A NOVEL ROLE FOR FETUIN-A IN THE PATHOPHYSIOLOGY OF

GLUCOCORTICOID-MEDIATED INSULIN RESISTANCE

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A NOVEL ROLE FOR FETUIN-A IN THE PATHOPHYSIOLOGY OF GLUCOCORTICOID-MEDIATED INSULIN RESISTANCE

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Edmond Y. Huang, son of Jack Huang and Katie Yen, was born in Tai-chung, Taiwan on July 24, 1983. Upon graduating from Gretchen Whitney High School in Cerritos, CA in 2001, Edmond chose to attend the University of California, San Diego. Edmond graduated from UC San Diego on June 12, 2005 with a Bachelor of Science degree in Biochemistry with an emphasis in Chemistry. After working for one year at Bachem California Inc. as a Production Chemist, Edmond elected to pursue a graduate degree in the field of Nutrition. In August 2006, Edmond enrolled into the Graduate Program at Auburn University. Here, he studied as a graduate research assistant in the Department of Nutrition and Food Science under the direction of Dr. Suresh T. Mathews in pursuit of a Master of Science degree in Nutrition.

THESIS ABSTRACT

A NOVEL ROLE FOR FETUIN-A IN THE PATHOPHYSIOLOGY OF GLUCOCORTICOID-MEDIATED INSULIN RESISTANCE

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Fetuin-A (also called alpha2-HS glycoprotein), a phosphorylated protein secreted by the liver, is a physiological inhibitor of the insulin receptor tyrosine kinase (IR-TK). Elevated plasma levels of fetuin-A have been observed in animal models of obesity and diabetes, with fetuin-A null mice exhibiting improved insulin sensitivity and resistance to weight gain. In humans, increased plasma fetuin-A levels have been strongly correlated with insulin resistance, fatty liver, and metabolic syndrome. Increasing evidence indicate that insulin resistance and metabolic syndrome are associated with elevated circulating and/or tissue levels of cortisol, the principal active glucocorticoid in humans. Recently dexamethasone (DEX), a synthetic glucocorticoid, was shown to up-regulate fetuin-A gene expression and protein levels in primary mouse hepatocytes. Since DEX has been implicated in insulin resistance in animal models and humans, it was of significant interest to investigate the role of fetuin-A in DEX-induced insulin resistance. Treatment of Hep3B human hepatoma cells with DEX significantly increased the synthesis and

secretion of fetuin-A, suggesting a possibility that glucocorticoids may regulate fetuin-A levels in humans. These findings were extended to the Zucker diabetic fatty (ZDF) rat, a model of extreme insulin resistance. ZDF rats exhibited a ~2-fold increase in corticosterone levels compared to age- (6 weeks old) and sex-matched lean controls. This increase was matched by a ~2-fold increase in plasma fetuin-A levels in ZDF rats. Consistent with these data, DEX treatment (1 mg/kg, i.p., once daily for 4 days) was associated with hyperinsulinemia and insulin resistance, analyzed by homeostasis model assessment (HOMA) in 4-week old, male Wistar rats. Concomitantly, DEX treatment significantly elevated hepatic fetuin-A gene expression and protein levels resulting in a ~2.2-fold increase in plasma fetuin-A levels. Administration of RU-486, a specific glucocorticoid receptor antagonist, restored insulin and HOMA-IR to normal and significantly decreased plasma fetuin-A levels in DEX-treated animals. Next, we sought to examine the role of endogenous glucocorticoids in regulating fetuin-A through the removal of adrenal glands. As expected, adrenalectomy significantly decreased circulating corticosterone and insulin levels, and effectively improved insulin sensitivity in Wistar rats. This improvement in insulin sensitivity was associated with a significant decrease in plasma fetuin-A levels. Furthermore, DEX-treated fetuin-A knockout mice showed improved insulin sensitivity compared to wild-type controls. Taken together, we demonstrate that circulating fetuin-A levels are regulated by glucocorticoids. Since fetuin-A is implicated in insulin resistance and metabolic syndrome, these studies suggest a novel role for fetuin-A in glucocorticoid-mediated insulin resistance.

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CHAPTER 1: INTRODUCTION

Data from the World Health Organization indicate that more than 1.6 billion adults across the globe are overweight, while roughly 400 million of those are considered obese. By 2015, it is projected that around 2.3 billion adults, or one-third of the world's current population, will be overweight (1). In Alabama alone, approximately 28.4% of adults are obese while an additional 34.8% of adults are overweight, ranking the state among the most obese in the nation (2). An increase in obesity has been associated with the development of numerous health problems, including: gallbladder disease, osteoarthritis, cardiovascular disease, and diabetes mellitus (3).

The International Diabetes Federation shows that diabetes currently affects 246 million people worldwide and is expected to affect 380 million by 2025. Each year, a further 7 million people develop this disease, while more than 3.8 million deaths per year are attributed to diabetes and its related complications. It is considered the fourth leading cause of global death by disease (4). Type 2 is the most prevalent form of diabetes, and a particularly strong association exists between weight gain and the development of type 2 diabetes mellitus irrespective of ethnicity or gender (5,6). Type 2 diabetes usually stems from insulin resistance, when the body does not effectively respond to the

glucoregulatory mechanisms of insulin. Pancreatic β-cells compensate for the detriment in insulin signaling by releasing more insulin to maintain glucose homeostasis until they lose their ability to secrete it, resulting in β-cell failure and subsequent diabetes. Many complications can arise from diabetes, including: heart disease, stroke, blindness, kidney disease, and limb amputations (7).

Obesity has been strongly implicated as a potential risk factor for type 2 diabetes and is positively associated with an attenuation of insulin sensitivity (8,9). It represents an expansion of adipose tissue resulting in the hypertrophic secretion of signaling molecules by the adipocyte, called adipokines. TNF-α is an adipokine that inhibits insulin receptor signaling by reducing receptor tyrosine kinase activity (10). It also stimulates lipolysis and increases circulating fatty acid concentrations, further exacerbating insulin signaling (11,12). Interleukin-6 (IL-6) is another adipokine that is increased in the adipocytes of obese subjects (13). The secretion of TNF-α and circulating plasma IL-6 have both been shown to be highly associated with obesity-associated insulin resistance (14). Additionally, other adipokines such as leptin, adiponectin, resistin, and retinol binding protein 4 (RBP4) have been suggested to play a pivotal role in the development of insulin resistance (11,15-21).

Another molecule that modulates insulin signaling is fetuin-A, a phosphorylated glycoprotein secreted by the liver. It is part of the cystatin family of proteins and a negative acute phase reactant (22). Fetuin-A has been shown to have sequence homology to its rat homolog pp63, a natural inhibitor of insulin receptor tyrosine kinase (IR-TK)

activity (23-25). Recombinant fetuin-A was shown to interact with the insulin receptor and inhibit insulin-stimulated receptor autophosphorylation and tyrosine kinase activity, leading to inhibition of the Ras/Raf/MAPK pathway (26,27). In rat liver and skeletal muscle, fetuin-A also inhibits tyrosine phosphorylation of the insulin receptor (25,27).

The human fetuin-A gene is located on chromosome 3q27, a type 2 diabetes susceptibility locus (28). Data from the Heart and Soul study strongly associate fetuin-A with an atherogenic lipid profile in non-diabetic subjects with coronary artery disease (29). Serum fetuin-A levels are also positively correlated with severity of atherosclerosis in peripheral vessels of patients with normal renal function (30). Additionally, Stefan *et al.* report that elevated fetuin-A levels in humans are positively associated with both insulin resistance and fat accumulation in the liver (31). On the other hand, mice null for the fetuin-A gene display improved insulin sensitivity and do not gain weight when fed a high fat diet (32). Also, these transgenic mice do not become obese or insulin resistant as a result of age (33). Overall, a growing body of evidence seems to implicate fetuin-A as highly associated with both insulin resistance and type 2 diabetes.

Glucocorticoids are a set of hormones that have also been strongly linked with the development of insulin resistance. Today, these compounds are the most common treatment for reducing inflammation and immune activation in rheumatoid arthritis, asthma, and allotransplantation (34). While glucocorticoids possess potent anti-inflammatory and immunosuppressive properties, they can also lead to adverse metabolic effects such as hyperlipidemia, central obesity, and insulin resistance (35). Within the

scope of diabetes and homeostatic glucose control, the detrimental effects of glucocorticoids are synergistic: they increase hepatic glucose production and attenuate peripheral insulin sensitivity to cause insulin resistance, contributing to extensive hyperglycemia (36). Patients with Cushing's Syndrome present an excessive amount of cortisol, the primary endogenous glucocorticoid in humans (37). These patients also demonstrate impaired glucose tolerance and are insulin resistant (38). The chronic administration of glucocorticoids has been associated with elevated insulin levels in both animal and human models (39,40), and euglycemic-hyperinsulinemic clamp studies reveal the presence of whole-body insulin resistance (41,42). Dexamethasone (DEX) is a synthetic glucocorticoid roughly fifty times more potent than cortisol (43). Therapeutically, it has been used as an anti-inflammatory agent, an antiemetic, and can also reduce pain and swelling (44-46). However, one of the adverse side effects of DEX administration is its ability to induce insulin resistance. In rat skeletal muscle, DEX impairs glucose transport with or without insulin (47). Similarly, dexamethasone attenuates insulin signaling and glucose transport in primary cultured rat adipocytes (48). Furthermore, a single-dose DEX injection leads to whole-body insulin resistance in rats (49).

A recent study demonstrates that DEX treatment up-regulates fetuin-A gene expression in both mouse hepatoma cells and primary murine hepatocytes (50). However, no study has investigated the relationship between DEX, fetuin-A, and insulin resistance. Thus, the objective of this study is to elucidate the role of fetuin-A as a potential mediator of glucocorticoid-induced insulin resistance.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Obesity

The National Institute of Diabetes and Digestive and Kidney Diseases report that roughly two-thirds of adults in the United States are overweight, and almost one-third are considered obese (51). The chief principle in the progression of obesity is the concept of energy balance: when energy intake exceeds energy expenditure, fat storage occurs and eventually results in weight gain and obesity (52). Thus, a widely discussed theory for its cause is that society is shifting more toward a sedentary lifestyle, one that promotes an increase in energy consumption coupled with a reduction in energy expenditure leading to eventual weight gain (53). The primary storage site for lipids in our body is subcutaneous fat tissue. However, when subcutaneous fat reaches a threshold beyond which it cannot store any more, excess lipids are redirected to other locations throughout the body, most notably as visceral fat in intra-abdominal areas, but also in insulin-sensitive tissues such as skeletal muscle and the liver. This results in the disruption of normal metabolic processes throughout the body (54). Obesity has been implicated as a major contributing factor in the development of various clinical disorders, including hypertension, atherosclerosis, dyslipidemia, insulin resistance, and diabetes (8,55). For example, a net retention of lipids within the liver has been associated with insulin resistance, obesity, and type 2 diabetes (56,57). Furthermore, excess fat deposition in

skeletal muscle has been shown to lead to diminished insulin sensitivity and a decrease in glucose uptake (58-61). These are all chief characteristics of type 2 diabetes.

2.2 Diabetes

The American Diabetes Association defines diabetes mellitus as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (62). Diabetes is the sixth leading cause of death in the United States (7), affecting approximately 7% of the U.S. population (63). Diabetes is associated with a host of metabolic complications, including: heart disease, stroke, atherosclerosis, limb amputation, and many others (7). There are three types of diabetes that have been well-characterized. Type 1 diabetes, previously known as juvenile diabetes, is usually diagnosed in children and is characterized by the body's inability to produce insulin (63). Gestational diabetes occurs during pregnancy, when hormones from the placenta block the action of insulin in the mother, leading to insulin resistance. Together, these two types comprise a scant 10% of the total number of diabetics in the world. The vast majority of cases reside in the third category: type 2 diabetes. As the most prevalent of the three, type 2 diabetes currently affects more than 240 million people worldwide, nearly 6% of the world's adult population. It is distinguished by the body's inability to respond to insulin, a condition known as insulin resistance. Previous studies attribute the hyperinsulinemia observed in relation to obesity to an attenuation in insulin clearance from circulation (64-66). Alternatively, other studies ascribe elevated plasma insulin to an expansion of β-cell mass and function, leading to an increase in insulin secretion, a process termed β-cell compensation (67-69). While the literature

seems to be divided on which mechanism predominates (70), both are indicative of a "pre-diabetic" state in which the body undergoes a period of hyperinsulinemia to overcome slight elevations in fasting blood glucose (71). Extensive β-cell compensation eventually leads to defects in its cellular mechanism such as mitochondrial dysfunction, ER stress, and glucolipotoxicity. These abnormalities result in progressive β-cell failure where insulin cannot be produced in sufficient quantities to maintain normal blood glucose, resulting in uncontrolled hyperglycemia (69).

2.3 Insulin action and signal transduction

The insulin receptor (IR) is a heterotetrameric membrane glycoprotein consisting of two extracellular α-subunits and two transmembrane β-subunits held together through disulfide bonds. Initially, there is a separation between the two β-subunits to prevent premature interaction between them. When insulin binds to the binding domain of the α-subunit, a conformational change is induced that brings the two α-subunits closer together resulting in the autophosphorylation of the β-subunits and activation of the intrinsic kinase activity. This triggers a cascade of signaling steps intracellularly beginning with the phosphorylation of the insulin receptor substrate (IRS) proteins, Shc (Src homology collagen) and APS (adapter protein with Pleckstrin-homology [PH] and Src-homology-2 [SH2] domains) (72). In particular, the phosphorylation of Tyr972 of the IR β-subunit creates a recognition site for interaction with phosphotyrosine binding (PTB) domains that are located on IRS-1 and -2 (73). Upon PTB binding, two tyrosine residues within IRS-1 (Tyr612 and Tyr632) undergo phosphorylation and serve as a docking site for phosphoinositide-3-kinase (PI3K), a lipid kinase with a host of signaling functions which

include: cellular growth and differentiation, synthesis and degradation of carbohydrates, proteins and lipids, and membrane trafficking (74). Upon binding to IRS-1 or -2, PI3K becomes activated and phosphorylates phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] at the 3-position of the inositol ring to yield phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and phosphatidylinositol 3,4-bisphosphate (PIP_2) (75,76). These act as second messengers and recruit phosphoinositide-dependent kinase 1 (PDK1) to partially activate protein kinase B (also known as AKT) by phosphorylating the latter. Alessi and colleagues have previously demonstrated that the complete activation of AKT is dependent upon its phosphorylation at two amino acid residues: Thr308 and Ser473 (77). While PDK1 has been shown to phosphorylate Thr308, the autonomous mechanism of phosphorylation of the Ser473 remains controversial (78). The expression of a dominant-negative kinase-deficient insulin receptor in the skeletal muscle of transgenic mice leads to impaired insulin sensitivity, glucose intolerance, and a diminished activation of IRS-1 and PI3K (79,80), demonstrating the vital role of these molecules in insulin signal transduction. After AKT is activated, it detaches from the plasma membrane to translocate GLUT4, an insulin-dependent glucose transporter that is highly expressed in adipose tissue and skeletal muscle, from the cytoplasm to the cell surface, allowing for the transport of glucose into the cell (81). AKT also governs the activity of glycogen synthesis via phosphorylation and subsequent inactivation of glycogen synthase kinase-3 (GSK3) to activate glycogen synthesis (82). Additionally, AKT affects the activity of 6-phosphofructose 2-kinase (PFK2), an enzyme responsible for one of the rate-limiting steps of glycolysis. Phosphorylation of PFK2 through AKT leads to the activation of glycolysis *in vitro* (83).

Insulin signaling also plays a dual role in the management of gene expression within the cell through the mitogen-activated protein kinase (MAPK) signaling cascade. Receptor autophosphorylation on tyrosine residues of the insulin receptor promotes the formation of a Shc-Grb2 complex (84). At the plasma membrane, the two molecules then associate with son-of-sevenless (SOS), a nucleotide exchange protein that converts inactive GDP-Ras to active GTP-Ras (85). Ras leads to the activation of MEKK which subsequently phosphorylates MEK-1/2 to activate MAPK and the manipulation of gene expression for various cellular processes (86).

As the most potent anabolic hormone known, insulin has many pleiotropic roles in the synthesis and storage of macronutrients such as carbohydrates, lipids, and proteins. It stimulates the uptake of glucose, amino acids, and fatty acids into cells, and increases the expression or activity of anabolic enzymes that catalyze glycogen, lipid, and protein synthesis. It also inhibits the expression or activity of enzymes that degrade those macronutrients and release them into circulation (87). In the hepatocyte, insulin inhibits the production and release of glucose by inhibiting the transcription of the gene encoding for phosphoenolpyruvate carboxylase, a rate-limiting enzyme in gluconeogenesis, leading to a decrease in hepatic glucose production (88). In adipose tissue, insulin acts as a down-regulator of lipolysis via its inhibition of hormone sensitive lipase (89).

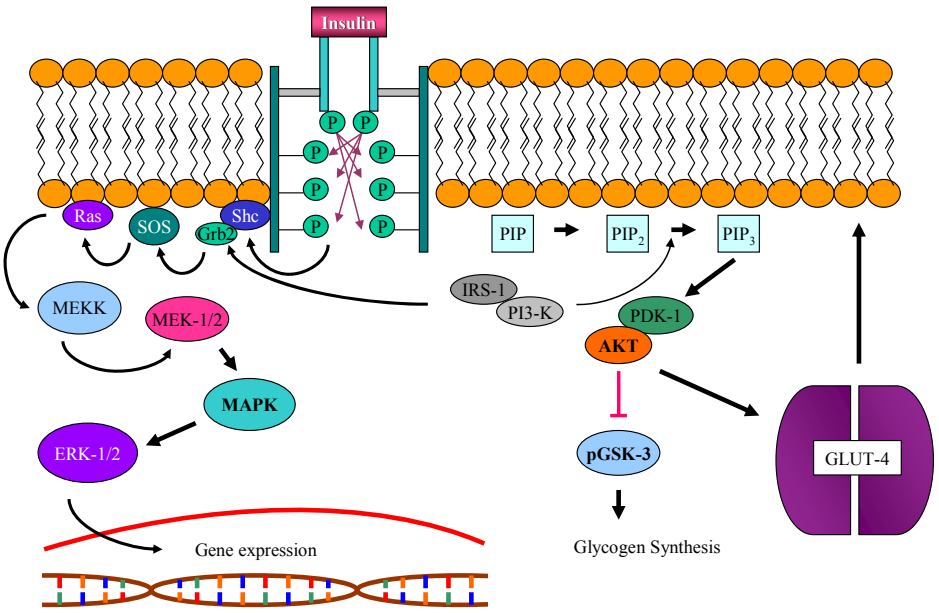


Fig. 1: Insulin signal transduction inside the skeletal muscle cell. The binding of insulin induces a conformational change in the insulin receptor, leading to the autophosphorylation of various tyrosine residues on the β subunit of the IR. PTB domains located on IRS proteins recognize and bind to these phosphorylated tyrosine residues, leading to the recruitment and activation of PI3K. This molecule then leads to the activation of AKT, ultimately resulting in the regulation of protein synthesis, glycogen synthesis, and translocation of GLUT4 to the plasma membrane for glucose uptake into the cell.

