Altered temporal changes in Ser312-phosphofetuin-A concentrations in response to a glucose challenge in a mouse model of diet-induced obesity and insulin resistance

by

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Abstract

Recent studies have implicated a role for the liver-secreted glycoprotein fetuin-A in insulin resistance. Fetuin-A negatively regulates insulin action by interacting with the activated insulin receptor and inhibiting insulin-stimulated insulin receptor autophosphorylation and tyrosine kinase activity. This study examined Ser312-fetuin-A phosphorylation status in diet-induced obese (DIO) C57Bl6 mice, and characterized the effect of an oral glucose challenge on temporal changes in Ser312-phosphofetuin-A in the DIO model. Serum Ser312-phosphofetuin-A concentrations were significantly elevated in DIO mice compared to their control counterparts, and showed a positive correlation with insulin resistance. In response to an oral glucose challenge (OGTT), plasma concentrations of Ser312-phosphofetuin-A levels demonstrated a significant temporal increase in mice fed regular chow. However, in the DIO model, plasma Ser312-phosphofetuin-A concentrations showed a significant temporal decrease. These findings demonstrate that Ser312-phosphofetuin-A concentrations are elevated in insulin resistant conditions, and suggest that phosphofetuin-A may be dynamically involved in the insulin response.

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1. Introduction

The modern prevalence of the Western lifestyle, one of high energy intake coupled with low physical activity, has triggered a substantial increase in the incidence of obesity and its associated metabolic complications. The escalation of obesity rates has reached epidemic proportions in the United States, with the Centers for Disease Control and Prevention (CDC) estimating that of United States adults aged 20 years and over, 34.2% were overweight (BMI = 25.0–29.9), 33.8% were obese (BMI = 30-39.9), and 5.7% were extremely obese (BMI ≥ 40) in 2007-2008 (1). Obesity has been associated with the development of numerous health problems, including: cardiovascular disease, osteoarthritis, cancer, hypertension, and diabetes mellitus (2). .

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (3). It is now well accepted that diabetes mellitus is one of the main threats to human health in the twenty-first century. Registered as the seventh leading cause of death in the United States (cited as the underlying cause in 71,382 death certificates) (4), diabetes is also the foremost cause of kidney failure, nontraumatic lower- limb amputations, and new cases of blindness among adults in the United States (5), but it is linked to many other serious complications including cardiovascular disease, stroke, hypertension, nephropathy, and neuropathy (6). The International Diabetes Federation (IDF) estimates that the number of

people with diabetes worldwide will increase from 336 million (8.3% of adults aged 20-79) in 2011 to 552 million (9.9%) by 2030 (7). The most accepted and unifying hypothesis connecting the pathophysiology of obesity and type 2 diabetes is a condition known as insulin resistance.

The etiology of insulin resistance, which may be defined as impaired insulin action despite normal or elevated levels of circulating insulin, is not yet well understood. Insulin action, specifically its blood glucose lowering effects, begins when the hormone binds to the insulin receptor, causing autophosphorylation and activation of the insulin receptor tyrosine kinase activity. Along with other downstream signaling components, these initial steps are frequently the target of various humoral factors that modulate insulin action, such as adipokines including leptin, adiponectin, resistin, and RBP-4; pro-inflammatory cytokines such as TNF- and IL-6, and others like PTP-1B (8-12). Alterations in serum concentrations of these humoral factors have been shown to influence whole body insulin sensitivity and insulin action, and their dysregulation is believed to play a pivotal role in the pathophysiology of metabolic conditions like obesity, type 2 diabetes, and insulin resistance. An emerging body of evidence has indicated a role for fetuin-A as a novel hepatokine in insulin resistance, obesity, and type 2 diabetes (13-16).

Since the fetuin-A gene is localized on chromosome 3q27 (17), a susceptibility locus for type 2 diabetes and metabolic syndrome (18; 19), it may be reasonably suspected of being associated with metabolic disorders. Two important longitudinal studies conducted recently have found positive associations between circulating fetuin-A and incident type 2 diabetes (13; 16). One of these associations was in a cohort from the

Health ABC study of older humans independent of physical activity, inflammatory biomarkers, and other measures of insulin resistance (13), while the other was of a sub-cohort of 2500 individuals from the EPIC-Potsdam study after adjusting for age, sex, BMI, waist circumference, lifestyle risk factors, glucose, triglycerides, HDL-cholesterol, gamma-glutamyl transferase, and hs-C-reactive protein (16). Together these two studies can be taken as evidence that fetuin-A is an independent risk factor for type 2 diabetes. Plasma fetuin-A levels were positively associated with maternal insulin resistance and higher in patients with gestational diabetes than in healthy pregnant women (20). Furthermore, higher plasma fetuin-A levels have been found to be associated with elevated risk of metabolic syndrome (21), decreased insulin sensitivity (15), increased visceral adiposity (22), liver fat accumulation (15), an atherogenic lipid profile (21), and a greater risk of both myocardial infarction and ischemic stroke (23). Interestingly, just as one single nucleotide polymorphism (SNP) was found to be associated with an increased risk for type 2 diabetes in French Caucasians (24), another was found to be protective against fatness in Swedish men (25). Moreover, in Danish whites, two different SNP's were positively correlated with incident type 2 diabetes and dyslipidemia while a third SNP was associated with increased insulin sensitivity (26). These data indicate that fetuin-A gene variations may contribute to the variety of metabolic traits in individuals, and it has even been suggested that the fetuin-A gene could be a type of "thrifty gene" that geneticist James Neel described decades ago (27; 28).

The leading explanation for fetuin-A's many associations with metabolism and insulin resistant conditions stems from studies demonstrating that fetuin-A is a negative regulator of insulin action. Fetuin-A interacts with the activated insulin receptor and

inhibits insulin-stimulated insulin receptor autophosphorylation and tyrosine kinase activity (29-31). Fetuin-A null mice displayed improved insulin receptor autophosphorylation, insulin sensitivity, and resistance to weight gain (32), and these mice were also protected against obesity and insulin resistance associated with aging (33). Human fetuin-A is a 55-59 kDa protein consisting of two subunits, an A chain (residues 19-300) and a B chain (residues 341-367), made up of 282 amino acids and 27 amino acids, respectively (28). These two subunits are connected by a single disulfide bond and, unless it has been removed by limited proteolysis post-translationally, a 40 amino acid "connecting peptide" (residues 301-340). The A chain has four glycosylation sites. There are two N-glycans attached to asparagine residues 138 and 158 and two O-glycans attached to threonine residues 238 and 252 (34). Also, fetuin-A can be phosphorylated at two sites: Ser120 of the A-chain and Ser312 of the connecting peptide. It has been shown that 77% of total phosphorylation occurs at Ser312, and that up to 20% of circulating fetuin-A is phosphorylated at least at one site (35). Phosphorylation is believed to be necessary for some biological activity; therefore, the heterodimeric form of fetuin-A lacking the connecting peptide's Ser312 residue is hypothesized to be inactivated (36). However, studies on phosphorylated fetuin-A and its role in insulin resistance are lacking. Studies from our laboratory have shown that niacin treatment lowers serum total fetuin-A and phosphofetuin-A concentrations in individuals with metabolic syndrome, and these changes correlated with the beneficial changes in serum lipids (37). Further, previous studies from our laboratory have shown increased plasma fetuin-A Ser312-phosphorylation in insulin resistant, leptin-signaling impaired Zucker diabetic fatty (ZDF) rats and in leptin-deficient *ob/ob* mice, compared to lean controls,

suggesting roles for leptin and insulin in the regulation of Ser312-phosphorylation of fetuin-A (38). All of these findings taken together suggest the possibility of a pathophysiological role for fetuin-A in diabetes, potentially through regulating insulin sensitivity. The goal of this study was to characterize fetuin-A and its phosphorylation in a mouse model of diet-induced obesity, and observe its dynamics during an oral glucose tolerance test. We hypothesized that fetuin-A and phosphofetuin-A will be higher in diet-induced obese, insulin resistant mice. Further, it was posited that fetuin-A and phosphofetuin-A concentrations may not be altered temporally in response to an oral glucose challenge.

2. Review of Literature

2.1 Obesity

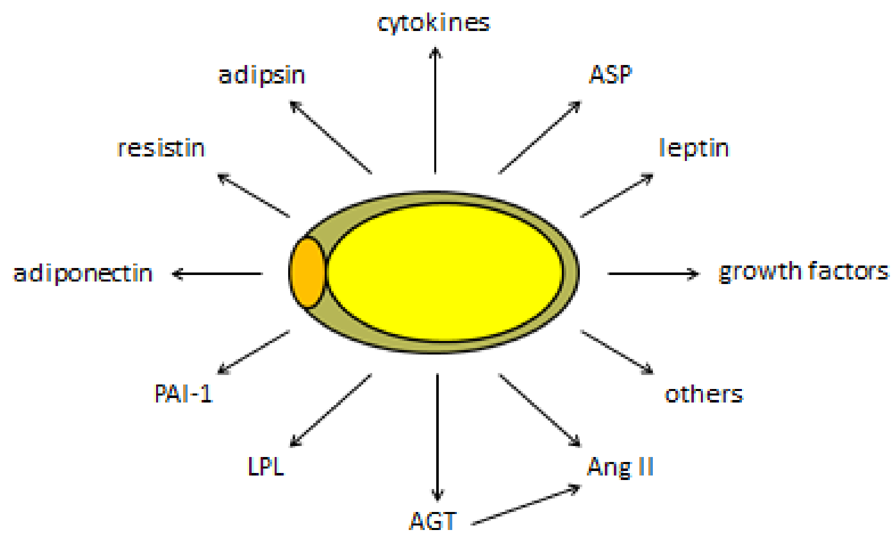
Obesity, clinically defined as a body mass index (BMI) greater than or equal to 30 kg/m^2 in adults 20 years and older, is highly prevalent in the modern world. According to the World Health Organization (WHO), the world's obesity rate has more than doubled since 1980. In 2008, over 200 million men and nearly 300 million women aged 20 years and over were obese (39). The escalation of obesity rates has reached epidemic proportions in the United States, with the Centers for Disease Control and Prevention (CDC) estimating that out of United States adults aged 20 years and over, 34.2% were overweight (BMI = 25.0–29.9), 33.8% were obese (BMI = 30-39.9), and 5.7% were extremely obese (BMI $\geq$ 40) in 2007-2008 (1). In 2010, Alabama was among the 12 states estimated to have an obesity rate greater than 30% (40). Another noteworthy aspect of the obesity epidemic is the increase in obesity prevalence in children. In the United States, obesity in children and adolescents aged 2-19 years has more than tripled over the past 40 years, bringing the total to 16.9% in 2007-2008 (41). Obesity increases the risk of a variety of diseases such as cardiovascular disease, cerebrovascular disease, certain types of cancer, diabetes mellitus, osteoarthritis, non-alcoholic fatty liver disease, and hypertension (42-45). Many of these diseases rank among the leading causes of mortality

around the world, accounting for millions of deaths per year (46). Thus, not only is obesity a significant contributor to deaths worldwide, but the high obesity rates of Alabama and the United States place these populations at high risk of dying from diseases associated with obesity.

In a few cases of obesity the excess adiposity can be caused solely by genes, endocrine disorders, medications, or psychiatric conditions; however, in the vast majority it is due to a combination of polygenetic susceptibility, excessive dietary intake, and insufficient energy expenditure (47-49). With the absence of significant change in the gene pool over the past few decades, discussion about the obesity epidemic generally attributes a causal role to the recent societal shift towards a sedentary lifestyle in an environment conducive to high energy intake (50). Given its complexity, treatment of obesity is contingent upon the individual and can include lifestyle modifications in diet and physical activity, pharmaceutical options, and/or surgical procedures (51; 52).

2.2 Adipose tissue and the pathophysiology of obesity

The role of adipocytes in metabolism was originally deemed a passive one, but it has recently become evident that adipose tissue is a major endocrine organ recognized as being an important component of metabolic control (53-58). Adipose tissue secretes a wide array of substances, referred to as adipokines and depicted in Fig. 2.1, which may exert autocrine, paracrine and/or endocrine functions (58). Although the functions of many of the over 50 adipokines identified to date are poorly understood, some of them greatly influence insulin sensitivity, glucose metabolism, and inflammation, and may provide a molecular link between obesity, insulin resistance, and the development of type 2 diabetes (59).



