PHOSPHORYLATION OF FETUIN-A, A PHYSIOLOGICAL INHIBITOR OF

INSULIN ACTION, REGULATED BY INSULIN AND LEPTIN

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PHOSPHORYLATION OF FETUIN-A, A PHYSIOLOGICAL INHIBITOR OF INSULIN ACTION, REGULATED BY INSULIN AND LEPTIN

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VITA

James B. Papizan, son of Bruce and Beverly Papizan, was born in Brooksville, FL on January 19, 1980. Upon graduating from Madison Central High School in Madison, MS, James chose to attend The University of Southern Mississippi on a music scholarship. After studying percussion performance for four years and performing abroad in such places as Paris, France and London, England, he switched his focus to Nutrition. James graduated from Southern Miss in 2005 with a B.S. in Nutrition and Dietetics and with minors in Music Performance and Chemistry. The summer succeeding his graduation, James married the beautiful Ansley Love Nored of Clinton, MS. In August of 2005, James and Ansley moved to Auburn, AL where James enrolled into Auburn University's Graduate School and pursued a Master of Science Degree in Nutrition in the Department of Nutrition and Food Science under the direction of Dr. Suresh T. Mathews.

THESIS ABSTRACT

PHOSPHORYLATION OF FETUIN-A, A PHYSIOLOGICAL INHIBITOR OF INSULIN ACTION, REGULATED BY INSULIN AND LEPTIN

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Fetuin-A, a liver secreted phosphoprotein and rat homologue of human 2-HS-glycoprotein (AHSG), is a physiological inhibitor of insulin action. Fetuin-A-null mice exhibit improved glucose tolerance and resistance to weight gain on a high-fat diet. Recent human data demonstrate a positive correlation of circulating fetuin-A levels with BMI, insulin resistance, and fatty liver. Since phosphorylation is critical for its inhibitory activity, circulating fetuin-A phosphorylation was assayed in animal models of insulin resistance, diabetes, and obesity. Using a phospho-specific (^{312}Ser) fetuin-A antibody, fetuin-A phosphorylation status was assayed in Zucker diabetic fatty (ZDF) rats, *ob/ob* and *db/db* mice, streptozotocin-treated rats, and in a rat model of diet-induced obesity (DIO). The leptin receptor-deficient, insulin resistant ZDF rats had significantly higher levels of phosphorylated fetuin-A compared to their lean counterparts. Likewise, the

leptin deficient, obese *ob/ob* mice had significantly higher levels of phosphorylated fetuin-A. Consistent with these results, central leptin administration to control and STZ-treated rats, as well as leptin treatment to human hepatoma cell lines, significantly decreased fetuin-A phosphorylation. To further characterize the regulation of fetuin-A phosphorylation, we demonstrate that insulin treatment downregulates phosphorylation in Hep3B and HepG2 cell lines. Similarly, DIO rats that exhibit elevated levels of insulin, albeit not to a significant degree, demonstrate significantly decreased levels of fetuin-A phosphorylation. We demonstrate that insulin and leptin decrease phosphorylation of fetuin-A. Accordingly, in conditions of mild to moderate elevation of insulin (as in the DIO model), fetuin-A phosphorylation is decreased, mitigating the inhibitory effect of fetuin-A on IR and allowing for increased insulin signaling. In conditions of extreme insulin resistance (ZDF and ob/ob models), where insulin is unable to exert its actions on insulin receptors, fetuin-A phosphorylation is increased, potentially exacerbating the diabetic phenotype. These findings suggest that fetuin-A may play a significant role in the regulation of insulin action.

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Chapter 1: Introduction

The incidence of obesity and its associated complications is escalating at an alarming rate worldwide. Obesity predisposes individuals to an increased risk of developing multiple diseases such as cardiovascular disease, arthritis, certain cancers, non-alcoholic fatty liver disease, and diabetes (1-3). The American Obesity Association defines obesity as an individual having a body mass index (BMI) equal to or greater than 30. The BMI is a measure of body fat based on a person's height and weight, i.e., [weight (kg)/height $(m)^2$]. According to The Alabama Center for Health Statistics, 24.5 percent of adults in Alabama are obese, making Alabama the second heaviest state in the nation. Since obesity is tightly correlated with the prevalence of diabetes, this has caused a relative neologism to creep into medical terminology: Diabesity – the twin epidemics. Therefore, the cornerstone of much research today is to elucidate the link between obesity and diabetes.

Obesity in Western society can partially be attributed to the high-fat diet and sedentary lifestyle. High-fat diet-induced obesity results in an increased secretion of adipocyte-derived nutrients such as free fatty acids (FFA), which have been implicated in the development of insulin-resistance, a hallmark of type 2 diabetes (4). Furthermore, hypertrophic adipocytes secrete less adiponectin, a hormone that has been demonstrated to increase insulin sensitivity (5). Obesity is also regarded as a chronic low-grade

inflammatory condition, and a growing amount of evidence has pointed to a link between insulin resistance and obesity-induced inflammation (3; 6). Cytokines associated with inflammation, e.g., tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1), have been shown to impair insulin action, resulting in diabetes (7-9). Human type 2 diabetes is characterized by two major characteristics: peripheral insulin resistance and impaired insulin secretion by the pancreatic β-cells (10;11). Normal blood glucose levels are maintained by adaptations of the β-cells as a compensatory response to insulin resistance. Manifestation of type 2 diabetes results only when the β-cells' adaptations fail to compensate for insulin resistance. Conversely, human type 1 diabetes is regarded as an autoimmune disease, characterized by the destruction of pancreatic β-cells, which results in the inability to produce insulin. Type 1 diabetes, or insulin-dependent diabetes mellitus (IDDM), was previously referred to as juvenile diabetes, as the onset of the disease usually occurs during childhood. Type 2 diabetes, or non insulin-dependent diabetes mellitus (NIDDM), was previously referred to as adult-onset diabetes, as the onset of the disease usually occurs during adulthood; however, as the prevalence of obesity in children continues to escalate in Western culture, more and more children are developing type 2 diabetes.

Insulin action begins with insulin binding to the insulin receptor (IR), which induces a conformational change thereby phosphorylating tyrosine residues located on the intracellular β-subunits in a process called autophosphorylation (12). This activates an intrinsic insulin-receptor tyrosine kinase (IR-TK), which phosphorylates the insulin receptor substrate (IRS) proteins, a critical node in insulin signaling (13). The IRS proteins are linked to the other two critical nodes in insulin signaling: the

phosphatidylinositol 3-kinase (PI3-K) pathway and the Ras-mitogen-activated protein kinase (MAPK) pathway, which are responsible for the metabolic and mitogenic actions of insulin, respectively (14) (Fig. 1). IRS proteins mediate insulin signaling and, like the IR, are activated by tyrosine phosphorylation and inhibited by serine phosphorylation and protein tyrosine phosphatases (PTPs) (9). While known modulators of insulin action act to sensitize the effects of insulin e.g., adiponectin (5; 15), leptin (16), and thiazolidindiones (TZDs) (17-19), others act to attenuate insulin signaling through mechanisms such as serine phosphorylation and protein tyrosine phosphatase activity. Serine kinases known to down-regulate insulin signaling include c-Jun-N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and S6-kinase by phosphorylating serine residues on IRS proteins. Other inhibitors of insulin action include PTP1B, suppressor of cytokine signaling-1 (SOCS1), and plasma-cell-membrane glycoprotein-1 (PC-1), which act at the level of the IR by either sterically blocking the IR/IRS interaction, or by modifying the IR-TK activity (20).

Another inhibitor acting at the level of the IR, specifically an IR-TK inhibitor, is fetuin-A, a 63 kD phosphorylated glycoprotein, synthesized and secreted primarily by the liver and belonging to the cystatin family of protease inhibitors (21). Fetuin-A was shown originally to be synthesized and secreted by normal rat hepatocytes as a phosphoprotein called PP^{63} (22). Later, PP^{63} was shown to be an inhibitor of IR-TK activity only in its phosphorylated form (23). Further investigations have identified bovine fetuin and human α2-HS-glycoprotein (AHSG) as homologs of rat fetuin-A (24-27).

Fetuin-A preferentially interacts with the activated insulin receptor, repressing insulin-induced IR autophosphorylation and subsequent downstream signaling activity *in*

vitro, in intact cells and *in vivo* (28). Fetuin-A null mice demonstrate improved insulin sensitivity, whole-body glucose utilization, and resistance to weight gain when fed a high fat diet (29). Additionally, these mice are protected against aging-induced obesity and insulin resistance (30). Interestingly, the gene for fetuin-A is localized on chromosome 3q27, a locus that has been shown to be associated with type 2 diabetes and cardiometabolic risk (31). While a polymorphism of the *AHSG* gene has been shown to be associated with type 2 diabetes, another polymorphism is associated with leanness in Swedish population (32). A growing body of human investigation studies points to a strong association of plasma fetuin-A levels with insulin resistance (33-35), an atherogenic lipid profile (34), and increased BMI and plasma triglyceride levels (36). Plasma concentrations of fetuin-A in humans range from 200-600 μg/ml, with an average plasma concentration of ~ 300 μg/ml in healthy adults (37). Fetuin-A exists in both phosphorylated and dephosphorylated forms in human plasma. Pioneering studies by Haglund et al have demonstrated that fetuin-A is phosphorylated on two serine residues, ^{120}Ser and ^{312}Ser, and that approximately 20% of circulating fetuin-A is phosphorylated (38). Phosphorylation of fetuin-A has been shown to be critical for its IR tyrosine kinase inhibitory activity (23). While ^{120}Ser-AHSG has been shown to be constitutively phosphorylated, ^{312}Ser-AHSG seems to exhibit regulatory covalent modifications, suggesting this site may be critical for its inhibitory activity (38).

While previous human studies have investigated the associations of fetuin-A in disease conditions, there are no reports examining its phosphorylation status. Therefore,

the goal of this study is to elucidate the phosphorylation status of fetuin-A in animal models of obesity, insulin resistance, and diabetes.

Chapter 2: Review of Literature

Obesity and inflammation

During recent decades, the incidence of obesity and its associated complications, including type 2 diabetes, has risen boundlessly, resulting in obesity-related health concerns associated with increased morbidity and mortality (39; 40). Equally alarming is the concomitant increase in childhood obesity, also resulting in obesity-related health threats, an occurrence that was inscrutable just a few decades ago (41). Adipose tissue was once considered an inert tissue, functioning solely as an energy storage depot. More recently, adipose tissue has been viewed as a dynamic endocrine system, secreting a vast array of adipokines with functions targeting the immune and metabolic systems (42; 43). Adiponectin, leptin, visfatin, resistin, and RBP4 are several adipokines thought to provide an important link or play a role in the development of obesity-induced insulin resistance (2; 3; 44;45). Increasing adiposity is also associated with elevated inflammatory responses, which is characterized by abnormal circulating levels of adipose-derived cytokines (3). Several of these cytokines, such as TNF-α and MCP-1, have been implicated in the development of impaired insulin action, resulting in insulin resistance and diabetes (5; 7; 46).

Diabetes

The American Diabetes Association defines diabetes as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (47). Three types of diabetes exist: type 1, type 2, and gestational diabetes. Type 1 diabetes, or IDDM, develops when the body's immune system attacks and destroys the pancreatic β-cells, the cells in which insulin is produced and secreted. In order to survive, individuals with type 1 diabetes must use exogenous insulin as a therapeutic in order to maintain euglycemia. Type 1 diabetes only constitutes 5-10 percent of all diagnosed cases of diabetes in the U.S., while type 2 diabetes, or NIDDM, comprises 90-95 percent of diagnosed cases. Insulin resistance, a complication characterized by the inability of peripheral tissue to respond to circulating insulin, usually preludes type 2 diabetes. As the β-cells' ability to respond to the body's demand for insulin attenuates, type 2 diabetes ensues. Gestational diabetes is a form of glucose intolerance that occurs during pregnancy, and women who develop gestational diabetes are at a greater risk for developing type 2 diabetes later in life. Currently there are over 20 million adults in the U.S. with diabetes, and, according to the Alabama Cooperative Extension System, Alabama leads the nation in diabetes with more than 440,000 individuals diagnosed with the disease. This prevalence is costing Alabama more than $3 billion dollars annually.

Insulin signal transduction

Insulin-mediated events control processes such as glucose uptake, lipid synthesis, protein synthesis, and glycogen deposition. These processes begin with insulin binding to

its IR, which is found in virtually all vertebrate tissues; however, the concentration of the receptors varies from tissue to tissue. The IR is a transmembrane, tetrameric protein that consists of two extracellular α-subunits and two intracellular β-subunits (48). The extracellular α-subunits contain the insulin-binding domain and controls the activity of the intracellular, intrinsic tyrosine kinases located on the β-subunits. Prior to insulin binding, a separation is maintained between the two intracellular tyrosine kinases, inhibiting transphosphorylation. Upon insulin binding to the extracellular α-subunits, the inhibitory activity is relieved, bringing the intracellular β-subunits and tyrosine kinases in close enough proximity for transphosphorylation (48). Once autophosphorylated, the IR recruits the IRS proteins, which have both pleckstrin-homology domains (PH domains) and phosphotyrosine binding domains (PTB domains) (49). Six IRS proteins have been identified and have been designated as IRS 1-6 (50; 51). IRS 1 and 2 are found ubiquitously, whereas IRS 3 is expressed in adipocytes and brain, and IRS 4 is limited to embryonic tissues. IRS 5 is found mostly in kidney and liver while IRS 6 is mostly expressed in skeletal muscle (51). The PTB domain of IRS proteins binds directly to the NPXpY-motif of the activated IR. The center and C-terminus of IRS proteins contain multiple potential tyrosine phosphorylation sites that, after phosphorylation by the IR, recruit intracellular molecules containing Src-homology-2 domains (SH2 domains) (49). Two examples of SH2 proteins that bind to phosphorylated IRS proteins are PI3K and Grb2. PI3K mediates the insulin-stimulated metabolic activities and contains a regulatory and a catalytic subunit, which upon activation by IRS proteins, converts phosphatidylinositol bisphosphate (PIP_2) to phosphatidylinositol triphosphate (PIP_3) (52). PIP_3 then recruits AKT/protein kinase B (AKT/PKB) and 3-phosphoinositide-dependent

kinase-1 (PDK-1) to the cell surface where PDK-1 activates AKT/PKB. The GTPase activating protein AKT substrate of 160 kDa (AS160) is one of the AKT/PKB targets responsible for GLUT-4 translocation and glucose uptake (52). Also, AKT/PKB directly phosphorylates GSK3, allowing for insulin-stimulated glycogen deposition. AKT/PKB also phosphorylates forkhead box 01 (FOX01), sequestering it in the cytoplasm, which inhibits gluconeogenesis (52). To coordinate insulin-stimulated mitogenic activities, the SH2 domain of the adaptor molecule, Grb2, binds to IRS proteins. Grb2 then associates with son-of-sevenless (SOS) to activate the Ras-Raf-Mek-MAPK pathway, which relays the message from the membrane to the nucleus (52).