2.4 Insulin resistance

In many models of insulin resistance, there is evidence indicating that attenuation of insulin signaling begins with the insulin receptor or its downstream targets. Understanding the mechanisms of insulin signaling forms the cornerstone to studying the development of insulin resistance, diabetes, and the risk of cardiovascular disease. Insulin resistance plays a central role as a precursor to the development of type 2 diabetes. It is characterized by the body's inability to respond to insulin and is a common phenomenon often associated with genetic predisposition, aging, a sedentary lifestyle, and primarily obesity (90). There seems to be a causal role for obesity in the pathogenesis of insulin resistance since weight gain worsens it while weight loss ameliorates it (91). Insulin resistance, obesity, and type 2 diabetes are positively associated with increased plasma free fatty acid concentrations (92-95).

It was originally assumed that the adipocyte was just a storage depot for fat in the body, but ever since Hotamisligil and colleagues showed that the cytokine TNF-α, produced by adipocytes, was able to induce insulin resistance (96), many others have gone on to identify other biologically active signaling molecules produced by fat cells, collectively labeled adipokines. These include leptin, interleukin-6, resistin, angiotensinogen, adiponectin, retinol-binding protein 4 (RBP-4), and others (20,97,98). These discoveries form the cornerstone of a plausible mechanism for the idea that excess fat storage is a notable contributing factor to reducing insulin signal transduction via the modulation of the secretion of adipokines, which may contribute to insulin resistance.

Of particular importance is the fact that TNF-α activates Jun terminal kinase 1 (JNK), a serine/threonine protein kinase. In both genetic and dietary animal models of obesity, JNK activity is increased in the liver, muscle, and adipose tissue. Conversely, the loss of JNK prevents insulin resistance (99). Activating JNK leads to the phosphorylation of insulin receptor substrate 1 (IRS-1) at a serine residue (Ser307 in rats/mice; Ser312 in humans) located on the C-terminal end of the PTB domain. This inhibits it and disrupts the association between the insulin receptor and IRS-1, rendering the latter molecule inactive and impairing the insulin signaling cascade (100,101). Another signaling molecule associated with TNF-α is IκKβ. Studies have shown that IκKβ can also impact insulin signaling through two mechanisms: 1) the phosphorylation of a serine residue on IRS-1, resulting in attenuated insulin signaling; and 2) the phosphorylation of Iκβ (inhibitor of NF-κβ), activating the transcription factor NF-κβ that stimulates the synthesis of pro-inflammatory molecules such as TNF-α and IL-6 (102,103). In similar fashion, Yuan and colleagues have previously demonstrated that the over-expression of IκKβ extenuates insulin signaling in cultured 3T3-L1 adipocytes, whereas its inhibition reverses insulin resistance (104).

The adipokine adiponectin can enhance the inhibition of hepatic glucose production as well as glucose uptake in fat and skeletal muscle. There is decreased expression of adiponectin in obese humans and mice (105), while the administration of adiponectin to obese and insulin resistant mice improves insulin sensitivity (106-108).

Resistin is another adipokine that has been shown to decrease insulin-dependent glucose transport *in vitro* and increase fasting blood glucose levels and hepatic glucose production *in vivo* (20,109-111). In addition, transgenic mice lacking resistin exhibit low fasting blood glucose levels due to a decrease in hepatic glucose production (112).

Aside from adipokines secreted by adipose tissue, circulating free fatty acids can also modulate glucose homeostasis. Patients with insulin resistance and type 2 diabetes frequently display abnormal lipid metabolism and elevations in both lipid deposition and concentration in the skeletal muscle (11,113). Free fatty acids have been shown to reduce glucose uptake, while a decrease in lipid levels improves insulin activity in the skeletal muscle, adipocyte, and the liver (114). They also affect downstream targets of the insulin receptor such as PI3-K activity in the skeletal muscle. An increase in plasma free fatty acids results in increased IRS-1 Ser307 phosphorylation, in turn leading to decreases in IRS-1 tyrosine phosphorylation, PI3-kinase activity, and glucose transport (115).

2.5 Negative regulators of insulin signaling

Within the context of insulin resistance, many molecules have been described as having a negative impact on insulin signaling. Since the active insulin receptor conformation is maintained through the phosphorylation of essential tyrosine residues, dephosphorylation of the receptor by protein tyrosine phosphatases (PTPs) can deactivate the receptor and reduce insulin signaling. Studies in human skeletal muscle show that protein tyrosine phosphatase-1B (PTP-1B) activity is increased in muscles from obese subjects, but is reduced in obese, diabetic subjects (116). Additionally, the

overexpression of PTP-1B reduces glucose uptake and GLUT-4 translocation to the cell membrane in primary rat adipocytes and Rat1 fibroblasts overexpressing human insulin receptors (117,118). Also, the overexpression of leukocyte antigen-related phosphatases (LAR) in mice has been shown to reduce both glucose disposal and uptake, causing whole-body insulin resistance (119).

Another negative modulator of insulin signaling is the suppressor of cytokine signaling (SOCS) family of proteins, which act as negative feedback regulators of cytokine signaling (120). Studies have indicated that SOCS-1, SOCS-3, and SOCS-6 can bind to the insulin receptor in cells (121,122). In addition, SOCS-3 attenuates insulin signaling both in vitro and in vivo (123,124). The overexpression of SOCS-1 and SOCS-3 in liver causes insulin resistance, while their suppression leads to markedly improved hepatic steatosis (125).

Plasma cell antigen 1 (PC-1) is a plasma membrane enzyme that is widely distributed in tissues, including the three major insulin-sensitive tissues: liver, adipose tissue, and skeletal muscle (126). Transgenic mice overexpressing PC-1 exhibited hyperglycemia, insulin resistance, and diabetes (127). Additionally, studies in human tissue have demonstrated that PC-1 is elevated in muscle and fat of insulin resistant patients (128,129). It has also been shown that PC-1 binds to amino acids 485-599 of the insulin receptor connecting domain, blocking IR autophosphorylation and subsequent insulin signaling (73,130).

The dual-specificity phosphatase Pten (phosphatase with tensin homology) is a negative regulator of insulin signaling through the PI3-K/AKT pathway (131). It has been shown to block MAPK phosphorylation in response to insulin stimulation by inhibiting IRS-1 phosphorylation and formation of the IRS1/Grb/Sos complex, leading to the suppression of cellular growth (132). Another phosphatase that modulates insulin signaling is SHIP-2. While the overexpression of this molecule does not affect insulin-induced tyrosine phosphorylation of the insulin receptor β-subunit, subsequent association of Shc and Grb2 is inhibited, resulting in a decrease of insulin-stimulated MAPK activity (133). A separate study has demonstrated that Pten, but not SHIP-2, suppresses insulin signaling through the PI3-K/AKT pathway in 3T3-L1 adipocytes (134), reinforcing the notion that SHIP-2 primarily affects the mitogenic pathway of insulin signaling.

2.6 Overview of fetuin A

A major phosphorylated glycoprotein was discovered in 1985 by Le Cam and others (22). This protein, named pp63 and secreted by rat hepatocytes, possessed strong structural similarity to the α-globulin family of proteins and was found to be negatively regulated during acute inflammation (22). Later on, this group characterized pp63 as a natural inhibitor of insulin receptor tyrosine kinase activity (25). Various homologs of rat pp63 exist, including bovine fetuin and human fetuin-A (also called α2-HS-glycoprotein or AHSG) (23). The human homolog was originally named fetuin; however, the discovery of a second member of the fetuin family, fetuin-B, prompted a name change from fetuin to "fetuin-A" (135). Like rat pp63, both bovine and human fetuin have been

characterized as having inhibitory effects on insulin receptor tyrosine kinase activity (24,136). Interestingly, the human fetuin-A gene (AHSG) is located on chromosome 3q27, a locus that has been associated with metabolic syndrome and type 2 diabetes (28,137-139). However, while a single-nucleotide polymorphism (SNP) of the fetuin-A gene is associated with type 2 diabetes in French Caucasians (140), a different group reports that an *AHSG* gene variant is associated with leanness among Swedish men (141). Lehtinen *et al.* have found that four SNPs in *AHSG* are nominally associated with coronary artery calcified plaque in European Americans with type 2 diabetes (142). Recently, two *AHSG* polymorphisms in Danish whites were shown to be associated with dyslipidemia and type 2 diabetes, while a Thr248Met SNP was associated with improved insulin sensitivity (143). These data suggest that *AHSG* gene variants may play a role in the development of various metabolic features.

The Heart and Soul Study was designed to investigate the influence of psychosocial factors on the progression of coronary artery disease (29). Non-diabetic outpatients were sequestered based on levels of serum fetuin-A. Results from this study demonstrated a strong association between higher human fetuin-A concentrations and an atherogenic lipid profile. Additionally, fetuin-A was also strongly associated with the metabolic syndrome, a constellation of metabolic risk factors associated with cardiovascular events and all-cause mortality (29). A recent (2007) study performed by Mori *et. al.* addressed the issue of fetuin-A and its association with arterial stiffness (144). Serum fetuin-A levels and stiffness parameter β for the common carotid artery were measured via ultrasound in 141 healthy subjects. Simple regression analysis showed

a significant ($p = 0.018$) positive correlation between fetuin-A and stiffness parameter β. All in all, this established a strong correlation between Ahsg and carotid arterial stiffness, independent of known atherogenic factors in healthy subjects (144).

Fetuin-A has been shown to interact with the insulin receptor (IR) and specifically inhibits insulin stimulated IR autophosphorylation (27). Studies with recombinant human fetuin-A have revealed that it inhibits the mitogenic, but not the metabolic, pathway of insulin signaling in cells overexpressing the human insulin receptor (26). Additionally, Mathews *et al.* demonstrated that recombinant human fetuin-A inhibited insulin-induced insulin receptor (IR) autophosphorylation in intact rat1 fibroblasts over expressing the human insulin receptor (the HIRcB cell line). In other words, the presence of fetuin-A in HIRcB cells disrupted insulin receptor signaling (27). Haglund *et al.* demonstrated through peptide fragment sequencing that human fetuin-A was phosphorylated at two sites, Ser120 and Ser312 (145). This finding showed for the first time that circulating fetuin-A was partially phosphorylated, and that it may potentially play a role in the signal transduction mechanism of insulin in cellular systems *in vivo*.

A strong association has been established between the consumption of a high-fat diet, the subsequent onset of obesity, and the development of diabetes (146). In a rat model of diet-induced obesity, a significant increase in fetuin-A gene expression has been observed (147). Given the interaction between fetuin-A and the insulin receptor, this leads to a possible association between fetuin-A and diabetes, potentially through the insulin signaling pathway. Fetuin-knockout (KO) mice exhibit increased basal and

insulin-stimulated phosphorylation of IR and increased activity of downstream signaling molecules such as mitogen-activated protein kinase (MAPK) and AKT in liver and skeletal muscle. Additionally, results from glucose and insulin tolerance tests showed enhanced insulin sensitivity and glucose tolerance when compared to controls. When fed a high-fat diet, these KO mice were resistant to weight gain, had significantly decreased body fat, and remained sensitive to insulin (32). Data from this study suggest that mice lacking fetuin-A are resistant to the adverse effects resulting from a high-fat diet, which include insulin resistance, glucose intolerance, and weight gain.

To lend further evidence of fetuin-A as a major player in diabetic symptoms, Stefan *et al.* performed a cross-sectional study on healthy Caucasians without type 2 diabetes, measuring liver fat accumulation and the degree of insulin sensitivity. Hepatic steatosis, or fatty liver, is common among individuals who are alcoholic, obese, and/or have diabetes. Results from the study showed a significant positive correlation between fetuin-A plasma levels and fasting insulinemia ($p = 0.01$). Also, plasma fetuin-A levels were significantly higher in those with impaired glucose tolerance when compared to controls ($p = 0.006$). A significantly negative correlation was observed when comparing plasma fetuin-A levels to insulin sensitivity, while liver fat showed a significant positive correlation with plasma fetuin-A (31). Thus, not only were fetuin-A levels associated with insulin resistance, but the accumulation of liver fat also seemed to be associated with plasma fetuin-A.

2.7.1 Glucocorticoids – functions and cellular mechanisms

As a whole, glucocorticoids are a class of hormones that possess a variety of functions in the body. Released from the cortex of the adrenal gland, these agents are secreted into the blood stream in a circadian pattern, peaking in the morning and diminishing at night (148). Cortisol is the main glucocorticoid present in humans and is an essential hormone for survival. It is released when the body faces acute duress, such as during fasting, inflammation, infection, or during the "fight or flight" response. Naturally, many of its effects are designed so that the body can mobilize resources for rapid energy production. Various effects of cortisol have been observed, including: an increase in blood pressure, increased liver and plasma proteins, suppression of the immune system, as well as elevated plasma free fatty acids, among others (149). However, arguably the most significant effect of glucocorticoids within the scope of diabetes research resides primarily in the liver: gluconeogenesis. In general, this process occurs either during fasting or exercise and is primarily used to restore blood glucose levels back to a normal physiologic range, between 70-150 mg/dL). However, glucocorticoid treatment has been shown to enhance glucose output and reduce glucose utilization in normal rats (150). Additionally, the dual effect of mobilizing extrahepatic amino acids for substrates coupled with an increase in enzyme production can potentially lead to a 6- to 10-fold elevation in glucose formation (149).

2.7.2 Glucocorticoids – production and regulation

In humans, the secretion of glucocorticoids in the body is controlled through the activity of the hypothalamic-pituitary-adrenal (HPA) axis. When either an intrinsic or

extrinsic stress is received, the hypothalamus releases corticotropin-releasing hormone (CRH, also known as CRF) which in turn releases adrenocorticotropic hormone (ACTH) from the pituitary gland into the bloodstream (151). However, to minimize the catabolic and immunosuppressive effects of this response when levels become too high, glucocorticoids directly inhibit the HPA axis primarily at the hippocampus by acting on the hypothalamus and the pituitary gland, in turn decreasing the levels of circulating ACTH (151,152).

While activation of the HPA due to stress leads to a negative inhibition of (119) glucocorticoid production, a partial resistance to the feedback inhibition of glucocorticoid release has also been shown (153). Sapolsky *et al.* have shown that in the hippocampus, acute stress decreases the number of glucocorticoid receptors, thereby augmenting the levels of circulating glucocorticoids and a resistance to feedback inhibition. After the stress response has been terminated, glucocorticoid levels decrease in association with diminishing levels of CRH. This ultimately results in the normalization of both glucocorticoid receptor concentration and the feedback inhibition system (154).

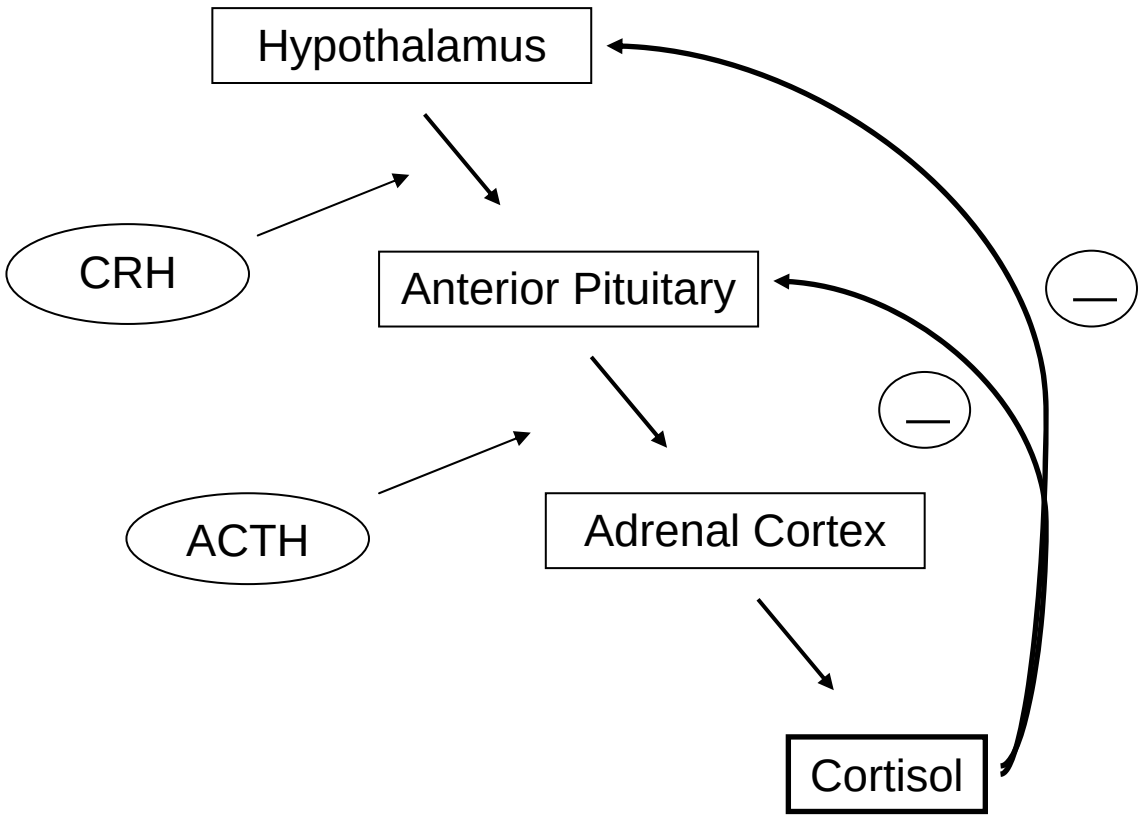


Fig. 2: Flowchart of glucocorticoid release and feedback inhibition. When the body experiences an acute stress, CRH neurons signal to the hypothalamus to release CRF, which acts on the pituitary gland to release ACTH. The latter hormone then travels to the adrenal cortex to stimulate glucocorticoid release, which can negatively feedback on its own production at both the hypothalamus and the anterior pituitary.

At the cellular level, glucocorticoids can readily permeate the cell membrane and bind to glucocorticoid receptors (GRs) located in the cytosol. The receptor is a ligand-activated transcription factor that is normally associated with heat-shock proteins (HSPs) in the absence of binding. Two types of glucocorticoid receptors have been well-characterized. Type 2, the classic glucocorticoid receptor, has low affinity for corticosterone and instead has a greater affinity for synthetic glucocorticoids such as dexamethasone (155). The type 1 receptor on the other hand, is a corticosterone-preferring receptor with high affinity for both corticosterone and the mineralocorticoid aldosterone (156). Once the glucocorticoid ligand binds to the GR, the complex undergoes a conformational change and subsequent dissociation from the HSPs, allowing both the receptor and its ligand to translocate into the nucleus (34). Within the nucleus, there are two types of mechanisms through which GRs can act. GRs bind to glucocorticoid response elements (GREs) to modulate transcription activity (157). A second proposed mechanism is characterized by interaction of the GR with other transcription factors without directly targeting specific DNA binding events, such as the involvement of GR with activating protein-1 (AP-1). AP-1 is a transcription factor that binds to specific target sequences within responsive promoters of DNA (158). While the GR itself does not bind to these promoters, deletion of AP-1 has been previously shown to abolish both AP-1-stimulated and GR-mediated repression of gene transcription, indicating a possible protein-protein interaction between the GR and AP-1 (159).