<u>Figure 2.1</u>: <u>*Secretions of the adipocyte*</u>. Adipocytes secrete a wide array of substances, termed adipokines. AGT, angiotensinogen; Ang II, angiotensin II; ASP, acylation stimulating protein; LPL, lipoprotein lipase; PAI-1, plasminogen activator inhibitor-1 (60).

Obesity is medically defined as "a state of increased body weight, more specifically adipose tissue, of sufficient magnitude to produce adverse health consequences" (61). It develops as the result of a positive energy balance, where energy intake is greater than energy expenditure (61). White adipose tissue, as opposed to brown adipose tissue (which uniquely expresses uncoupling protein (UCP) -1 and is responsible for thermogenetic energy expenditure), normally functions as the primary storage site of dietary lipids; however, as it accumulates more mass during positive energy balance its lipid storage potential is reduced (53; 62). This results in a fatty acid surplus that disrupts metabolic pathways and endocrine function in many tissues while in circulation and also by forming destructive ectopic fat deposits in organs like the liver, spleen, and pancreas. (53; 54; 56; 63). In obesity, adipose tissue may not receive adequate tissue circulation, which can lead to a hypoxic state and tissue necrosis (53). Consequently, macrophage

infiltration and dysfunctional cytokine secretion are also characteristic of excessive fat accumulation. The site of adiposity is thought to be important, in that intra-abdominal fat is more highly correlated with type 2 diabetes than gluteal or subcutaneous fat, possibly because of it being more lipolytically active due to its complement of adrenergic receptors (64). All of this taken together illustrates an overall metabolic shift in obesity, especially central obesity, towards a disease-prone state of lipid overflow, insulin resistance, chronic low-grade inflammation, enlarged adipocytes, systemic oxidative stress, and altered endocrine function (53; 56; 60).

2.3 Diabetes

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (3). The prevalence of diabetes has steadily risen in recent decades (65), and the International Diabetes Federation (IDF) estimates that the number of people with diabetes worldwide will increase from 336 million (8.3% of adults aged 20-79) in 2011 to 552 million (9.9%) by 2030 (7). According to the American Diabetes Association (ADA), 18.8 million United States citizens had diagnosed cases of diabetes in 2010, accompanied by an estimated 7 million more that are undiagnosed (6). In Alabama, 11.1% of the state's population had diagnosed diabetes in 2010 (66). Diabetes, especially when poorly managed, is a major threat to individual wellness and can ultimately cause death; it was the seventh leading cause of death in both the United States (cited as the underlying cause in 71,382 death certificates) and the state of Alabama (1,313) in 2007 (4). Not only is diabetes the foremost cause of kidney failure, nontraumatic lower- limb amputations, and new cases of blindness among adults in the United States (5), but it is linked to many other serious

complications including cardiovascular disease, stroke, hypertension, nephropathy, and neuropathy (6). According to the ADA, a clinical diagnosis of diabetes can be confirmed in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis and/or a random plasma glucose ≥ 200 mg/dl by a measurement of the percentage of hemoglobin that is glycated (A1C) ≥ 6.5%, a fasting plasma glucose (FPG) ≥ 126 mg/dl, or a 2-hour plasma glucose ≥ 200 mg/dl during a 75 g oral glucose tolerance test (OGTT) (3). Alarmingly, additional to the diagnosed and undiagnosed cases of diabetes in 2010, an estimated 79 million Americans have a condition that is associated with an increased risk for developing diabetes called prediabetes, which has been clinically defined as having impaired fasting glucose (IFG, FPG = 100-125 mg/dl) or impaired glucose tolerance (IGT, OGTT = 140 -199 mg/dl) (3; 6).

There are many different classifications of diabetes mellitus; however, type 2 diabetes accounts for at least 90% of all cases. The other two major categories of diabetes are type 1 and gestational diabetes. Type 1 diabetes presents as absolute insulin deficiency from autoimmune destruction of pancreatic beta cells (67), and gestational diabetes is a temporary condition during pregnancy (68). Pathologically, the hyperglycemia in type 2 diabetes is the result of substandard insulin activity due to varying degrees of two metabolic issues: insulin resistance and defective insulin secretion. The etiology of type 2 diabetes is not yet fully understood, and it is likely to have multiple causes (3). In regard to nature verses nurture, type 2 diabetes is believed to be a heritable condition that can be brought on by lifestyle factors (69). The recent rise in diabetes occurrence is largely attributed to the increasing prevalence of sedentary lifestyle, overconsumption, and obesity (70), and it is widely considered to be a

preventable disease (71). There is no cure for diabetes currently available. A variety of effective treatment methods focus on assisting the body with glycemic control in order to reduce the risk of diabetic hyperglycemia, prevent long-term complications, and allow for a relatively stable, normal life (72).

2.4 Insulin action and signaling

2.4.1 Insulin action

Insulin, a peptide hormone secreted by pancreatic beta cells, is a crucial regulatory component of energy metabolism (73). Insulin secretion decreases in response to hypoglycemia, hyperinsulinemia, and increased catecholamine concentrations, while it is stimulated by hyperglycemia, elevated plasma concentrations of amino acids, and increased nonesterified fatty acids (NEFA) (74; 75). Insulin serves to lower glucose levels in circulation by enhancing glucose uptake into skeletal muscle and adipose tissue, with skeletal muscle accounting for up to 75% of this insulin-stimulated glucose uptake (76). Furthermore, it increases glycogen synthesis and inhibits glycogenolysis in muscle, thus enhancing glucose utilization. Also, insulin inhibits hepatic gluconeogenesis, preventing the liver from producing endogenous glucose (77). Insulin exerts anabolic effects in the metabolism of proteins and lipids as well, promoting lipogenesis and protein synthesis while inhibiting the expression or activity of the enzymes responsible for their catabolism (76). In addition to its metabolic effects, insulin is also capable of mitogenic activity, promoting cell growth and differentiation (76).

2.4.2 Insulin Signaling

The insulin receptor (IR) consists of two extracellular α-subunits and two transmembrane β-subunits, which are held together through disulfide bonds to yield a

heterotetrameric membrane protein (78). Insulin binds extracellularly, inducing a conformational change in the α-subunits that ultimately causes the β-subunits to undergo autophosphorylation (78; 79). Now activated, the intrinsic tyrosine kinase activity of the IR triggers an intracellular signaling cascade, beginning with the phosphorylation of insulin receptor substrate (IRS) proteins 1-4, Src homology collagen (Shc), GRB2-associated-binding protein 1 (Gab1), proto-oncogene protein c-Cbl (named after **C**asitas **B**-lineage **L**ymphoma), and adapter protein with Pleckstrin-homology and Src-homology-2 domains (APS) (80; 81). All of these proteins provide specific docking sites for other signaling proteins containing SH2 domains (80). These events lead to the activation of downstream signaling molecules via three main pathways: the IRS/phosphatidylinositol 3-kinase (PI3K) pathway, the Cbl-associated protein (CAP)/Cbl pathway, and the RAS/mitogen-activated protein kinase pathway (MAPK) pathway (82). An overview of these pathways is provided in Fig. 2.2.

Insulin is a major hormone, whose binding to the insulin receptor transduces the signal intracellularly through several signaling elements. Of these, IRS-1 and -2 mediation appears to be the most important in regard to both carbohydrate metabolism and somatic growth (80; 83; 84). Phosphorylation of Tyr972 on the IR β-subunit allows for its interaction with phosphotyrosine binding (PTB) domains on IRS-1 and -2 (85). Upon PTB binding, IRS-1 residues Tyr612 and Tyr632 become phosphorylated and are then used as a docking site for phosphoinositide-3-kinase (PI3K) (86). PI3K is a lipid kinase with numerous functions in cellular growth and differentiation, macronutrient synthesis and degradation, and membrane trafficking (81). Activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate to yield phosphatidylinositol 3,4,5-

trisphosphate (PIP3) and phosphatidylinositol 3,4-bisphosphate (PIP2), which proceed as second messengers ultimately causing the phosphorylation of the downstream effector AKT (87; 88). Now active, AKT detaches from the plasma membrane and translocates glucose transporter 4 (GLUT4) from the cytoplasm to the cell surface. GLUT4 is highly expressed in skeletal muscle and adipose tissue, and when this transporter becomes abundant on these cell surfaces it allows for an insulin-dependent enhancement of glucose uptake (89). AKT is also thought to be instrumental in the mechanism of insulin's promotion of glycogen synthesis and glycolysis, by phosphorylating glycogen synthase kinase-3 and 6-phosphofructose 2-kinase, respectively (90; 91). The CAP/Cbl pathway is believed to work in parallel with the PI3K/Akt pathway in managing GLUT4 translocation, given that the translocation does not occur without the adaptor protein CAP (92; 93). In this CAP-dependent pathway, Cbl is phosphorylated by the insulin receptor and then recruits CrkII-C3G to lipid rafts, where C3G activates the GTP-binding protein TC10 (94). TC10's involvement in actin's spatial arrangement is thought to complement PI3K signaling in GLUT4 translocation (95; 96). Mitogenic insulin signaling is believed to be facilitated by the RAS/MAPK pathway (97). In this pathway, IR autophosphorylation promotes the formation of the Shc-Grb2 complex, which then associates with son-of sevenless (SOS) (98). SOS activates RAS, which ultimately leads to the activation of MAPK to influence DNA synthesis, cell growth, proliferation, and survival, and gene expression in a variety of tissues (97; 99; 100).

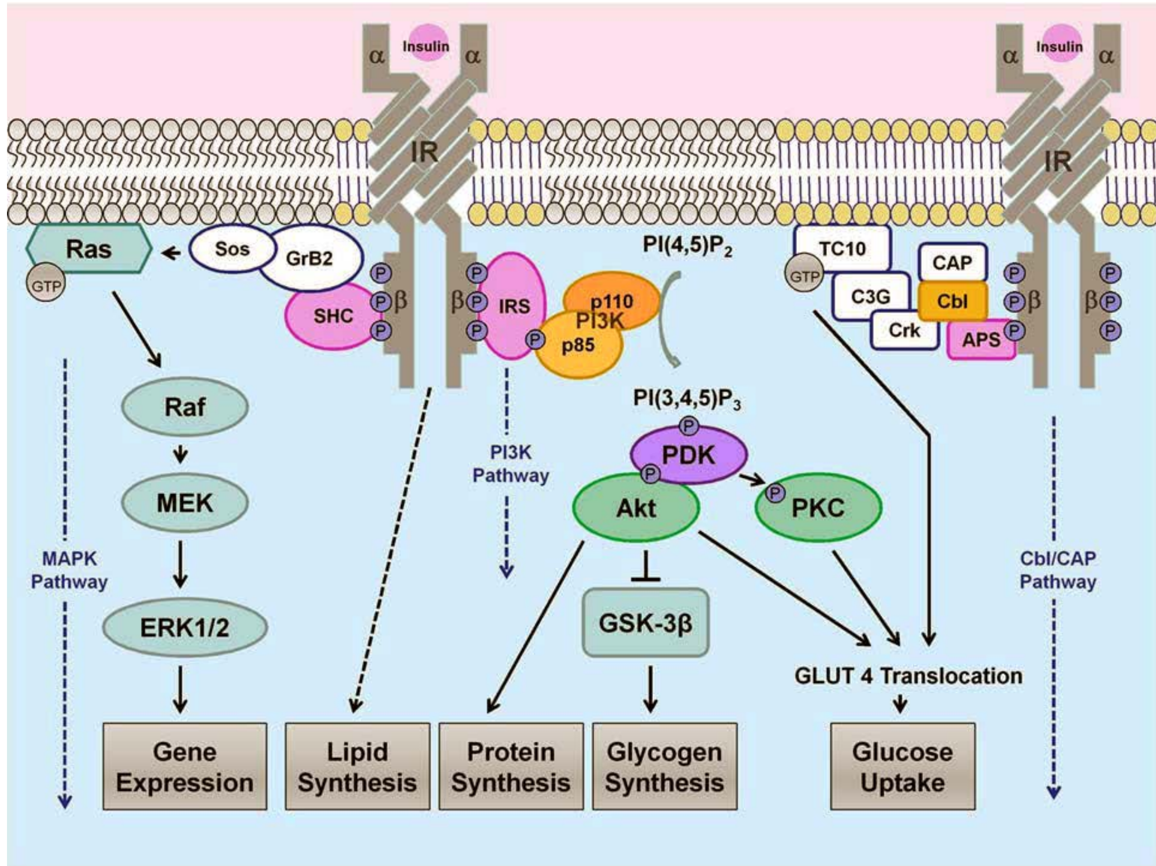


Figure 2.2: *Insulin Signaling via PI3K, Cbl/CAP, and MAPK*. When insulin binds its receptor on the cell membrane, it triggers the activation of three main intracellular signaling pathways: PI3K, Cbl/CAP, and MAPK (101).

