate students to monitor and discuss project issues and scientific progress.

Individual: Teayoun Kim **Title:** Post-Doc Fellow **Dept/ Affiliation:** NUFS
Roles / Responsibilities:

Dr. Kim will perform and oversee assays for total fetuin, insulin, and other humoral factors. Kim will also perform Western blotting to quantitate phospho-fetuin levels.

Individual: Felipe Araya and Robert Bowers **Title:** Doctoral Students **Dept/ Affiliation:** KINE
Roles / Responsibilities:

Mr. Araya and Mr. Bowers will work with Dr. Grandjean to collect all human participant data. These students will also assist with biochemical and statistical analyses.

Individual: A. Jack Mahurin, D.O. **Title:** Medical Doctor **Dept/ Affiliation:** Baptist Health - Montgomery
Roles / Responsibilities:

Dr. Mahurin is a faculty memeber in the Baptist Family Medicine Residency Program in Montgomery, AL. He is also a Visiting Clinical Professor in the Department of Kinesiology at Auburn University. Dr. Mahurin will be responsible for screening volunteers who wish to join the study. He will be present for all graded exercise testing and will interpret all graded exercise test results. Dr. Mahurin has also agreed to provide medical supervision and advice as needed throughout the project.

11. **LOCATION OF RESEARCH. List all locations where data collection will take place. Be as specific as possible.**

Preliminary screening of volunteers, physiological assessments, exercise training and blood collection will occur in Memorial Coliseum 2092 - the Exercise Technology Laboratory. Blood storage and blood analyses will occur in rooms 134 and 148 of the Poultry Science Building (These rooms are adjoining laboratory spaces directed by Dr. Mathews). Nutritional counselling will occur in the conference room housed in the Poultry Science Building room 102B or in MC 2092.

12. **PARTICIPANTS.**

a. **Describe the participant population you have chosen for this project.**

Apparently healthy, weight-stable, obese and physically-inactive male volunteers will be partitioned into those meeting three or more of the criteria for MetS as described by the third report of the National Cholesterol Education Program Adult Treatment Panel (NCEP ATP III) and exhibiting elevated fasting blood glucose levels versus those meeting no MetS criteria other than obesity and/or abdominal girth (control). Up to 25 participants per group will be included in this project. All volunteers will be screened to include only those with the following characteristics: 1) 30 to 65 years of age; 2) non-smokers; 3) no documented cardiovascular or metabolic disease; 4) not currently taking medication known to alter lipid or glucose metabolism; 5) practicing no regular leisure time physical activity or strenuous vocational activity over the previous six months; 6) weight stable over the previous 6 months; 7) obese (BMI > 30 kg · m2 or % fat > 30, and waist girth > 88 cm); 8) no signs or symptoms of latent heart disease, and; 9) no conditions that would preclude treadmill walking.

What is the minimum number of participants you need to validate the study? 40

What is the maximum number of participants you will include in the study? 100

b. **Describe the criteria established for participant selection. (If the participants can be classified as a "vulnerable" population, please describe additional safeguards that you will use to assure the ethical treatment of these individuals.)**

The criteria we will use to determine eligibility for participation are described by the NCEP ATP III and outlined above in 12a. Every effort will be made to recruit a culturally diverse sample. Female participants will not be recruited to reduce the confounding of gender on the blood and metabolic variables of interest in this study. This investigation is viewed as an initial step in examining the responses of novel regulators of insulin action to lifestyle modifications in humans. We plan future investigations that will build on these initial results and will include females, children and adolescents.

c. **Describe all procedures you will use to recruit participants.** ***Please include a copy of all flyers, advertisements, and scripts and label as Appendix B.***

Male participants will be recruited from Auburn University and the Auburn-Opelika and surrounding communities. Participants will be recruited by word of mouth and flyers posted at Auburn University and the surrounding community. Advertisements will also be listed in local publications such as newspapers and organizational newsletters. All advertisements, flyers, bulletins, and public appearances will be informational only.