2.7.3 11β-hydroxysteroid dehydrogenase

The tissue-specific mediator of glucocorticoid action is 11β-hydroxysteroid dehydrogenase, an enzyme that drives the interconversion of 11-hydroxy (active) and 11-keto (inactive) glucocorticoids. Two isozymes have been characterized in detail: 11β-HSD1 and 11β-HSD2. The type 1 enzyme is widely found in various organs throughout the body, including the liver, adipose tissue, and skeletal muscle (160). In humans, its role is to convert inactive cortisone to active cortisol via 11-ketoreductase activity. On the other hand, the type 2 enzyme in humans is responsible for converting active cortisol (corticosterone in rodents) to inactive cortisone (11-dehydrocorticosterone in rodents).

In the liver, 11β-HSD1 has been shown to counter the effects of insulin through the up-regulation of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in the gluconeogenic pathway (161,162). Conversely, the inhibition of 11β-HSD1 leads to a decrease in blood glucose concentrations in hyperglycemic mice (162). The dysregulation of 11β-HSD1 expression and activity has also been observed in diabetic animal models and humans. In a mouse model of obesity and diabetes (*ob/ob* mouse), hepatic 11β-HSD1 activity was reduced when compared to lean control animals. However, there was an elevation in both the liver and plasma corticosterone levels, as well as an inverse relationship between 11β-HSD1 expression and body weight (163), implicating the enzyme as a possible contributing factor to obesity-induced glucocorticoid production. Furthermore, in obese humans, an elevation of adipose 11β-HSD1 activity was associated with obesity, insulin resistance, and other aspects of the metabolic syndrome (164-166).

Carbenoxolone, a derivative of liquorice, is a non-selective inhibitor of 11β-HSD types 1 and 2 in humans. Walker *et al.* have shown that oral carbenoxolone increases insulin sensitivity and decreases glucose production in the liver (167). A later study by the same group reveals that the inhibition of 11β-HSD1 in the liver of type 2 diabetic patients reduces their glucose production rate during hyperglucagonemia, but has no effect on gluconeogenesis or lipid profiles in these patients, perhaps due to the relatively mild potency of carbenoxolone as a non-selective 11β-HSD inhibitor (168,169). In contrast, the selective inhibition of 11β-HSD1 leads to a lowering of blood glucose levels and improved insulin sensitivity in various mouse models of type 2 diabetes (162,170,171). 11β-HSD1 knockout mice express lower levels of TNF-α along with reduced visceral fat accumulation when on a high-fat diet, while isolated adipocytes from these mice exhibit higher basal and insulin-stimulated glucose uptake (172). Additionally, these mice have an improved lipid profile, hepatic insulin sensitization, and are resistant to high-fat induced hyperglycemia (173,174).

2.7.4 Glucocorticoids and insulin resistance

Since glucocorticoids have potent anti-inflammatory and immunosuppressive properties, their uses in remedial settings today include allergic and hematological disorders, and renal, intestinal, liver, and skin diseases (42). However, when given in excess, glucocorticoids can lead to features commonly associated with the metabolic syndrome, such as obesity, hyperlipidemia, and insulin resistance (175). A prime example of glucocorticoid excess and its effects is manifested in patients with Cushing's syndrome. This endocrine disorder is characterized by a chronic exposure to excess

glucocorticoids produced by the adrenal cortex. Patients with Cushing's syndrome generally demonstrate central obesity, arterial hypertension, impaired glucose tolerance, diabetes, and hyperlipidemia (37). These factors can all lead to elevated risks of cardiovascular disease and mortality in these patients. Dexamethasone is a synthetic glucocorticoid roughly fifty times as potent as cortisol (43). It is widely used today to treat many inflammatory and autoimmune conditions, such as rheumatoid arthritis, inflammatory bowel, and asthma (34). In spite of its potent therapeutic properties, adverse side effects of dexamethasone and other glucocorticoid treatments have also been well documented. For instance, an increased risk of developing gestational diabetes is reported in women receiving glucocorticoids for threatened pre-term delivery (176). Patients with hypercortisolism show a 30-40% risk of developing diabetes mellitus (177). Also, the inclusion of glucocorticoids as part of the standard therapy after organ transplantation are believed to be responsible for the development of post-transplant diabetes mellitus (PTDM) in up to 40% of renal transplant cases (178). When given in excess, dexamethasone adversely affects muscle catabolism (179), resulting in both increased adiposity (180,181) and insulin resistance. (39,182). As a result, many researchers have treated animals with dexamethasone to study the effects and parameters of insulin resistance. A study by Qi *et al.* shows that although a single-dose of dexamethasone (1 mg/kg body weight) is not associated with hyperinsulinemia or hyperglycemia, a euglycemic-hyperinsulinemic clamp study reveals a decrease in glucose infusion rate, indicating poor glucose disposal in cells, a hallmark characteristic of insulin resistance (49). Furthermore, dexamethasone treatment leads to an increased level of lipoprotein lipase (LPL) in the heart. This is indicative of an increase in the ability of

LPL to facilitate fatty acid delivery to the heart, leading to excessive triglyceride storage in the organ (49). Overall, these results illustrate that dexamethasone induces whole-body insulin resistance as well as an alteration of fatty acid metabolism in the heart.

A separate paper describes the effects of dexamethasone on GLUT2 protein levels and its gene expression in the pancreatic islet cells of male Sprague-Dawley rats. Data from this study reveal that the inclusion of dexamethasone alone decreases GLUT2 protein by ~65% while no effect on GLUT2 mRNA is observed. In contrast, palmitic acid alone induces a 40% decrease in GLUT2 mRNA, but does not consistently affect protein expression. Dexamethasone only minimally affects the GLUT2 translation rate, but the half-life of the protein is decreased by 50%, indicating a post-translational degradation mechanism. Lastly, the inclusion of both dexamethasone and palmitic acid decreases glucose-induced insulin secretion (183). These results demonstrate that in isolated pancreatic β-cells, a combination of palmitic acid and dexamethasone can both diminish GLUT2 expression and attenuate glucose-induced insulin secretion, two common features seen in type 2 diabetes.

2.8 Objectives/hypotheses of thesis research

Recently, Woltje *et al.* demonstrated that dexamethasone treatment increased fetuin-A gene expression in both Hepa1-6 mouse hepatoma cells and primary murine hepatocytes (50). Additionally, they showed that the fetuin-A promoter contained glucocorticoid response elements through which dexamethasone modulated fetuin-A levels, and that these promoter sequences were highly conserved in mouse, rat, and

human fetuin-A genes (50). However, no current studies to date have examined fetuin-A levels in glucocorticoid-based insulin resistant models. Thus, the overall objective of this study was to examine the relationship between glucocorticoids, fetuin-A, and insulin resistance.

We hypothesized that both cells and animals treated with dexamethasone will express an elevated level of fetuin-A. In addition, dexamethasone treatment will lead to diminished insulin sensitivity as assessed through HOMA-IR and the insulin tolerance test. However, the absence of fetuin-A will result in improved insulin sensitivity, irrespective of dexamethasone treatment.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell culture experiments

Human Hep3B and HepG2 hepatoma cells (ATCC, Manassas, VA) were cultured in 100 mm tissue culture dishes (BD Falcon, San Jose, CA) using Improved Modified Eagle's Medium (IMEM) (Mediatech, Herndon, VA), supplemented with heat-inactivated 10% (v/v) fetal bovine serum, and penicillin streptomycin (1%) in a 37 °C incubator containing 5% CO_2. After growing to confluency, cells were washed with Dulbecco's phosphate buffered saline (Invitrogen, Grand Island, NY) and starved overnight in serum-free IMEM containing penicillin streptomycin (1%) and 0.1% bovine serum albumin (Fisher Scientific, Fairlawn, NJ). Cells were then washed with PBS, treated with either DEX or vehicle (ethanol), and incubated overnight in serum-free IMEM. Media and cells were collected 24 hours after the treatment and stored in -20 °C for protein and gene expression analysis, respectively.

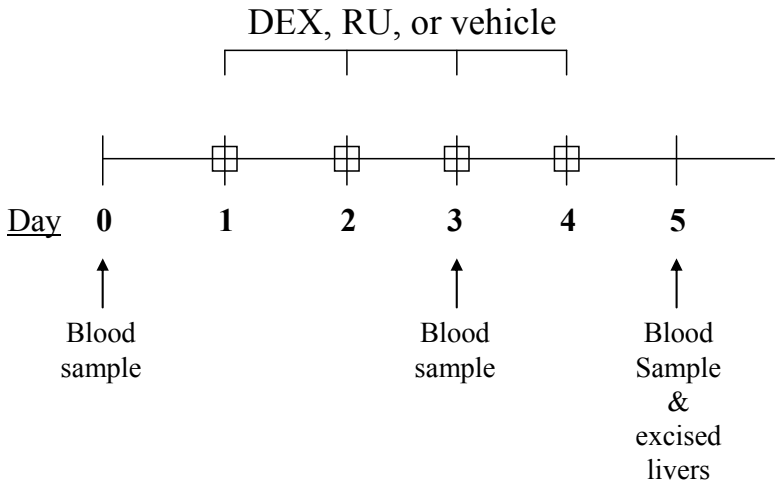
3.2 Animals

Protocols for animal care, testing, and euthanasia were evaluated and approved by the Institutional Animal Care and Use Committee at Auburn University. Eight week old male Wistar rats were purchased from Charles River Laboratories (Indianapolis, IN).

Animals were maintained on a 12-hour light/dark cycle and fed standard rodent chow and water *ad libitum*.

3.3 Dexamethasone / RU-486 treatment

Rats were injected with DEX (1 mg/kg body weight) (Sigma, St. Louis, MO), RU-486 (50 mg/kg body weight) (Sigma), or vehicle (saline for DEX, ethanol for RU-486), once daily for four days. Animals in the DEX+RU group received RU injection two hours prior to DEX treatment. All injections were administered intraperitoneally. Blood samples were taken from the lateral saphenous vein 24 hours before the initial treatment, on the third day, and on the fifth day of treatment, for glucose, insulin, and Western blot assays. Rats were sacrificed 24 hours after the final injection and truncal blood was collected from each animal. Liver sections were excised and flash-frozen by immersion in liquid nitrogen and stored in a -80˚C freezer until use.

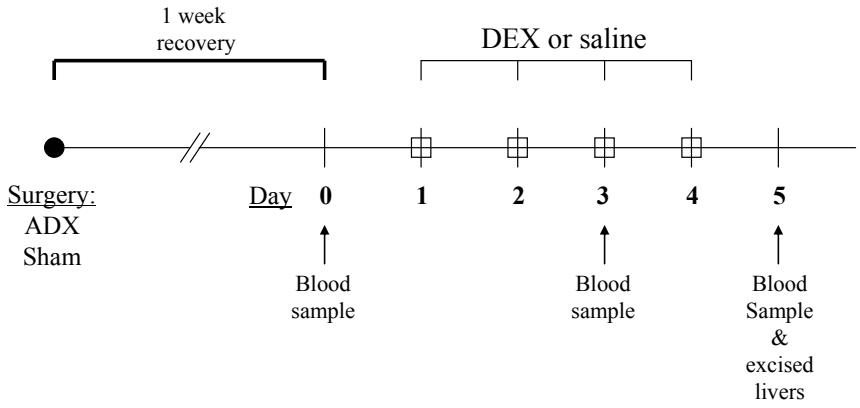


3.4 Adrenalectomy

Wistar rats were randomly assigned to either adrenalectomized (ADX) or sham-operated groups. Standard rodent chow and water were provided *ad libitum*. After acclimatization, animals were anesthetized with an intraperitoneal injection of a ketamine (70 mg/kg)/xylazine (5 mg/kg) cocktail. Assurance of complete anesthesia was confirmed by pedal and corneal reflexes. A sterile ophthalmic lubricant (Akwa Tears) (Akorn Inc, Buffalo Grove, IL) was applied to the cornea to prevent drying. The hair on the back of the lumbar area was shaved and a 3 cm dorsal midline skin incision was made at the level of the 1st to 3rd lumbar vertebra. After cuts were made through the muscle wall both adrenal glands were located and exteriorized along with the surrounding fat pad with a pair of straight mouse-toothed thumb forceps. The adrenal glands were then excised by blunt dissection and discarded. The muscle incisions were then sutured shut with monofilament synthetic absorbable suture (Covidien Syneture, Mansfield, MA). The skin incision was then closed with surgical staples. Sham rats were operated similar to the ADX rats described above, with the exception that their adrenal glands were located but not removed. Both rat groups were given standard rat chow. In addition to regular drinking water, a bottle of 0.9% NaCl and 0.15% KCl (w/v) was provided *ad libitum* .

After a seven-day recovery period, both ADX and sham-operated rats were injected intraperitoneally with DEX or saline, once daily for four days. Blood samples were taken from the lateral saphenous vein 24 hours before the initial treatment, on the third day, and again on the fifth day of treatment, for glucose, insulin, and Western blot assays. Rats were sacrificed 24 hours after the final injection and truncal blood was

collected from each animal. Liver sections were excised and flash-frozen by immersion in liquid nitrogen and stored in a -80˚C freezer until use.



3.5 Fetuin-A null mice

Fetuin-A null mice, back-crossed over ten generations onto C57B/6J background, were kindly provided by Dr. Willi Jahnen-Dechent (Aachen University, Germany). After acclimatization, fetuin-A null mice were set up for breeding. Age- and sex-matched C57Bl/6 mice (Charles River Laboratories, Indianapolis, IN) were used as controls. Eight-week old, male, fetuin-A null mice and C57Bl/6 mice were injected with 1 mg DEX per kilogram body weight in a total volume of 100 µL, or an equivalent volume of saline, intraperitoneally, once daily for four days. Twenty-four hours after the final injection, mice were subject to either an insulin tolerance test (ITT) or glucose tolerance test (GTT). After completion of these assays, mice were euthanized using carbon dioxide followed by cervical dislocation.

3.6 Insulin tolerance test (ITT)

Following a four-hour fast, mice were injected intraperitoneally with 0.50 U/kg regular human insulin. Blood samples were obtained at 0, 15, 30, and 60 minutes from the tail vein and blood glucose was measured with an Accu-Chek glucometer (Roche Diagnostics, Indianapolis, IN).

3.7 Mouse tail DNA preparation

Sections (5-6 mm) of the tail from fetuin-A null and wild-type mice were clipped and stored in -20ºC. Purification of total DNA from rodent tails was performed using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland) according to manufacturer's instructions. The DNA was further purified by mixing it with an equal volume of phenol:choloroform:isoamyl alcohol. DNA purity, determined by calculating the optical density ratio at wavelengths of 260 and 280 nm, and DNA concentrations were measured with a spectrophotometer (DU530 UV/Vis) (Beckman Coulter, Fullerton, CA).

3.8 Polymerase chain reaction (PCR) and genotyping

PCR mixtures contained 1x PCR buffer (Bio-Rad, Hercules, CA), 1 M Betaine (Sigma, St. Louis, MO), 200 μM each dNTP (Bio-Rad), 0.4 μM WT forward primer, 0.3 μM WT reverse primer, 0.2 μM knockout forward primer, 2.5 U of Taq DNA Polymerase (Promega, Madison, WI), and 4 mM $MgCl_2$ (Bio-Rad) in a total volume of 25 μL. Specific primer sequences are provided (Table 1). The thermal cycling protocol was 71ºC for 2 minutes; mouse-tail DNA was then added. This was followed by 40 cycles of 96ºC

for 10 seconds, 60ºC for 2 minutes, and 71ºC for 2 minutes. 5x loading buffer (Sigma, St. Louis, MO) was added to the PCR tubes and samples loaded into a 1% agarose gel. Gel was visualized with ethidium bromide (Fisher Scientific, Fairlawn, NJ) staining under UV light. Reference amplicons were 2.0 kb for WT and 0.6 kb for fetuin-$A^{-/-}$ mice, respectively.

3.9 Biochemical assays

Glucose levels were determined using an Accu-Chek glucometer (Roche Diagnostics, Indianapolis, IN). Plasma insulin levels were measured by a rat insulin ELISA assay kit (Linco Research, St. Charles, MO) according to the manufacturer's instructions. Rat insulin was used as a standard. The homeostasis model assessment (HOMA) method (184) was used as a measure of insulin resistance, calculated using non-fasted values of blood glucose and plasma insulin:

$$\text{HOMA-IR} = [\text{Insulin } (\mu\text{U/mL}) \times \text{Glucose (mmol/L)}] / 22.5$$

3.10 Protein quantification

Protein concentrations in Hep3B and HepG2 cell culture media were assayed with the Bradford method using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

3.11 Corticosterone assay

To evaluate the success of surgical adrenalectomies, plasma samples from adrenalectomized rats were assayed for corticosterone levels by an Enzyme Immunoassay

(EIA) Kit (Diagnostic Systems Laboratories, Webster, TX) according to manufacturer's instructions. Briefly, rat corticosterone standards, controls, and unknowns were added into a 96-well plate. Enzyme conjugate solution was then added to each well and briefly shaken. Rat corticosterone antiserum was then added and the wells incubated at room temperature on a microplate shaker. The plate was washed, TMB chromogen solution was added, and the plate was read at 450 nm on a microplate reader (Bio-Tek, Winooski, VT). Corticosterone concentrations were calculated based on a four-parameter curve-fit of the assayed standard results.

3.12 Western blotting

Plasma samples from animal experiments were diluted (1:100 in PBS) before loading onto the gel. Cell culture media and animal plasma samples were mixed with 3X SDS-buffer and run on a 4-20% SDS-PAGE gel (NuSep, Austell, GA). SDS-PAGE gels were transferred to a 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 in either 5% milk (non-fat dry milk; Bio-Rad, Hercules, CA) or 1% BSA (Fisher Scientific, Fairlawn, NJ), both dissolved in Western blot wash solution (10 mM Tris pH 7.4, 100 mM NaCl, 0.1% Tween-20). The membranes were then incubated with antibodies specific to either human fetuin-A (Immunostar Inc., Hudson, WI) or phosphorylated Ser312 fetuin-A (custom generated with the epitope "HTFMGVVSLGSPS(PO_4)GEVSHPR" and affinity purified; Affinity BioReagents, Golden, CO) and incubated with either SuperSignal West Femto or Pico chemiluminescent substrate (Pierce, Rockford, IL) for 5 minutes. The blot was then

imaged using the UVP Bioimaging System and LabWorks software package (UVP, Upland, CA). Relative area densities were quantified using the Un-Scan It software package, v.5.1 (Silk Scientific, Orem, UT).