2.4 Insulin Resistance

Insulin resistance is a physiological condition where insulin's effects on glucose uptake, metabolism, and/or storage are blunted (43). It is characterized by glucose intolerance and hyperglycemia accompanied by increased plasma concentrations of insulin (compensatory hyperinsulinemia), along with dyslipidemia: elevated free fatty acid and lowered high density lipoprotein (HDL) concentrations (82). As peripheral tissues, including adipose, muscle, and liver, become resistant to insulin, its effects on them are impaired, resulting in increased lipolysis in adipose tissue, reduced insulin-dependent glucose uptake into skeletal muscle, reduced glycogen synthesis, and increased

hepatic glucose production (43). Compensatory hyperinsulinemia is pitted against a gradual increase in severity of tissue insulin resistance until at some point the pancreatic beta cells break down and are unable to secrete enough insulin to regulate glucose metabolism (102). Inadequate expansion of β cell mass or dysfunctional β cell mass response to glucose are currently believed to be the two possible scenarios leading to beta cell failure (103). The evidence surrounding insulin resistance shows that it usually manifests with an “unhealthy” cluster of symptoms including central obesity, glucose intolerance, dyslipidemia, and hypertension that promotes the development of diseases such as type 2 diabetes mellitus, cardiovascular disease, cancer, hypertension, polycystic ovarian syndrome, and nonalcoholic fatty liver disease (104).

The phenomenon of insulin resistance is not yet fully understood. For instance, how does it develop systemically, affecting the whole body? The findings from multiple studies in tissue-specific knockout mice indicate that there is collaboration between tissues (104). This collaboration seems to be fascinatingly complex; involving mediation not only by classical hormones and the nervous regulation, but also several new classes of bioactive molecules produced and secreted by a variety of cell types, advancing our understanding of it is of critical importance in the elucidation of insulin resistance.

Mechanistic theories of insulin resistance within tissues are in the process of being characterized. While mutations in the insulin receptor gene affecting synthesis, degradation, and function have been seen in patients with severe insulin resistance (105), these are not the cause of the majority of insulin resistance in the general population where abnormalities in insulin receptor activity and defects in the downstream insulin signaling cascade have been noted (106; 107). Insulin receptor autophosphorylation and

tyrosine kinase activity have been found to be decreased in muscle and adipose tissues of insulin-resistant patients (108-111). Impaired IRS-1 phosphorylation and PI3K activation accompanies the reduced tyrosine kinase activity of obese and type 2 diabetic patients (112). Furthermore, many studies have implicated defects in intracellular signal transduction. These include c-Jun NH2-terminal kinase (JNK)-induced serine phosphorylation and thus inhibition of IRS-1 (113; 114), and also alterations in the activity of phosphotyrosine phosphatase-1B (PTP-1B), phosphatase and tensin homolog (PTEN), and SH2 domain-containing inositol 5-phosphatase 2 (SHIP2) lipid phosphatase (115; 116). Excessive adiposity is commonly implicated in the current understanding of cellular and molecular mechanisms of insulin resistance, which has also come to include humoral factors secreted by adipose tissue, the liver, and possibly muscle tissue (8).

2.4.1 Lipid overload and insulin resistance

There is a strong association between obesity, insulin resistance, and type 2 diabetes; however, the mechanisms involved appear to be complex and are not yet well understood. Insulin resistance is a pathophysiological hallmark of obesity, and it seems to be a major precursor in the development of type 2 diabetes (53; 54). While weight gain worsens insulin resistance, weight loss ameliorates it (117). Importantly, excess intra-abdominal (central) adiposity appears to be a key component in the pathogenesis of metabolic disorders and a more significant factor than the absolute amount of adipose tissue (53; 118; 119). In obese individuals, because their adipose tissue is resistant to the anti-lipolytic effect of insulin, it releases increased amounts of free fatty acids and glycerol into circulation (120). These circulating free fatty acids have been shown to decrease tyrosine phosphorylation of IRS-1 in skeletal muscle, which suppresses

downstream PI3K activity and thus reduces glucose uptake (121). Furthermore, lipid excess leads to increased intracellular amounts of fatty acid metabolites such as diacylglycerol (DAG) that leads to serine/threonine phosphorylation of IRS-1 and IRS-2, which renders them unable to activate PI3K, ultimately diminishing insulin signal transduction (122). Moreover, decreasing circulating lipid levels improves insulin activity in skeletal muscle, adipose tissue, and the liver (123). Not only does fat accumulation in the liver induce insulin resistance, impairing insulin's suppression of hepatic gluconeogenesis (124), but obesity-associated hepatic steatosis is associated with increased liver inflammation. The inflamed liver can be a primary source of systemic factors, including tumor necrosis factor (TNF)- α, interleukin (IL)-6, and IL-1β, that contribute to the development of insulin resistance (125).

2.4.2 Humoral factors and insulin resistance

There is a surplus of evidence in support of the theory that the activity of humoral factors is involved in the development of weight gain and insulin resistance. These humoral factors include adipokines such as adiponectin, resistin, leptin, and retinol binding protein-4 (RBP-4), proinflammatory cytokines such as TNF-α and IL-6, and other proteins that are involved in the development of insulin resistance (8; 126-128). Adiponectin, which has insulin-sensitizing, fat-burning, and anti-inflammatory properties, is one of the most abundant adipokines (129; 130). Ablation of the gene coding for adiponectin in mice causes lipid accumulation and severe insulin resistance (131), and administration of adiponectin to obese, insulin resistant mice improves their insulin sensitivity (132). Accumulating evidence suggests that the high molecular weight form of adiponectin is the most physiologically active, and is thus chiefly responsible for

these effects (133). Adiponectin has been found to be decreased in abdominally obese individuals, in contrast with the elevated levels of other adipocyte-secreted proteins like resistin, leptin, and RBP-4 (134). Resistin has been shown to increase hepatic glucose output in vivo and reduce insulin-dependent glucose transport in vitro (135-139). Leptin is speculated to have a possible effect on insulin resistance as well, due to its ability to stimulate PI3K activity in human muscle, adipose, and liver tissue (140; 141). RBP-4 induces insulin resistance by enhancing expression of the gluconeogenic enzyme phosphoenylpyruvate carboxykinase in the liver through a retinol-dependent mechanism, and also by reducing PI3K activty. Also, RBP-4 knockout mice exhibit enhanced insulin sensitivity (101).

Pro-inflammatory cytokines, such as TNF-α and IL-6 are produced by adipocytes and macrophages, serving as a link between chronic inflammation, obesity, insulin resistance, and diabetes (142; 143). Both cytokines upregulate inflammation by acting through classical receptor-mediated processes stimulating the JNK and IκB kinase-β (IKK-β)/nuclear factor-κB (NF-κB) pathways. Both of these pathways cause IRS serine phosphorylation, inhibiting insulin signaling, while simultaneously promoting the expression of inflammatory mediators that can lead to insulin resistance (144-147). TNF-α has been shown to induce insulin resistance, and mouse models lacking TNF-α function have improved insulin sensitivity and glucose homeostasis (74; 148); (149).

Many other proteins have regulatory roles on insulin activity, and under pathophysiological conditions they may contribute to insulin resistance. PTP-1B, which is ubiquitously expressed and stimulated by high blood glucose, has been demonstrated to have the capacity to dephosphorylate the insulin receptor (12). PTP-1B levels and activity

have been shown to be elevated in tissue culture models associated with insulin resistance (150), and overexpression of PTP-1B in rat adipocytes reduces glucose uptake and GLUT-4 translocation (151). Leukocyte antigen-related (LAR) phosphatases have been shown to cause whole body insulin resistance when overexpressed in mice by reducing glucose uptake (152). Plasma cell antigen (PC-1), a plasma membrane enzyme exhibiting the potential to inhibit insulin receptor autophosphorylation, is found to be elevated in muscle and fat tissue of insulin resistant humans (153). In addition, mice genetically altered to overexpress PC-1 are diabetic, exhibiting hyperglycemia and insulin resistance (154). Pten is a negative regulator of insulin signaling via PI3K, acting by inhibiting IRS-1 phosphorylation and thus blocking MAPK activation (155). Conversely, SH2B1 is considered to be an endogenous insulin sensitizer because of its ability to inhibit tyrosine dephosphorylation of IRS proteins (156). It is understood to play a key role in maintaining both body weight and glucose metabolism in mice, with its genetic deletion yielding obesity and insulin resistance (157).

2.6 Fetuin-A

2.6.1 Fetuin-A history and terminology

Fetuin is a plasma protein that was originally discovered in 1944 by Kai Pedersen in fetal bovine serum (158). Then, in 1961, the human form of fetuin, alpha2-Heremans Schmid glycoprotein (AHSG), was named after its co-discoverers (159). Decades would pass before the two proteins were recognized as orthologs in 1990 (160). With an abundance of other fetuin orthologs being discovered in other species, like rat phosphorylated protein 63 (pp63) (161), fetuin has been deemed the preferred name in

order to reduce confusion. It has been called fetuin-A ever since the year 2000, when a second member of the fetuin family was identified and named fetuin-B (162).

2.6.2 Structure of fetuin-A

The human fetuin-A gene spans approximately 8.2 kb of chromosome 3, composed of seven exons divided by six introns (163). Fetuin-A gene transcription produces a single 1.8 kb mRNA strand that is then translated into a polypeptide chain of 367 amino acids (164). Human fetuin-A is a 55-59 kDa protein consisting of two subunits, an A chain (residues 19-300) and a B chain (residues 341-367), made up of 282 amino acids and 27 amino acids, respectively (28). These two subunits are connected by a single disulfide bond and, unless it has been removed by limited proteolysis post-translationally, a 40 amino acid "connecting peptide" (residues 301-340) (164). This limited proteolysis of the connecting peptide could explain the observation that in contrast to all other species orthologs studied to date except other higher primates, human fetuin-A exists in two forms with different apparent molecular weights and isoelectric points (28). Also, since recombinant human fetuin-A made in insect cells only features the single strand form, it is believed that proteolysis is a post-secretory event (165). The A chain has four glycosylation sites. There are two N-glycans attached to asparagine residues 138 and 158 and two O-glycans attached to threonine residues 238 and 252 (34). Also, fetuin-A can be phosphorylated at two sites: Ser120 of the A-chain and Ser312 of the connecting peptide. It has been shown that 77% of total phosphorylation occurs at Ser312, and that up to 20% of circulating fetuin-A is phosphorylated at least at one site (35). Phosphorylation is believed to be necessary for some biological activity; therefore,

the heterodimeric form of fetuin-A lacking the connecting peptide's Ser312 residue is hypothesized to be inactivated (36).

The three-dimensional structure of fetuin-A still needs to be fully characterized, but it has been reported as globular with a diameter of ~55 Å (28). Its secondary structure is known to be characterized by six disulfide linkages. One between the first and the last cysteine residues of the amino acid sequence connecting the A subunit to the B subunit, and five others linked consecutively between cysteines in the A chain (28; 166). The pattern of these disulfide bridges yields two cystatin-like domains, so fetuin-A is classified as a member of the cystatin superfamily (167). These cystatin-like domains make up two of a tripartite structure (illustrated in Fig. 2.3); the third domain is a unique, proline-rich COOH-terminal domain (30). Human, porcine, ovine, murine, and bovine forms of fetuin-A show ~75% amino acid sequence similarity (168). The mouse and rat orthologs exhibits 60% and 56% amino acid sequence similarity with human fetuin-A, respectively (161; 169). Fig. 2.4 depicts the alignment of mouse, rat, and human fetuin-A sequences. Furthermore, fetuin-A orthologs in rat, sheep, pig, mouse and human exhibit 80–90% homology at the cDNA level (170; 171). In the genomes of all of these species, and in other vertebrates that have been found to have orthologs, the 12 cysteine residues are highly conserved. This suggests that the six-disulfide arrangement may contribute to a common structure between species, probably implicating it in fetuin-A's functional role (28; 36).