What is the maximum number of potential participants you plan to recruit? 500

d. **Describe how you will determine group assignments (e.g., random assignment, independent characteristics, etc.).**

Volunteers who meet the operationally-defined criteria outlined above will be continuously accrued into the study. For the first project, volunteers will be partitioned into those meeting three or more of the criteria for MetS as described by the NCEP ATP III (waist girth > 40", triglyceride concentrations > 150 mg/dl with or without HDLcholesterol < 40 mg/dl, fasting glucose 100 – 126 mg/dl, and either systolic or diastolic blood pressure >140 / 90 mmHg) versus those meeting no MetS criteria other than obesity and/or abdominal girth (no MetS control group).

e. **Describe the type and amount and method of compensation for participants.**

Participants completing the baseline phase of this study (addressing specific aims 1 and 2 outlined in #9 of this protocol) will receive a token payment of $50.00. Participants completing the weight loss phase of this study (addressing specific aim 3 outlined in part 9 of this protocol) will receive a token payment of $100.00. Participants completing the post-weight loss phase (addressing specific aim 4) will receive $50.00. To be sure, volunteers completing all data measures will receive up to $200.00 total. Compensation will be disbursed as the phase is completed. Compensation not be prorated; however, participants withdrawing early from this study will receive the benefits outlined in #16 of this protocol.

13. **PROJECT DESIGN & METHODS. Describe the procedures you will plan to use in order to address the aims of this study. (NOTE: Use language that would be understandable to a layperson. Without a complete description of all procedures, the Auburn University IRB will not be able to review protocol. If additional space is needed for #13, part b, save the information as a .pdf file and insert after page 6 of this form.)**

a. Project overview. (Briefly describe the scientific design.)

The baseline phase of this study will compare daily variation and acute responses to exercise in markers of IR and related humoral factors. The weight loss and post-weight loss phase will characterize temporal and total changes in markers of IR and related humoral factors with an 8 to 10% weight loss from initial body weight. We will also examine the interaction of weight loss on acute humoral responses to exercise.

b. Describe all procedures and methods used to address the purpose.

Participant Recruitment & Selection: Male volunteers from the Auburn-Opelika area will be recruited through multiple media outlets and posted advertisements. All volunteers will be initially screened by phone interview to include only those with the following characteristics: 30 to 65 years of age, non-smokers, no documented cardiovascular or metabolic disease, not currently taking medication known to alter lipid or glucose metabolism, practicing no regular leisure time physical activity or strenuous vocational activity over the previous six months and weight stable over the previous 6 months. Volunteers meeting these criteria will be asked to visit our lab for an information meeting and to undergo additional preliminary measurements. After reviewing the requirements of the study and signing an institutionally-approved informed consent document, volunteers will have their height, weight and waist circumference measured and a provide a finger stick sample of blood to determine total cholesterol, HDL-cholesterol, triglyceride and glucose concentrations. Those volunteers meeting the preliminary criteria for participation will be asked to return to the lab in order to undergo a physiological assessment that includes a physician screening, DXA scan for determining total and regional body fat, and a standardized graded exercise test on a treadmill to assess their heart rate, ECG, blood pressure, and respiratory gas (VO_2, VCO_2) responses to exercise and to determine their cardiovascular fitness (VO_2max). Results will be used to include only those who meet the additional characteristics: obese (BMI > 30 kg · m2 or % fat > 30, waist girth > 88 cm); no signs or symptoms of latent heart disease, and; no conditions that would preclude treadmill walking.

Volunteers will be partitioned into those meeting three or more of the criteria for MetS as described by the NCEP ATP III and having elevated fasting blood glucose levels (waist girth > 40", triglyceride concentrations > 150 mg/dl with or without HDL-cholesterol < 40 mg/d, fasting glucose 100 – 126 mg/dl, and either systolic or diastolic blood pressure >140 / 90 mmHg) versus those meeting no MetS criteria other than obesity and/or abdominal girth (control). Copies of the Initial Screening Survey questions, Health and Lifestyle History Questionnaire, Physician Screening form and Physiological Data Collection forms are found in appendix C.)