INTRACELLULAR, INSULIN-DEPENDENT SIGNALING PATHWAYS

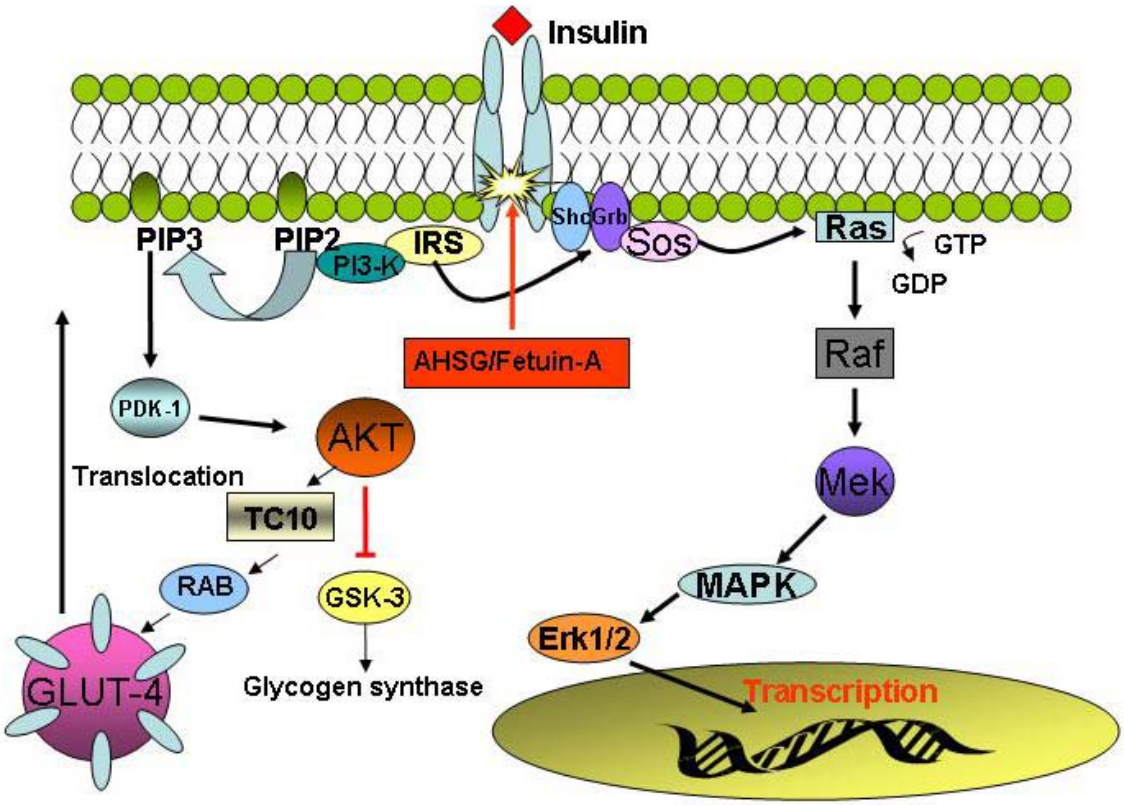

FIG. 1. Metabolic and mitogenic responses to insulin. In the presence of insulin, the IR becomes phosphorylated, and IRS proteins are recruited to the IR to mediate the PI3-K and MAPK pathways. Fetuin-A is shown here inhibiting the IR-TK activity, thus repressing insulin-mediated events.

Insulin resistance

Insulin resistance is characterized by an attenuated ability of peripheral tissue to respond to circulating insulin. While the underlying cellular mechanisms of insulin resistance have not been comprehensively characterized, multiple factors have been implicated in its manifestation. The first link between obesity-induced inflammation and insulin resistance was reported by Hotamisligil and co-workers (7). Their findings illustrated an increase in TNF-α mRNA in adipose tissue of different rodent models of diabetes and obesity, and that neutralization of TNF-α in these animals significantly increased peripheral glucose uptake in response to insulin. Corresponding human studies showed increased TNF-α expression in adipose tissue of obese individuals, with decreased expression following weight loss (53). As a mechanism to explain inflammation-induced insulin resistance, TNF-α has been shown to activate JNK and IκK kinase β (IKKβ), which have both been demonstrated to phosphorylate serine residues on IRS-1, attenuating insulin action (54-56). Insulin-dependent tyrosine phosphorylation of IRS proteins is a critical step in insulin action, as these proteins connect IR activation to essential downstream kinase events leading to glucose uptake and gene expression. In the event where IRS proteins are absent in the liver of rodents, a systemic insulin resistant, glucose intolerant phenotype results, confirming the indispensability of these proteins (57). Further, in animal models of diet-induced obesity (DIO), JNK activation and IRS serine phosphorylation has been shown to be upregulated (58; 59). Drugs that act to sensitize insulin action have been shown to increase IRS-1 tyrosine phosphorylation (60), and endogenous insulin sensitizers, such as adiponectin, have been demonstrated to

reduce serine phosphorylation, and concomitantly increase tyrosine phosphorylation of IRS-1, increasing insulin-dependent downstream cellular events (61). These findings illustrate the importance of IR activation and its subsequent communication with post-receptor signaling molecules.

Negative modulators of insulin action

A small number of insulin resistance cases have demonstrated mutations of the IR gene, suggesting insulin resistance occurs at the level of the receptor. Conversely, in the vast majority of patients with insulin resistance, the IR gene has been found to be normal, indicating a post-receptor phenomenon (62). However, several molecules have recently been identified that act at the level of the receptor, attenuating insulin action. PTP-1B acts as a negative regulator of insulin action by dephosphorylating tyrosine residues on the IR. Furthermore, PTP-1B expression in muscle and adipose tissue is increased in obese humans and rodent models, and overexpression of PTP-1B results in severe insulin resistance and decreased glucose uptake (63; 64). Other proteins, such as the suppressors of cytokine signaling (SOCS-1 and -3), mitigate insulin signaling by binding to essential kinase domains of the IR, thereby inhibiting the IR/IRS interaction (65; 66). This is of particular interest since SOCS-1 and -3 have been found to be increased in different rodent models of obesity and insulin resistance (67), and knockdown of these proteins demonstrate lower glucose levels and increased insulin sensitivity (68). Another protein responsible for contributing to insulin resistance by interacting with the IR is plasma-cell-membrane glycoprotein-1 (PC-1). PC-1 is an ectoenzyme present in the cell membrane that exhibits pyrophosphatase and phosphodiesterase activities (69). Maddux and co-

workers have demonstrated that PC-1 directly interacts with the α-subunit of the IR, and overexpression of PC-1 is sufficient to induce insulin resistance and hyperglycemia (70; 71). Further, PC-1 has been shown to be elevated in insulin-resistant tissues and fibroblasts (72; 73). PC-1 does not block insulin binding to the IR, rather it abates the IR's insulin-induced conformational change that leads to IR-TK activity and autophosphorylation (70; 74).

Fetuin-A: Introduction

Fetuin-A (also known as α2-HS-glycoprotein, AHSG), a 63 kD phosphorylated glycoprotein, synthesized and secreted primarily by the liver and belonging to the cystatin family of protease inhibitors, is a natural inhibitor of IR-TK activity (23). Fetuin-A preferentially interacts with the activated insulin receptor, repressing insulin-induced IR autophosphorylation and subsequent downstream signaling activity *in vitro*, in intact cells, and *in vivo* (28). Mice lacking fetuin-A demonstrate improved insulin sensitivity, whole-body glucose utilization, and resistance to weight gain when fed a high fat diet (29). Additionally, these mice are protected against aging-induced obesity and insulin resistance (30). Interestingly, the gene for fetuin-A is localized on chromosome 3q27, a locus that has been shown to be associated with type 2 diabetes and cardiometabolic risk (31). While a polymorphism of the *AHSG* gene has been shown to be associated with type 2 diabetes, another polymorphism is associated with leanness in Swedish population (32). A growing body of human investigation studies point to a strong association of plasma fetuin-A levels with insulin resistance (33-35), an atherogenic lipid profile (34), and increased BMI and plasma triglyceride levels (36). Recently, a second member of the

fetuin family has been described, which designated the previously mentioned fetuin (AHSG, fetuin, pp63) as fetuin-A (75). Fetuin-B demonstrates domain homology, conservation of cysteine resisdues, and chromosomal assignments with fetuin-A; however, fetuin-A and –B exhibit significant differences in amino acid sequence, protein structure, and gene expression, suggesting discrete functions for the proteins (75).

Structure

Human α2-HS-glycoprotein (AHSG), named after the discoverers Heremans, Schmid, and Bürgi (76; 77), is the species homologue of fetuin-A, first reported by Pederson as the most abundant protein in fetal bovine serum (78). Fetuin-A belongs to the class of cysteine protease inhibitors of the cystatin superfamily (79), which encompasses proteins that exhibit several cystatin-like domains. Fetuin-A is composed of two chains, a large A-chain and a smaller B-chain, that are linked by a short connecting peptide and an interchain disulfide bridge. It has been shown that the two chains are encoded by a single mRNA transcript (80), and the connecting peptide is partially or completely cleaved before the protein is released into circulation (81). Characterization of the A-chain revealed that it consists of 282 amino acid residues, while exhibiting 29% α-helix, 24% β-pleated sheet, and 26% reverse turns (82). Four glycosylation sites were also identified; two *N*-glycosylation sites were observed on ^{138}Asn and ^{158}Asn, while two *O*-glycans were identified on ^{238}Thr and ^{252}Thr. The B-chain also has been characterized, consisting of 27 amino acid residues and a trisaccharide consisting of sialic acid, galactose, and *N*-acetylgalactosamine *O*-glycosidically linked to ^{328}Ser (83). The 40 amino acid connecting peptide joins the A-chain at ^{282}Leu and the B-chain at ^{323}Thr.

Some controversy exists as to whether the connecting peptide is posttransitionally cleaved by limited proteolysis (83; 84), or if the A-chain retains 39 of the 40 amino acids of the connecting peptide, resulting in a single-chain circulating protein (81; 85).

Fetuin-A exists in both phosphorylated and dephosphorylated forms in human plasma. Haglund et al, have demonstrated that fetuin-A is phosphorylated on two serine residues, ^{120}Ser and ^{312}Ser, two of the phosphorylation sites suggested by Jahnen-Dechent et al. (85), and that approximately 20% of circulating fetuin-A is phosphorylated (38). Phosphorylation of fetuin-A has been shown to be critical for its IR tyrosine kinase inhibitory activity (23). While ^{120}Ser has been shown to be constitutively phosphorylated, ^{312}Ser seems to exhibit regulatory covalent modifications, suggesting this site may be critical for its inhibitory activity (38). Furthermore, these two phosphorylation sites are conserved in human, mouse, rat, sheep, pig, and cow (82; 83; 86; 23; 87; 25; 88) (Fig. 2.).

The disulfide loops present in fetuin-A and its similarity with cystatins has been elucidated by Kellerman *et al*. (81). They report the presence of 12 half-cysteine residues on the molecule, and that 11 of them are present on the large A-chain while only one resides on the smaller B-chain, forming six disulfide bridges. The first and last half-cysteine residues engage to form a disulfide loop, connecting the A- and B-chains at ^{14}Cys and ^{140}Cys. The other five disulfide loops are found on the A-chain, which exhibit a similar disulfide loop arrangement found in those of the cystatin family, hence fetuin-A's classification in the cystatin superfamily. From the amino- to the carboxyl-terminus, fetuin-A consists of three domains. Domains 1 and 2 (D1-2), located on the A-chain, are

cystatin-like domains, whereas domain 3 (D3) is of a hydrophobic nature, composed of a proline-rich region and the connecting peptide (81) (Fig. 3).

In an effort to determine the conserved regions of fetuin-A, the cDNA's encoding human (82; 83), rat (23; 86), mouse (87), cow (25), sheep, and pig (88) fetuin have been cloned and sequenced, and comparisons between these species reveals strong similarity (89). Dziegielewska and Brown have shown that human fetuin-A shares 64, 65, 64, 56, and 59% sequence homology with sheep, pig, cow, rat, and mouse fetuin, respectively. Of particular interest is the conservation of the Asn-linked *N*-glycans on the A-chain, and ^{120}Ser and ^{312}Ser of the A-chain and connecting peptide, respectively, which, as mentioned previously, have been reported to be phosphorylated (38; 85). The conservation of these posttranslationally modified sites may serve as sites of regulation of fetuin-A's functions.