3.13 Liver homogenates

Frozen liver tissues from Wistar rats were homogenized in tissue lysis buffer containing the following: 50 mM HEPES, pH 7.4; 100 mM sodium pyrophosphate; 100 mM sodium fluoride; 10 mM EDTA; 10 mM sodium orthovanadate; 2 mM PMSF; 1% Triton X-100; Complete Mini protease inhibitor, 1 tablet per 10 mL solution (Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged at 4ºC, and the supernatant was retained. Bradford protein quantification and Western blotting were then performed as described previously.

3.14 Total RNA isolation

Frozen liver tissues excised from male Wistar rats were homogenized in Trizol reagent (Invitrogen, Grand Island, NY), transferred to a QIA shredder column (Qiagen, Germantown, Maryland), and RNA was isolated according to manufacturer's instructions. Any potential DNA contaminants were removed using DNase I (Qiagen, Germantown, Maryland). The concentration of RNA was determined with a spectrophotometer (DU530 UV/Vis) (Beckman Coulter, Fullerton, CA), while the purity was ascertained by calculating the optical density ratio at wavelengths of 260 and 280nm.

3.15 cDNA synthesis

Extracted RNA was synthesized into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Each RNA sample was diluted with nuclease-free water to yield a normalized quantity of 1 μg RNA. This amount was then added to a tube containing iScr. buffer and iScr. reverse transcriptase (Bio-Rad, Hercules, CA). Tubes were placed in a thermocycler (Bio-Rad, Hercules, CA) and run using the following protocol: 25ºC for 5 minutes, 42ºC for 30 minutes, and 85ºC for 5 minutes. The synthesized cDNA was then stored in a -20ºC freezer.

3.16 Real-time PCR gene expression

To assess fetuin-A gene expression, cDNA was added to iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) along with forward and reverse primers in a final reaction volume of 25 μL. Quantitative real-time PCR was carried out using the MyiQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA). Specific primer sequences are provided (Table 2). The thermal cycling protocol was 95ºC for 3 minutes followed by 40 cycles of 95ºC for 15 seconds, 58ºC for 30 seconds, and 72ºC for 30 seconds. To account for variability in the quality and quantity of total RNA used, gene expression of fetuin-A was normalized to that of β-actin mRNA as an endogenous control.

3.17 Statistical analysis

Experimental results were expressed as mean ± standard error of the mean (SEM). Comparisons between various treatments and/or groups were carried out using the

unpaired Student's t-test or one-way analysis of variance (ANOVA) where appropriate. Differences were considered to be statistically significant if 'p value' was less than 0.05. Statistical analysis was performed using GraphPad InStat v.3 (GraphPad, San Diego, CA).

CHAPTER 4: RESULTS

4.1 Elevated fetuin-A levels in Hep3B and HepG2 human hepatoma cells

Fetuin-A is produced and secreted by the liver and undergoes post-translational modifications. Human fetuin contains an 18-amino-acid N-terminal signal peptide and is therefore secreted into media (185,186). While the rat hepatoma cell line H4IIE does not secrete fetuin, other rat hepatoma cell lines such as FTO-2B and Fao have been shown to secrete it (187). The human hepatoma cell lines Hep3B and HepG2 have also been shown to synthesize fetuin-A (145,188). Earlier studies by Woltje *et al* (50) identified a glucocorticoid response element in the promoter of fetuin-A which was responsive to DEX treatment, resulting in increased fetuin-A expression in primary murine hepatocytes. To confirm these effects, fetuin-A levels were assayed after a 24-hour DEX treatment in human Hep3B and HepG2 cells. Real-time PCR results indicated an elevation of relative gene expression of fetuin-A at 5 μM and 125 μM DEX treatment (Fig. 3). However, no changes in relative gene expression of fetuin-A were observed in HepG2 cells (data not shown). Fetuin-A secreted into media was assessed by Western blot. DEX treatment induced maximal secretion of fetuin-A at 1.0 and 5.0 μM in Hep3B cells (Fig. 4). No significant differences in fetuin-A secretion were observed in HepG2 cells (Fig. 5).

4.2 Elevated corticosterone and fetuin-A levels in a ZDF rat model

The primary glucocorticoid in rodents, corticosterone, has also been associated with insulin resistance (189,190). To delineate our hypothesis that glucocorticoids are associated with fetuin-A levels in animals, corticosterone and fetuin-A levels were measured in 6-week-old Zucker Diabetic Fatty (ZDF) rats. Although ZDF rats exhibited glucose levels comparable to lean controls at six weeks of age (ZDF: 151 ± 8 mg/dL, Lean: 128 ± 7 mg/dL, NS) (Fig. 6), they were hyperinsulinemic (ZDF: 8.982 ± 2.247 ng/mL, Lean: 0.524 ± 0.148 ng/mL, $p < 0.05$) (Fig. 7) and insulin resistant as determined by HOMA-IR (84.54 ± 25.4, n = 6) when compared with lean controls (3.84 ± 0.9, n = 6) ($p = 0.01$). ZDF rats had significantly higher plasma corticosterone levels than lean controls (Fig. 8). A concomitant elevation in fetuin-A, though not significant ($p = 0.10$), was also observed in the plasma of these rats, suggesting that elevated corticosterone levels in ZDF rats may be associated with fetuin-A levels and insulin resistance (Fig. 9).

4.3 Increased fetuin-A expression in DEX-treated Wistar rats

Glucocorticoids such as dexamethasone have been shown to induce insulin resistance in both animals and humans (40,42,43,47), and also to up-regulate fetuin-A gene expression (50). Since fetuin-A is a natural inhibitor of insulin receptor tyrosine phosphorylation (24,27) and was associated with insulin resistance and fat accumulation in the liver (31,191), it was logical to pose the question: Does DEX treatment increase fetuin-A levels and thereby contribute to an insulin resistant state? To assess the effect of glucocorticoids on insulin resistance, 4-week-old male Wistar rats were given an intraperitoneal injection of DEX (1 mg/kg body weight) once daily for four days. In

addition, a separate group of animals was injected with RU-486 (50 mg/kg) two hours prior to DEX. RU-486 is a glucocorticoid receptor antagonist used to counter the effects of elevated glucocorticoid levels. It has been used in patients with Cushing's syndrome who exhibit hypercortisolism (192), and has been shown to reduce characteristics associated with type 2 diabetes in animals, such as hyperglycemia and insulin resistance (161,193).

4.3.1 Body weight changes

DEX-treated and RU-treated rats did not exhibit significant differences in body weight when compared to saline. However, a significant decrease ($p < 0.01$) in body weight was observed in the DEX+RU -treated rats on day 5 (153.0 ± 2.8 g) compared to saline-treated rats (181.9 ± 4.2 g). Additionally, day 5 body weights of saline-treated rats (181.9 ± 4.2 g) were significantly greater ($p < 0.05$) than their baseline (day 0) weights (158.4 ± 2.7 g) (Fig. 10).

4.3.2 Glucose and insulin levels

Blood glucose levels were not altered with either DEX-, RU-, or DEX+RU – treatment compared to saline-treated controls (Fig. 11). On the contrary, plasma insulin was significantly elevated on day 3 in DEX-treated rats (2.372 ± 0.603 ng/mL) when compared to saline treatment (0.773 ± 0.093 ng/mL) ($p < 0.01$). Though not statistically significant, DEX-treated rats maintained elevated insulin levels (2.326 ± 0.573 ng/mL) on day 5 of the experiment when compared to saline treatment (1.445 ± 0.281 ng/mL). As expected, DEX-treated rats that were also given RU exhibited a significant decrease in

plasma insulin on both day 3 (0.584 ± 0.210 ng/mL) and day 5 (0.560 ± 0.068 ng/mL) when compared to animals treated with only DEX (Day 3: 2.372 ± 0.603 ng/mL, $p < 0.01$; Day 5: 2.326 ± 0.573 ng/mL, $p < 0.01$) (Fig. 12).

4.3.3 HOMA-IR

The homeostasis model assessment of insulin resistance (HOMA-IR) is a calculated estimate of relative insulin resistance. First described by Matthews *et al.*, it is based on plasma insulin and glucose values to assess contributions of insulin resistance and deficient cell function to hyperglycemia (184). DEX injection in Wistar rats led to a significant increase ($p < 0.05$) in non-fasted HOMA-IR (20.6 ± 5.8) when compared to the saline-treated group on day 3 (6.6 ± 0.9). On day 5, a trend towards significance was observed in these groups (DEX: 22.0 ± 5.5, Saline: 12.9 ± 2.6) though this difference was not statistically significant. The DEX-treated rats that were also given RU showed a significant decrease ($p < 0.05$) in non-fasted HOMA-IR on day 3 (4.8 ± 1.9) when compared to DEX-treated rats (20.6 ± 5.8). Day 5 non-fasted HOMA-IR was also significantly decreased ($p < 0.01$) in DEX+RU rats (4.9 ± 0.6) when compared to DEX-treatment alone (22.0 ± 5.5). (Fig. 13).

4.3.4 Fetuin-A gene expression in DEX-treated Wistar rats

To evaluate fetuin-A gene expression, liver samples were excised from DEX- and RU- treated Wistar rats. RNA was isolated, reverse transcribed to cDNA, and quantitative real-time PCR was performed. No significant changes in fetuin-A gene expression were observed among treatment groups relative to the housekeeping gene, β-actin (Fig. 14).

4.3.5 Plasma fetuin-A concentrations in DEX-treated Wistar rats

Wistar rat blood samples were obtained on days 0, 3, and 5 for quantitation of fetuin-A. Plasma, diluted 1:100 in saline, was separated on SDS-PAGE, transferred to nitrocellulose, and incubated with human fetuin-A specific antibodies. Though fetuin-A has a molecular weight of 48 kD, its apparent molecular weight on a 4-20% SDS-PAGE gel was ~60 kD. The increase in molecular weight was due to post-translational modifications including N- and O-linked glycosylations, and phosphorylation. In our hands, plasma fetuin-A consistently demonstrated a molecular weight of ~60 kD. Additionally, anti-human fetuin-A antibody demonstrated cross-reactivity with rat fetuin-A. Densitometric analysis showed a significant increase in fetuin-A levels on day 5 of the experiment ($p < 0.01$). While not statistically significant, RU injection in DEX-treated rats decreased fetuin-A levels relative to DEX alone. Interestingly, rats injected with RU alone exhibited a significant increase in plasma fetuin-A on day 5 (Fig. 15).

4.3.6 Insulin receptor (IR) autophosphorylation in Wistar rats

To study the effects of DEX treatment on hepatic insulin signaling, liver tissues were homogenized, run on SDS-PAGE, and immunoblotted with antibody specific to three phosphorylated tyrosine residues (Tyr1158, Tyr1162, Tyr1163) of the insulin receptor. Though not statistically significant, DEX-treated animals exhibited a ~52% decrease in IR phosphorylation status relative to saline controls ($p = 0.07$) (Fig. 16).

4.3.7 Summary

Taken together, these results indicate that DEX treatment induces hyperinsulinemia and insulin resistance in Wistar rats. Concomitant increases in fetuin-A gene expression and protein levels, and a decrease in IR activation were observed. Findings from this experiment suggest that treatment with RU-486, a glucocorticoid receptor antagonist, protects Wistar rats against DEX-induced insulin resistance and prevents an increase of fetuin-A gene expression and protein levels.

4.4 Decreased fetuin-A expression in adrenalectomized (ADX) Wistar rats

Since DEX-treatment increased plasma fetuin-A levels, it was of significant interest to understand the effects of adrenalectomy (the surgical removal of adrenal glands) on fetuin-A levels. Adrenalectomized or sham-operated rats were allowed a 1-week recovery period. During this time, both water and salt solutions (0.9% NaCl and 0.15% KCl) were provided *ad libitum*.

4.4.1 Body weights and corticosterone levels in ADX and sham rats

Following the recovery period (Day 0), significant ($p < 0.01$) decreases in body weights were observed in ADX animals compared to sham controls (Fig. 17). To determine the effectiveness of adrenalectomy, circulating levels of corticosterone were measured. As expected, plasma corticosterone levels were significantly lower in ADX rats (Fig. 18).

4.4.2 Glucose and insulin levels

Blood glucose levels were not significantly different among surgical procedures or treatment (Fig. 19). However, a significant increase ($p < 0.05$) in insulin was observed on day 3 of DEX-treated sham rats (2.183 ± 0.158 ng/mL) compared to baseline values (1.023 ± 0.146 ng/mL). However, these levels were attenuated by day 5 (DEX: 1.434 ± 0.059 ng/mL vs. Saline: 1.023 ± 0.146 ng/mL). Though not statistically significant, ADX rats had lower baseline (Day 0) insulin levels (0.455 ± 0.030 ng/mL) when compared with sham-controls (1.023 ± 0.146 ng/mL). Similarly, insulin levels tended to be lower in saline-injected adrenalectomized rats compared to sham controls. With DEX treatment on day 3 (DEX: 1.943 ± 0.396 ng/mL vs. Saline: 0.694 ± 0.127 ng/mL, $p < 0.05$) and day 5 (DEX: 2.000 ± 0.328 ng/mL vs. Saline: 0.735 ± 0.111 ng/mL, $p < 0.05$), insulin levels were both significantly elevated compared to saline-treated ADX rats (Fig. 20).

4.4.3 HOMA-IR

Non-fasted HOMA-IR values exhibited a similar pattern as insulin levels in ADX rats. DEX treatment significantly elevated HOMA-IR when compared to baseline sham values on day 3 in sham controls (DEX: 19.9 ± 1.6 vs. Baseline: 9.3 ± 1.4, $p < 0.01$) but not day 5 (DEX: 12.3 ± 0.5 vs. Baseline: 9.3 ± 1.4). In ADX rats, DEX treatment led to a significant increase in HOMA-IR for both day 3 (DEX: 16.4 ± 3.0 vs. Baseline: 3.4 ± 0.3, $p < 0.001$) and day 5 (DEX: 15.0 ± 2.5 vs. Baseline: 3.4 ± 0.3, $p < 0.001$) of treatment compared to baseline (Day 0) values (Fig. 21).

4.4.4 Fetuin-A levels in ADX rats

Western blot analysis was performed using anti-fetuin-A antibody to assess the effects of adrenalectomy on plasma fetuin-A. ADX Wistar rats showed a significant decrease ($p < 0.05$) in fetuin-A levels (0.21 ± 0.03) when compared to sham-operated controls (1.00 ± 0.30 relative density units) (Fig. 22). Furthermore, unlike sham controls, adrenalectomized rats failed to respond to DEX, demonstrating significantly lower levels of fetuin-A (Fig. 23). Similarly, DEX treatment in ADX rats did not alter fetuin-A levels (Fig. 24). However, sham rats demonstrated significantly elevated levels of total ($p < 0.001$) (Fig. 25) and phosphorylated fetuin-A (Fig. 26).

4.4.5 Summary

Together, these results indicate that adrenalectomy improves insulin sensitivity. DEX treatment induces insulin resistance in both sham and ADX rats to similar degrees. Additionally, these findings demonstrate that metabolites secreted from the adrenal glands may play a significant role in the regulation of fetuin-A protein expression.

4.5 Improved insulin sensitivity and glucose tolerance in DEX-treated fetuin-A knockout mice

Since DEX treatment increased fetuin-A expression in Wistar rats, we hypothesized that fetuin-A null mice would be resilient to DEX-induced insulin resistance.

4.5.1 DNA genotyping

Fetuin-A null mice were identified by genotyping using standard PCR protocols. Wild-type (WT) C57Bl/6 mice produced an amplicon of approximately 2.0 kb, whereas fetuin-A KO mice produced an amplicon of 0.6 kb (Fig. 27).

4.5.2 Insulin tolerance test

An insulin tolerance test (ITT) was administered to assess the degree of insulin responsiveness in DEX-treated (1 mg/kg) fetuin-A KO mice. With a single i.p. injection of regular human insulin (0.50 U/kg), a significant ($p < 0.05$) difference was observed in glucose clearance between DEX-treated WT and KO mice at both 30- and 60- minute time points (Fig. 28). Additionally, DEX-treated WT mice exhibited attenuated insulin sensitivity when compared to saline-treated WT controls at the 60-minute time point ($p < 0.05$).

4.5.3 Summary

These results demonstrate that DEX-treated mice are more insulin resistant than saline-treated controls, whereas fetuin-A knockout mice injected with DEX are protected against DEX-induced insulin resistance.

CHAPTER 5: DISCUSSION

Diabetes, currently affecting over 22 million people in the U.S., is characterized by a deterioration of efficient homeostatic glucose control by insulin, either through a deficiency in insulin production or the disruption of insulin signaling known as insulin resistance. If left unchecked, diabetes can lead to a host of metabolic problems such as blindness, stroke, and cardiovascular disease (7). Insulin signaling can be characterized into three "critical nodes of signaling:" the insulin receptor and IRS proteins; PI3-kinase and its catalytic subunits; and the AKT isoforms (72). These "nodes" are frequently the target of various humoral factors that modulate insulin action, such as free fatty acids; adipokines including leptin, adiponectin, resistin, and RBP-4; and inflammatory cytokines such as TNF-α and IL-6 (11,15,194-197).

Additionally, several physiological regulators of the insulin receptor have been identified. Increased expression of protein tyrosine phosphatases PTP-1B, LAR, and SHP-2 have been demonstrated in muscle and adipose tissues of obese animals and humans (198). Further, PTP-1B knockout mice exhibit improved insulin sensitivity, increased energy expenditure, and are resistant to diet-induced obesity, demonstrating an important role for PTPs in the regulation of insulin signaling and energy metabolism (199,200).

Another molecule that regulates proximal insulin signaling and which may play a contributing role in insulin resistance is PC-1, a glycoprotein with enzymatic phosphodiesterase activity. It is expressed in various tissues and has been shown to inhibit insulin receptor tyrosine kinase activity (201-203). Moreover, this inhibition seems to be through the binding of PC-1 to the α-subunit of the insulin receptor, thus interfering with IR movement and autophosphorylation of the β-subunits (73,130), thereby disrupting insulin signaling. PC-1 activity in fibroblasts from patients with type 2 diabetes are positively correlated with insulin resistance (204), while the overexpression of PC-1 leads to insulin resistance and diabetes in mice (127).

Fetuin-A, a glycoprotein secreted by the liver, is a negative acute phase reactant (188). Fetuin-A has been shown to interact with the insulin receptor and disrupt both IR autophosphorylation and inhibit IR tyrosine kinase activity (24,27). Additionally, recombinant fetuin-A inhibits the insulin-stimulated mitogenic signaling pathway in cells overexpressing the insulin receptor (26). In humans, fetuin-A is associated with insulin resistance and fat accumulation in the liver (31). On the other hand, fetuin-A knockout mice demonstrate improved insulin signaling and are resistant to diet-induced obesity (32,33).

Excess amounts of glucocorticoids have also been known to cause insulin resistance in both animals and humans despite their therapeutic anti-inflammatory effects (205-209), with dexamethasone (DEX) as a prime example (47,48,169,178). However, the exact mechanisms through which glucocorticoids induce insulin resistance are still

not clear. Interestingly, DEX up-regulates fetuin-A gene expression in mouse hepatoma cells and primary murine hepatocytes (50). Fetuin-A interacts with the insulin receptor and has previously been shown to have IR inhibitory activity (24-27). In this study, we have addressed the relationship between DEX, fetuin-A, and insulin resistance both in cell culture and in animal models of insulin resistance.