Baseline Procedures: Participants will report to the Exercise Technology Laboratory after two days of a moderated diet (described below) and physical activity and after a 10- to 12-hr overnight fast. After obtaining a body weight, a catheter will be inserted into an antecubital vein and a fasting blood sample will be obtained. Next, participants will undergo a 2-hr oral glucose tolerance test (OGTT) by consuming 75 g of a standard carbohydrate syrup. Blood samples will be obtained at 20-min intervals for determining glucose, insulin, and free fatty acid (FFA) responses. Fasting blood samples will be obtained again on days 5 and 7 of the moderated diet in order to determine daily variation in phospho-fetuin and related humoral markers of IR. On day 7, participants will undergo a single bout of treadmill walking at 60 to 70% of their VO_2max (moderate intensity) in order to expend 500 kcals of energy. Exercise intensity and caloric expenditure will be monitored by heart rate and respiratory gas analysis at regular intervals throughout the exercise session. Blood samples will be obtained before and immediately and 24 hrs after the exercise session. Blood sampling procedures 24 hrs after the exercise session will include an OGTT in order to determine changes in IR. The single blood samples obtained on days 5 and 7 will be 10 ml each (0.75 tbsp). The OGTT will include one 10 ml sample and six 7 ml samples totaling 52 mls - or 3.5 tbsps). Copies of the Exercise Session Worksheet and Blood Sampling Record are found in appendix C.

Diet and Physical Activity Records: All participants will record their dietary consumption and habits over a baseline 3-day period. (The 3 days of diet recording will occur over two week days and one weekend day and will be completed between the volunteer's initial information meeting and when they arrive at the lab for the physician screening and physiological assessment.) Dietary records will be analyzed by commercially-available software (Nutrition Calc +2.0, McGraw Hill, Boston, MA) and participants will be counseled to maintain a similar caloric and nutrient intake and to refrain from moderate or vigorous physical activity in the days throughout blood sampling. Participants will maintain physical activity and dietary records in order to account for any changes in caloric consumption or expenditure that could potentially influence the experimental results. Records will be collected and reviewed at each participant visit during the first phase of data collection. The daily dietary record and physical activity record and an average of the body weights will be used to estimate baseline daily energy requirements.

Please see figure 1 on page 6a for an illustration of the baseline procedures. (Copies of the Daily Dietary Record, food exchange catalog and Physical Activity Record are found in appendix C.)

c. **List all instruments used in data collection. (e.g., surveys, questionnaires, educational tests, data collection sheets, outline of interviews, scripts, audio and/or video methods etc.)** ***Please include a copy of all data collection instruments that will be used in this project and label as Appendix C.***

Initial Screening Survey
Health & Lifestyle History Questionnaire
Physician Screening Form
Physiological Data Collection Form (for preliminary screening)
Blood Sampling Record (for determining plasma volume changes with daily variation and acute exercise responses)
Exercise Session Worksheet (for monitoring intensity and caloric expenditure with experimental exercise sessions)
Daily Diet Record Form (for 3- and 7-day records)
American Dietetic Association Food Exchange Program Pamphlet
Physical Activity Record
Weekly Exercise Log
Weekly Weight Record and Weight Loss Chart (for tracking weight loss)
Monthly Information Form (for participant counseling)

d. **Data Analysis: Explain how the data will be analyzed.**

Multiple between condition/group factors [2 conditions (MetS and no MetS); 2 groups (EXER and DIET)] and a within condition/group factor (multiple sample points) repeated measures ANOVAs will be employed to determine significant findings. Pearson product moment correlations will be used to determine significant relationships between physical and biochemical variables that may occur with the interventions. Because this study is exploratory in nature, significance will be accepted at a comparison-wise error rate of $p = 0.05$.