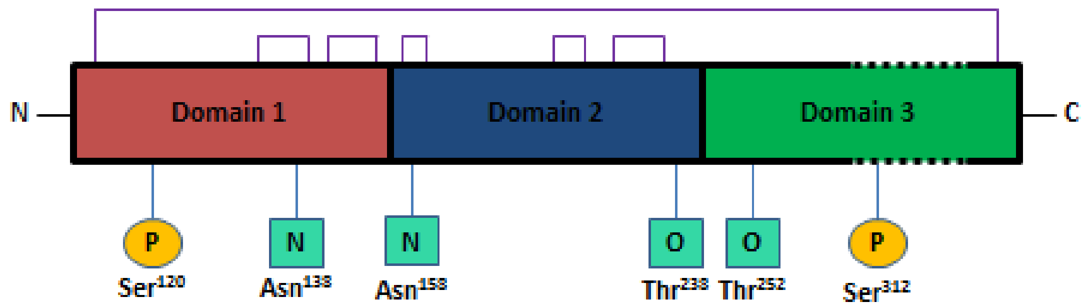


Figure 2.3: *The tripartite domain structure of human fetuin-A*. Domains 1 (res 34-138) and 2 (res 140-246) are cystatin-like domains, while Domain 3 contains a proline-rich region. The six disulfide bridges are shown as purple lines connecting at their related cysteine residues. The A subunit (residues 19-300) is connected to the B subunit (residues 341-367) by the 40 residue connecting peptide shown as the dashed portion of Domain 3. Post-translational modifications are shown at their relative locations.

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Human fetuin-A:    sp_P02765 MKSLVLLLCLAQLWGCHSAPHGPGLIYRQPNCDDPETEEAALVAIDYINQN  51
Mouse fetuin-A:    sp_P29699 MKSLVLLLCFAQLWGCQSAPQGTGLGFRELACDDPEAEQVALLAVDYLNNH
Rat fetuin-A  :    sp_P24090 MKSLVLLLCFAQLWSCQSAPQGAGLGFRELACDDPETEHVALIAVDYLNKH

                   NLPWGYKHTLNQIDEVKVWPQQPSGELFEIEIDTLETTCHVLDPTPVARCSVRQLKEHAVE 111
                   HLLQGFKQVLNQIDKVKVWSRRPFGVVYEMEVDTLETTCHALDPTPLANCSVRQLTEHAVE
                   HLLQGFRQILNQIDKVKVWSRRPFGEVYELEIDTLETTCHALDPTPLANCSVRQQAEHAVE

                   GDCDFQLLKLDGKFSVVYAKCDSSPDSAEDVRKVCQDCPLLAPLNDTRVVHAAKAALAAFN 172
                   GDCDFHILKQDGQFRVMHTQCHSTPDSAEDVRKLCPRCPLLTPFNDTNVVHTVNTALAAFN
                   GDCDFHILKQDGQFRVLHAQCHSTPDSAEDVRKFCPRCPILIRFNDTNVVHTVKTALAAFN

                   AQNNGSNFQLEEISRAQLVPLPPSTYVEFTVSGTDCVAKEATEAAKCNLLAEKQYGFCKAT 233
                   TQNNGTYFKLVEISRAQNVPLPVSTLVEFVIAATDCTAKEVTDPAKCNLLAEKQHGFCKAN
                   AQNNGTYFKLVEISRAQNVPFPVSTLVEFVIAATDCTGQEVTDPAKCNLLAEKQYGFCKAT

                   LSEKLGGAEVAVTCTVFQTQPVTSQPQPEGANEAVPTPVVDPDAPPSPPLGAPGLPPAGSP 294
                   LMHNLGGEEVSVACKLFQ-----TQPQPANANAVGPVPT----ANAALPAD---------P
                   LIHRLGGEEVSVACKLFQ-----TQPQPANANPAGPAPTVGQAAPVAPPAG---------P

                   PDSHVL--LAAPPGHQLHRAHYDLRHTFMGVVSLGSPSGEVSHPRKTRTVVQPSVGAAAGP 353
                   PASVVVGPVVVPRGLSDHRTYHDLRHAFSPVASVESASGETLHSPK---VGQP---GAAGP
                   PESVVVGPVAVPLGLPDHRTHHDLRHAFSPVASVESASGEVLHSPK---VGQPGDAGAAGP