CONSERVED SERINE RESIDUES ON FETUIN-A

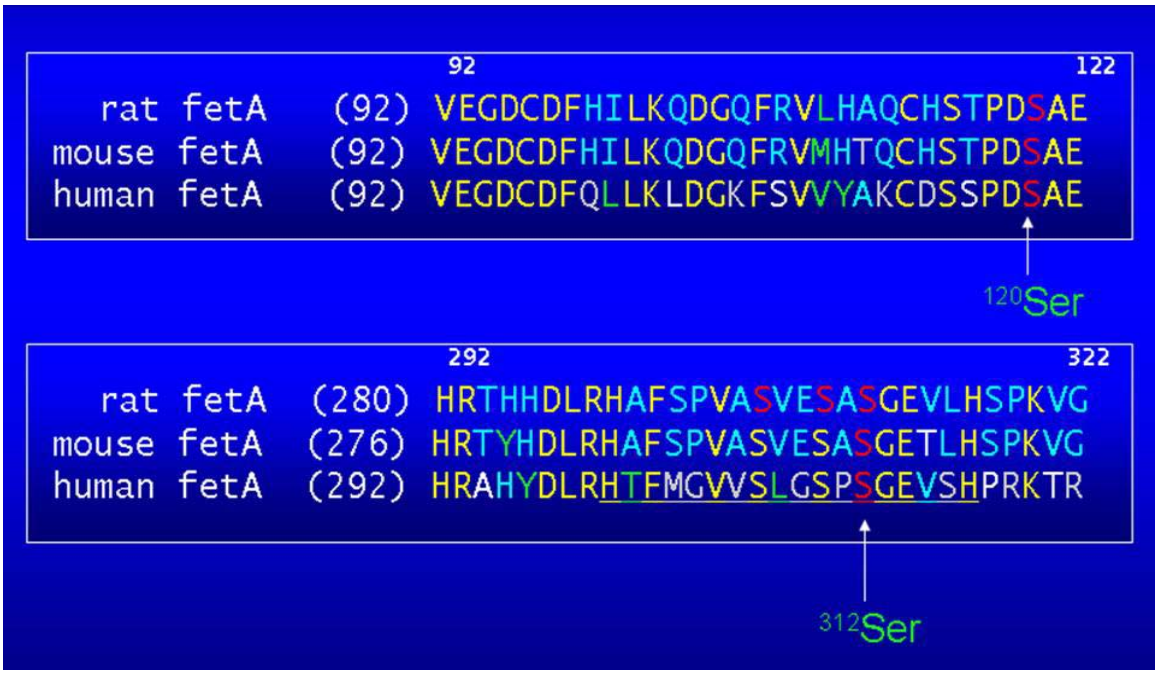

FIG. 2. Conserved serine residues. Fetuin is phosphorylated on ^{120}Ser and ^{312}Ser, and the sequence alignment of rat, mouse, and human are shown. Conserved ^{120}Ser and ^{312}Ser residues are indicated by arrows. The underlined sequence denotes the peptide from which phospho-^{312}Ser-fetuin-A antibody was generated.

PROTEIN STRUCTURE OF HUMAN AHSG/FETUIN-A

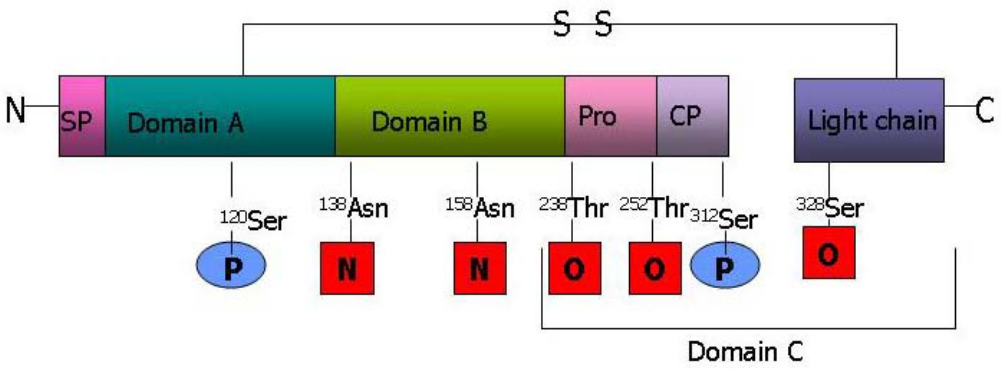

FIG. 3. Fetuin Protein structure (81; 85). The A-chain, comprising the signal peptide (SP), domains A and B, a proline rich segment (Pro) and 39 of the 40 amino acids of the connecting peptide (CP), is linked to the B-chain, or light chain, by a single disulfide bond. The conserved *N*- and *O*-linked glycosylation and phosphorylation sites are represented by N, O, and P, respectively.

IR-TK inhibition

Fetuin-A was first identified as a 63-kD phosphoprotein (PP^{63}), secreted from isolated rat hepatocytes (22). The same group later characterized PP^{63} as a natural inhibitor of the IR-TK, and showed that only the phosphorylated protein was active as an IR-TK inhibitor (23). Subsequently, a search for related protein sequences revealed that human AHSG and bovine fetuin shared 56 and 60% amino acid sequence identity, respectively, to that of rat PP^{63}, indicating its classification as a mammalian fetuin (24). Srinivas et al. (90) demonstrated that AHSG is specific for the tyrosine kinase activity of the IR, inhibiting insulin-induced autophosphorylation and tyrosine kinase activity *in vitro* and *in vivo.* This group further demonstrated that AHSG inhibits insulin-stimulated IRS-1 tyrosine phosphorylation and its subsequent association with PI3-K. Inhibition of insulin-mediated mitogenesis in rat hepatoma cells with ASHG was also demonstrated. To assess whether AHSG competes with insulin for binding sites on the α-subunit of the insulin receptor, Srinivas et al, performed competitive binding studies using ^{125}I-labeled insulin in the presence or absence of AHSG. AHSG was found not to alter specific insulin binding to the IR (90). Further characterization of AHSG's inhibitory effects has been elucidated. Srinivas et al. (91), using recombinant AHSG, illustrated that insulin-stimulated GRB2 association with IRS-1 was blunted. Furthermore, the insulin-dependent GRB2/Sos complex, which docks to IRS-1, was found to be inhibited by more than 75% in the presence of AHSG. AHSG also inhibited the insulin-dependent tyrosine phosphorylation of Shc and the Ras-GTP formation. Consequently, phosphorylation of the downstream targets Raf and MEK were also inhibited in the presence of ASHG. However, AHSG failed to blunt the metabolic effects of insulin, i.e., glucose uptake and

amino acid transport (91). Similar reports of the inhibitory activity of human recombinant AHSG have been illustrated (92). Kalabay et al. expressed ASHG in insect cells using the baculovirus system and showed that the purified protein had the connecting peptide intact. The connecting peptide contains the ^{312}Ser residue reported to be phosphorylated, and phosphorylation has been shown to be critical for AHSG's inhibitory effects.

The paradox lies in the concept that AHSG inhibited PI3-K (90), but was without effect on glucose uptake (91). PI3-K has been shown to be critical in insulin-induced glucose uptake (93) and has been well characterized since then (20). One explanation was that AHSG's inhibition of PI3-K was not complete and, therefore, remaining activity of PI3-K may have been sufficient to mediate glucose uptake. Another contributing factor could have been the degree of activity, i.e., phosphorylation of AHSG. AHSG's effects on insulin-stimulated glucose uptake are currently under investigation and preliminary studies by Kim et al. demonstrate that phosphorylated AHSG inhibits insulin-stimulated glucose uptake in skeletal muscle cells and adipocytes (94).

The homology of fetuins, human AHSG, and PP^{63} have been reported, and similar inhibitory effects of PP^{63} and human AHSG have been demonstrated (23; 90-92). Subsequently, bovine fetuin, like its homologs AHSG and PP^{63}, was reported to act as a negative regulator of insulin action by inhibiting the IR-TK activity (95). Bovine fetuin completely blocks insulin-stimulated autophosphorylation *in vitro* and significantly blunted IR-TK activity in HIRcB cells. Additionally, bovine fetuin completely abrogated insulin-stimulated DNA synthesis in rat H-35 hepatoma cells. These studies demonstrate a common IR-TK inhibitory function for the fetuin homologs; however, the studies were carried out in cells overexpressing human IR's, not in legitimate targets of insulin such as

adipocytes. Chen et al. showed, in a physiological perspective, that human AHSG, indeed, inhibits insulin-dependent mitogenic activity in rat adipose cells but not glucose uptake (96).

Mathews et al. have demonstrated the time-course and specificity of inhibition, its interaction with IR, and described a probable physiological role for AHSG (28). They demonstrated that incubation of 1.8 μM AHSG in HIRcB cells is sufficient to inhibit insulin-induced IR autophosphorylation by over 80%. This inhibitory effect was abated to half in 60 minutes and had no effect on insulin-like growth factor-1-induced cognate receptor (IGF-IR) autophosphorylation. To further characterize the specificity of AHSG, Mathews et al. treated HIRcB cell lysates, in the presence or absence of insulin, with increasing concentrations of AHSG. Immunoprecipitation with anti-AHSG antibody demonstrated that AHSG preferentially interacts with the activated insulin receptor; only in the highest concentration (4.0 μM) did AHSG interact with the IR in basal conditions, while concentrations as low as 0.9 μM AHSG interacted with the activated IR. This investigation also illustrated that AHSG interacts with β-subunits of the IR, and insulin-dependent conformational change of the β-subunits was not required for AHSG's inhibitory actions. To demonstrate this, HIRc B cells were treated with trypsin, which results in proteolysis of the IR α-subunit at ^{576}Arg-^{577}Arg, making the IR-TK activity constitutively active (97). To confirm the insulin-binding site was removed by trypsin, ^{125}I-labled insulin was unable to bind to IRs from trypsin-treated cells, while bound to IRs from control cells. AHSG (0.1 μM) completely inhibited both insulin-stimulated and trypsin-activated IR-TK activity. To corroborate these findings *in vivo*, 2 μM/120 μg bolus AHSG was injected into rats at the portal vein in the presence or absence of insulin.

ASHG inhibited insulin-stimulated IR autophosphorylation and IRS-1 tyrosine phosphorylation in intact rat liver and hind-limb muscle, further characterizing and adding physiological relevance to AHSG's inhibitory activity (28).

The human *AHSG* gene is localized on chromosome 3q27, a recently established type 2 diabetes susceptibility locus (98). Additionally, Kissebah et al. have demonstrated a quantitative trait locus on chromosome 3q27 strongly linked to the metabolic syndrome (31) Mice null for the *Ahsg* gene are fertile and exhibit no gross abnormalities with the exception of ectopic microcalcifications in a minority of females breeders (99). Mathews et al. explored the role of fetuin in insulin action in fetuin-deficient mice (29). Fetuin knockout (KO) mice demonstrated enhanced basal and insulin-stimulated IR autophosphorylation and increased MAPK and Akt phosphorylation. The KO mice also exhibited increased glucose tolerance and insulin sensitivity, and when challenged with a high-fat diet, remained insulin sensitive, exhibited decreased fat mass, and were resistant to weight gain. Additionally, the fetuin KO mice demonstrated protection from obesity and insulin resistance associated with aging (30). These findings illustrate a role for fetuin in regulating insulin sensitivity, weight gain, and fat accumulation and demonstrate a novel therapeutic target for combating the deleterious symptoms of type 2 diabetes, obesity, and insulin resistance.

AHSG gene

The cDNA encoding AHSG has been cloned and chromosomally mapped to 3q27 (80). Osawa et al. (100) have described the structure and organization of the *AHSG* gene by comparing human AHSG cDNA sequence (80), to the genetic sequence of rat PP^{63}

(101). A total of 8,748 bp were delineated from PCR fragments, and sequence analysis revealed the presence of seven exons and six introns, which spanned 8.2 kb. The ATG start codon was found on the first exon, while the TAG stop codon was found on the seventh exon at nucleotide positions 7787 and 7789, relative to the ATG start codon. Further analysis and comparisons indicated that the positions of the intron interruptions corresponded exactly with that of rat PP^{63} (fetuin) gene as described by Falquerho et al (101). These findings, along with conserved protein sequences, demonstrate the identity of human AHSG and rat fetuin. Osawa et al. further demonstrate the presence of the CCAAT enhancer binding protein (C/EBP)-binding sequence (TTATGCAAT) and hepatocyte nuclear factor (HNF)-binding sequence (TGTTTGC) on the promoter. The C/EBP family of transcription factors is involved in metabolism and differentiation of cells, especially in liver and adipose tissue (102), while HNF is involved in the transcription of various liver specific genes (103). Furthermore, C/EBP and hepatocyte nuclear factor 3β (HNF-3β) have been demonstrated to mediate dexamethasone-induced upregulation of the *AHSG* gene (104). Treatment with dexamethasone to primary rat hepatocytes increased C/EBP protein levels and transcription of *AHSG.* Accordingly, it was demonstrated that C/EBP and HNF-3β bind to the steroid response unit (SRU), which is highly conserved in rat, mouse, and human within the promoter, resulting in enhanced transcription of *AHSG*. Dexamethasone has been well characterized as an insulin antagonist, opposing the effects of insulin (105). The study by Woltje et al. suggests that dexamethasone-mediated upregulation of *AHSG* via C/EBP and HNF-3β, may contribute to dexamethasone-induced insulin resistance. In addition, regulation of the *AHSG* gene in response to high fat (HF) and low fat (LF) diets has been reported in

rats (106). Osborne-Mendel (OM) and S5B/PI rats differ in their susceptibility to obesity when challenged with a HF diet. S5B/PI rats remain lean, while OM rats exhibit a much higher propensity to weight gain. Further analyses demonstrate that the S5B/PI rats exhibit an impaired expression of *AHSG*, further implicating AHSG in the involvement of insulin resistance and fat accumulation.

Human Studies

Polymorphisms of human AHSG have been reported (107; 108). One study reports differences in six nucleotide arrangements at amino acid positions: 84,107, 172, 230, 238, and 252 of AHSG cDNA obtained by RT-PCR (107). Positions 107, 230, and 238 give rise to amino acid substitutions, while 84, 172, and 252 do not. Interestingly, amino acid positions ^{230}Thr and ^{238}Thr harbor two of the *O*-linked glycosylates (See Fig. 2). A single nucleotide polymorphism (SNP) in the 5′ region has been identified and associated with insulin-mediated inhibition of lipolysis and stimulation of lipogenesis, as well as two additional SNPs being associated with circulating cholesterol (108), providing more evidence for AHSG/fetuin-A's role in fat accumulation. Further, in a Swedish population, substitutions at Thr230Met and Thr238Ser, two of the variations described by Osawa et al. (107), are associated with decreased AHSG protein levels, which correspond to an increased propensity for leanness among men (32). These results are in concert with findings from the *Ahsg* KO mice, which report resistance to weight gain in *Ahsg* null mice when fed a high fat diet (see reference 29). Other investigations report *AHSG* SNPs associated with type 2 diabetes in French Caucasians and increased adipocyte β2-adrenoceptor function (109; 110), further demonstrating AHSG/fetuin-A's role in regulating body mass and adiposity.