14. **RISKS & DISCOMFORTS: List and describe all of the reasonable risks that participants might encounter if they decide to participate in this research.** ***If you are using deception in this study, please justify the use of deception and be sure to attach a copy of the debriefing form you plan to use and label as Appendix D.***

The primary risk associated with respiratory gas analysis for specification of VO2peak and exercise calorimetry is with contamination of the mouthpiece and tubing. The risks associated with maximal exercise testing are comparable to those faced when performing vigorous exercise. These include occasional abnormal blood pressure responses, the possibility of fainting, potentially abnormal heart beats, heavy and difficult breathing, and in rare instances heart attack or death. According to the reports published in the American College of Sports Medicine's Guidelines for Exercise Testing & Prescription (7th Edition) the mortality rate of maximal exercise testing is minimal with an observed rate of 0.5 deaths per 10,000 (0.005%) tests performed. The incidence of an untoward event of a medical injury is 8 events per 10,000 (0.08%).

According to the American College of Sports Medicine the risks associated with sub-maximal exercise are even lower than the risks associated with maximal exercise testing (31). Possible risks are the same as the maximal exercise test, and are listed above.

Electrocardiography (ECG) used during the maximal GXT will use electrodes on the torso. This requires scrubbing the skin and wiping with an alcohol pad. The possible risks associated with this include an allergic reaction to the pad or wipe and abrasion or minor cuts to the scrubbed area.

Blood sampling may impose minor bruising, swelling, and itching of the affected area. As with any similar procedure that disrupts the skin barrier, there is an increased risk of infection.

There is a risk for breaching (not maintaining) confidentiality of participant information.

15. PRECAUTIONS. Describe all precautions you have taken to eliminate or reduce risks that were listed in #14.

Every effort will be made to minimize all of the physiologic risks inherent in this study through preliminary screening, identification of contraindications to exercise testing, adherence to standards of practice for graded exercise testing that are published by the American College of Sports Medicine (ACSM Guidelines 7th ed., 2006), and personal monitoring of each test.

All graded exercise tests will be performed by Dr. Grandjean, Mr. Araya and Mr. Bowers. Dr. Jack Mahurin will be present for these tests and will provide interpretation of the test results. Dr. Grandjean has current ACLS Provider credentials. All individuals hold current CPR certification. Investigators will closely follow emergency plans and procedures that have been established for the Exercise Technology Laboratory which are on file at the OHSR and have been approved by the current board.

All respiratory gas analysis will be performed with cleaned and disinfected pneumotachs and mouthpieces. The pneumotachs and mouthpieces will be cleaned with soap and water and disinfected with a 5% bleach solution. This equipment will be rinsed and allowed to air dry before reuse. Pneumotachs and mouthpieces will not be reused at the same testing session.

Universal precautions will be observed for each blood sample and the following procedures will be used to help minimize any potential risks to the participant and investigator: the technician will use surgical latex gloves; the antecubital area will be cleansed with an alcohol pad prior to puncturing the skin; all blood draw equipment and instruments will be sterile; and all punctured sites will be properly dressed with antiseptic and bandage following each sample collection. Dr. Grandjean will oversee all phlebotomy procedures. All investigators have completed (last year) and will complete (this year and before initiating this study) the updated training in blood sampling, blood sample handling, and blood-borne pathogens posted on Open WebCT by the Auburn University Biosafety Office. (A BUA has been submitted to the Office of Safety and Risk Management.)

All participant file information will be maintained as described in #17. Investigators will not discuss any participant information concerning this study with any other participant.

The nutritionist will meet with the participants at least twice at the beginning of the study and more times if needed. The nutritionist will meet at least one time per month with the participants and more times if needed. The nutritionist will be available by phone and by e-mail throughout the study.

16. BENEFITS.

a. List all realistic benefits participants can expect by participating in this study.

The benefits of participation in the initial screening include the determination of cardiovascular fitness, body composition, and blood markers of metabolic health.

The benefits of participation include a greater understanding of how one's body responds to an oral glucose challenge and aerobic exercise. All participants will receive education regarding the appropriate strategies for weight loss and weight maintenance through physical activity and dietary interventions. Participants will receive personalized recommendations, counseling and supervision during a weight loss intervention. All participants will receive a study summary that includes a synopsis of their personal results and how they compared to their assigned group and the cohort as a whole.

b. List all realistic benefits for the general population that may be generated from this study.