                   VVPPCPGRIRHFKV 367
                   VSPMCPGRIRHFKI
                   VAPLCPGRVRYFKI
```

Figure 2.4: *Amino acid sequence alignment of fetuin-A from Homo sapiens, Mus musculus, and Rattus norvegicus*. Multiple sequence alignment was performed using Phylogeny Inference Package (Phylip). The displayed sequences include the signal peptide (amino acids 1-18). These sequnces are further processed into the mature form. Phosphorylation sites Ser120 (Ser138 including the 18 aa signal peptide) and Ser 312 (Ser330 including the 18 aa signal peptide) are highlighted in yellow.

2.6.3 Fetuin-A and biological significance

Fetuin-A is produced and secreted into circulation by hepatocytes and osteoblasts (36; 172), but why? The functional role of fetuin-A has been discussed in hundreds of articles since it was discovered over 60 years ago. The longest-lived theory, drawing originally from speculation that fetuin-A is the active substance in fetal bovine serum, is that it plays an important role in growth and development (173). Further evidence in favor of this role is that in the developmental stage of multiple species, fetuin-A has been detected to be highly concentrated in specific organs like bone marrow, brain, and liver, until it drops off soon after birth. This suggests that it may be a significant element of the development of these organs (174-179). The evidence for fetuin-A's role in growth and development is offset by the finding that it is not essential for embryogenesis given the viability of mice lacking the gene (180). Also, during the third trimester of pregnancy, fetuin-A circulates at its highest concentrations, which can reach up to 10-22 g/L (181). In adult humans, fetuin-A is released into circulation at a mean serum concentration of 0.6 g/L (182). Fetuin-A is highly abundant in bone matrix, and is thought to have a possible role in bone formation and resorption (183; 184). In cases of Paget's disease, an affliction of increased bone turnover with disordered and thickened bone, patients have lower levels of fetuin-A (185). Fetuin-A has also been implicated in insulin and growth factor signaling (29; 186-188).

There are many other possible biological roles of fetuin-A. It has been identified as a negative acute phase reactant in humans, having a significantly decreased circulatory presence in the case of infection or cancer (182; 189; 190). In contrast, plasma fetuin-A levels were increased in patients after ischemic stroke or cattle after trauma (23; 191),

implying that fetuin-A may also function as a positive acute phase reactant. It may act as an opsonizing agent (192). Fetuin-A has been implicated in lipid binding, associating with a variety of lipids to form a particle similar to high density lipoproteins (193). It is also thought to act as a stimulator of lipogenesis (194). There is a growing body of evidence to show that fetuin-A has a role in the cardiovascular system, possibly having dual functionality as an atherogenic factor and/or an inhibitor of vascular calcification (195). Interestingly, it has been suggested that fetuin-A may be a physiological modulator of the insulin receptor (171).

It is possible that fetuin-A could have multiple biological roles, especially between species since species-specific differences in its gene regulation have been observed (191; 196-198). Further, phosphorylation status of fetuin-A has been shown to be associated with its biological activity (199). However, none of the fetuin-A secreted by osteoblasts is phosphorylated (36). On top of this divergence, the limited proteolysis that appears to only occur in humans and/or the structural segmentation of fetuin-A's tripartite structure could explain the protein's apparent diversity of function (200).

2.6.4 Fetuin-A is associated with metabolic disorders

Since the fetuin-A gene is localized on chromosome 3q27 (17), a susceptibility locus for type 2 diabetes and metabolic syndrome (18; 19), it is reasonably suspected of being associated with metabolic disorders. Two important longitudinal studies conducted recently have found positive associations between circulating fetuin-A and incident type 2 diabetes (13; 16). In a cohort from the Health ABC study of older humans fetuin-A was associated with incident diabetes independent of physical activity, inflammatory biomarkers, and other measures of insulin resistance (13). Similarly, in a sub-cohort of

2500 individuals from the EPIC-Potsdam study, fetuin-A was associated with incident diabetes, after adjusting for age, sex, BMI, waist circumference, lifestyle risk factors, glucose, triglycerides, HDL-cholesterol, gamma-glutamyl transferase, and hs-C-reactive protein (16). Together these two studies can be taken as evidence that fetuin-A is an independent risk factor for type 2 diabetes. There are also multiple associations between circulating fetuin-A levels and insulin resistance found in the literature. Plasma fetuin-A levels were positively associated with maternal insulin resistance and elevated in patients with gestational diabetes than in healthy pregnant women (20). Fasting plasma fetuin-A levels were found to be higher in patients with IGT than individuals with normal glucose tolerance (15). A correlation has been found between serum fetuin-A levels and insulin resistance in non-diabetics (14). In obese children, not only were higher fetuin-A levels correlated with insulin resistance, nonalcoholic fatty liver disease, and features of the metabolic syndrome, but fetuin-A levels were decreased after weight loss (201). Furthermore, higher plasma fetuin-A levels have been found to be associated with elevated risk of metabolic syndrome (21), decreased insulin sensitivity (15), increased visceral adiposity (22), liver fat accumulation (15), an atherogenic lipid profile (21), and a greater risk of both myocardial infarction and ischemic stroke (23).

2.6.5 Fetuin-A gene variations are associated with metabolic traits

Since 1977, the fetuin-A gene has been known to be characterized by high genetic variation (202). In humans, more than 30 gene variants have been identified in different ethnic groups (203). Depending on the variation, fetuin-A's conformation can be altered, which is likely the case in Arg299Cys where an additional cysteine residue is present (203). Also, variation in the promoter has been found to impact transcriptional activity

and thus serum concentrations of fetuin-A (204). Interestingly, just as one single nucleotide polymorphism (SNP) was found to be associated with an increased risk for type 2 diabetes in French Caucasians (24), another was found to be associated with leanness in Swedish men (25). Moreover, in Danish whites, two different SNP's were positively correlated with incident type 2 diabetes and dyslipidemia while a third SNP was associated with increased insulin sensitivity (26). A different SNP has been shown to be associated with a resistance to body fat accumulation through a significant increase in beta2-adrenoreceptor sensitivity and thus subcutaneous adipose lipolysis (205), and yet another SNP was reported to be associated with insulin-mediated lipolysis (206). These data indicate that fetuin-A gene variations may contribute to the variety of metabolic traits in individuals, and it has even been suggested that the fetuin-A gene could be a type of "thrifty gene" that geneticist James Neel described decades ago (27; 28).

2.6.6 Fetuin-A is a physiological regulator of insulin action

In agreement with fetuin-A's many associations with metabolism and insulin resistant conditions, there is a growing body of evidence suggesting that fetuin-A plays a role in the regulation of insulin action. Fetuin-A has been identified as a natural inhibitor of the IR autophosphorylation and IR tyrosine kinase activity, both *in vitro* and *in vivo* (29; 186). This inhibition has been extended to other species including mice, sheep, pigs, and cattle (31; 169). Interestingly, fetuin-A has been shown to interact directly with the IR, preferring its activated form; however, it does not compete with insulin binding (171; 207). Fetuin-A null mice displayed increased basal and insulin-stimulated IR phosphorylation, and also increased activity of downstream signaling molecules such as MAPK and AKT in their liver and skeletal muscle. They also exhibited improved insulin