Several reports demonstrate decreased levels of AHSG are associated with leanness and resistance to weight gain. Other studies reciprocate these findings by showing increased AHSG levels positively correlate with body mass and insulin resistance. Gestational diabetes is defined as carbohydrate intolerance that is first recognized during pregnancy. Kalabay et al., show that gestational diabetes patients have significantly higher levels of fetuin-A than do healthy pregnant women and non-pregnant controls (35). Data from the Heart and Soul Study indicate that fetuin-A levels are positively associated with the metabolic syndrome as well as an atherogenic lipid profile (34). Mori et al. have compared fetuin-A levels between patients with type 2 diabetes and non-diabetic controls (36). There were no significant differences in fetuin-A levels between non-diabetic and type 2 diabetic groups; however, fetuin-A levels were highly associated with HOMA, BMI, and triglyceride levels in the non-diabetic group. Additionally, fetuin-A levels were shown to be associated with insulin resistance and a fatty liver in individuals without diabetes (33).

These human reports imply a significant role of fetuin-A in regulating body mass, fat accumulation, and insulin resistance. However, these studies have only examined total fetuin-A levels. Since phosphorylation of fetuin-A has been has been shown to be a critical regulator of its IR-TK inhibitory activity, assessment of fetuin-A phosphorylation in conditions of diabetes, obesity, and insulin resistance can significantly contribute to our understanding of the role of AHSG in these conditions.

Objectives and hypotheses

The objective of the present study was to elucidate the phosphorylation status of fetuin-A in animal models of diabetes, obesity, and insulin resistance. Fetuin-A is a

physiological inhibitor of the IR-TK, and phosphorylation has been shown to be critical for its inhibitory activity. Human studies demonstrate that fetuin-A is positively correlated with BMI, insulin resistance, fatty liver, and an atherogenic blood profile; however, no studies have examined fetuin-A phosphorylation in obese, diabetic, or insulin resistant conditions. The ^{120}Ser and ^{312}Ser phosphorylation sites on fetuin-A are conserved in human, mouse, rat, pig, sheep, and cow, which suggests a regulatory role at these sites. The majority of the phosphorylation has been shown to occur at ^{312}Ser. Accordingly, we have generated an anti-phospho-^{312}Ser-fetuin-A antibody in order to assay fetuin-A phosphorylation in animal models of obesity, diabetes, and insulin resistance. The animal models in the present study are well characterized and widely used in scientific investigations.

We hypothesize that in conditions of extreme insulin resistance or frank diabetes animals will exhibit increased phospho-fetuin-A levels. Additionally, in conditions of slightly elevated diabetic parameters, i.e., insulin, leptin, and glucose levels, animals will exhibit decreased phospho-fetuin-A levels as a compensatory mechanism to allow for increased insulin action.

CHAPTER 3: PHOSPHORYLATION OF FETUIN-A, A PHYSIOLOGICAL INHIBITOR OF INSULIN ACTION, REGULATED BY INSULIN AND LEPTIN

ABSTRACT

Fetuin-A, a liver secreted phosphoprotein and rat homologue of human 2-HS-glycoprotein (AHSG), is a physiological inhibitor of insulin action. Fetuin-A-null mice exhibit improved glucose tolerance and resistance to weight gain on a high-fat diet. Recent human data demonstrate a positive correlation of circulating fetuin-A levels with BMI, insulin resistance, and fatty liver. Since phosphorylation is critical for its inhibitory activity, circulating fetuin-A phosphorylation was assayed in animal models of insulin resistance, diabetes, and obesity. Using a phospho-specific (^{312}Ser) fetuin-A antibody, fetuin-A phosphorylation status was assayed in Zucker diabetic fatty (ZDF) rats, *ob/ob* and *db/db* mice, streptozotocin-treated rats, and in a rat model of diet-induced obesity (DIO). The leptin receptor-deficient, insulin resistant ZDF rats had significantly higher levels of phosphorylated fetuin-A compared to their lean counterparts. Likewise, the leptin-deficient, obese *ob/ob* mice had significantly higher levels of phosphorylated fetuin-A. Consistent with these results, central leptin administration to control and STZ-treated rats, as well as leptin treatment to human hepatoma cell lines, significantly decreased fetuin-A phosphorylation. To further characterize the regulation of fetuin-A phosphorylation, we demonstrate that insulin treatment downregulates phosphorylation in

Hep3B and HepG2 cell lines. Similarly, DIO rats that exhibit elevated levels of insulin, albeit not to a significant degree, demonstrate significantly decreased levels of fetuin-A phosphorylation. We demonstrate that insulin and leptin decrease phosphorylation of fetuin-A. Accordingly, in conditions of mild to moderate elevation of insulin (as in the DIO model), fetuin-A phosphorylation is decreased, mitigating the inhibitory effect of fetuin-A on IR and allowing for increased insulin signaling. In conditions of extreme insulin resistance (ZDF and ob/ob models), where insulin is unable to exert its actions on insulin receptors, fetuin-A phosphorylation is increased, potentially exacerbating the diabetic phenotype. These findings suggest that fetuin-A may play a significant role in the regulation of insulin action.

INTRODUCTION

Insulin resistance is characterized by an attenuated ability of peripheral tissue to respond to circulating insulin. Individuals with insulin resistance have a higher propensity for developing type 2 diabetes. Of the reported 20 million people who suffer from diabetes in the U.S., 90-95% is affected by type 2 diabetes; *ergo*, enhancing insulin action in this population is of paramount importance. Though the underlying cellular mechanisms leading to insulin resistance have not been clearly delineated; several physiological regulators of insulin action, including free fatty acids (4), TNFα (7), leptin (111), adiponectin (112), resistin (113), and RBP4 (45), have been implicated in modulating insulin sensitivity. Some of these molecules have been demonstrated to inhibit insulin action at the level of the insulin receptor (IR), thus decreasing downstream, insulin-dependent signaling events. Fetuin-A (also known as α2-HS-glycoprotein, AHSG), a 63 kD phosphorylated glycoprotein, synthesized and secreted primarily by the liver and belonging to the cystatin family of protease inhibitors, is a natural inhibitor of IR tyrosine kinase activity (21).

Fetuin-A preferentially interacts with the activated insulin receptor, repressing insulin-induced IR autophosphorylation and subsequent downstream signaling activity *in vitro*, in intact cells and *in vivo* (28). Mice lacking fetuin-A demonstrate improved insulin sensitivity, whole-body glucose utilization, and resistance to weight gain when fed a high fat diet (29). Additionally, these mice are protected against aging-induced obesity and insulin resistance (30). Interestingly, the gene for fetuin-A is localized on chromosome

3q27, a locus that has been shown to be associated with type 2 diabetes and cardiometabolic risk (31). While a polymorphism of the *AHSG* gene has been shown to be associated with type 2 diabetes, another polymorphism is associated with leanness in Swedish population (32). Increased plasma fetuin-A levels have been reported in insulin resistance in humans. Further, a growing body of human investigation studies point to a strong association of plasma fetuin-A levels with insulin resistance (33-35), an atherogenic lipid profile (34), increased BMI and plasma triglyceride levels (36). Plasma concentrations of fetuin-A in humans range from 200-600 μg/ml, with an average plasma concentration of ~ 300 μg/ml in healthy adults (37). Fetuin-A exists in both phosphorylated and dephosphorylated forms in human plasma, and studies by Haglund et al, have demonstrated that fetuin-A is phosphorylated on two serine residues, ^{120}Ser and ^{312}Ser, and that approximately 20% of circulating fetuin-A is phosphorylated (38). Phosphorylation of fetuin-A has been shown to be critical for its IR tyrosine kinase inhibitory activity (22). While ^{120}Ser has been shown to be constitutively phosphorylated, ^{312}Ser seems to exhibit regulatory covalent modifications, suggesting this site may be critical for its inhibitory activity (38).

While previous human studies have investigated the associations of fetuin-A in disease conditions, there are no reports examining its phosphorylation status. Therefore, the aim of this study is to elucidate the phosphorylation status of fetuin-A in obesity, insulin resistance, and diabetes.

MATERIALS AND METHODS

Animals: Animal use and euthanasia protocols were reviewed and approved by Auburn University's Institutional Animal Care and Use Committee. Five-week-old male Zucker diabetic fatty (*fa/fa*) and lean Zucker (*fa/-)* rats were purchased from Charles River Laboratories (Indianapolis, IN). Rats were housed on a 12-hour light/dark cycle and fed a standard chow diet *ad libitum* with free access to water. Blood samples were collected from the saphenous vein bimonthly. At the time of sacrifice, a section of the liver was rapidly removed and stored in RNA later for gene expression analysis. Male Wistar rats were purchased from Charles River Laboratories and were treated with or without streptozotocin (30 mg/kg intravenously via the tail vein) and/or central leptin (5 μg ICV/day/10 days), resulting in four groups: Control, STZ-treated, leptin-treated, and STZ-leptin-treated. Pooled plasma samples from *ob/ob* and *db/db* animals were obtained from Jackson Laboratories (Bar Harbor, ME). For the high-fat fed rodent model, we obtained frozen liver tissues and plasma samples from our collaborator, Dr. Catherine Jen, Professor and Head, Department of Nutrition and Food Science at Wayne State University, MI. For this model, Wistar rats were fed a 40% high-fat diet or standard rat chow for 6 weeks**.**

Antibodies: Rabbit polyclonal phospho-specific anti-^{321}Ser-Fetuin-A antibody was generated using the following peptide: HTFMGVVSLGSP**S**(PO_4)GEVSHPR. Antibody-containing serum was affinity purified using the above peptide (Affinity BioReagents, Golden, CO). Pan-fetuin-A antibody was purchased from INCSTAR (Stillwater, MN).

Metabolic studies: Blood glucose levels were determined bimonthly using an Accu-Chek glucometer (Roche Diagnostics). Insulin and leptin levels were measured by ELISA using rat insulin or leptin as a standard (Linco Research Inc.).

Cell culture: Hep-3B and Hep-G2 cells were cultured in improved modified Eagle's medium (IMEM) supplemented with 10% fetal bovine serum. Cells were grown to confluence then starved overnight in a serum-free 0.1% BSA IMEM. The cells were then washed with PBS, treated for 24 hours with insulin (0.01 and 1.0 μM) (Roche Diagnostics) or leptin (1.0 and 100 nM) (R&D Systems, Minneapolis, MN), and maintained in serum-free media. Cells were then washed and treated again with insulin or leptin. Media was collected 6 hours later for SDS-PAGE and Western analysis.

SDS-PAGE and Western analysis: Plasma from whole blood samples was diluted in sterile saline to 1:100 and was quantified using the Bradford protein assay. Normalized samples were boiled in SDS-buffer and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human fetuin-A (INCSTAR) and ^{312}Ser-Fetuin-A (Affinity BioReagents). Media samples from cell culture were similarly assayed.

Quantitative RT-PCR analysis: Total RNA was isolated from rat liver tissue using an RNeasy kit (QIAGEN). cDNA was prepared from 1 μg of RNA using the iQ^{TM} SYBR Green Supermix (BioRad). Quantitative real-time PCR was performed on an iCycler real-time PCR detection system (BioRad), using the following primers: Fetuin forward 5′ACGTGGTCCACACTGTCAAA′3, reverse 5′CGCAGCTATCACAAACTCCA′3 (Invitrogen). Expression level were standardized to β-actin by calculating ΔCt (ΔCt = Gene Ct - β-actin Ct). Gene expression was determined using the ΔΔCt method (114).

Partial purification of IR autophosphorylation and IR-TK activity: IR's were partially purified from whole liver tissue of high-fat fed and chow-fed rats using wheat germ agglutinin (WGA)-agarose columns and eluted with 0.3 mol/l *N*-acetylglucosamine. Total protein was normalized to 1 μg/ml using the Bradford protein assay. IR autophosphorylation was carried in the presence or absence of insulin using [γ-^{32}P]ATP in combination with 5 mmol $MnCl_2$, 50 μmol/l ATP, 50 mmol/l HEPES, pH 7.6, and 0.1% Triton X-100. IR-TK activity was assayed by quantitation of phosphorylation on exogenous poly($Glu^{80}Tyr^{20}$) (115).

Statistical analyses: Data are presented as means ± SE. Statistical significance was determined using a one-way ANOVA and unpaired *t* tests where appropriate. Significance was declared for *P* values < 0.05. (GraphPad Instat).

RESULTS:

Phospho-specific anti-^{312}Ser fetuin antibody: Phospho-specific antibody was tested for its ability to detect fetuin ^{312}Ser- phosphorylation. Fetuin was purified from HepG2 cell-media on a Jacalin column and was dephosphorylated by addition of calf intestinal alkaline phosphatase (CIAP) (Promega) in 10 mM Tris HCl, pH 8.0 for 2 h at 37º C. Samples, with and without treatment of CIAP, were assayed for phosphorylation using Western blotting. We show that the phospho-specific anti-^{312}Ser fetuin antibody detects only phosphorylated fetuin, while the pan-fetuin antibody detects both phosphorylated and dephosphorylated fetuin (Fig. 4).