Fetuin A and phospho-fetuin A have been shown to play an integral and unique role in animal models of insulin resistance. This study could potentially implicate phosphorylated fetuin-A as a critical factor in the pathophysiology of insulin resistance and the metabolic syndrome in humans. Understanding the molecular mechanisms of insulin resistance and MetS may help us identify phosphofetuin-A as a therapeutic target for developing targeted lifestyle intervention, pharmaceutical agents, and identifying genetic links.

17. **PROTECTION OF DATA.**

a. **Will data be collected as anonymous?** ☐ **Yes** ☑ **No** ***If "YES", go to part "g".***

b. **Will data be collected as confidential?** ☑ **Yes** ☐ **No**

c. **If data is collected as confidential, how will the participants' data be coded or linked to identifying information?**

Participant information will be maintained by name and code list. Each participant will be identified with a code that signifies their group (MetS = A; control = B), their weight loss intervention (Exercise = 1; Diet = 2), and their participant number as they accrue into the study. For example, John Doe is in the MetS group, exercise weight loss intervention and was the 30th participant to meet eligibility requirements would have a code number A-1-30. Further numbering will identify the blood sample point (Pre, IPE = immediately post-exercise, 24H = 24 after exercise, OGTT1, 2 etc, month 1, 2, 3 etc).

d. **Justify your need to code participants' data or link the data with identifying information.**

Identifying information and codes must be linked in order to determine responses and changes within a participant over time.

e. **Where will code lists be stored?**

The code list will maintained in duplicate. One copy will be stored on a password-protected computer in Dr. Grandjean's office (MC 2070). The second copy will be maintained on a password-protected computer in Dr. Mathew's office (Poultry Science 101). It is necessary to maintain two copies so that the Co-PIs can coordinate blood sample handling, storage and analyses.

f. **Will data collected as "confidential" be recorded and analyzed as "anonymous"?** ☐ **Yes** ☑ **No**

g. **Describe how the data will be stored (e.g., hard copy, audio cassette, electronic data, etc.), where the data will be stored, and how the location where data is stored will be secured in your absence.**

Participant files containing hard copy data will be stored in a locked file cabinet in MC 2084. Hard copies of diet records may also be stored in a locked file cabinet in Dr. Mathew's office - Poultry Science 101. Electronic files of participant data will be stored on password-protected computers in the Co-PI's offices. Electronic files will be stored in separate digital "folders" from the location of the code list. The electronic files will be consistently updated and shared between the Co-PIs via e-mail attachment. All data will be maintained in rooms that are locked when not in use.

h. **Who will have access to participants' data?**

All individuals listed as key personnel will have access to participant files (hard copy and electronic). Key personnel will have access to electronic files on lab computers by permission from Co-PIs.

i. **When is the latest date that the data will be retained?**

We plan on retaining all information until manuscripts prepared from this data are accepted for publication. We will provide any information that the IRB and OHSR require for monitoring this project and apply for project renewal until project completion. Current funding is for a 3-year period.

j. **How will the data be destroyed? (NOTE: Data recorded and analyzed as "anonymous" may be retained indefinitely.)**

Hard copy data will be shredded and electronic data will be erased from the lab computers.

PROTOCOL REVIEW CHECKLIST

All protocols must include the following items:

☑ **1.** Research Protocol Review Form (All signatures included and all sections completed)

☑ **2.** Consent Form or Information Letter (examples are found on the OHSR website)

☑ **3.** Appendix A "Reference List"

☑ **4.** Appendix B if flyers, advertisements, generalized announcements or scripts are used to recruit participants.

☑ **5.** Appendix C if data collection sheets, surveys, tests, or other recording instruments will be used for data collection. Be sure to mark each of the data collection instruments as they are identified in section # 13, part c.

☐ **6.** Appendix D if a debriefing form will be used.

☐ **7.** If research is being conducted at sites other than Auburn University or in cooperation with other entities, a letter from the site / program director must be included indicating their cooperation or involvement in the project. NOTE: If the proposed research is a multi-site project, involving investigators or participants at other academic institutions, hospitals or private research organizations, a letter of IRB approval from each entity is required prior to initiating the project.