sensitivity, resistance to high-fat diet-induced weight gain, and lower free fatty acid and triglyceride levels compared with wild-type control mice (32). Furthermore, these mice were protected against obesity and insulin resistance associated with aging (33). Additionally, in a rat model of diet-induced obesity, a significant increase in fetuin-A gene expression has been observed (208). Studies have linked fetuin-A into the growing molecular map of inflammation and insulin sensitivity. The expression of fetuin-A is downregulated by proinflammatory cytokines such as TNF-α, IL-1, and IL-6 (209). Hennige et al. have recently shown that fetuin-A may play a role in the upregulation of TNF-α and IL-1β, while suppressing the production of insulin-sentizing adiponectin (210). Previously in our lab, Papizan and Mathews have shown increased plasma fetuin-A Ser312-phosphorylation in insulin resistant, leptin-signaling impaired Zucker diabetic fatty (ZDF) rats and in leptin-deficient *ob/ob* mice, compared to lean controls, suggesting roles for leptin and insulin in the regulation of Ser312-phosphorylation of fetuin-A (38). All of these findings taken together suggest the possibility of a pathophysiological role for fetuin-A in diabetes, potentially through regulating insulin sensitivity.

2.7 Objectives and hypothesis

The goal of this study was to characterize fetuin-A and its phosphorylation status in a mouse model of diet-induced obesity, and observe its dynamics during an oral glucose tolerance test. We hypothesized that fetuin-A and phosphofetuin-A will be higher in diet-induced obese mice, and that they will not decrease in response to the oral glucose challenge.

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3. Altered temporal changes in Ser312-phosphofetuin-A concentrations in response to a glucose challenge in a mouse model of diet-induced obesity and insulin resistance

Abstract

Fetuin-A, a liver-secreted protein with the capacity to negatively regulate insulin action, has been implicated in the growing molecular framework of obesity and insulin resistance. Phosphorylation has been hypothesized to be critical for its ability to inhibit insulin receptor tyrosine kinase activity. This study examined total fetuin-A and Ser312-phosphofetuin-A concentrations in diet-induced obese (DIO) C57Bl6 mice, and characterized their temporal dynamics during an oral glucose tolerance test. No significant impact of high-fat diet feeding on total fetuin-A concentration was found. However, plasma Ser312-phosphofetuin-A concentrations were significantly elevated in DIO mice compared to their control counterparts, and showed a positive correlation with insulin resistance. In response to an oral glucose tolerance test, plasma concentrations of Ser312-phosphofetuin-A showed a significant temporal increase in mice fed regular chow, whereas in the DIO model, Ser312-phosphofetuin-A showed a significant temporal decrease. Thus, Ser312-phosphofetuin-A concentrations are elevated in insulin resistant conditions, and phosphofetuin-A may be dynamically involved in the regulation of blood glucose concentrations following an oral glucose load.

Introduction

Insulin resistance is a pathophysiological hallmark of obesity, and a major precursor in the development of type 2 diabetes (1; 2). Various humoral factors, including adiponectin, resistin, RBP-4, TNF-α, IL-6, and PTP-1B, have been shown to act as physiological regulators of insulin action by influencing whole body insulin sensitivity, suggesting a potential role of these molecules in the development of insulin resistance and diabetes (3; 4). Recent studies suggest the liver-secreted, phosphorylated glycoprotein, fetuin-A, may be a component of the molecular framework of insulin resistance as well.

Fetuin-A has been demonstrated to be a potent inhibitor of insulin receptor tyrosine kinase activity, thus having the capacity to act as a negative regulator of insulin action (5-10). It is believed to directly interact with the β subunit of the insulin receptor, in a manner preferential to its activated form noncompetitive with insulin binding, inhibiting insulin-stimulated insulin receptor autophosphorylation (6; 11). Fetuin-A gene expression has been observed to increase in rat models of diet-induced obesity (12-14). Studies on fetuin-A null mice showed improved insulin sensitivity and resistance to weight gain (15). Further, these mice were also protected against obesity and insulin resistance associated with aging (16). In humans, serum fetuin-A concentrations were positively correlated to obesity, insulin resistance, metabolic syndrome, and accumulation of liver fat (13; 17-21). Recently, fetuin-A has also been designated as an independent risk factor for the development of type 2 diabetes (22; 23).

Fetuin-A has been shown to undergo post-translational modifications including N- and O-glycosylation and phosphorylation (24; 25). Approximately 20% of the

circulating plasma fetuin-A is phosphorylated on Ser120 and Ser312, with the majority of phosphorylation (~ 77%) on Ser312 (25). Only the phosphorylated form of the protein was shown to be active as an inhibitor of insulin receptor tyrosine kinase and receptor autophosphorylation (5; 26). Further, insulin and IL-1β were shown to differentially regulate fetuin-A phosphorylation, with insulin dramatically decreasing the phosphorylation of fetuin-A, and IL-1β markedly diminishing the mRNA transcription and modifying the N-glycosylation process of fetuin-A (27). However, our understanding of fetuin-A phosphorylation status in insulin resistance is limited (28; 29). In this study, we have evaluated Ser312-fetuin-A phosphorylation status in diet-induced obese, insulin resistant C57Bl6 mice, and characterized the effect of an oral glucose challenge on temporal changes in Ser312-phosphofetuin-A in this model.

Materials and Methods

Animal subjects

The animal use and euthanasia protocols implemented in this study were approved by Auburn University's Institutional Animal Care and Use Committee. Five-week old male C57Bl/6 mice were purchased from Charles River Laboratories (Indianapolis, IN). Mice were housed on a 12-hour light/dark cycle with free access to water. Mice in the control group (n=8) were fed a low-fat diet (10% kcals from fat, Research Diets, New Brunswick, NJ). Mice in the high-fat group (n=8) were fed a high-fat diet (45% kcals from fat, Research Diets, NJ). Mice were fed the above diets *ad libitum* with free access to water for a period of 8 weeks. Body weight was recorded at baseline and weekly during the study period. At the end of the 8th week, the mice were subjected to a 6-hour fast overnight, after which an oral glucose tolerance test was administered (2 mg/g). Blood samples were collected by snipping less than 1 mm of the tail, and gently stroking the tail. Blood samples were obtained at 0, 15, 30, 60, and 120 minutes, centrifuged, and serum samples were stored at -80^{o} C.

Metabolic analysis

Blood glucose levels were determined using an Accu-Chek glucometer from Roche Diagnostics (Indianapolis, IN). Insulin levels were measured by a mouse enzyme-linked immunosorbent assay (ELISA) from Crystal Chem (Downers Grove, IL). HOMA values were calculated according to (30), using the following formula: Insulin (μU/mL) × Glucose (mmol/L)/22.5. Insulin to glucose ratio was calculated as Insulin (μU/mL)/Glucose (mmol/L). Total adiponectin was measured with a commercial mouse ELISA kit from ALPCO (Salem, NH).

SDS-PAGE and Western analysis

For total fetuin-A, serum from whole blood samples was diluted in sterile saline 1:100 and quantified using the Bradford protein assay. Samples were boiled in a 1:1 sample:Laemmli Sample Buffer (Bio-Rad, Hercules, CA) mixture, and separated on 4-20% SDS-PAGE with Tris-Glycine-SDS buffer (IBI, Foxboro, MA). We used 10-well precast gels from NuSep (Bogart, GA) for the fasting and fed samples, and 18-well Criterion Precast gels from Bio-Rad (Hercules, CA) for the OGTT samples. Separated proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) by the semi-dry transfer method using transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol). Membranes were blocked overnight in 5% milk (non-fat dry milk; Bio-Rad, Hercules, CA) and immunoblotted with polyclonal goat anti-mouse fetuin-A (R&D Systems, Inc., Minneapolis, MN) for total fetuin-A analyses or a custom rabbit anti-human phosphorylated Ser312 fetuin-A antibody, which was generated using the epitope "HTFMGVVSLGSPS(PO4)GEVSHPR" and affinity purified using non-phospho and phospho peptides (Affinity BioReagents, Golden, CO). Blots were developed using the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) for total fetuin-A, and SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) for Ser312-phosphofetuin-A, and imaged using the UVP BioImaging and LabWorks software package (UVP, Upland, CA). Relative area densities were quantified using the UN-SCAN IT software package, v.6.1 (Silk Scientific, Orem, UT).

Statistical analyses

Data are presented as means ± SE. Statistical significance was determined using one-way ANOVA and unpaired t tests where appropriate. Correlation was evaluated