First, we characterized the effects of DEX treatment on fetuin-A gene expression, synthesis, and secretion. Our studies showed that DEX treatment in Hep3B cells led to an increase in fetuin-A gene expression while no differences were observed in HepG2 cells. Earlier studies have demonstrated that the transcription of PEPCK, an enzyme involved in gluconeogenesis, is induced by glucocorticoids and inhibited by insulin in H4IIE cells. On the other hand, glucocorticoids repress PEPCK transcription while no effect is observed after insulin treatment in HepG2 cells (210,211). Thus, the inconsistencies observed in our experiment may be the result of differences in cellular machinery between various cancer cell lines. This may warrant the use of primary hepatocytes as an effective model to most extensively mimic actual physiological conditions. Concomitant increases in fetuin-A protein levels secreted into media were also observed. Although DEX treatment was administered in a dose-dependent manner, both gene expression and protein levels of fetuin-A were saturable at a dosage (5 μM) in Hep3B cells. These observations are consistent with an earlier study in primary murine hepatocytes, where a 5.0 μM DEX concentration elicited the highest amount of gene transcription (50). Interestingly, gene transcription and protein levels in our studies did not match consistently. This may be due to the dependence of protein secretion on both the

transcription of fetuin-A and post-translational modifications that mediate its secretion into the cell culture media, leading to a delayed secretory response relative to gene transcription.

Recently, Woltje *et al.* examined regulatory elements on the fetuin-A gene promoter and identified a putative glucocorticoid response element (GRE). A sequence analysis of the fetuin-A promoter regions in human, mouse, and rat genes revealed a high degree of sequence similarity. All three species' promoter sequences contained a putative GRE along with a C/EBP-β and an HNF-3 binding site. The mouse and rat response elements showed a 96.4% sequence homology, while the human sequence showed 79.6% sequence identity, suggesting a common regulatory mechanism between all three species.

Based upon the finding that DEX treatment up-regulated fetuin-A gene expression in primary murine hepatocytes (50), we sought to examine the status of glucocorticoids and plasma fetuin-A in an animal model of insulin resistance. The Zucker diabetic fatty (ZDF) rat is an animal model of type 2 diabetes. These animals have a genetic mutation in the leptin receptor and are characterized by overt hyperglycemia, hyperlipidemia, impaired glucose tolerance, and insulin resistance (212). At 6 weeks of age, ZDF rats are normoglycemic, but have a ~2-fold increase in β-cell mass and exhibit hyperinsulinemia (213), indicative of an insulin resistant state. Our findings showed that plasma samples from 6-week-old ZDF rats demonstrated significantly elevated ($p < 0.05$) corticosterone levels when compared to lean controls. Concurrently, plasma fetuin-A levels were also higher in these animals. These results are consistent with other studies showing positive

associations of corticosterone with insulin resistance (164,190). Further, these findings suggest that the increase in corticosterone levels may contribute to increased plasma fetuin-A concentrations in ZDF rats, which may play a role in insulin resistance (24,27).

Next, we sought to examine the role of fetuin-A in glucocorticoid-induced insulin resistance in Wistar rats. As reported earlier, no significant changes in body weight were observed with DEX treatment (214,215). However, DEX-treated rats given RU had significantly lower body weights than saline-treated controls. Although food intake was not monitored in this study, our findings are consistent with other investigators who have previously reported that treatment with RU and DEX together led to a significant decrease in body weight (216,217).

Our results also showed that DEX treatment significantly increased ($p < 0.01$) plasma insulin and HOMA-IR. The addition of RU to DEX-treated animals led to a significant decrease in both insulin and HOMA-IR. Earlier research by Liu *et al.* had demonstrated that RU-486 (RU) attenuated glucocorticoid-induced insulin resistance (161). This supports the concept of DEX administration leading to insulin resistance in rats, and that this effect is mediated through the glucocorticoid receptor. Of the several methods used for determining insulin resistance, such as the homeostasis model assessment for insulin resistance (HOMA-IR), quantitative insulin-sensitivity check index (QUICKI), fasting glucose-to-insulin ratio (FGIR), and the euglycemic-hyperinsulinemic clamp, the latter clamp method is considered the gold standard (218,219). However, this is more labor intensive, time-consuming, and less comfortable

than other indirect methods of analysis. Thus, earlier studies have been performed to demonstrate the validity and accuracy of surrogate measures (HOMA-IR, QUICKI, etc.) in evaluating insulin sensitivity (184,220-222). For our studies, HOMA-IR was chosen as the preferred method for determining insulin resistance. HOMA-IR is a calculation of insulin sensitivity using fasting plasma glucose and insulin values (184) and has been utilized in many other studies to gauge insulin resistance (223-226). Since blood was collected during experimental treatment, HOMA-IR calculations utilized in our experiments were based on non-fasted levels of both glucose and insulin.

Since DEX treatment induced insulin resistance and RU treatment abrogated DEX-induced insulin resistance, we assayed fetuin-A gene expression and plasma levels to understand the role of fetuin-A as a possible contributing factor to this effect. Interestingly, fetuin-A gene expression was not significantly different among treatment groups. However, increased plasma fetuin-A was observed after DEX treatment in Wistar rats; this corroborates well with the notion that fetuin-A is associated with insulin resistance (31,33). On the other hand, plasma fetuin-A decreased when RU was given to DEX-treated rats. This fits our hypothesis that RU treatment ameliorates DEX-induced insulin resistance, possibly through the disruption of glucocorticoid receptor signaling leading to a decrease in fetuin-A. However, rats treated with RU alone demonstrated significantly elevated plasma fetuin-A levels on day 5, but this did not result in elevated plasma insulin or HOMA-IR. In the circulation, approximately 20% of fetuin-A is phosphorylated at two sites: Ser120 and Ser312 (145). Also, it has been shown that the phosphorylation of fetuin-A is critical for its tyrosine kinase inhibitory activity (185).

Thus, a possible rationalization for this observed inconsistency may be that although total fetuin-A levels were high, relative levels of phosphorylated fetuin-A to total levels were low enough that the IR inhibitory effects in these animals were negligible. Interestingly, plasma fetuin-A was significantly elevated in RU-treated rats despite a low level of gene expression in these animals. While this may imply a diminished level of protein synthesis, a possible explanation for the elevated plasma levels could be due to a delayed degradation of plasma fetuin-A. This compels further examination into the mechanisms of fetuin-A synthesis, secretion, and degradation in these studies.

Since DEX treatment increased fetuin-A gene expression and protein levels with a concomitant increase in insulin resistance, it was of significant interest to examine insulin receptor activation in DEX-treated Wistar rats. The insulin receptor, along with the IRS family of proteins, has been considered one of the "critical nodes" for the propagation of insulin signal transduction. It is a critical junction for cross-talk between pathways that modulate the activity of other signaling systems (72). Earlier studies have demonstrated that dexamethasone treatment can lead to whole body insulin resistance and can disrupt this node of insulin signaling (49,215). DEX treatment in primary rat skeletal myocytes leads to a significant decrease in insulin-stimulated IRS-1 content and phosphorylation, with no effect on IRS-1 serine phosphorylation status (227). In primary rat adipocytes, DEX abates basal and insulin-stimulated glucose uptake, lowers IRS-1 expression, and decreases both PI3K and AKT content (48). In addition, it has been shown to impair insulin-stimulated glucose transport by inhibiting GLUT4 translocation (228), and reduces AKT and GSK-3 phosphorylation in skeletal muscle (39). Fetuin-A has been

shown to interact with the insulin receptor to reduce autophosphorylation of tyrosine residues on the receptor (27). Since fetuin-A expression was increased after DEX-treatment, it was our hypothesis that insulin receptor activation may be impaired. Accordingly, liver IR phosphorylation status was examined by immunoblotting with anti-phospho IR antibody (Tyr1158, Tyr1162, Tyr1163). Our data showed a near-significant reduction ($p = 0.07$) in the phosphorylation of these tyrosine residues, which have previously been shown to be crucial for insulin signal transduction (229,230). This suggests that the DEX-mediated increase in fetuin-A may be involved in both the abatement of IR autophosphorylation and the concomitant decrease in insulin sensitivity.

Overall results from this experiment indicate that glucocorticoid administration leads to insulin resistance and increased levels of fetuin-A, while the administration of RU improves insulin sensitivity and prevents the increase of plasma fetuin-A in these animals. While RU-treated rats exhibit significantly elevated fetuin-A, insulin and HOMA-IR are not increased, suggesting that other factors such as fetuin-A phosphorylation may play a role in its natural IR inhibitory activity. This warrants further investigation into the status of phosphorylated fetuin-A in DEX- and RU-treated Wistar rats.

Surgical adrenalectomies (ADX) have been performed in various studies to observe its effects on insulin sensitivity and glucose metabolism in rodent models of diabetes and insulin resistance (231-233). It has been shown to curtail hyperinsulinemia and hyperglycemia in both *ob/ob* mice (234,235) and Wistar rats (236). As expected, our

findings showed that adrenalectomy led to a significant decrease in both body weight and corticosterone levels, which were consistent with other studies (237-239). Also, earlier studies by White *et al* have shown that adrenalectomized rats exhibit a decrease in body weight which can be reversed with aldosterone treatment, a type 1 receptor agonist (156). While blood glucose levels were not significantly different between ADX and sham-operated controls, plasma insulin levels and HOMA-IR were both lower in ADX rats (saline-injected) compared to sham controls (saline-injected). DEX treatment resulted in a significant increase in both insulin and HOMA-IR in the ADX and sham groups. Studies by Solomon *et al.* and Yukimura *et al.* have demonstrated elevated plasma insulin in adrenalectomized rats with glucocorticoid administration (240,241). Since HOMA-IR is based on a calculation of glucose and insulin values, we have shown that DEX-treated rats exhibit elevated HOMA-IR when compared to saline-treated controls due to their hyperinsulinemic profile, despite glucose levels being similar to that of controls. Earlier findings have also demonstrated that adrenalectomy improves insulin sensitivity (242,243), while the injection of glucocorticoids reverses the effects of adrenalectomy (244,245).

Along with improved insulin sensitivity, ADX rats also exhibited a decrease in plasma fetuin-A compared to sham-controls in both saline- and DEX-treated groups. Fetuin-A has been shown to be associated with insulin resistance (31), whereas fetuin-A knockout mice demonstrate improved insulin sensitivity (32,33). In ADX rats, the decrease in fetuin-A protein expression was consistent with an improvement in insulin sensitivity. Interestingly, DEX treatment in ADX rats did not lead to elevated plasma

fetuin-A, suggesting that adrenal gland-secreted metabolites may play a pivotal role in the glucocorticoid-mediated up-regulation of fetuin-A. This notion is supported by the fact that corticosteroids can bind two types of receptors: type 1 and type 2. The type 1 receptor has a high affinity for both corticosterone and the mineralocorticoid aldosterone. On the other hand, the type 2 receptor has a higher affinity for synthetic glucocorticoids such as dexamethasone. Adrenalectomized rats do not produce the corticosteroids necessary to activate the type 1 and type 2 receptors. Therefore, our observation that treatment with DEX, a type 2 receptor agonist, does not elicit an increase in fetuin-A may suggest a cooperative effect of both type 1 and type 2 receptors in mediating the increase in plasma fetuin-A detected in our earlier experiment.

No significant decrease in fetuin-A was observed when comparing DEX- and saline-treated ADX rats. On the other hand, sham-operated rats treated with DEX displayed significantly elevated levels of both total and phosphorylated fetuin-A. Earlier studies have revealed that the phosphorylation of fetuin-A is critical for its IR inhibitory activity (185). Along with fetuin-A, HOMA-IR was also elevated in DEX-treated sham-operated rats. Taken together, these results demonstrate that glucocorticoids increase fetuin-A with concomitant elevations in insulin and HOMA-IR, possibly mediated through pathways drawn from the adrenal glands.

The fetuin-A knockout mouse was first generated by Jahnen-Dechent *et al.* as a model to study ectopic mineralization (246). Since fetuin-A is shown to be an inhibitor of insulin receptor tyrosine kinase activity and is also associated with insulin resistance and

the metabolic syndrome (24,29,31), it was of significant interest to utilize the fetuin-A knockout mouse model in studying the pathogenesis of insulin resistance. Mathews *et al.* have previously showed that fetuin-A knockout mice are protected from obesity and insulin resistance when fed a high-fat diet (32). The same group later demonstrated that insulin sensitivity was not affected in fetuin-A knockout mice as a result of aging (33). Based on these findings, we hypothesized that fetuin-A knockout mice given DEX would retain a sustained degree of insulin sensitivity. The insulin tolerance test (ITT) is based on an intraperitoneal injection of insulin and a measurement of the animal's glucose response over time. It is an effective tool used to assess whole-body insulin sensitivity in animals (33,106,247,248). Thus, we utilized this method in evaluating the effects of DEX on insulin signaling in our study. Results showed that fetuin-A knockout mice treated with DEX exhibited improved insulin sensitivity compared to wild-type DEX-treated controls. Additionally, DEX treatment in wild-type mice led to significantly impaired insulin signaling compared to wild-type saline-treated controls, which was consistent with our earlier results demonstrating that DEX induced insulin resistance in animals. Together, these findings demonstrate the efficacy of DEX in inducing insulin resistance, while gene knockout of fetuin-A prevents the disruption of insulin signaling. This suggests that fetuin-A may play a critical role in the development of DEX-induced insulin resistance.

The metabolic syndrome is a cluster of metabolic abnormalities that increase the risk for cardiovascular disease. According to the National Cholesterol Education Program's Adult Treatment Panel III report (NCEP-ATP III), these factors include:

abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, and insulin resistance (249). Fetuin-A has been shown to be strongly associated with features of the metabolic syndrome, such as an atherogenic lipid profile, hyperlipidemia, liver fat accumulation, and insulin resistance (29,31). Moreover, adiponectin levels, which are inversely correlated with coronary heart disease (250), are shown to be suppressed by fetuin-A in animals and humans (251). Patients with Cushing's syndrome have hypercortisolemia and exhibit many characteristics of the metabolic syndrome, including obesity, hypertension, dyslipidemia, and glucose intolerance (169). These, along with elevated levels of cortisol, suggest that glucocorticoids may be a contributing factor in the development of these features (252).

Endogenous glucocorticoids are produced through the hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine feedback circuit (160). In response to acute stress, neurons stimulate the hypothalamus to release corticotropin releasing factor (CRF), which then travel to the anterior pituitary to secrete ACTH. This hormone then acts on the adrenal cortices to mediate glucocorticoid production (153). Studies have demonstrated that increased HPA and glucocorticoid activity are both consistently correlated with obesity, hyperglycemia, and insulin resistance (253-255). However, an even more important notion may be the inter-conversion of glucocorticoids between inactive and active states.

Two key enzymes that mediate tissue-specific glucocorticoid activity are 11β-hydroxysteroid dehydrogenase (11β-HSD) types 1 and 2. Type 1 converts inactive

glucocorticoids to their active forms and is widely expressed in insulin-sensitive tissues such as the liver and adipose (256). On the other hand, type 2 is responsible for the conversion of active to inactive glucocorticoids and is more widely expressed in the kidney, an aldosterone-selective target tissue (42). In adipose tissue, 11β-HSD1 activity is consistently associated with obesity, insulin resistance, and other features of the metabolic syndrome (164,166,175). Conversely, 11β-HSD1 activity in the liver is decreased in obese Zucker rats and *ob/ob* mice (163,257). Obese, insulin resistant humans exhibit elevated cortisol generation from its inactive form in both skeletal muscle and adipose without significant increases in plasma cortisol concentrations (166,258). This suggests that the tissue-specific activity of 11β-HSD1 in converting inactive to active glucocorticoids is a greater contributing factor to the development of features associated with the metabolic syndrome than circulating levels of glucocorticoids (259). Interestingly, the liver-specific overexpression of 11β-HSD1 in mice leads to mild insulin resistance, dyslipidemia, and hypertension without an increase in fat mass (260). Moreover, overexpressing 11β-HSD1 selectively in adipose tissue of mice leads to the development of visceral obesity that is exacerbated by a high-fat diet. In addition, these animals become diabetic, insulin resistant, and exhibit hyperlipidemia (261). On the other hand, the transgenic overexpression of 11β-HSD2 in adipocytes protects mice from diet-induced obesity and weight-gain due to reduced fat mass accumulation (262). Whole-body 11β-HSD1 knockout mice exhibit increased liver insulin sensitivity, improvements in both lipid profile and glucose tolerance, and are also resistant to weight gain and diabetes (172,173). Non-specific inhibitors of 11β-HSD such as carbenoxolone have been shown to increase glucose uptake in the liver and improve insulin sensitivity in type 2

diabetics (168) and healthy subjects (167). Furthermore, the selective inhibition of 11β-HSD1 lowers blood glucose levels and leads to improved hepatic insulin sensitivity in hyperglycemic mice (162,170). These findings suggest that the enzymatic regulation of 11β-HSD activity may be a potent avenue of therapeutic strategies for the treatment of various features associated with the metabolic syndrome. Additionally, our results have demonstrated that glucocorticoids elevate fetuin-A levels and impair insulin signaling. On the other hand, fetuin-A knockout mice exhibit protective effects against insulin resistance similar to those seen in the targeted inhibition or genetic knockout of 11β-HSD1. Accordingly, the tissue-specific activity of the 11β-HSD enzymes may be of significant interest in prospective investigations elucidating the role of fetuin-A in glucocorticoid-mediated insulin resistance.

Recently, the sphingolipid ceramide was shown to be a molecular intermediate linking glucocorticoid-, saturated fat-, and obesity-induced insulin resistance (263). Dexamethasone treatment in mice induced the expression of genes necessary for ceramide biosynthesis and promoted ceramide accumulation in the liver. Concomitant increases in both fasting blood glucose and insulin, along with impaired glucose disposal were also observed. On the other hand, treatment with myriocin, an inhibitor of ceramide biosynthesis, significantly ameliorated DEX-induced glucose intolerance in these animals, suggesting that enzymes required for ceramide biosynthesis could be a potential drug target for glucocorticoid-induced insulin resistance. These results parallel our findings that demonstrate fetuin-A as a central mediator of DEX-induced insulin resistance. Additional experiments in this area could potentially involve assessing the

degree of ceramide expression and circulating levels in DEX-treated fetuin-A knockout mice to provide further insight into the mechanisms of glucocorticoid-mediated insulin resistance.