☐ **8.** Written evidence of acceptance by the host country if research is conducted outside the United States.

AUBURN UNIVERSITY INSTITUTIONAL REVIEW BOARD for RESEARCH INVOLVING HUMAN SUBJECTS

REQUEST for PROTOCOL REVISION

For Information or help completing this form, contact: **THE OFFICE OF HUMAN SUBJECTS RESEARCH**, 307 Samford Hall
Phone: 334-844-5966 **e-mail:** hsubjec@auburn.edu **Web Address:** http://www.auburn.edu/research/vpr/ohs/index.htm

Complete this form using Adobe Acrobat Writer (versions 5.0 and greater).

1. **PROTOCOL NUMBER:** 07-210 MR 710 2. **DATES OF STUDY:** **FROM:** 10/10/2007 **TO:** 10/09/2008

3. **REQUESTED DATE FOR PROTOCOL CHANGE TO TAKE EFFECT:** 06/01/2008

4. **PROJECT TITLE:** Alterations in Phosphorylated Fetuin-A, a Novel Regulator of Insulin Action, in Insulin Resistance and Metabolic Syndrome: Effects of Lifestyle Modification

5.

Peter W. Grandjean	Assoc Prof	KINE	4-1462	grandpw@auburn.edu
PRINCIPAL INVESTIGATOR	**TITLE**	**DEPT**	**PHONE**	**E-MAIL**

2050 Memorial Coliseum
ADDRESS FOR CORRESPONDENCE

PI SIGNATURE

6. **Describe all research activities that have occurred up to this point.**

We are currently collecting data on seven participants that meet the NCEP criteria for "metabolic syndrome" as described in the original protocol. These participants have completed baseline experimentation and are undergoing the weight loss portion of the protocol. All data is being collected as described in the IRB-approved protocol. Screening of potential participants is ongoing.

7. **Use the space below to describe the requested changes to your research protocol. Please include an explanation and/or rationale for each of the changes you have requested.**

As part of the stated objectives we outlined in the previously-approved protocol, we proposed to assay daily variation and the effects of acute exercise on fetuin-A levels and phosphorylation status. Our aim is to correlate these responses to insulin sensitivity as measured by the homeostasis model assessment (HOMA), the quantitative insulin sensitivity check (QUICKI) indexes, and an oral glucose tolerance test (OGTT).

In order to expand the characterization beyond men with "metabolic syndrome", we would like to include a group of aged-matched men of normal weight (BMI between 20 and 25 kg/m2). These men would meet the criteria we previously outlined for being apparently healthy, weight-stable, and physically-inactive. These volunteers will undergo the same screening and preliminary assessments we described in our original protocol. The normal weight me will undergo all of the baseline experimental procedures but will not undergo the weight loss or post-weight loss experimentation.

8. **Identify any changes in the anticipated risks and / or benefits to the participants.**

There are no changes to the anticipated risks. The normal weight men will not benefit from weight loss; however, they will accrue the same benefits of participation as the metabolic syndrome participants with respect to the the initial screening. Benefits include the determination of cardiovascular fitness, body composition, and blood markers of metabolic health. The benefits of participation in the baseline experimentation include a greater understanding of how one's body responds to an oral glucose challenge and aerobic exercise. All participants will receive a study summary that includes a synopsis of their personal results and how they compared to their assigned group and the cohort as a whole.

9. **Identify any changes in the safeguards or precautions that you will use to address the changes in the anticipated risks.**

No changes are anticipated...

Additional documentation includes a new consent document specifically designed for the normal weight volunteers.

10. **Attach any additional supporting documentation you feel may assist the IRB in evaluating your request for protocol revisions.**

11. **If research is being conducted at sites other than Auburn University or in cooperation with other entities, a letter from the site / program director must be included acknowledging their acceptance of the proposed changes.**

12. **Attach a copy of the "stamped" IRB approved consent form you are currently using.**

13. **Attach a revised copy of the consent document that includes updated information regarding the requested changes. (Be sure to review the OHSR website for current consent document guidelines and updated contact information.)**