Phospho-fetuin and total fetuin levels in rodent models of diabetes and obesity:

Zucker Diabetic Fatty Rats (ZDF):

Male ZDF rats, having a defect in the long-form of the leptin receptor (OB-R), exhibit significantly higher body weights at ages 6 weeks through 12 weeks compared to lean controls; however, by week 14 there was no significant difference in body weights between the two groups (Fig. 5). Although at 6 weeks there was no apparent difference in blood glucose levels, levels began to escalate at 7 weeks and plateau at 10 weeks in the ZDF rats (Fig. 6). Conversely, plasma insulin levels were significantly elevated at 6 weeks, indicating insulin resistance, and began to decline at 9 weeks, suggesting β cell failure (Fig. 7). Calculation of the homeostasis model assessment of insulin resistance (HOMA-IR) indicates that the ZDF rats were significantly more insulin resistant than their lean controls at week 6 (84.54 ± 25.4 vs. 3.84 ±0.9) ($p = 0.01$) and week 8 (247.56 ± 11.06 vs. 3.53 ± 1.12) ($p < 0.001$). After week 8, the ZDF rats began to exhibit β cell failure indicated by declining insulin levels, suggesting a shift from insulin resistance to overt diabetes. This decline in circulating insulin would alter the HOMA calculation; therefore, insulin resistance data was not calculated for 9 through 14 weeks of age. Since previous reports have only assayed total circulating levels of fetuin in diabetic and insulin resistant conditions, it was of interest to ascertain the phosphorylation of fetuin-A in the ZDF rat, a genetic model of insulin resistance and progressive β-cell failure, leading to diabetes. Using the phospho-fetuin anti-^{312}Ser antibody, we found plasma phospho-fetuin levels to be significantly increased in 6-week old ZDF rats compared to lean controls (Fig. 8). However, by 14 weeks there were no appreciable differences in plasma phospho-fetuin between the groups (Fig. 9). Similarly, there were no significant differences in total plasma fetuin levels at 6 or 14 weeks (Fig. 10, 11), despite an escalating trend in total plasma fetuin in the ZDF rats by 14 weeks (Fig. 11). Since

phospho-fetuin levels in the 6-8 weeks old, insulin and leptin resistant ZDF rat were significantly elevated, it was of interest to examine phosphorylation status of fetuin in other animal models of obesity and diabetes.

Obese ob/ob and diabetic db/db mouse models:

The *ob/ob* and *db/db* mice have mutations in the leptin gene and the long-form of the leptin receptor (ObR), respectively (98; 116). Pooled plasma samples from 8 week old *ob/ob* mice and *ob/+* littermate controls and *db/db* mice and *db/+* littermate controls were purchased form Jackson Laboratories, ME. Both of these mice exhibit hyperinsulinemia (Fig. 12). The *db/db* mouse is hyperleptinemic while the *ob/ob* is devoid of circulating leptin due to mutations in the long form of the leptin receptor and the leptin gene, respectively (Fig. 13). Consistent with the ZDF rat, the *ob/ob* mouse demonstrated significantly increased phosphorylation of fetuin, compared with the *ob/+,* lean littermates. No significant changes in fetuin-A phosphorylation was observed in the *db/db* mice (Fig. 14). We did not observe any significant differences of total plasma fetuin levels between the groups (Fig. 15). Taking into account that both leptin-signaling impaired ZDF rats and leptin-deficient *ob/ob* mice demonstrated increased plasma phospho-fetuin levels, we postulated that leptin might play a role in the regulation of fetuin's phosphorylation status. This hypothesis was tested in a streptozotocin (STZ) model of type 1 diabetes.

Streptozotocin (STZ) type 1 diabetic rat model:

STZ-treated male Wistar rats, a model for type 1 diabetes, were injected with leptin (5 μg/day/10 days) icv to ascertain the effects of *in vivo* leptin treatment on plasma phospho-fetuin and total fetuin levels in type 1 diabetes. Glucose and insulin levels of

STZ-treated rats indicated that STZ-treatment induced a type 1 diabetic phenotype (Fig. 16, 17). In addition, central leptin administration to STZ-treated animals completely normalized blood glucose levels without altering insulin levels. STZ-treated animals exhibited decreased levels of phospho-fetuin, while leptin administration to STZ-treated animals seemed to further repress phosphorylation. Similarly, leptin administration to control animals significantly decreased fetuin phosphorylation (Fig. 18), consistent with the *ob/ob* and ZDF models. Total plasma fetuin levels were significantly elevated in the STZ animals compared to control and leptin-treated STZ animals. Likewise, total plasma fetuin levels were elevated in the leptin-treated control group, albeit not to a significant degree (Fig. 19).

High fat-fed rat model:

Rats fed a high fat diet exhibit hyperinsulinemia, hyperlipidemia, hyperleptinemia, and impaired insulin signaling and glucose metabolism (29). Therefore, our goal was to distinguish the differences in levels of phospho-fetuin and total plasma fetuin in animals fed a high fat or low fat diet. Rats on a 40% high-fat diet for six weeks demonstrated increased leptin levels and normal glucose levels but elevated insulin levels, suggesting insulin resistance as indicated by an elevated HOMA index (Table 1). Rats fed the high fat diet also demonstrated significantly decreased levels of phospho-fetuin while total plasma fetuin levels were unaltered compared to a low fat diet (Fig. 20, 21).

Because phosphorylated fetuin inhibits IR-TK activity, we hypothesized that the high fat-fed rats, which exhibit decreased fetuin-A phosphorylation, would demonstrate no change in or increased IR-TK activity compared to chow-fed rats. Insulin receptors

from whole liver tissue were purified in wheat-germ agglutinin columns, and IR-TK activity was assayed. Extreme care was taken to preserve the integrity and phosphorylation status of the purified receptors by addition of protease and phosphatase inhibitors to all buffers and carrying out the entire purification procedure at 4ºC. Similar studies have been conducted by other investigators to assay the phosphorylation status of insulin receptors (29; 115). While the results were not significant, the high fat-fed rats demonstrated a trend toward increased basal IR-TK activity and insulin-stimulated IR-TK activity compared to chow-fed rats (Fig. 22), indicating that in high-fat feeding a counter regulatory mechanism occurs, possibly in order to enhance insulin action.

Effects of insulin- and leptin-treated Hep3B cells: The human hepatocellular carcinoma cell line, Hep3B, has been shown to express a functional leptin receptor (OB-R) (117) as well as synthesize and secrete fetuin in its phosphorylated form. In an attempt to corroborate our *in vivo* findings in intact cells, we treated Hep3B cells with different concentrations of leptin or insulin. Western blot analysis of fetuin secreted into the cell media indicated that leptin (1 and 100 nM) and insulin (0.01 and 1.0 μM) considerably abated the amount of phospho-fetuin without any appreciable change in fetuin levels (Fig. 23).

DISCUSSION:

A considerable amount of evidence has shown fetuin to act as an inhibitor of insulin receptor autophosphorylation and tyrosine kinase activity (90; 92). Phosphorylation of fetuin has been shown to be necessary for its inhibitory actions (23; 92). Recently, human fetuin was shown to be phosphorylated on ^{120}Ser and ^{312}Ser. These sites are conserved in human, pig, cow, sheep, rat, and mouse (82; 83; 86; 23; 87; 25; 88), suggesting these sites may be critical for fetuin's physiological activity. Human data show fetuin levels are increased with elevated BMI, plasma insulin and triacylglycerides, insulin resistance, and metabolic syndrome (33-36); however, no data exist that evaluate phosphorylation status in these conditions. Accordingly, we have addressed whether fetuin phosphorylation is altered in animal models of obesity, insulin resistance, and diabetes. Here, we report that insulin and leptin downregulate fetuin phosphorylation *in vivo* and in intact cells.

The ZDF rat is an animal model of impaired leptin signaling and extreme insulin resistance due to a mutation in the long form of the leptin receptor (118). In the ZDF rat, we observed an increase in plasma phospho-fetuin at 6 and 8 (data not shown) weeks of age. However, the magnitude of the increase in plasma phospho-fetuin at 8 weeks was not as great as at 6 weeks and was completely normalized by 10 weeks. The ZDF rats are overtly insulin resistant and demonstrate impaired leptin action, resulting in increased levels of plasma phospho-fetuin. At 6 weeks of age, the ZDF rat demonstrates euglycemia, similar to the high fat fed rat; however, these two rat models exhibit a

dichotomous relationship regarding fetuin phosphorylation status. The increased phospho-fetuin levels in the ZDF rat could be attributed solely to the impairment of leptin action. The ZDF rat also demonstrates an attenuating progression of phospho-fetuin and an increasing trend in total plasma fetuin levels as it ages.

The *ob/ob* mouse, a model of obesity and severe insulin resistance due to a mutation in the leptin gene, similarly exhibited increased plasma phospho-fetuin levels. An important result here is that both the ZDF and *ob/ob* animals, exhibiting impaired and no leptin signaling, respectively, demonstrate elevated levels of plasma phospho-fetuin. However, we observed no increase in phospho-fetuin levels in *db/db* mice, which, like the ZDF rats, also have a mutation in the long form of the leptin receptor. Pooled plasma samples from *ob/ob* and *db/db* mice were collected at 8 weeks of age; therefore, a possible explanation is that phospho-fetuin levels may have already been normalized by 8 weeks of age in the *db/db* mice. Our studies demonstrate that fetuin phosphorylation status in ZDF rats, which was elevated in 6 weeks, began to return to control levels beginning at 9 weeks of age. At 10 weeks of age, ZDF rats demonstrate significantly elevated blood glucose levels (559.67 ± 25.04 vs. 128.67 ± 27.06) and a failure of the β-cells to cope with rising glucose levels. Similarly, in the *db/db* model, it may be possible that fetuin phosphorylation status returned to control levels by 8 weeks of age.

Since we observed an increase in plasma phospho-fetuin levels in leptin-impaired animals, we investigated the effects of central leptin administration on fetuin phosphorylation. STZ-treated rats exhibited increased total plasma fetuin levels and decreased fetuin gene expression (data not shown), whereas central leptin administration normalized blood glucose, total plasma fetuin levels, and gene expression in STZ-treated

rats. The decrease in total plasma fetuin levels, as seen in the STZ-leptin-treated rats, could be attributed to the normalization of blood glucose levels, i.e., hyperglycemia can lead to nonenzymatic glycation of intracellular and extracellular proteins resulting in advanced glycation end products (AGEs) (119). AGEs are compounds that have been implicated in many of the deleterious symptoms of diabetes, affecting protein turnover, transcriptional factors, and gene expression, to name a few (119). We speculate this putative mechanism could also be the reason why we observed an increasing trend in the levels of total plasma fetuin in the ZDF rat. Despite an increase in total plasma fetuin, STZ-treated rats demonstrated decreased phospho-fetuin levels ($P = 0.08$), and central leptin administration further repressed phospho-fetuin levels. Similarly, control animals treated with leptin exhibited decreased phospho-fetuin levels to a significant degree ($P = 0.017$). These results, combined with data from the *ob/ob* and ZDF animals, suggest that leptin decreases plasma phospho-fetuin.

The high fat-fed rats exhibited modest elevation of leptin and insulin levels, yet maintained euglycemia, suggesting a trend toward insulin resistance. While the increased levels of insulin, leptin, and HOMA were not significant in the high fat fed rats, there was a significant decrease in plasma phospho-fetuin levels. We speculate that in conditions of euglycemia and slightly elevated levels of insulin and leptin, as in the high fat-fed model, a compensatory mechanism represses fetuin-A phosphorylation in order to enhance insulin action. Phosphorylated fetuin has been shown to be an IR-TK inhibitor, thus it would be logical to assume animals that demonstrate decreased phosphorylated fetuin would exhibit increased IR-TK activity. Accordingly, we show that the high fat-fed rats demonstrate a trend toward increasing both basal and insulin-stimulated IR-TK activity.

To further characterize insulin and leptin's role in regulating fetuin phosphorylation, Hep3B cells were treated with insulin or leptin. Confirming our animal data, both insulin and leptin treatment resulted in downregulation of fetuin phosphorylation.

In this study, we report, for the first time, that both levels and phosphorylation status are altered in animal models of obesity, insulin resistance, and diabetes. As observed in the high fat-fed model, conditions of slightly elevated insulin and leptin levels, as in mild insulin resistance or in the pre-diabetic stage, fetuin phosphorylation is decreased. This is further confirmed by our experiments in intact cells, where insulin and leptin repress fetuin phosphorylation. The decreased fetuin phosphorylation, as observed in the high fat-fed rats, may result as a compensatory mechanism in an attempt to enhance insulin signaling. However, with increased or extreme insulin resistance or leptin signaling impairment, as in the ZDF and *ob/ob* models, molecular mechanisms in the downregulation of fetuin phosphorylation is impaired, leading to significantly elevated levels of phosphorylated fetuin.

CONCLUSION

Diabetes affects more than 20 million adults in the U.S., and Alabama leads the nation with over 440,000 cases, resulting in an estimated $3 billion dollars annually in health care costs in Alabama alone. Several elements have been implicated in the development of diabetes, including obesity and genetics, and many physiological modulators such as free fatty acids, TNFα, MCP-1, leptin, adiponectin, etc. have been shown to regulate insulin action. Fetuin-A, also known as human AHSG, has been shown to be a negative regulator of insulin action by acting as a physiological inhibitor of the IR-TK. Phosphorylation status of fetuin-A has been shown to be critical for its inhibitory activity. Human data demonstrate that circulating levels of total fetuin-A positively correlate with BMI, insulin resistance, fatty liver, and an atherogenic blood profile. Furthermore, the gene for fetuin-A is localized on chromosome 3q27, a locus that has been shown to be associated with the development of type 2 diabetes. While previous human studies have investigated the associations of fetuin-A in disease conditions, there are no reports examining its phosphorylation status. Therefore, the aim of this study was to elucidate the phosphorylation status of fetuin-A in obesity, insulin resistance, and diabetes.

We show that high fat-fed animals, which exhibit euglycemia and slightly elevated levels of insulin and leptin, repress fetuin phosphorylation as a possible compensatory mechanism to enhance insulin action. Conversely, ZDF and *ob/ob* animals are leptin-signaling impaired and, as indicated by HOMA, are extremely insulin

resistance and therefore are unable to repress fetuin-A phosphorylation, which would possibly exacerbate the diabetic symptoms.