CHAPTER 6: CONCLUSIONS

Diabetes is a disease that affects nearly 250 million people around the world with an estimated annual cost of approximately $175 billion. Various factors may predispose a person to developing diabetes, such as a high-fat diet, obesity, and ethnicity. In addition, inflammatory cytokines such as TNF-α and IL-6 can contribute to insulin resistance, a hallmark feature of type 2 diabetes. Among other roles, fetuin-A acts as a natural inhibitor of insulin signaling through the disruption of insulin receptor tyrosine autophosphorylation. It is highly associated with obesity, insulin resistance, dyslipidemia, and features of the metabolic syndrome (29,31,191,264). Furthermore, serum fetuin-A is also associated with carotid arterial stiffness (144), atherosclerosis (30), C-reactive protein (251), and negatively represses high molecular weight adiponectin (251). Polymorphisms in the fetuin-A gene have been shown to be associated with type 2 diabetes (140), dyslipidemia (143), and leanness (265) among varying populations, suggesting that fetuin-A has modulatory effects on whole body metabolism from a genetic standpoint. Conversely, fetuin-A knockout mice are protected against diet-induced obesity and weight gain (32). Given that fetuin-A is associated with insulin resistance and that dexamethasone increases fetuin-A gene expression and decreases peripheral insulin sensitivity, we sought to assess the possible role of fetuin-A as a mediator of glucocorticoid-induced insulin resistance.

We first demonstrated that DEX up-regulated both gene expression of fetuin-A and protein secretion in human Hep3B and HepG2 hepatoma cell lines. In ZDF rats, an animal model of gross insulin resistance and diabetes, both corticosterone and fetuin-A levels were elevated. DEX injection in male Wistar rats led to a significant increase in plasma insulin, HOMA-IR, and fetuin-A. On the other hand, treatment with the glucocorticoid receptor antagonist RU-486 ameliorated DEX-induced increases of these factors, implicating fetuin-A as a possible target of glucocorticoid action. Adrenalectomized rats exhibited improved HOMA-IR, while saline- and DEX-treated rats demonstrated significantly lower fetuin-A levels compared to their respective sham-operated controls. DEX-treated wild-type mice developed whole-body insulin resistance, while fetuin-A knockout mice injected with DEX showed a level of insulin sensitivity that was comparable to saline-treated controls.

These results demonstrate that DEX induces insulin resistance in animals with a concomitant increase in plasma fetuin-A. Surgical adrenalectomy improves insulin sensitivity and also lowers fetuin-A levels, while mice lacking fetuin-A are protected against DEX-induced disruption of insulin signaling. Overall, our findings lend support for a novel mechanistic function of fetuin-A in the pathophysiology of glucocorticoid-mediated insulin resistance.

Gene	Sequence
mf-wt3prime1s (amplifies WT and KO)	5’-ACT CTT CAT TCT CCT AAG GTG G-3’
mf-wt3prime1as (amplifies only WT)	5’-TAT GCC TTC TCA CAG CAC CG-3’
pGKneo3prime1s (amplifies only KO)	5’-TTG AAT GGA AGG ATT GGA GC-3’

Table 1: Primer sequences used for polymerase chain reaction for fetuin-A knockout mouse DNA genotyping.

Gene	Sequence
AHSG (forward)	5’-ACG TGG TCC ACA CTG TCA AA-3’
AHSG (reverse)	5’-CGC AGC TAT CAC AAA CTC CA-3’
β-actin (forward)	5’-CCT CTA TGC CAA CAC AGT GC-3’
β-actin (reverse)	5’-CAT CGT ACT CCT GCT TGC TG-3’

Table 2: Primer sequences used in real-time PCR for fetuin-A and β-actin gene expression.

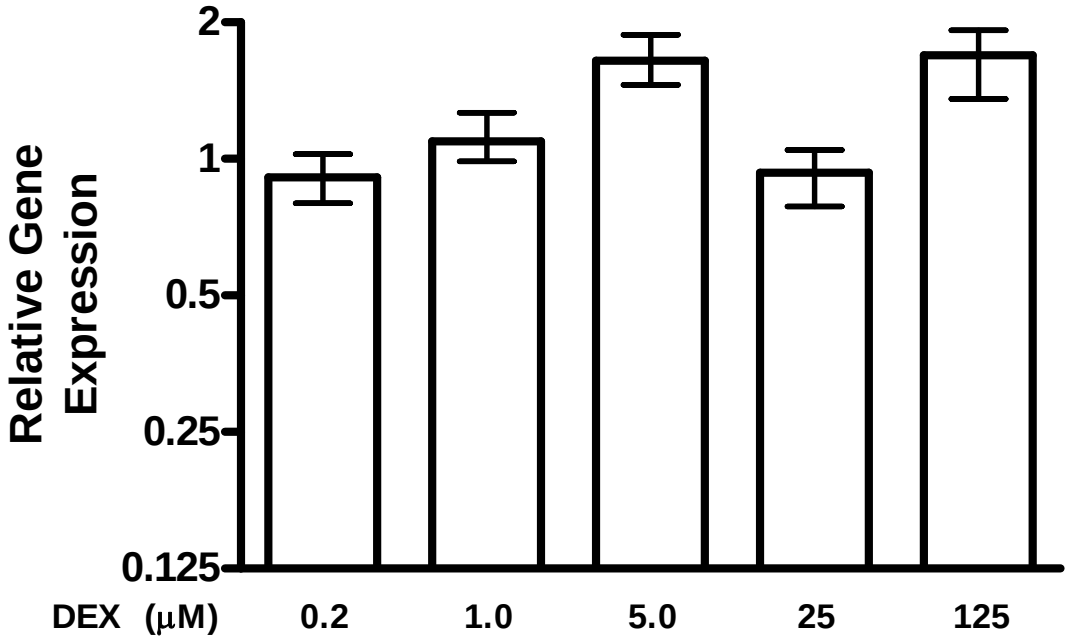


Fig. 3: Fetuin-A gene expression in human Hep3B hepatoma cells. Confluent Hep3B cells were serum-starved overnight, then treated with dexamethasone (DEX). Cells were collected 24 hours after treatment. Total RNA was isolated from Hep3B cells. Real-time PCR was then performed, in triplicate, for fetuin-A gene expression. Data are expressed as relative gene expression of fetuin-A ± SEM using REST 2005 software. The relative gene expression of β-actin was 1.0.

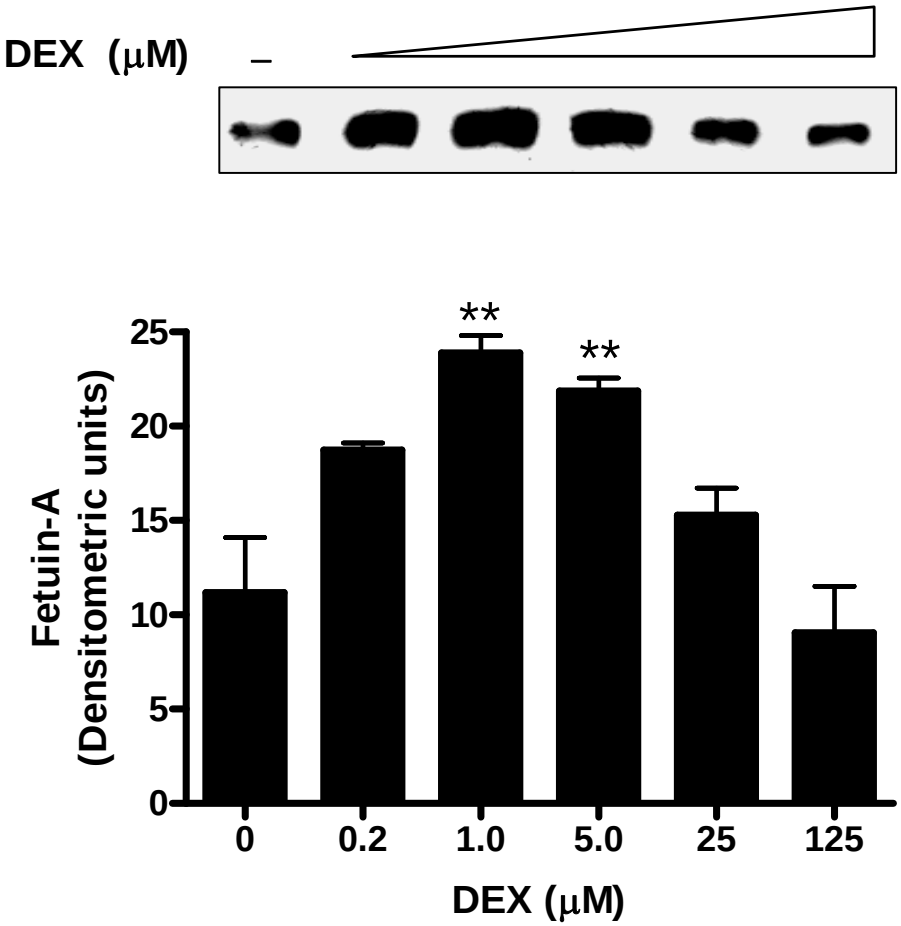


Fig. 4: Effect of DEX treatment on fetuin-A secretion in human Hep3B hepatoma cells. Confluent Hep3B cells were serum-starved overnight, then treated with dexamethasone (DEX). Media was collected 24 hours after treatment. Proteins secreted into the cell culture media were separated by SDS-PAGE, and immunoblotted with anti-human fetuin-A antibody, and chemiluminescence was detected using LabWorks Image Acquisition software. Data from Western blot analysis are expressed as arbitrary densitometric units and represent the mean of three independent experiments. A representative western blot is shown. ** $p < 0.01$ vs. 0 μM DEX.

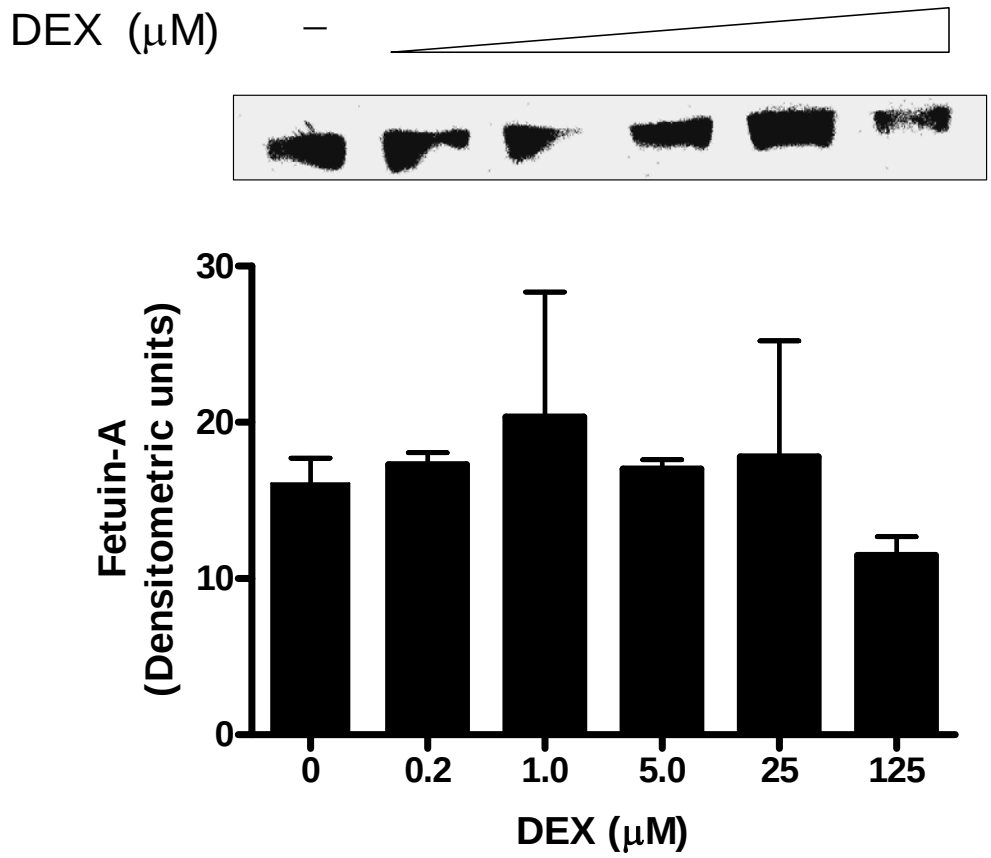


Fig. 5: Effect of DEX treatment on fetuin-A secretion in human HepG2 hepatoma cells. Confluent HepG2 cells were serum-starved overnight, then treated with dexamethasone (DEX). Media was collected 24 hours after treatment. Proteins secreted into the cell culture media were separated by SDS-PAGE, and immunoblotted with anti-human fetuin-A antibody, and chemiluminescence was detected using LabWorks Image Acquisition software. Data from Western blot analysis are expressed as arbitrary densitometric units and represent the mean of two independent experiments. A representative western blot is shown. Results are not statistically significant between treatments.

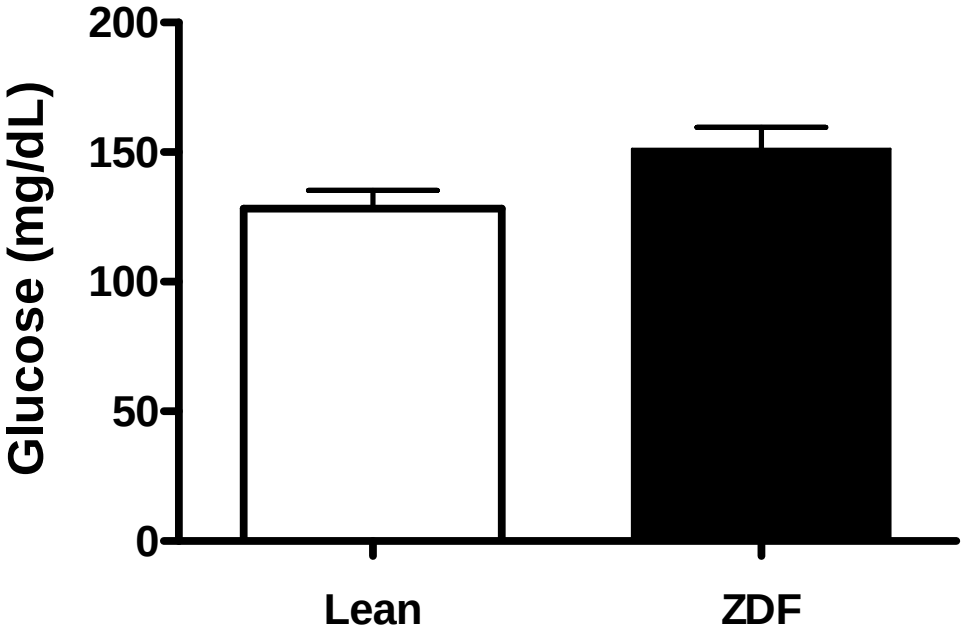


Fig. 6: Blood glucose concentrations in Zucker diabetic fatty rats. Blood samples were obtained from 6-week-old adult, male ZDF rats from the lateral saphenous vein. Glucose levels were measured with a glucometer. Data are expressed as mean ± SEM (n=6 per group) and are not statistically significant.

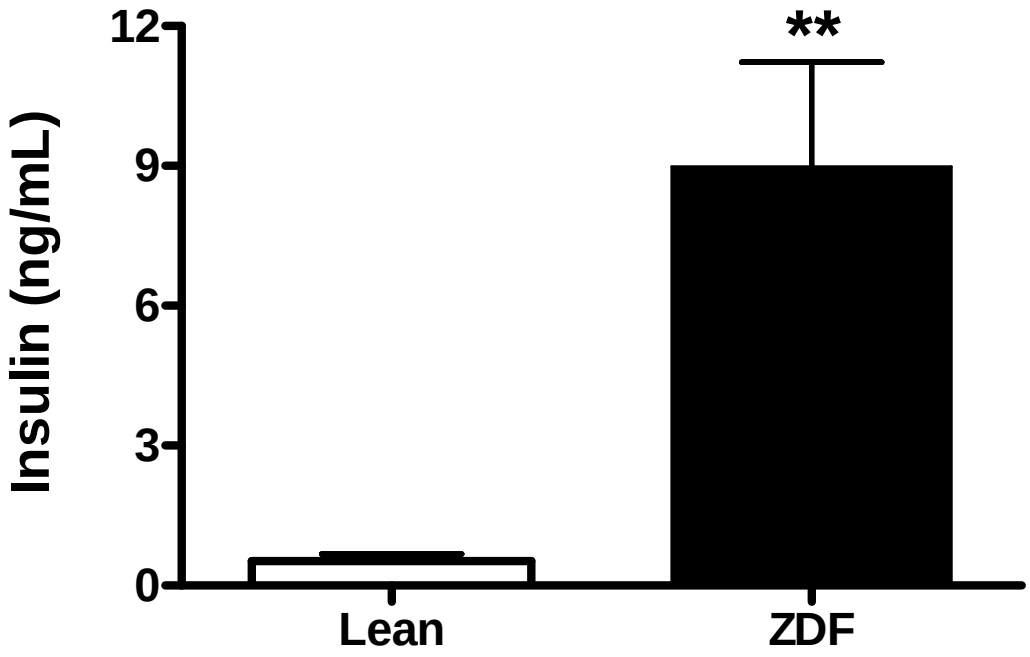


Fig. 7: Plasma insulin concentrations in Zucker diabetic fatty rats. Blood samples were obtained from 6-week-old adult, male ZDF rats from the lateral saphenous vein. Plasma insulin concentrations were assayed with ELISA. Data are expressed as mean ± SEM (n=6 per group). ** $p < 0.05$.

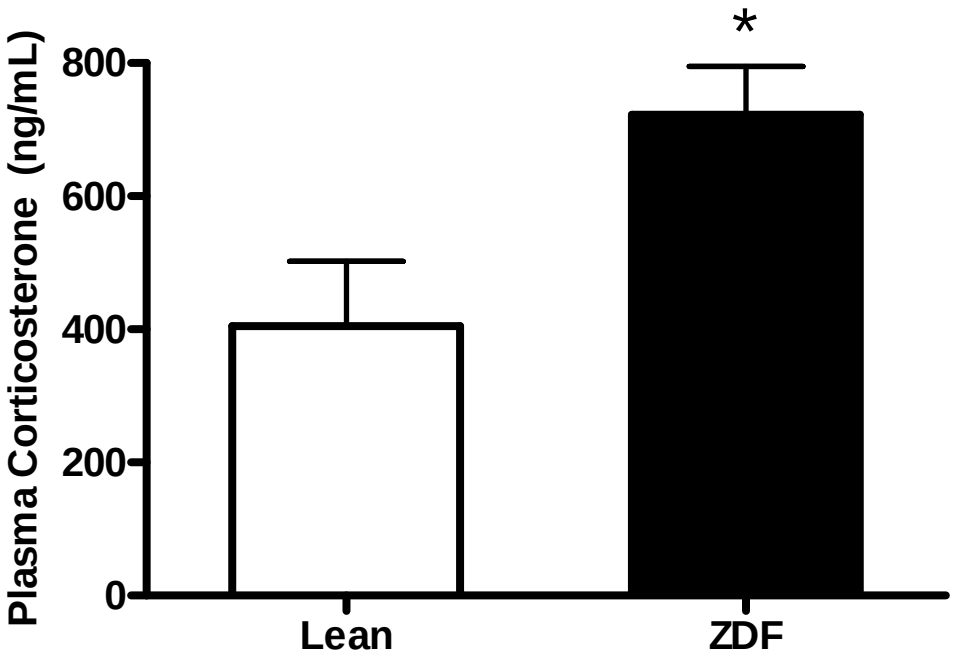


Fig. 8: Plasma corticosterone levels in Zucker diabetic fatty rats. Blood samples were obtained from 6-week-old adult, male ZDF rats. Plasma corticosterone levels were quantitated through ELISA. Data are expressed as mean ± SEM (n=5 per group).
* $p < 0.05$.