using Pearson product-moment correlation coefficient. Statistical significance was declared for p values less than 0.05. Statistical analysis was performed using GraphPad Prism v. 5 (GraphPad, San Diego, CA).

Results

4.1 High-fat diet-induced body weight changes and metabolic indices

Beginning with the second week, body weights of mice fed a high-fat diet (45% kcals from fat) were significantly higher compared with mice fed a low-fat diet (10% kcals from fat) (Fig.4.1). At the end of 8 weeks, mice fed the high-fat diet had gained a significantly greater amount of weight than the low-fat fed control mice (39.7 ± 1.1 g versus 29.1 ± 0.6 g, respectively). As expected, at the end of 8 weeks of HF feeding, fasting (12-hour) blood glucose (HF: 169.4 ± 14.8 mg/dL vs. LF: 132.8 ± 6.9 mg/dL, $p = 0.041$) and insulin concentrations (HF: 0.93 ± 0.13 ng/mL vs. LF: 0.46 ± 0.06 ng/mL, $p = 0.005$) were significantly higher compared to LF-fed mice (Fig. 4.2). Insulin concentrations were significantly elevated in the fed condition in HF-fed mice, compared to LF-fed mice. In addition to the observed hyperinsulinemia, HF-fed mice demonstrated significantly higher HOMA-IR index and insulin/glucose ratio indicating that the HF-fed mice were insulin resistant (Fig. 4.2). Total adiponectin was not significantly different between LF- and HF-fed mice (Fig. 4.3). Total fetuin-A levels were not significantly different between the LF- and HF-fed groups. However, in LF-fed mice, total fetuin-A levels were significantly elevated in the fed state compared to the fasted condition (Fig. 4.4A). Further, Ser312-phosphofetuin-A levels were found to be significantly elevated in both the fasted and fed conditions in high-fat mice compared to LF-fed mice (Fig. 4.4B). Furthermore, serum Ser312-phosphofetuin-A levels were positively correlated with body weight ($r = 0.64$, $p = 0.008$), insulin ($r = 0.58$, $p = 0.018$), and HOMA-IR ($r = 0.59$, $p = 0.016$) (Table 4.1).

4.2 Fetuin-A response to an oral glucose load

Since phosphofetuin-A concentrations were altered with high-fat feeding, we wished to characterize fetuin-A and phosphofetuin-A changes in response to an oral glucose load. Therefore, following 8 weeks of feeding a high-fat diet, mice were administered an oral glucose tolerance test. High fat-fed mice demonstrated significantly elevated area under the curve for glucose ($Glucose_{AUC}$, $p < 0.001$) and for insulin ($Insulin_{AUC}$, $p < 0.001$) (Fig. 4.5 & 4.6), indicative of glucose intolerance and insulin resistance. The area under the curve for fetuin-A and phosphofetuin-A were not significantly altered (Fig.4.5). Surprisingly, we observed temporal changes in phosphofetuin-A levels that were statistically significant. In the low fat-fed control mice, phosphofetuin-A levels were significantly elevated at the 30 ($p < 0.01$), 60 ($p < 0.001$), and 120-minute ($p < 0.001$) time points, compared to baseline (time zero). Conversely, in the high fat-fed mice, phosphofetuin-A levels showed a significant decrease at 15 ($p < 0.01$) and 30-minute ($p < 0.01$) time points (Fig.4.6B), compared to baseline (time zero) (Fig. 4.6D). By 120 minutes, phosphofetuin-A levels were comparable to levels at baseline (time zero). Though total fetuin-A concentrations showed a similar trend in temporal changes as phosphofetuin-A, these were not different statistically. These findings, showing temporal alterations in phosphofetuin-A following an oral glucose load that are divergent depending on insulin sensitive versus insulin resistant state, are novel, and suggest that phosphofetuin-A may play a potential role in the regulation of insulin action.

Discussion

The C57Bl/6 mouse, when fed a high-fat diet, provides a well-established and commonly used research model for simulating obesity, insulin resistance, and type 2 diabetes (31). After 8 weeks on the high-fat diet, our mice were insulin resistant. Of the several methods used for determining insulin resistance, the euglycemic-hyperinsulinemic clamp is considered the gold standard (32; 33); however, it is more laborious and time-consuming than other indirect methods of analysis. HOMA-IR, a valid and accurate alternative to the clamp, evaluates insulin sensitivity using fasting plasma glucose and insulin values, and has been used in several studies to gauge insulin resistance (30; 34-37). By conducting HOMA-IR calculations, we concluded that our high-fat fed mice had become insulin resistant, and were therefore useful for studying type 2 diabetes.

Fetuin-A has been shown to have an inhibitory effect on insulin action by interacting with the activated insulin receptor and inhibiting insulin receptor autophosphorylation and IR-TK activity *in vitro* and in peripheral tissues in animals (5; 6; 9; 38). Human fetuin-A is phosphorylated on Ser120 and Ser312, both of which lie in the sequence Ser-Xaa-Glu/Ser(P), which is a conserved recognition motif for phosphorylation of secreted proteins (25; 39). While 2 other potential serine phosphorylation sites have been identified in the rat, serine-310 and serine-307 (40), the conserved Ser312 phosphorylation site has been shown to account for 2 of the 3-3.6 molecules of protein-bound phosphate of rat fetuin-A. This study has clearly shown that, Ser312-phosphofetuin-A, but not total fetuin-A concentrations, were significantly increased in the obese, insulin resistant mice, compared to LF-fed mice, both in the fasted

and fed conditions. Further, our findings from this study have indicated that serum Ser312-phosphofetuin-A concentrations were strongly correlated with body weight, insulin, and HOMA-IR. These findings may have a significant impact in fetuin-A biology, since phosphorylation has been previously shown to be critical for fetuin-A's inhibitory activity (5; 26). Thus, assessment of serum Ser312-phosphofetuin-A concentrations may offer better insights into understanding the molecular framework of insulin resistance than total fetuin-A concentrations.

In this study, we did not observe alterations in total adiponectin concentrations with HF-feeding. Further, no correlations were observed between fetuin-A or Ser312-phosphofetuin-A and total adiponectin concentrations. Previous studies have shown that fetuin-A was negatively correlated to total adiponectin in humans (41-43), and in mice treated with fetuin-A, adipose tissue Adipoq mRNA expression and circulating adiponectin levels were decreased (43). While the duration of the HF-feeding period, the composition of the diet, and/or the small sample size may have contributed to the current lack of correlation of total fetuin-A or Ser312-phosphofetuin-A to total adiponectin, additional studies are required to elucidate the significance and role of phosphofetuin-A with HMW adiponectin, obesity and insulin resistance.

Administration of an oral glucose tolerance test yielded interesting findings on the dynamics of Ser312-phosphofetuin-A concentrations in LF- versus HF-fed mice. Ser312-phosphofetuin-A concentrations showed a significant and temporal increase at 30, 60, and 120-minute time points, following an oral glucose load in LF-fed mice. On the contrary, in the obese, insulin resistant HF-fed mice, Ser312-phosphofetuin-A concentrations were significantly decreased at the 15 and 30-minute time points,

following the oral glucose load, which returned to levels comparable to baseline at the 120-minute time-point. In this, we provide evidence for the first time that phosphofetuin-A may be implicated in the normal mechanism of insulin action. It may be speculated that in normal mice (LF-fed), the increased Ser312-phosphofetuin-A concentration, following a glucose load, enhances interaction of fetuin-A with the activated IR (6), and inhibits insulin receptor autophosphorylation and tyrosine kinase activity, thereby blunting the insulin response. In the obese, insulin resistant HF-fed mice, the temporal decrease in Ser312-phosphofetuin-A concentrations could possibly reflect a compensatory mechanism to improve insulin action.

There are several limitations to this study. First, fetuin-A and Ser312-phosphofetuin-A concentrations were assessed by Western blotting techniques, which is only semi-quantitative. Secondly, our Ser312-phosphofetuin-A antibody was generated against human Ser312-phosphorylated fetuin-A epitope. It is hypothesized that phosphorylation sites, mechanisms, and functions arising from phosphorylation mechanisms are similar in mice and humans. Causality cannot be implied due to the lack of mechanistic evidence in this study. Despite these limitations, our data suggests that dysregulation of phosphofetuin-A is a feature of insulin resistance, and further investigation into the nature of this association is warranted. Fetuin-A does not require the proximal 576 amino acids of the alpha subunits of the IR (6); thus further experimentation to elucidate the mechanisms by which fetuin-A interacts with the IR beta subunit, how this complex is internalized, and the dynamics of regulation of insulin receptor activation need to be understood. Future studies characterizing the role of this “hepatokine” in metabolism and its associated pathophysiological conditions may lead to potential therapies to treat diseases like type 2 diabetes.