Since phosphorylation of fetuin has been shown to be critical for its activity as an inhibitor of insulin action, our studies indicating that both levels and phosphorylation status of fetuin are altered in obesity, insulin resistance, and diabetes may constitute an important element in the pathogenesis of obesity, insulin resistance, and diabetes. That fetuin levels are correlated with the metabolic syndrome or increased cardiometabolic risk in humans suggests that phosphorylated fetuin can be an anti-diabetic drug target. Development of antibodies, vectors, antisense oligonucleotides, siRNA, shRNA or a small molecule targeting fetuin and/or its phosphorylation status; or identifying potential kinases, phosphatases or upstream regulators of fetuin phosphorylation, etc., may serve to ameliorate the deleterious symptoms of insulin resistance, obesity, diabetes and cardiovascular diseases.

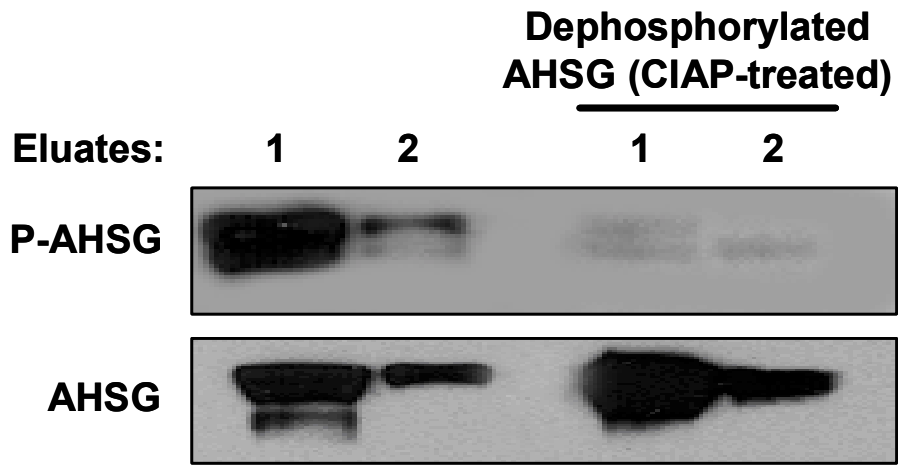

FIG. 4. Specificity of phospho-fetuin (^{312}Ser) antibody: AHSG secreted into the media from human HepG2 hepatoma cells was purified, and eluted fractions were assayed by Western blotting for phospho-AHSG (upper panel) and AHSG (lower panel).Treatment with calf intestinal alkaline phosphatase (CIAP) dephosphorylated phospho-AHSG up to 90%. Anti-^{312}Ser-AHSG antibody specifically detected the phosphorylated form of AHSG, while pan-AHSG antibody detected both phosphorylated and dephosphorylated AHSG.

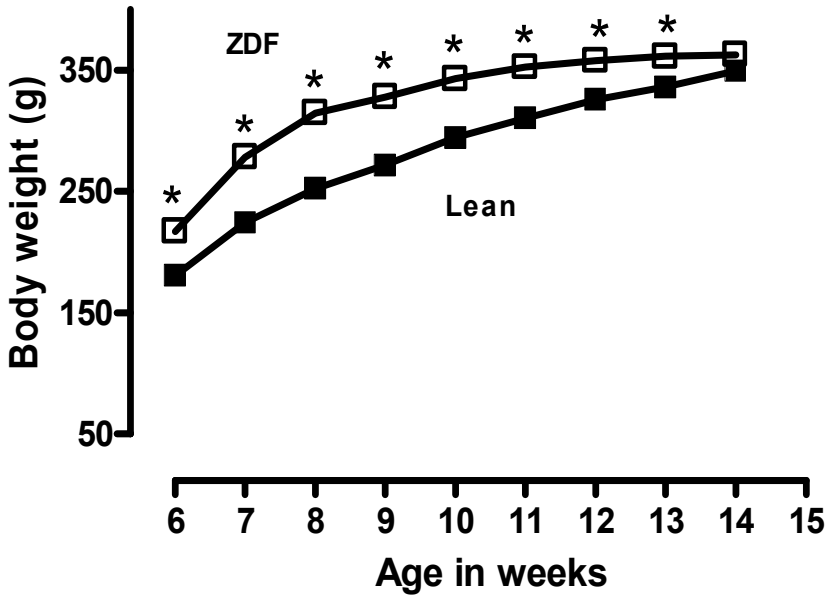

FIG. 5. Body weights of ZDF and lean control rats. Zucker diabetic fatty rats and its lean controls were fed standard rat chow. Values shown are weekly mean body weights (g) ± SEM for each group (n=6). * $p < 0.05$.

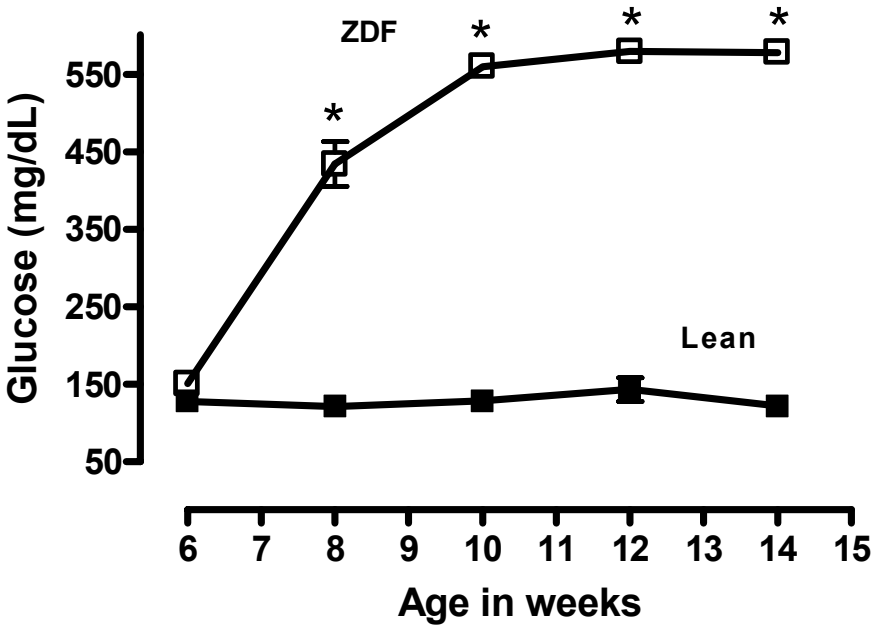

FIG. 6. Glucose levels in ZDF rats. Glucose levels were assayed with a handheld Accu-Chek glucometer (Roche Diagnostics) starting at 6 weeks of age through 15 weeks of age. Values shown are bimonthly mean glucose readings (mg/dL) ± SEM for each group (n=6). * $p < 0.05$

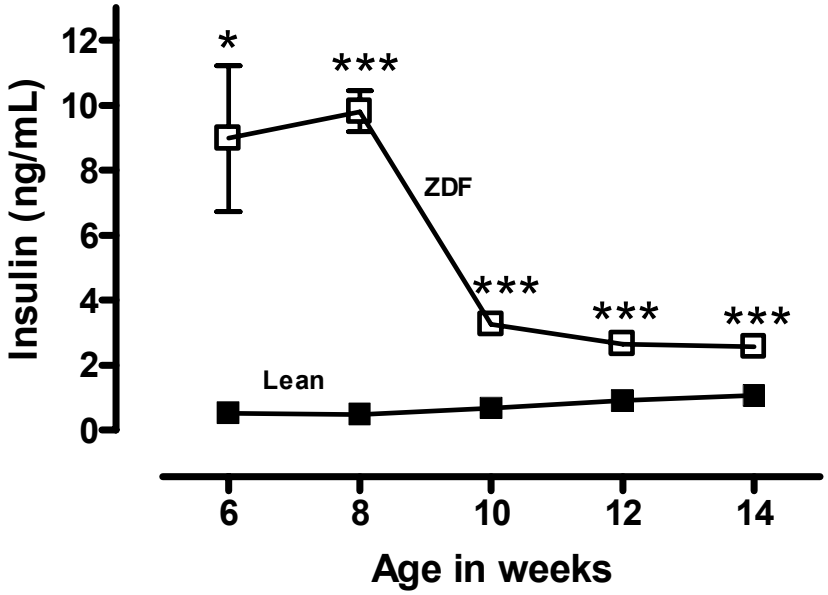

FIG. 7. Insulin levels in ZDF rats. Insulin levels were assayed by ELISA (Linco Research Inc.) using rat standards as a control. Values shown are bimonthly mean insulin readings (ng/mL) ± SEM for each group (n=6). * $p < 0.05$;
*** $p < 0.001$

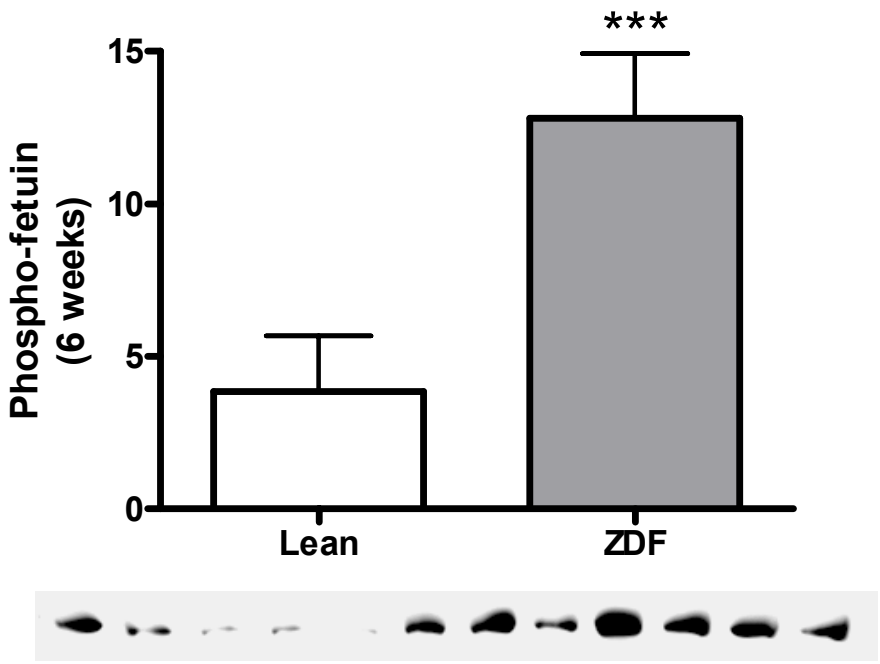

FIG. 8. Phosphorylation of fetuin in insulin resistant, obese ZDF rats (6 weeks). Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human ^{312}Ser-Fetuin-A (Affinity BioReagents). Values shown are mean densitometric quantification units from Western blot analysis ± SEM for each group (n=6) at 6 weeks. *** $p < 0.001$

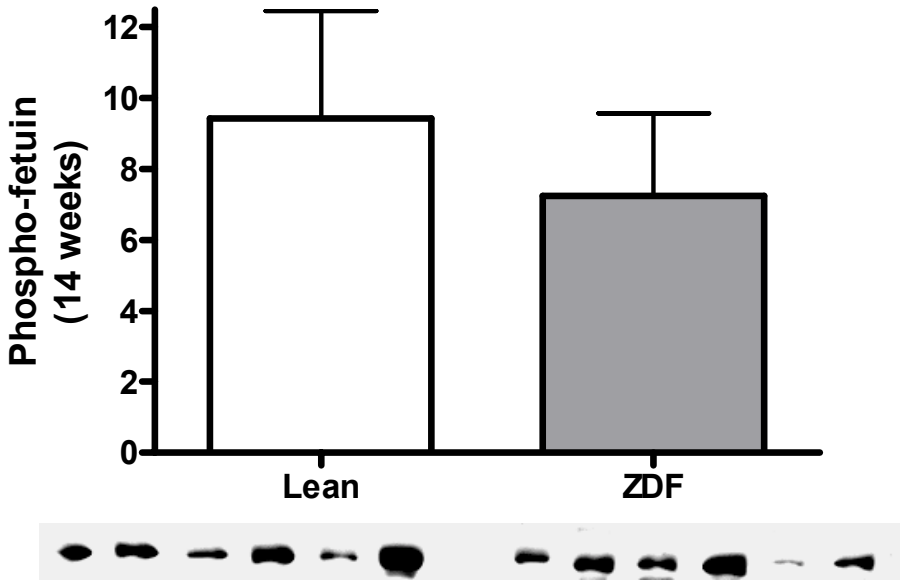

FIG. 9. Phosphorylation of fetuin in insulin resistant, obese ZDF rats (14 weeks). Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human ^{312}Ser-Fetuin-A (Affinity BioReagents).Values shown are mean densitometric quantification units from Western blot analysis ± SEM for each group (n=6) at 14 weeks.

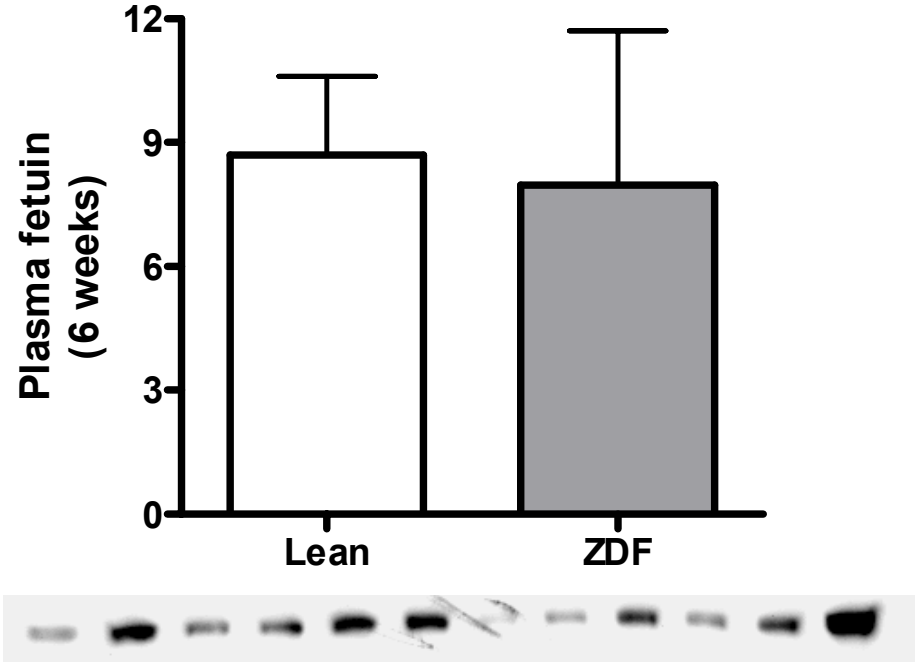

FIG. 10. Total plasma fetuin levels in insulin resistant, obese ZDF rats (6 weeks). Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human fetuin-A (INCSTAR). Values shown are mean densitometric quantification units from Western blot analysis ± SEM for each group (n=6) at 6 weeks.