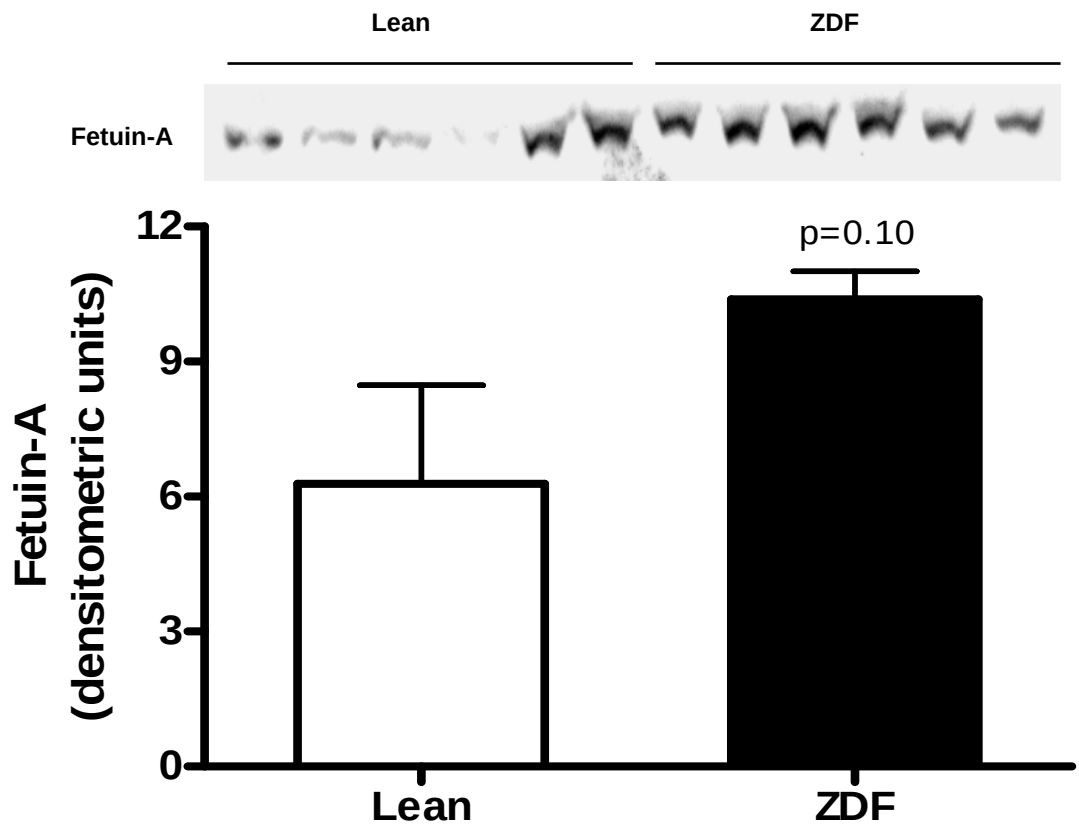


Fig. 9: Fetuin-A levels in Zucker diabetic fatty rats. Blood samples were obtained from 6-week-old adult, male ZDF rats. Plasma samples were diluted 1:100 in saline, run on SDS-PAGE, immunoblotted, and visualized for fetuin-A as described previously. Data are expressed as mean ± SEM (n = 6 per group) and are not statistically significant (p=0.01).

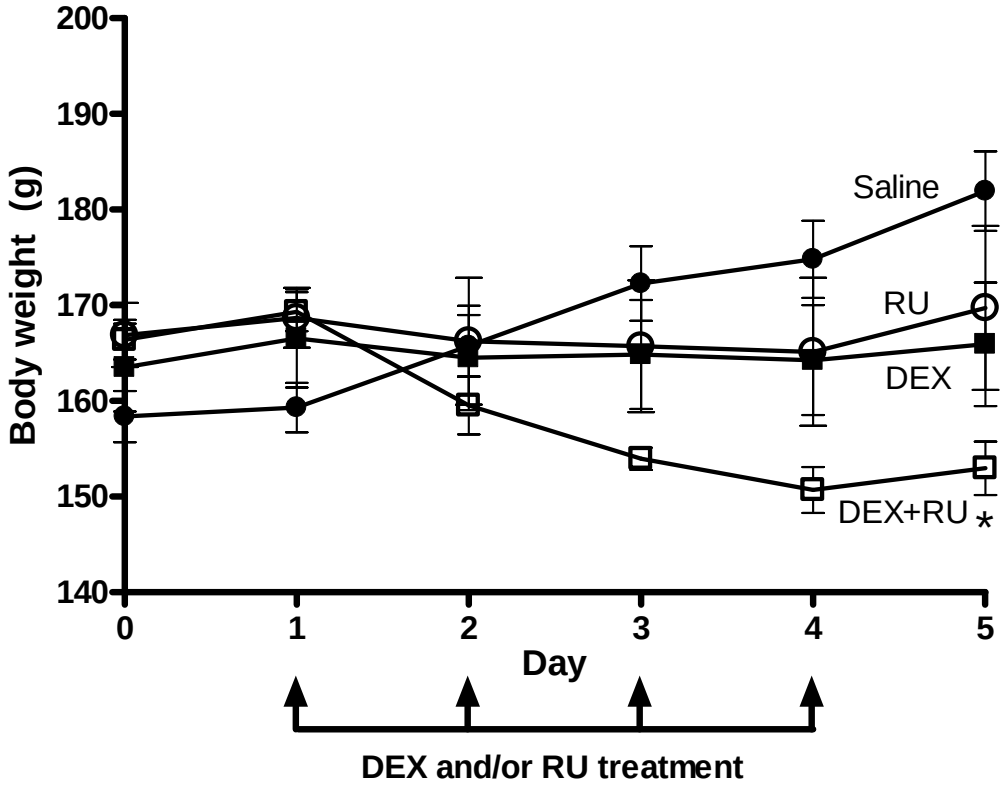


Fig. 10: Body weights in 4-week-old Wistar rats. Body weights were measured just before treatment on each day shown. Data are expressed as mean ± SEM. * $p < 0.05$ vs. same-day Saline. DEX: dexamethasone treatment; RU: RU-486 treatment; DEX+RU: dexamethasone and RU-486 treatment; Saline: vehicle treatment.

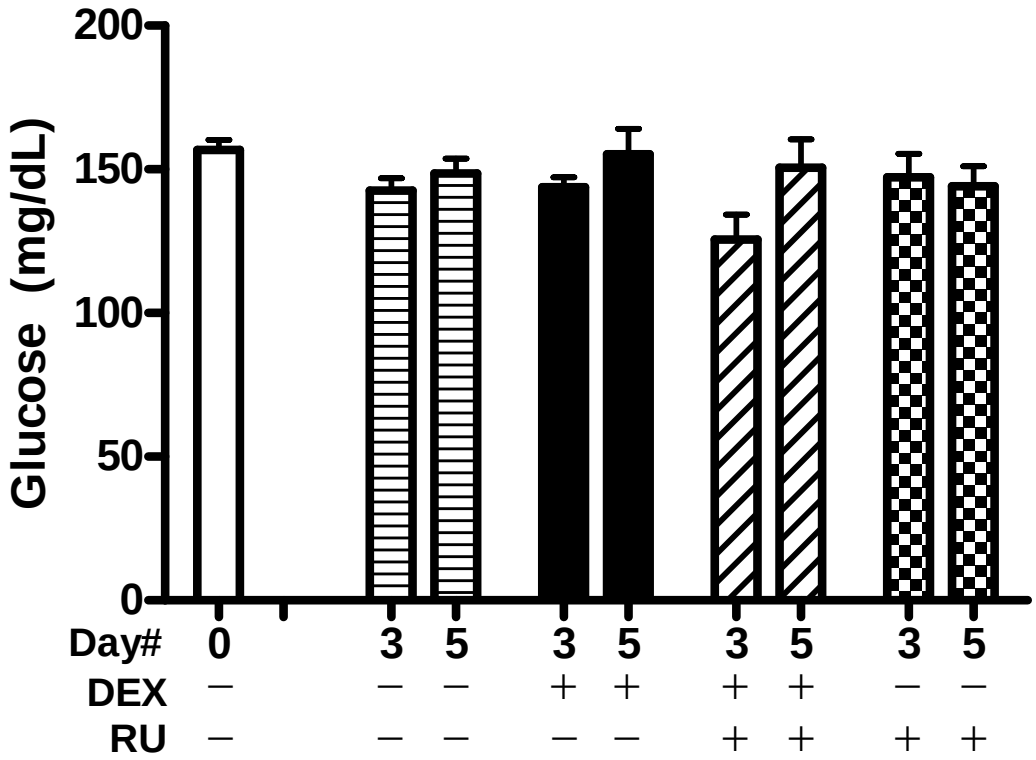


Fig. 11: Blood glucose concentrations in DEX- and/or RU-treated Wistar rats. 4-week-old, male, Wistar rats were injected with dexamethasone (DEX, 1 mg/kg body weight) or vehicle (saline), once daily for 4 days. RU-treated rats were injected RU-486 (RU, 50 mg/kg body weight) 2h prior to DEX or saline injection, once daily for 4 days. All injections were administered intraperitoneally. Blood samples were collected from the lateral saphenous vein on day 0 (baseline), and following the commencement of treatment, on days 3 and 5. Glucose levels were measured with a glucometer. Data shown are mean ± SEM (n=5 or 6 in each group). Means shown are not statistically significant between treatments and/or days.

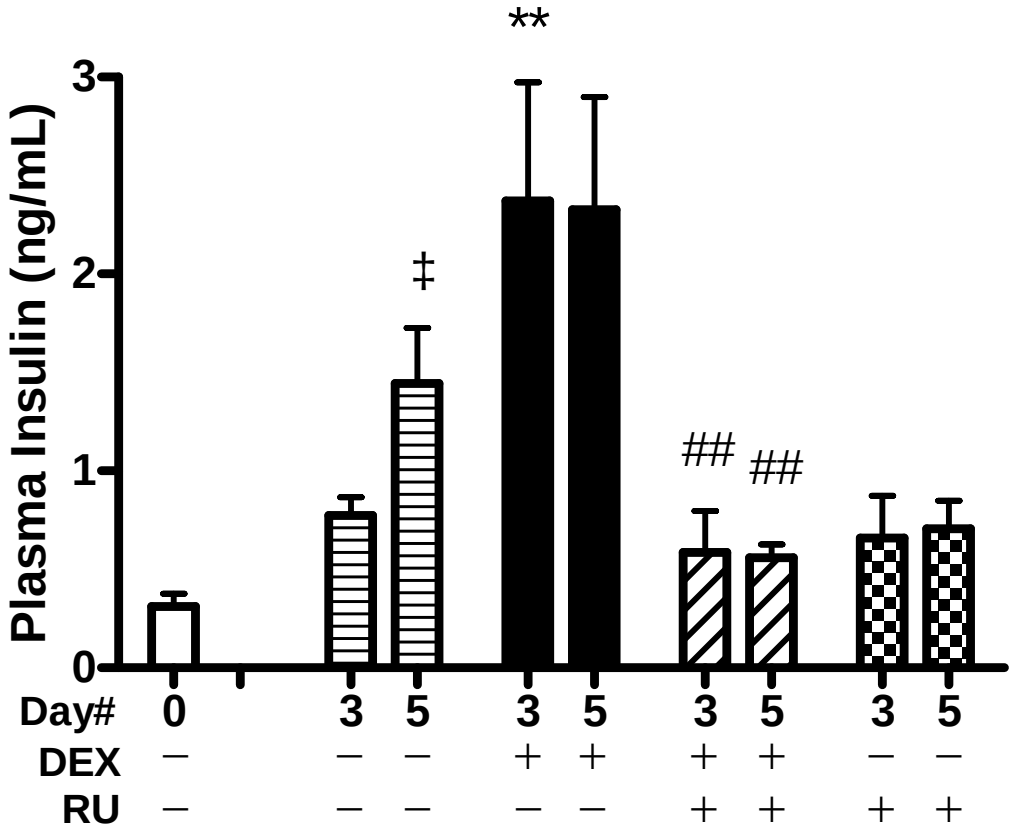


Fig. 12: Plasma insulin concentrations in DEX- and/or RU-treated Wistar rats. 4-week-old, male, Wistar rats were injected with dexamethasone (DEX, 1 mg/kg body weight) or vehicle (saline), once daily for 4 days. RU-treated rats were injected RU-486 (RU, 50 mg/kg body weight) 2h prior to DEX or saline injection, once daily for 4 days. All injections were administered intraperitoneally. Blood samples were collected from the lateral saphenous vein on day 0 (baseline), and following the commencement of treatment, on days 3 and 5. Plasma insulin concentrations were assayed with ELISA. Data shown are mean ± SEM (n=5 or 6 in each group). ‡ $p < 0.05$ vs. Day 0; ** $p < 0.01$ vs. same-day Saline; ## $p < 0.01$ vs. same-day DEX.

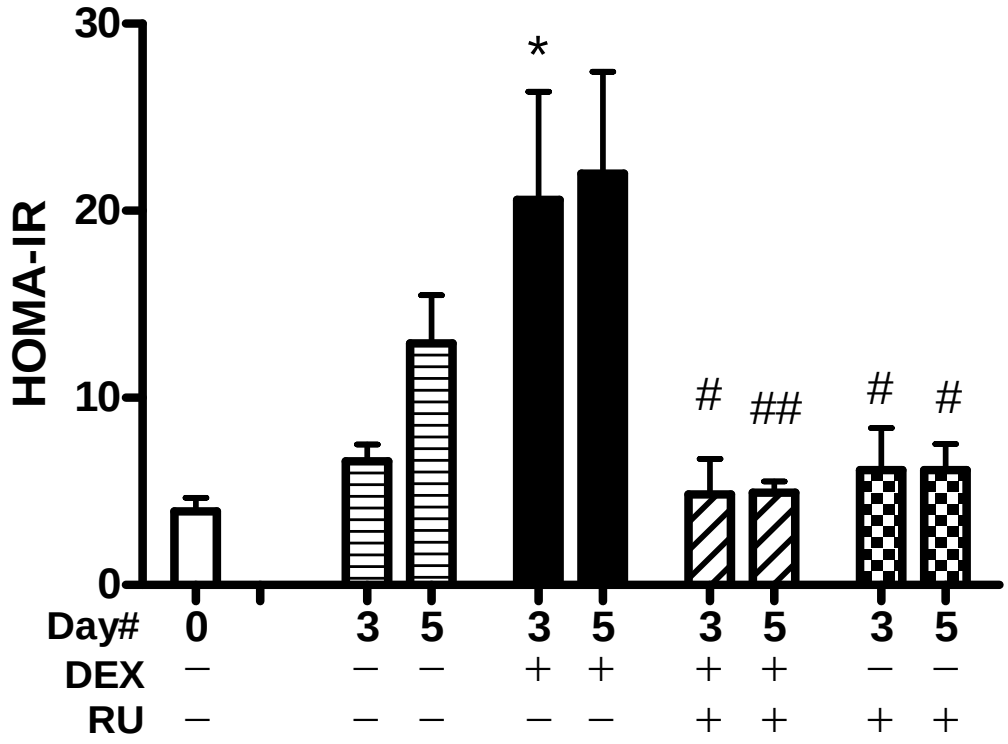


Fig. 13: HOMA-IR in DEX- and/or RU-treated Wistar rats. 4-week-old, male, Wistar rats were injected with dexamethasone (DEX, 1 mg/kg body weight) or vehicle (saline), once daily for 4 days. RU-treated rats were injected RU-486 (RU, 50 mg/kg body weight) 2h prior to DEX or saline injection, once daily for 4 days. All injections were administered intraperitoneally. Blood samples were collected from the lateral saphenous vein on day 0 (baseline), and following the commencement of treatment, on days 3 and 5. HOMA-IR using non-fasted blood samples was calculated as:

$$\text{HOMA-IR} = [\text{Insulin } (\mu\text{U/mL}) \times \text{Glucose (mmol/L)}] / 22.5$$

Data are expressed as mean ± SEM (n=5 or 6 in each group). * $p < 0.05$ vs. same-day Saline; # $p < 0.05$, ## $p < 0.01$ vs. same-day DEX.

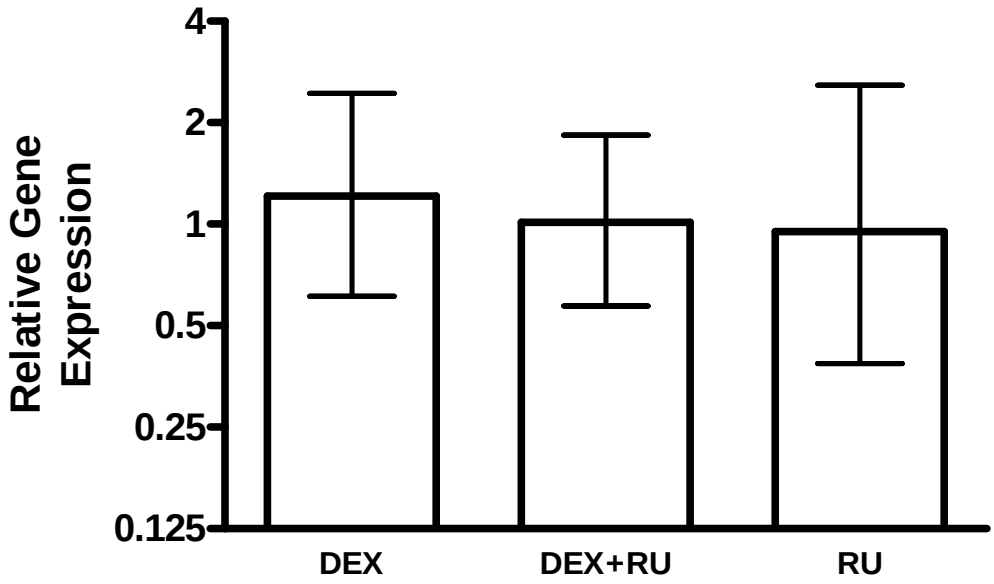


Fig. 14: Fetuin-A gene expression in DEX- and/or RU-treated Wistar rats. 4-week-old, male, Wistar rats were injected with dexamethasone (DEX, 1 mg/kg body weight) or vehicle (saline), once daily for 4 days. Two other groups were injected RU-486 (RU, 50 mg/kg body weight) 2h prior to DEX or saline injection, once daily for 4 days. All injections were administered intraperitoneally. Total RNA was isolated from excised rat livers (n=6). Real-time PCR was then performed, in triplicate, for fetuin-A gene expression. Data are expressed as relative gene expression of fetuin-A ± SEM using REST 2005 software. The relative gene expression of β-actin was 1.0.