Table 4.1: *Correlation of fetuin-A, phosphofetuin-A, and adiponectin with body weight, insulin, glucose, HOMA-IR, and insulin/glucose ratio*.

	Body weight	**Insulin**	**Glucose**	**HOMA-IR**	**Insulin/Glucose**
Fetuin-A	0.46	0.32	0.01	0.33	0.27
Phosphofetuin-A	0.64**	0.58*	0.14	0.59*	0.48
Adiponectin	-0.10	-0.21	-0.06	-0.19	-0.20

Data are shown as ‘r value’ representing Pearson product-moment correlation coefficient (n = 16, for each parameter). HOMA-IR, Homeostasis model assessment-insulin resistance index, * $p < 0.05$, **$p < 0.01$.

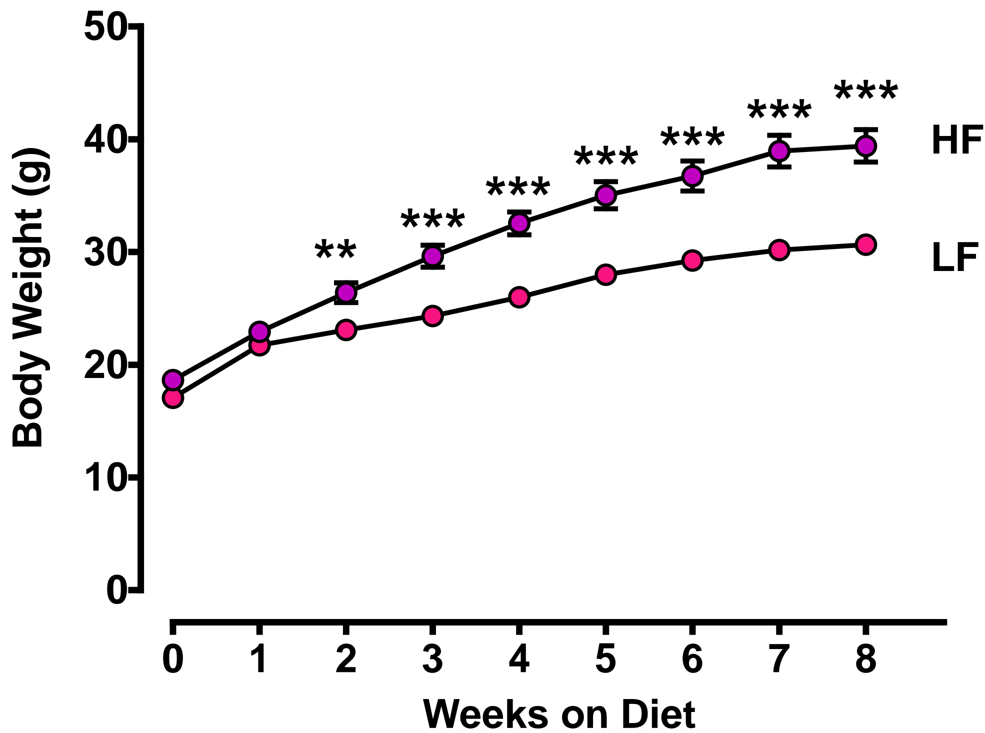
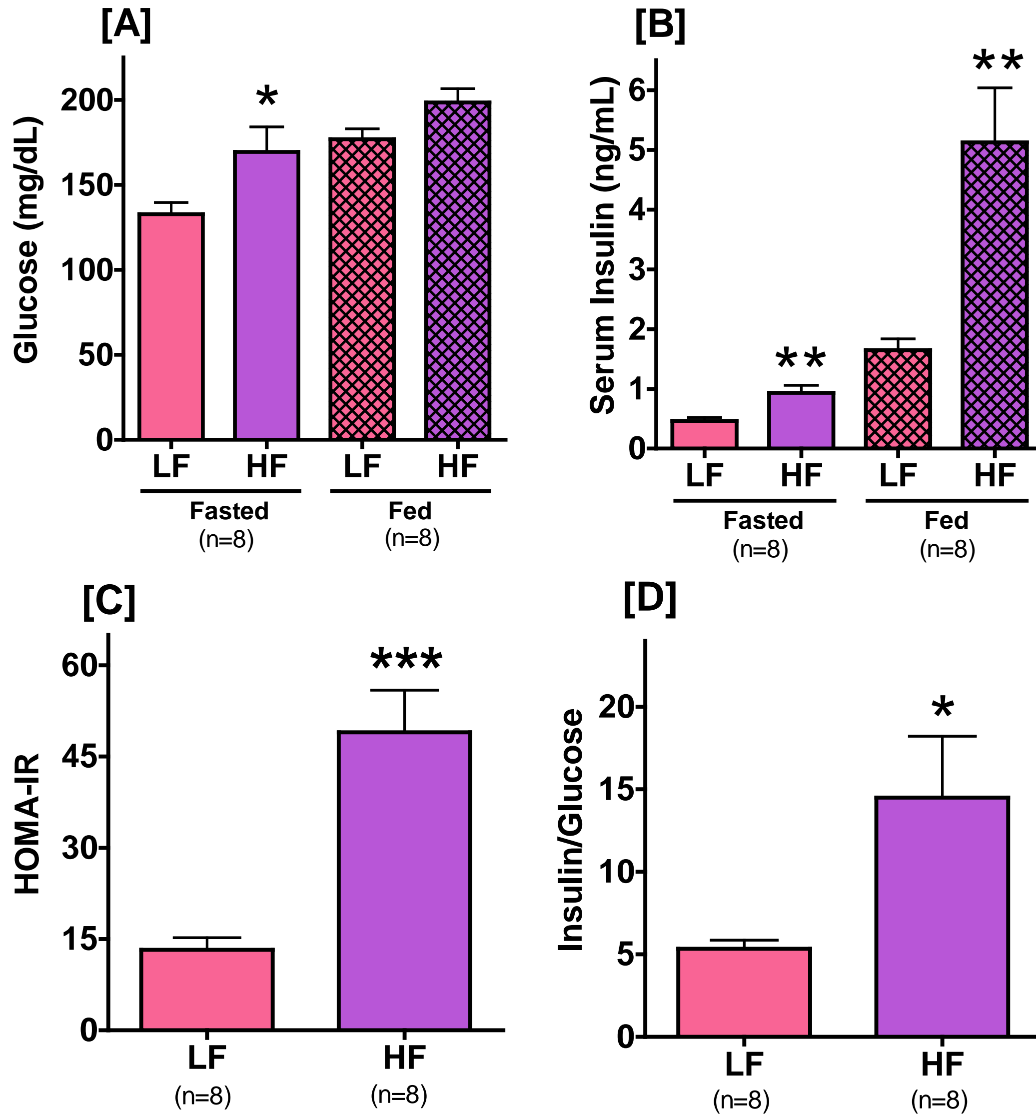
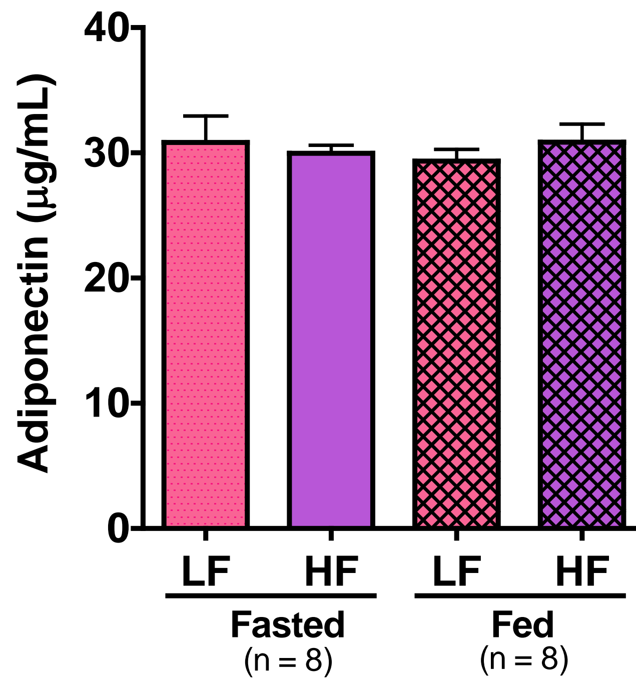


***Fig. 4.1**: Body weight changes in C57BL/6 mice fed a high-fat diet*. C57Bl/6 mice were fed either a low-fat diet (LF) (10% kcals from fat, Research Diets, New Brunswick, NJ) or high-fat diet (HF) (45% kcals from fat, Research Diets), *ad libitum*, for a period of 8 weeks (n=8 per group). Body weight was recorded at baseline and weekly during the study period. Data are shown as mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$.



<u>Fig. 4.2</u>: *<u>Metabolic markers of insulin resistance</u>*. Following 8 weeks of feeding C57Bl/6 mice either a low-fat (LF) or a high-fat (HF) diet, blood glucose (A) and serum insulin (B) were assessed in the fasted (12-hour overnight fast) and fed conditions. HOMA-IR (C) and insulin/glucose ratio (D) were assessed as surrogate measures of insulin resistance in the fasted state. Data are shown as mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.



<u>Fig. 4.3</u>: *<u>Serum total adiponectin in HF-fed C57Bl/6 mice</u>*. Serum total adiponectin levels were assessed in the fasted (12-hour overnight fast) and fed conditions in mice fed a LF- or HF-diet for a period of 8 weeks. Data are shown as mean ± SEM.

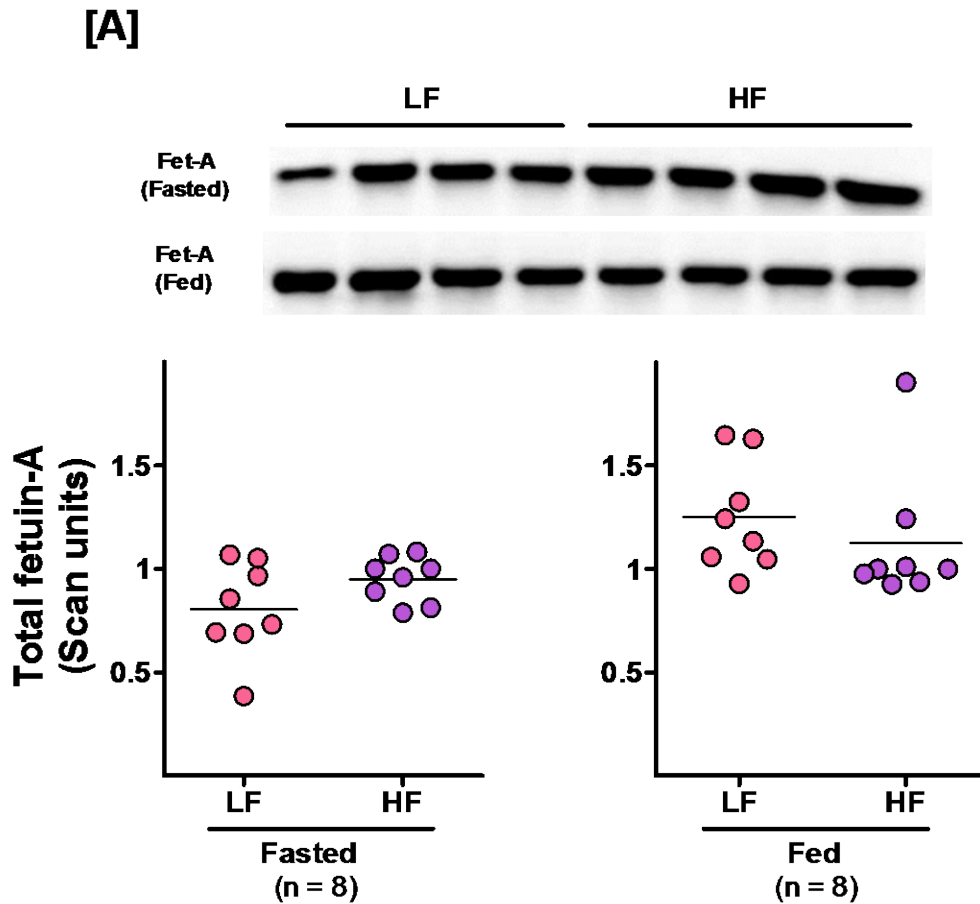


Fig. 4.4A: *Scatter plot of serum total fetuin-A concentrations in HF-fed C57Bl/6 mice*. Following 8 weeks of feeding a LF- or HF-diet, blood samples were obtained in the fasted (12-hour overnight fast) and fed conditions. Serum fetuin-A concentrations were assessed by Western blotting (representative blots shown, upper panels). Scatter plot representing densitometric analyses of total fetuin-A bands are depicted (bottom panel). Horizontal lines indicate mean values.

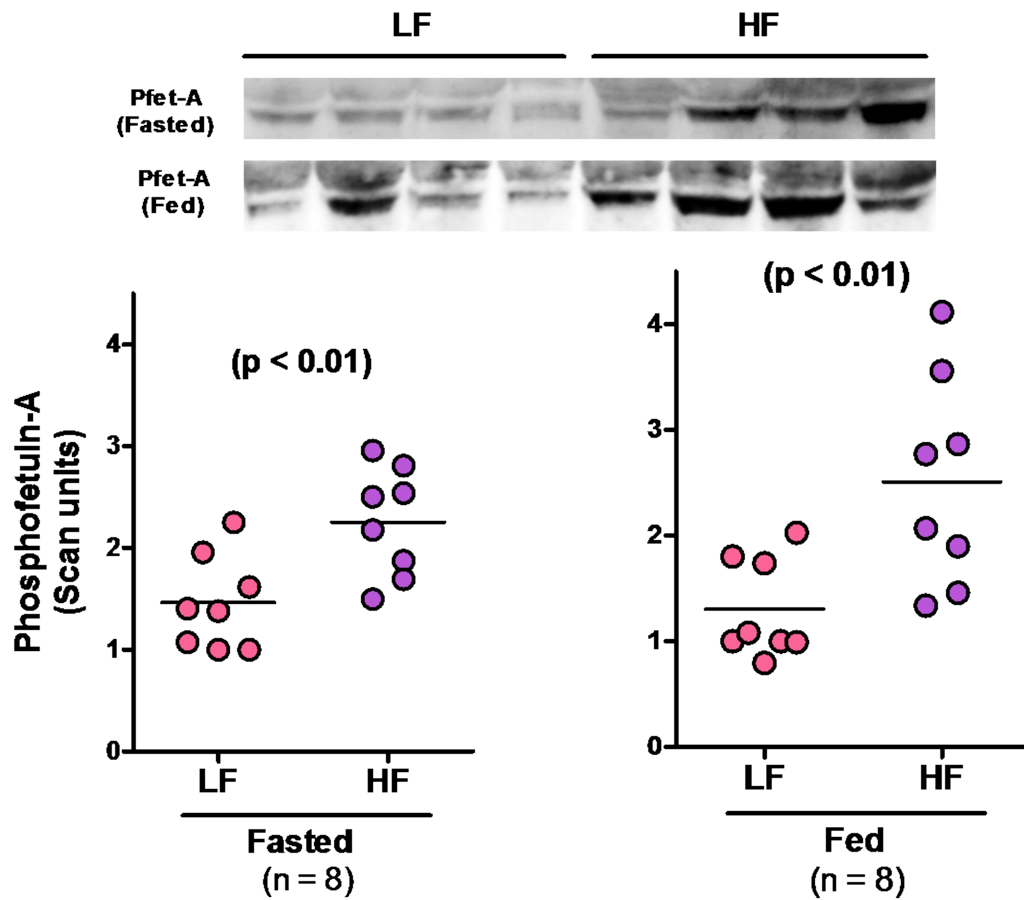


Fig. 4.4B: *Scatter plot of serum Ser312-phosphofetuin-A concentrations in HF-fed C57Bl/6 mice*. Following 8 weeks of feeding a LF- or HF-diet, blood samples were obtained in the fasted (12-hour overnight fast) and fed conditions. Serum Ser312-phosphofetuin-A concentrations were assessed by Western blotting (representative blots shown, upper panels). Scatter plot representing densitometric analyses of Ser312-phosphofetuin-A bands are depicted (bottom panel). Horizontal lines indicate mean values. $P < 0.01$, compared to LF in either fasted or fed conditons.

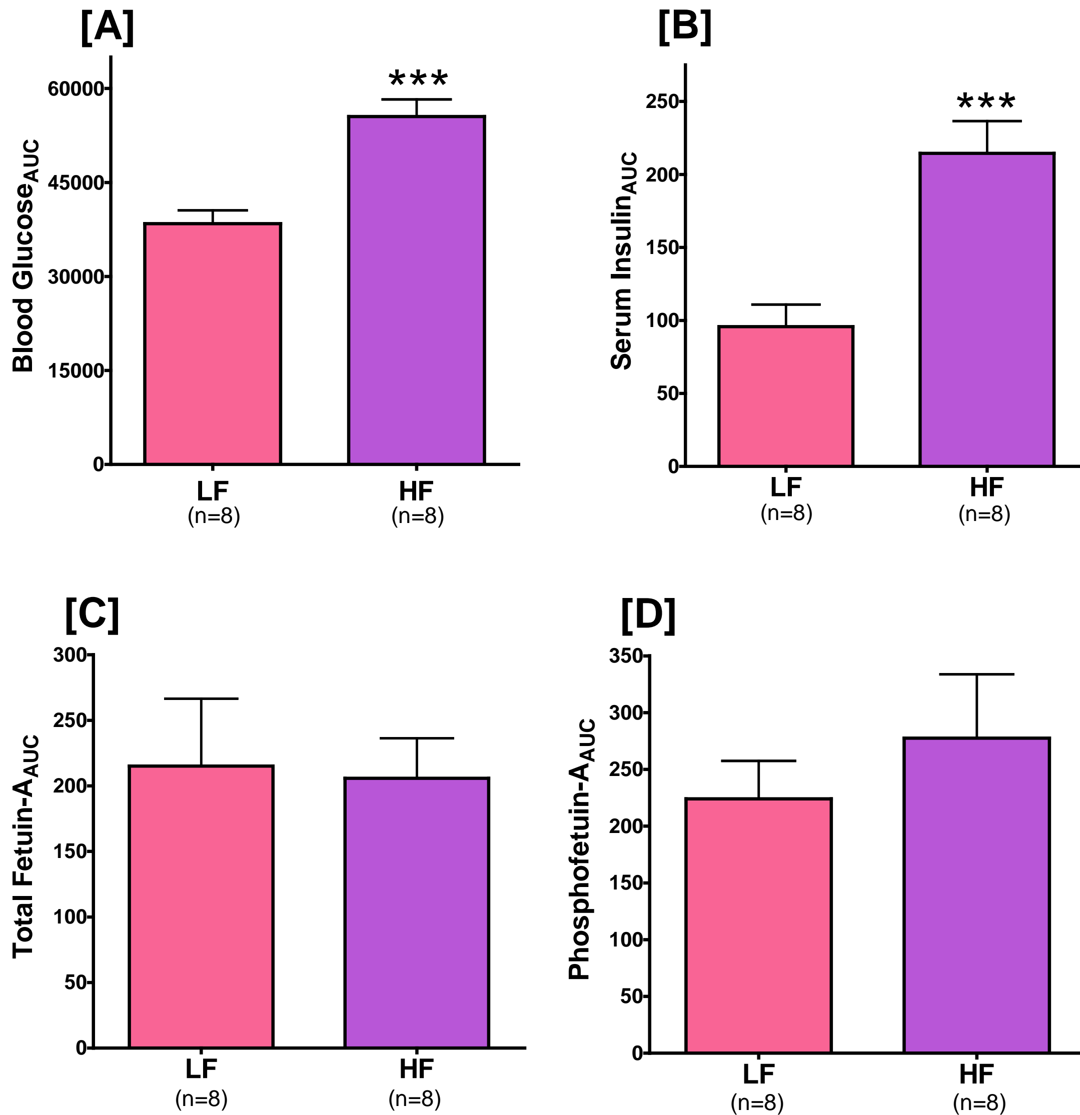


Fig. 4.5: *Area under the curve - Glucose tolerance test*. Following 8 weeks of feeding a LF- or HF-diet, C57Bl/6 mice were fasted for 6-hours and an oral load of glucose (2 mg/g) was administered. Blood samples were collected from the tail vein at 0, 15, 30, 60, and 120 minutes. The area under the curve (AUC) for blood glucose (A), serum insulin (B), total fetuin-A (C), and Ser312-phosphofetuin-A (D) were assessed. Data are shown as mean ± SEM. ***$p < 0.001$.

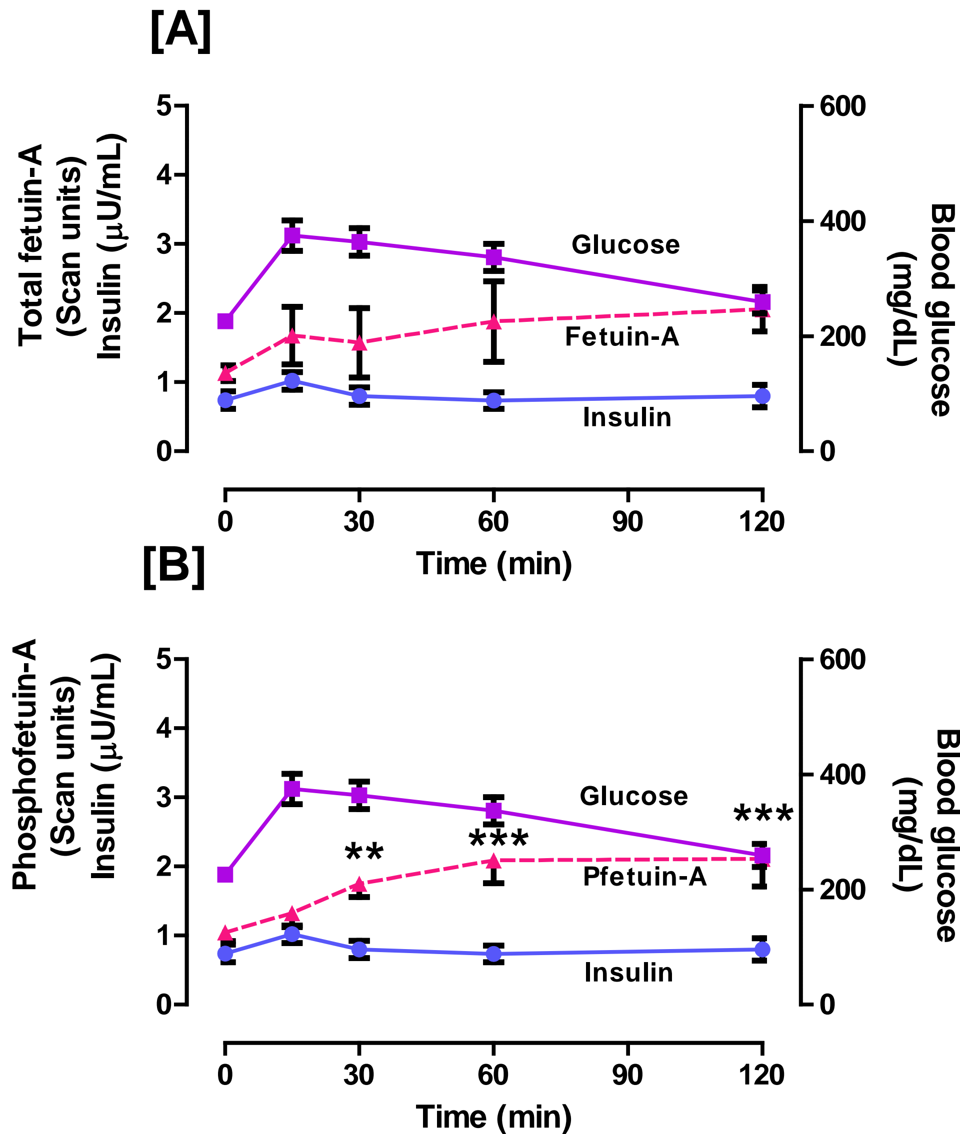
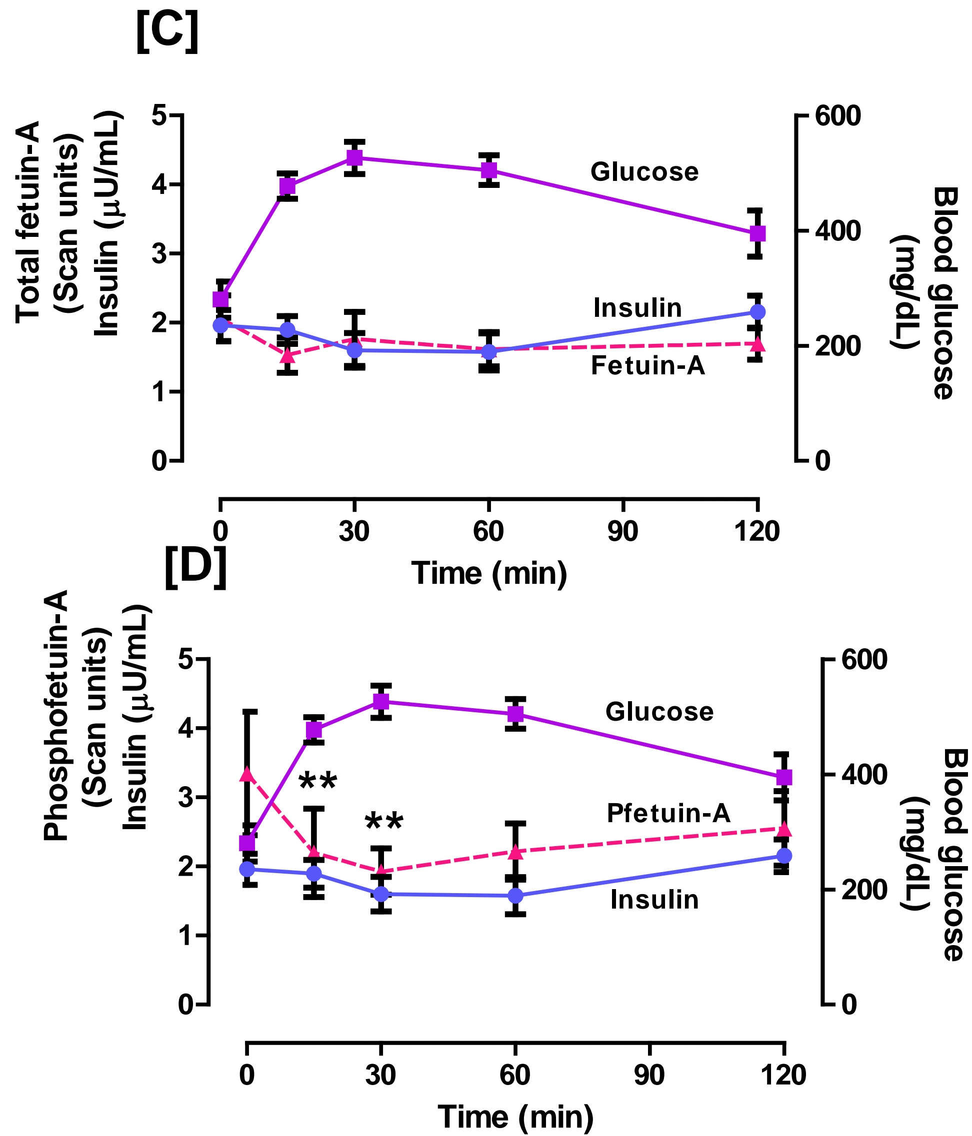


Fig. 4.6A,B: *Temporal alterations of total fetuin-A and phosphofetuin-A relative to blood glucose and serum insulin during an OGTT in LF-fed mice*.*C57Bl/6 mice, fed a low-fat (LF) diet for 8 weeks, were subjected to an oral glucose load (2 mg/g), following a 6-hour fast. Blood samples were obtained at 0,15,30,60, and 120-minute time points. Temporal alterations in blood glucose, serum insulin, and total fetuin-A concentrations (A), and phosphofetuin-A concentrations (B) are depicted. Data are shown as mean ± SEM, **p < 0.01, ***p < 0.001 for changes in phosphofetuin-A (Pfetuin-A) concentrations, compared to 0 time-point.*



<u>Fig. 4.6C,D</u>: *<u>Temporal alterations of total fetuin-A and phosphofetuin-A relative to blood glucose and serum insulin during an OGTT in HF-fed mice</u>.C57Bl/6 mice, fed a high-fat (HF) diet for 8 weeks, were subjected to an oral glucose load (2 mg/g), following a 6-hour fast. Blood samples were obtained at 0,15,30,60, and 120-minute time points. Temporal alterations in blood glucose, serum insulin, and total fetuin-A concentrations (A), and phosphofetuin-A concentrations (B) are depicted. Data are shown as mean ± SEM, **p < 0.01 for changes in phosphofetuin-A (Pfetuin-A) concentrations, compared to 0 time-point.*

4. Conclusion

Over the past few decades, a substantial increase in the incidence of obesity and type 2 diabetes has been observed, primarily due to the increasing prevalence of the Western lifestyle, consumption of a high caloric diet coupled with a sedentary lifestyle. These twin epidemics represent a serious threat to the health of Alabamians, the population of the United States, and for the rest of the world. Insulin resistance, which is implicitly linked into the pathophysiology of both obesity and type 2 diabetes, has been shown to be associated with the dysregulation of several humoral factors, including adiponectin, resistin, adiponectin, RBP-4, TNF-α, IL-6, and PTP-1B. Emerging evidence has implicated fetuin-A as a novel hepatokine that has been linked to obesity, insulin resistance, and type 2 diabetes.

Fetuin-A, a liver-secreted humoral factor, has been shown to act as a natural inhibitor of insulin signaling by binding to the activated insulin receptor and disrupting its intracellular function at the level of autophosphorylation, and thus it is believed to have a role in metabolism. Circulating levels of fetuin-A are positively correlated with obesity, insulin resistance, metabolic syndrome, and liver fat accumulation, and have been shown to be an independent risk factor for incident type 2 diabetes. About 20% of circulating fetuin-A is phosphorylated, and phosphorylation has been shown to be critical for its inhibition of insulin receptor autophosphorylation. However, there are limited studies on phosphorylated fetuin-A and its role in insulin resistance.

This study examines fetuin-A phosphorylation status and temporal alterations in response to a glucose load, in a mouse model of obesity and insulin resistance. Ser312-phosphofetuin-A concentrations were elevated in C57Bl/6 mice fed a high-fat diet for 8 weeks compared to low-fat fed controls. Also, this study shows for the first time that there appears to be temporal alterations in Ser312-phosphofetuin-A serum concentrations during an OGTT. In normal, low-fat diet fed mice we observed a significant temporal increase in Ser312-phosphofetuin-A concentrations at 30, 60, and 120-minute time-points, following an oral glucose load. On the contrary, in high-fat diet-induced obese and insulin resistant mice, Ser312-phosphofetuin-A concentrations showed a significant temporal decrease 15 and 30 minutes after an oral glucose load. Our findings suggest that a) under normal conditions phosphofetuin-A may be dynamically involved in the negative regulation of the insulin signal, and b) in high-fat diet-induced insulin resistance, the observed temporal decrease in phosphofetuin-A concentrations during an OGTT may be a compensatory mechanism to improve insulin action. Ser312-phosphofetuin-A concentrations were strongly correlated with body weight, insulin, and insulin resistance as HOMA-IR. Our findings, indicating that phosphorylation status of fetuin-A is associated with insulin resistance and that phosphofetuin-A levels are dynamically altered following an oral glucose load, suggest that measurements of Ser312-phosphofetuin-A may offer a better correlation to insulin resistance than total fetuin-A concentrations. Further studies should focus on elucidating the association of phosphofetuin-A with the pathophysiology of insulin resistance, obesity, and type 2 diabetes, and its interplay with other humoral factors associated with these disorders.

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