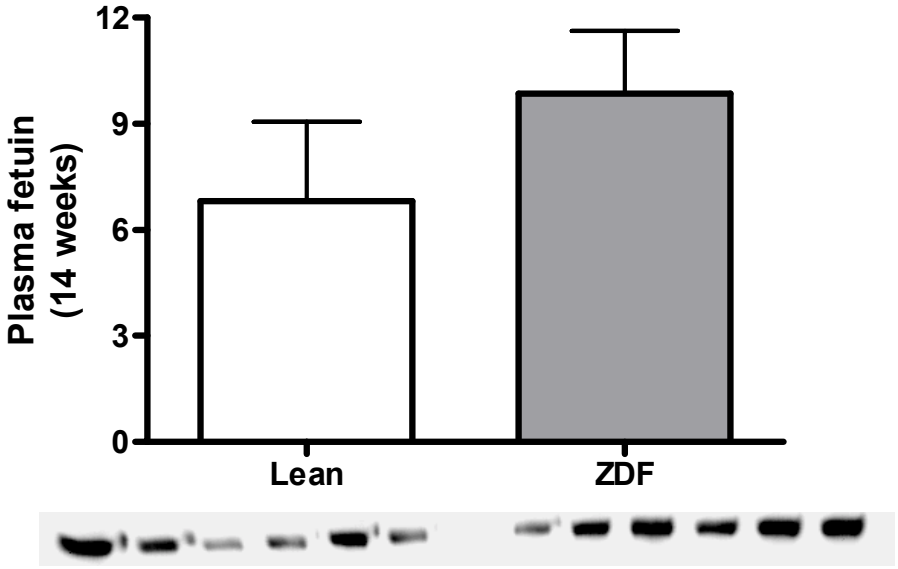

FIG. 11. Total plasma fetuin levels in insulin resistant, obese ZDF rats (14 weeks). Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human fetuin-A (INCSTAR). Values shown are mean densitometric quantification units from Western blot analysis ± SEM for each group (n=6) at 14 weeks.

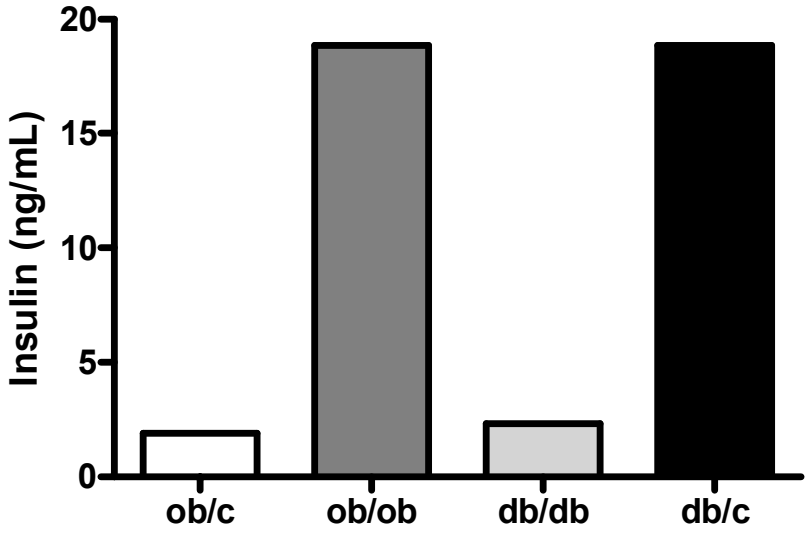

FIG. 12. Insulin levels in *ob/ob* and *db/db* mice. Insulin levels were assayed by ELISA (Linco Research Inc.) using mouse standards as a control. Values shown are insulin levels (ng/mL) assayed from pooled plasma samples.

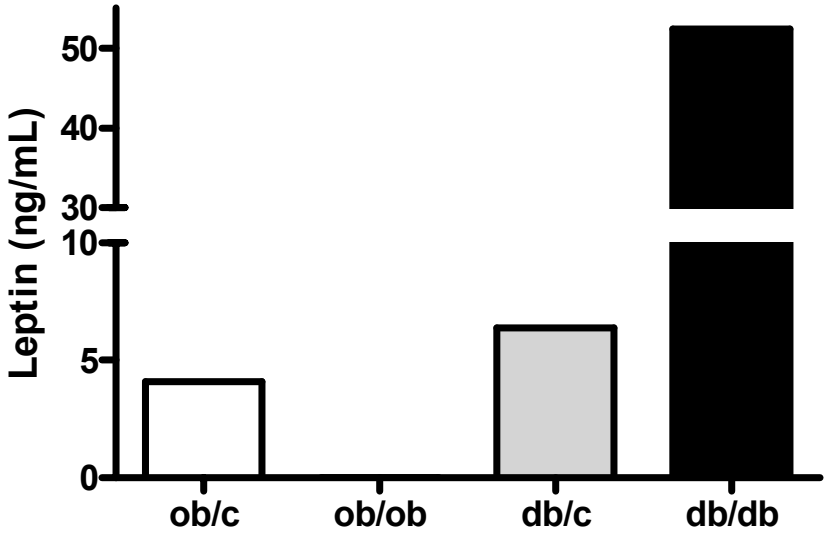

FIG. 13. Plasma leptin levels in *ob/ob* and *db/db* mice. Leptin levels were assayed by ELISA (Linco Research Inc.) using mouse standards as a control. Values shown are leptin levels (ng/mL) assayed from pooled plasma samples.

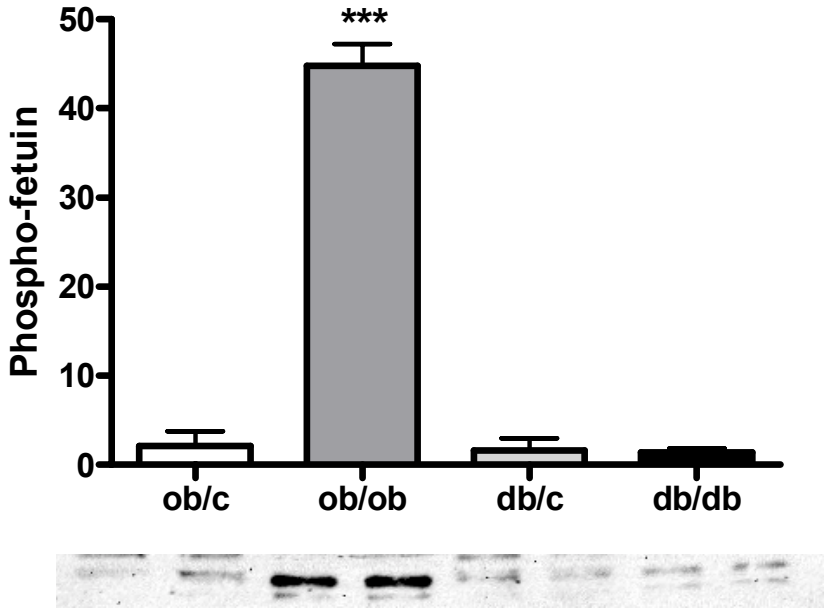

FIG. 14. Phosphorylation of fetuin in obese, diabetic *ob/ob* and *db/db* mice. Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human ^{312}Ser-Fetuin-A (Affinity BioReagents).Values shown are mean densitometric quantification units from Western blot analysis ± SEM from pooled plasma samples run in duplicates. *** $p < 0.001$.

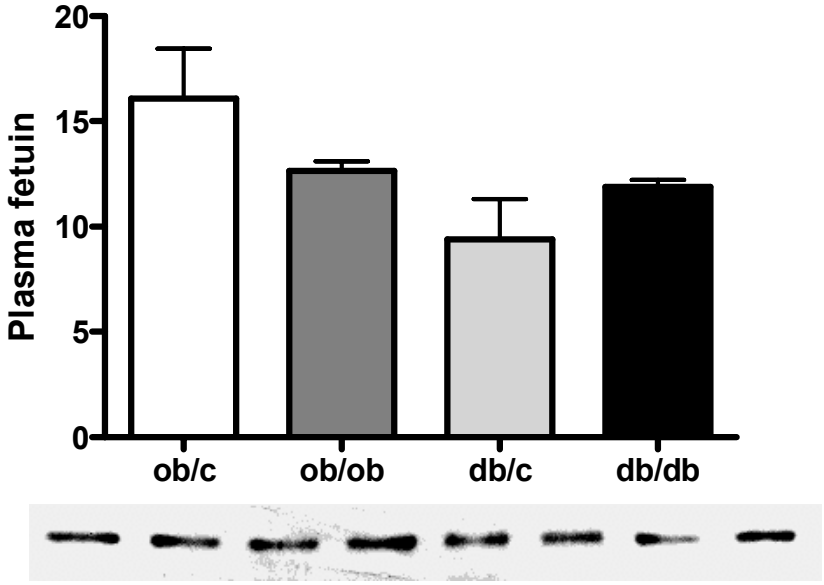

FIG. 15. Total plasma fetuin levels in obese, diabetic *ob/ob* and *db/db* mice. Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human fetuin-A (INCSTAR). Values shown are mean densitometric quantification units from Western blot analysis ± SEM from pooled plasma samples run in duplicates.

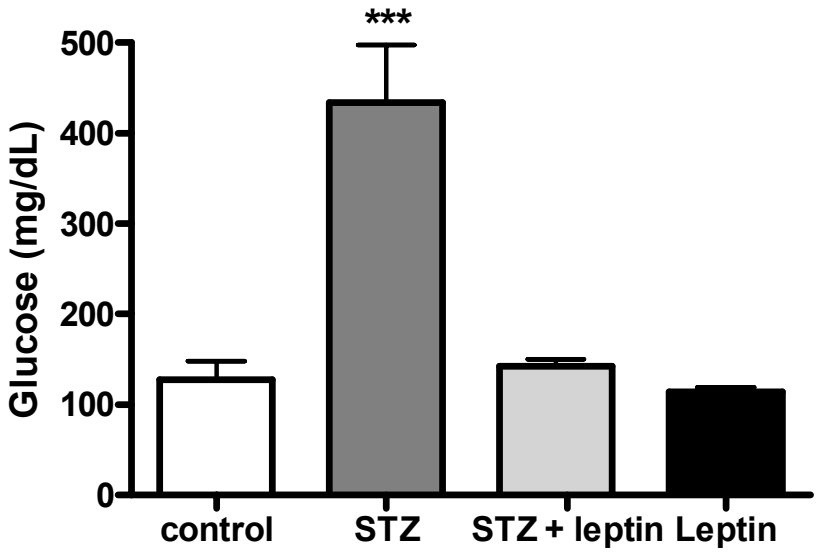

FIG. 16. Glucose levels in STZ-treated rats. Glucose levels were assayed with a handheld Accu-Chek glucometer (Roche Diagnostics). Values shown are mean glucose readings (mg/dL) ± SEM for each group (n=5). *** $p < 0.001$

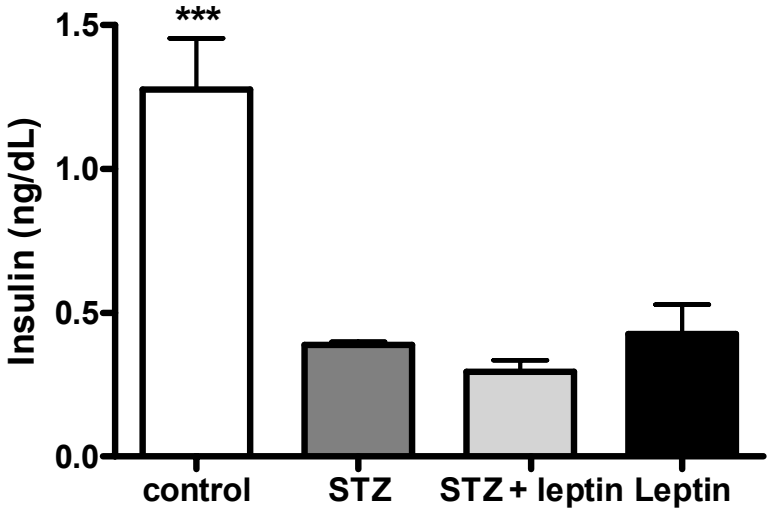

FIG. 17. Insulin levels in STZ-treated rats. Insulin levels were assayed by ELISA (Linco Research Inc.) using rat standards as a control. Values shown are mean insulin readings (ng/dL) ± SEM for each group (n=5). *** $p < 0.001$

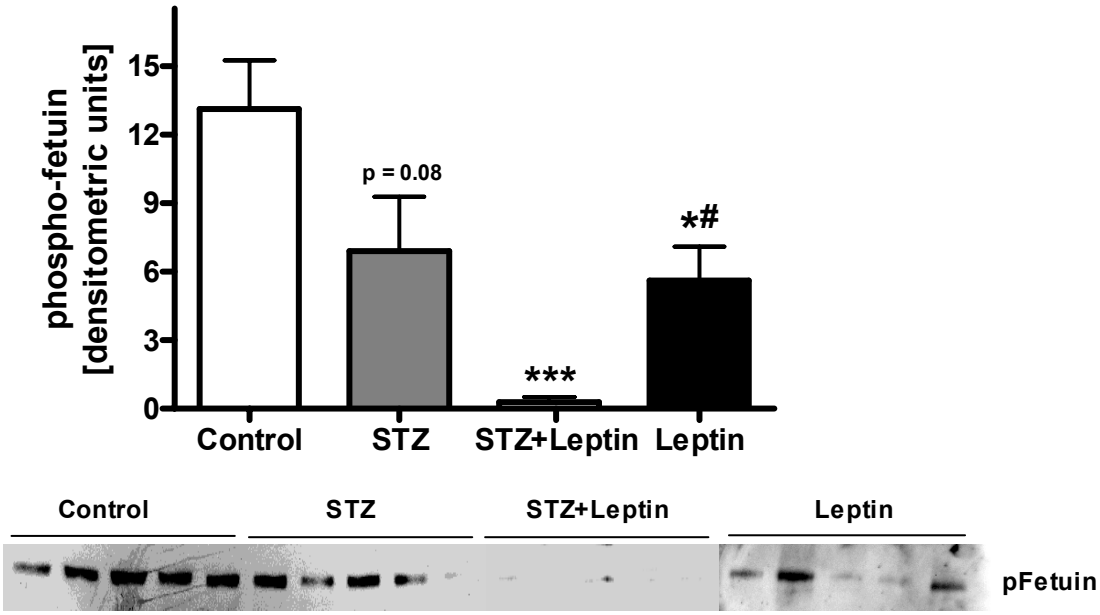

FIG. 18. Phosphorylation of fetuin in STZ-treated rats. Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human ^{312}Ser-Fetuin-A (Affinity BioReagents). Values shown are mean densitometric quantification units from Western blot analysis ± SEM for each group (n=5). * $p < 0.05$ compared to control; # $p < 0.05$ compared to STZ+Leptin; *** $p < 0.001$ compared to control and STZ.

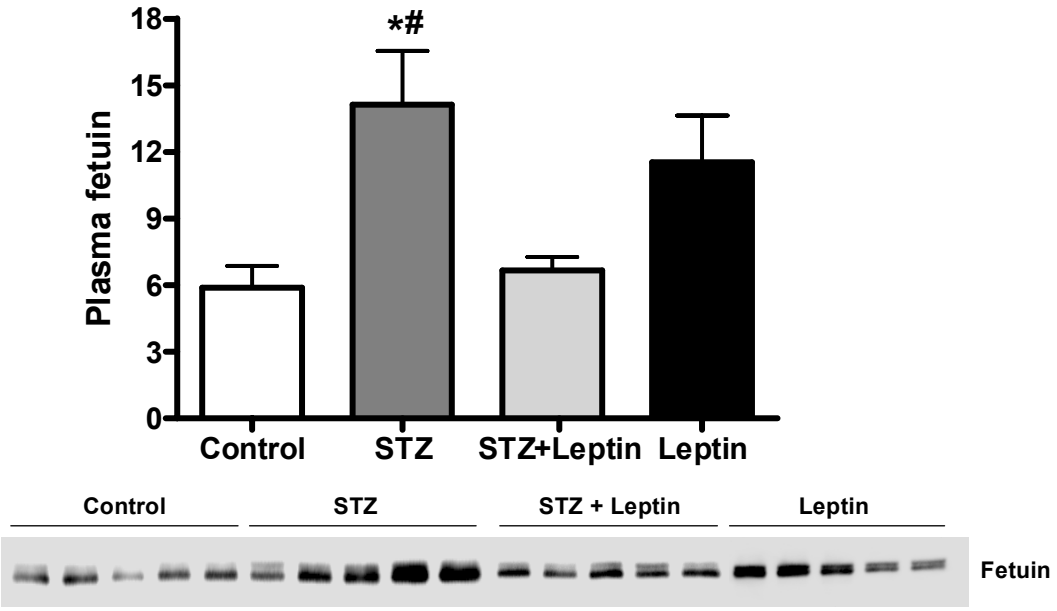

FIG. 19. Total plasma fetuin levels in STZ-treated rats. Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human fetuin-A (INCSTAR). Values shown are mean densitometric quantification units from Western blot analysis ± SEM for each group (n=5). * $p < 0.05$ compared to control and STZ+Leptin; # $p < 0.05$ compared to Leptin.

TABLE 1
Metabolic indices in high fat-fed and chow-fed rats

	n	Glucose (mg/dL)	Insulin (ng/mL)	HOMA	Leptin (ng/mL)
Chow	5	139.2 ± 30.7	1.22 ± 0.09	9.5 ± 1.0	1.4 ± 0.9
High fat	5	154.0 ± 11.3	1.73 ± 0.27	16.2 ± 3.8	2.2 ± 1.0

Values shown are means ± SEM. Glucose levels were assessed with a glucometer. Insulin and leptin levels were determined using ELISA. HOMA, an index of insulin resistance, was calculated as: [glucose (mmol/L) x insulin (μU/mL)/22.5].

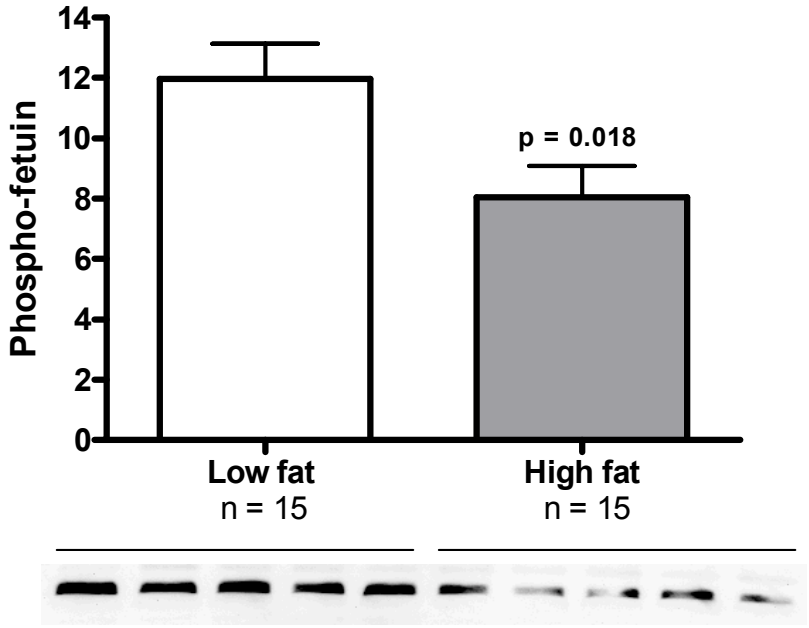

FIG. 20. Phosphorylation of fetuin in high fat-fed rats. Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human ^{312}Ser-Fetuin-A (Affinity BioReagents). Values shown are mean densitometric quantification units from Western blot analysis ± SEM for each group (n=5).

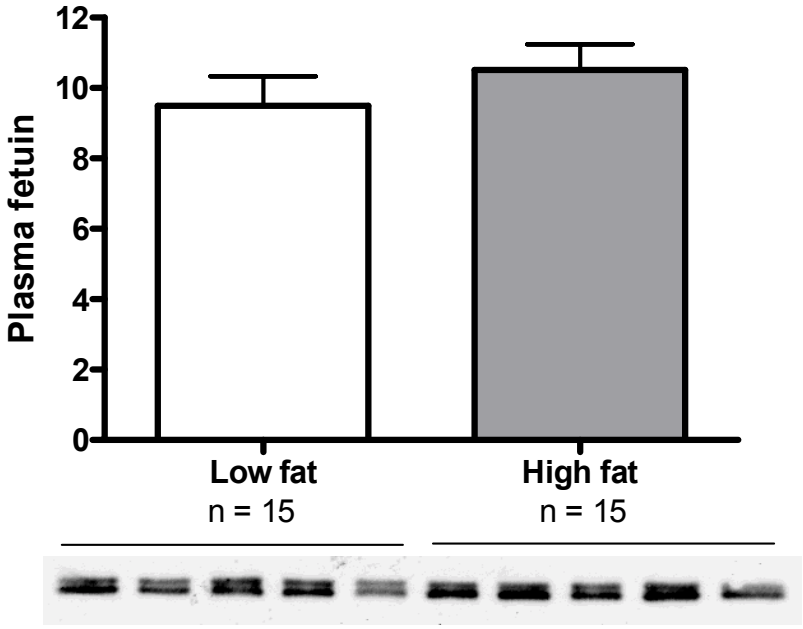

FIG. 21. Total plasma fetuin levels in high fat-fed rats. Plasma samples were taken and separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human fetuin-A (INCSTAR). Values shown are mean densitometric quantification units from Western blot analysis ± SEM for each group (n=5).

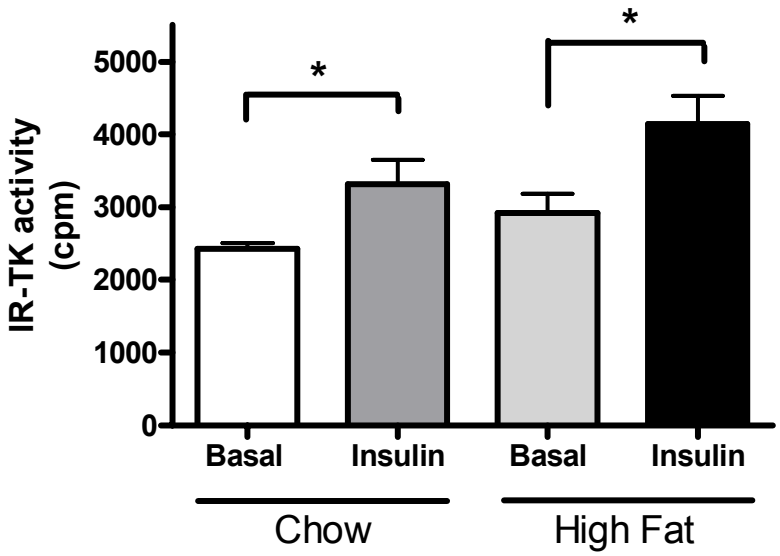

FIG. 22. Insulin receptor tyrosine kinase activity in high fat-fed rats. IR's were partially purified from whole liver tissue of high-fat fed and chow-fed rats using wheat germ agglutinin (WGA)-agarose columns. IR autophosphorylation was carried in the presence or absence of insulin using [γ-^{32}P]ATP in combination with 5 mmol $MnCl_2$, 50 µmol/l ATP, 50 mmol/l HEPES, pH 7.6, and 0.1% Triton X-100. IR-TK activity was assayed by quantitation of phosphorylation on exogenous poly($Glu^{80}Tyr^{20}$). Values shown are the mean (blank) ± SEM in each group (n=6). * $p < 0.05$.

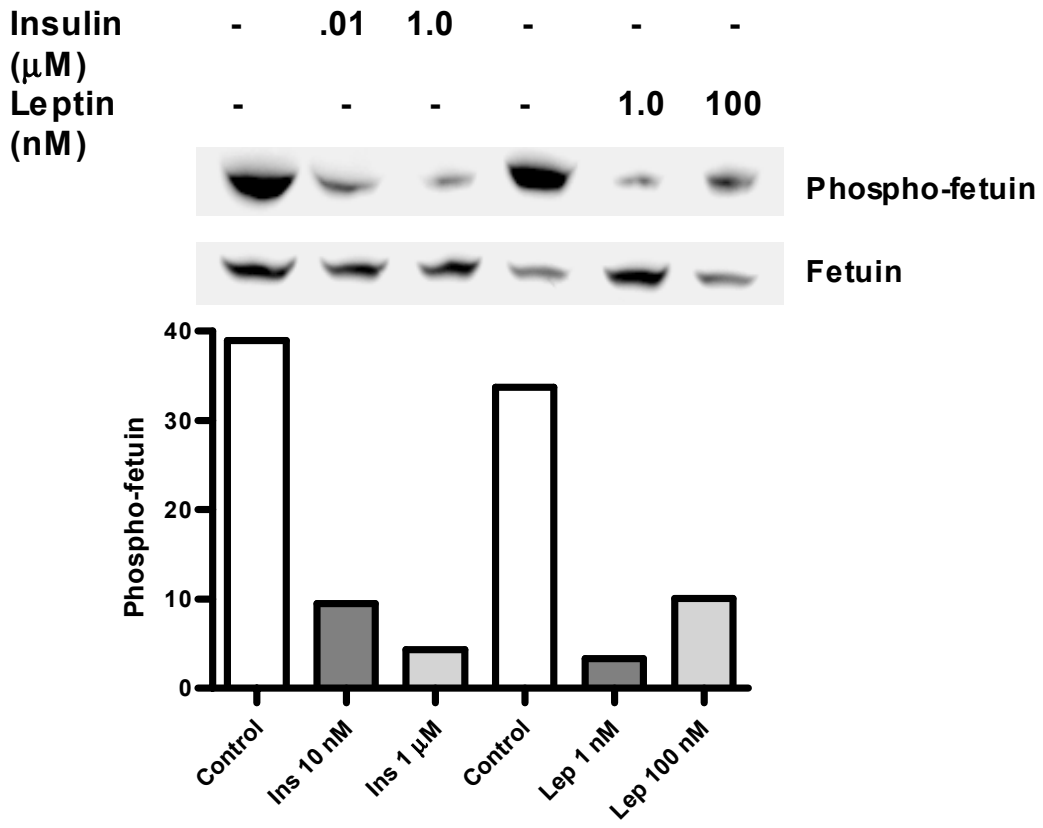

FIG. 23. Regulation of fetuin phosphorylation in Hep-3B human hepatoma cells. Hep-3B cells were grown to confluence and starved overnight. Cells incubated in the appropriate concentrations of insulin or leptin for 24 hours in serum free media. Cells were then washed, and fresh media with or without insulin or leptin was reapplied. Media was collected 6 hours later and assayed for fetuin and phospho-fetuin.

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