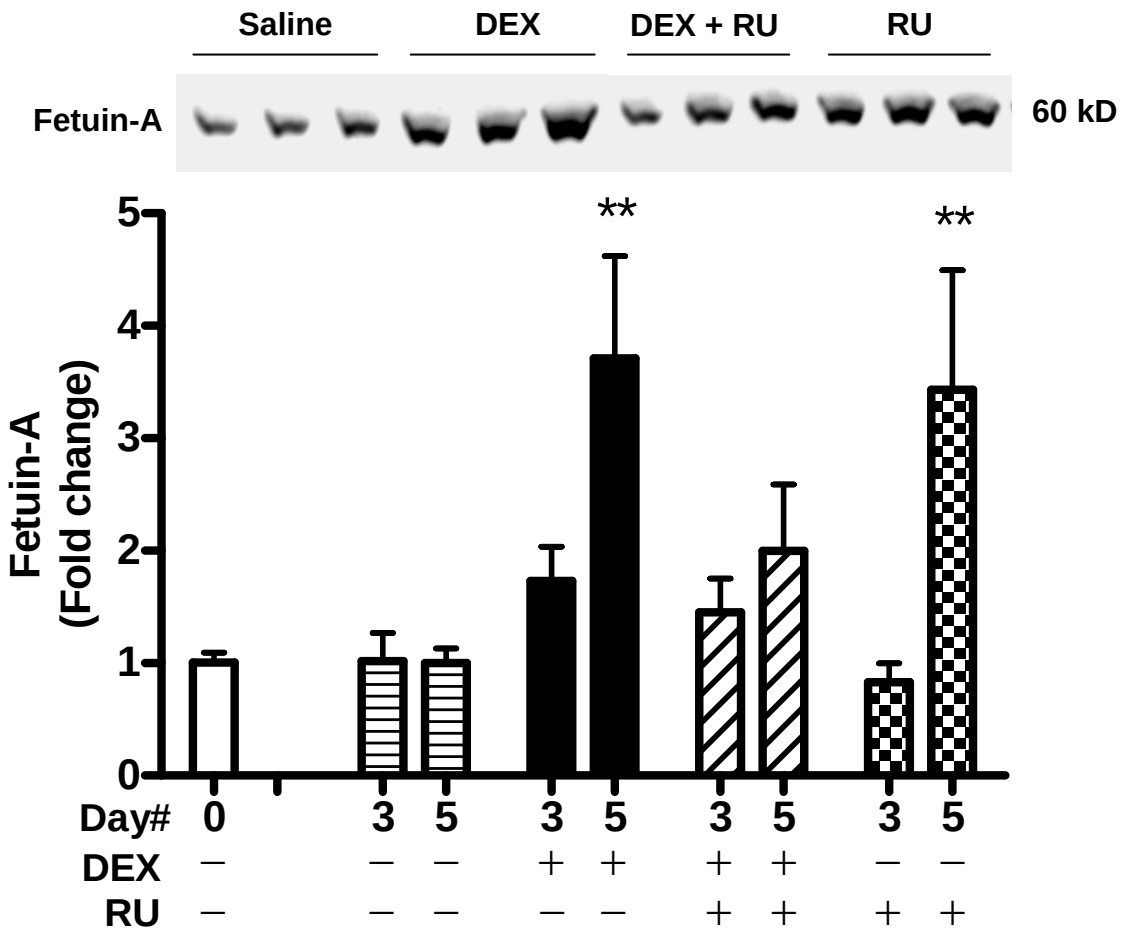


Fig. 15: Plasma fetuin-A levels in DEX- and/or RU-treated Wistar rats. 4-week-old, male, Wistar rats were injected with dexamethasone (DEX, 1 mg/kg body weight) or vehicle (saline), once daily for 4 days. RU-treated rats were injected RU-486 (50 mg/kg body weight) 2h prior to DEX or saline injection, once daily for 4 days. All injections were administered intraperitoneally. Blood samples were collected from the lateral saphenous vein on day 0 (baseline), and following the commencement of treatment, on days 3 and 5. Data are expressed as mean ± SEM (n=5 or 6 in each group). A representative Western blot from Day 5 is shown. ** $p < 0.01$ vs. Saline.

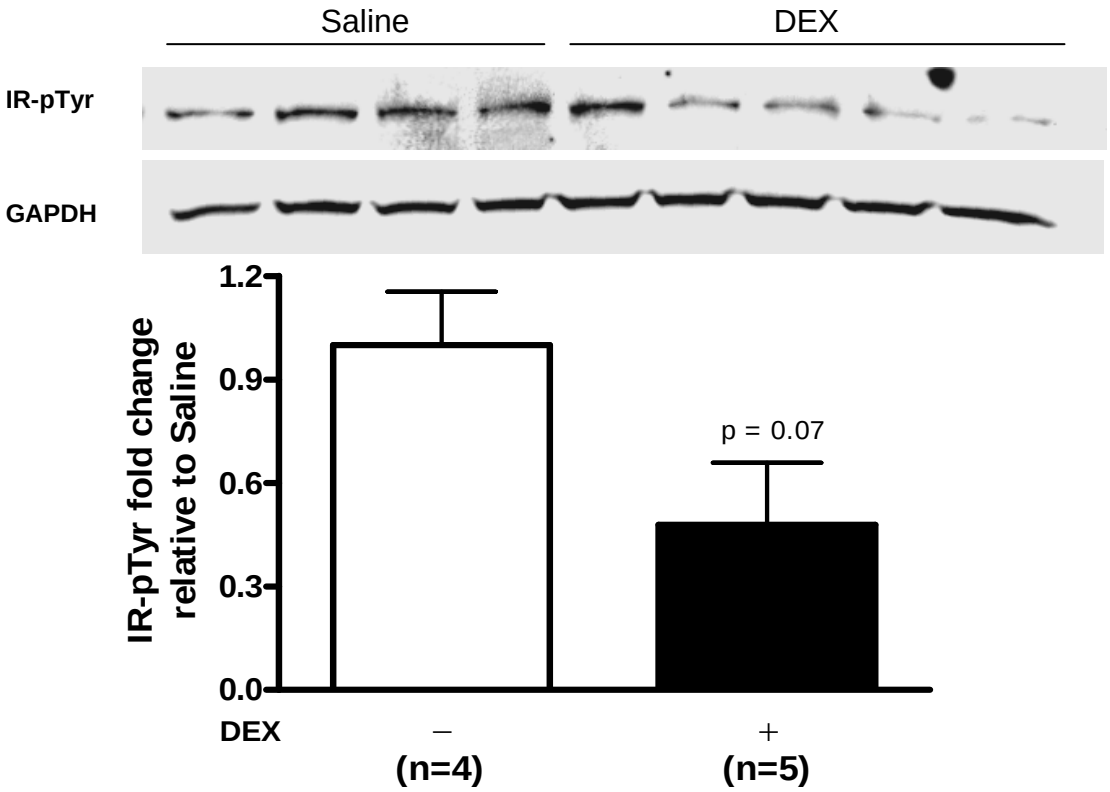


Fig. 16: Insulin receptor tyrosine phosphorylation (IR-pTyr) levels in DEX- or saline-treated rats. 4-week-old, male, Wistar rats were injected with dexamethasone (DEX, 1 mg/kg body weight) or vehicle (saline), once daily for 4 days. Liver tissues were homogenized, run on SDS-PAGE, and immunoblotted with anti-IR phospho-Tyrosine antibody. Chemiluminescence was detected using LabWorks Image Acquisition software. GAPDH was visualized as a loading-control. Data are expressed as mean ± SEM (n=4 or 5 in each group).

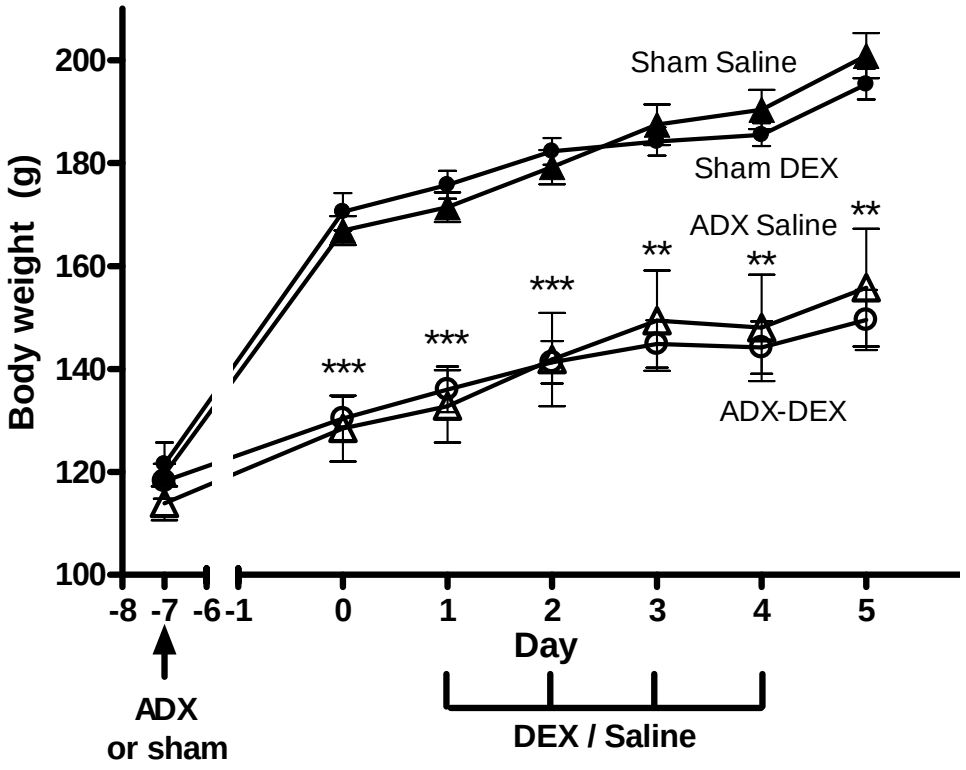


Fig. 17: Body weight changes in adrenalectomized (ADX) Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Following a recovery period of 1 week, rats were injected either dexamethasone (DEX, 1 mg/kg body weight) or saline once daily for 4 days, as described previously. Data are expressed as mean ± SEM (n=5 or 6 in each group).
** $p < 0.01$, *** $p < 0.001$ vs. Sham groups.

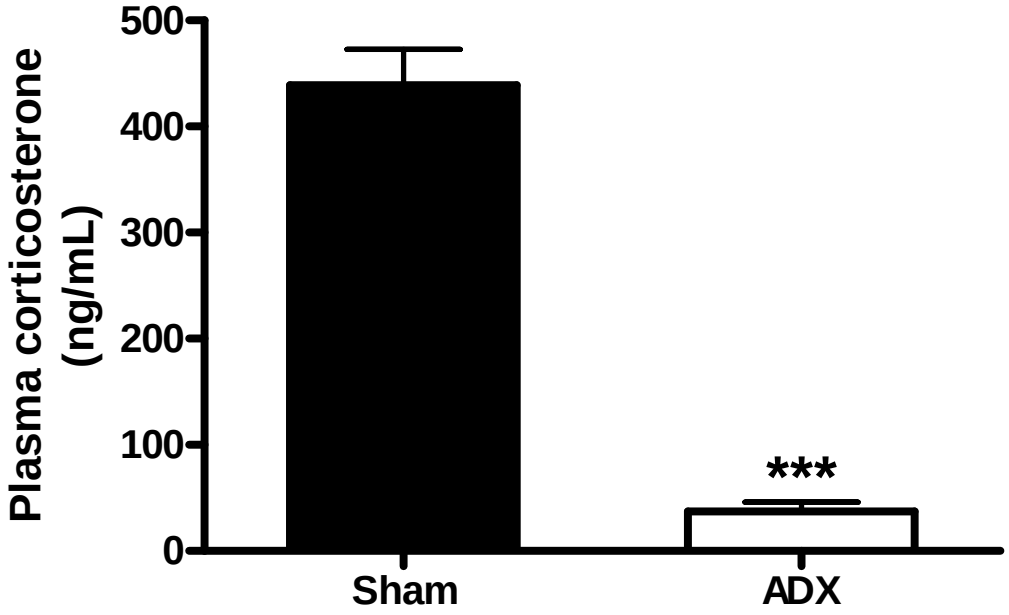


Fig. 18: Plasma corticosterone concentrations in adrenalectomized (ADX) Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Blood samples for the corticosterone assay were collected at approximately 9:00 am on Day 0. Data are expressed as mean ± SEM (n=10 or 11 in each group). *** $p < 0.001$.

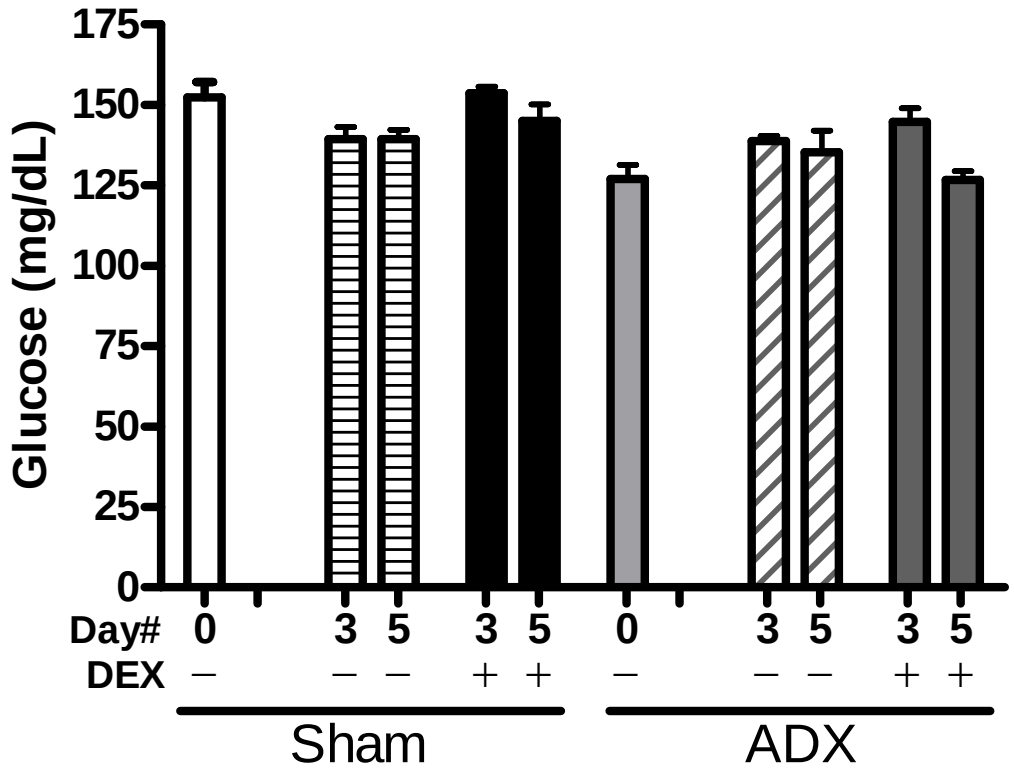


Fig. 19: Blood glucose concentrations in adrenalectomized (ADX) Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Following a recovery period of 1 week, rats were injected either dexamethasone (DEX, 1 mg/kg body weight) or saline once daily for 4 days, as described previously. Glucose levels were measured with a glucometer. Data are expressed as mean ± SEM (n=5 or 6 in each group). Means shown are not statistically significant between treatments and/or days.

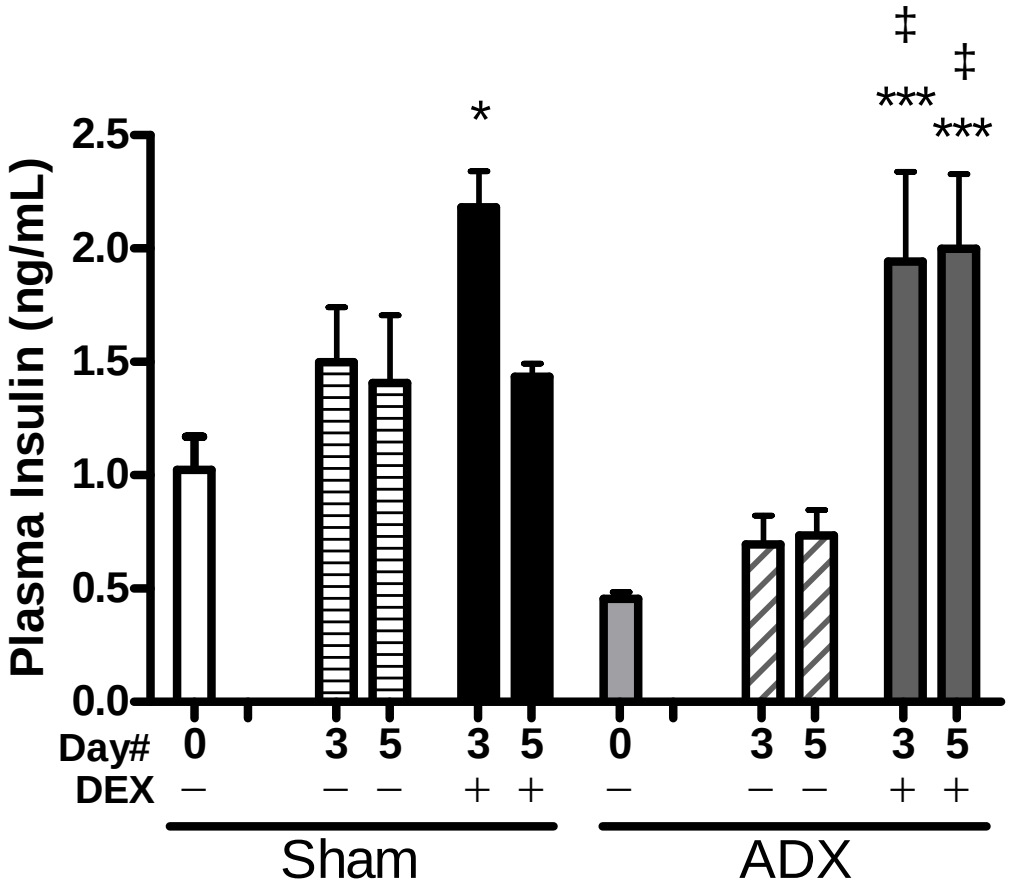


Fig. 20: Plasma insulin levels in adrenalectomized (ADX) Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Following a recovery period of 1 week, rats were injected either DEX (1 mg/kg body weight) or saline once daily for 4 days, as described previously. Insulin was assayed with ELISA. Data are expressed as mean ± SEM (n=5 or 6 in each group). ‡ $p < 0.05$ vs. same-day Saline [ADX]; * $p < 0.05$, *** $p < 0.001$ vs. Day 0 [respective surgical group].

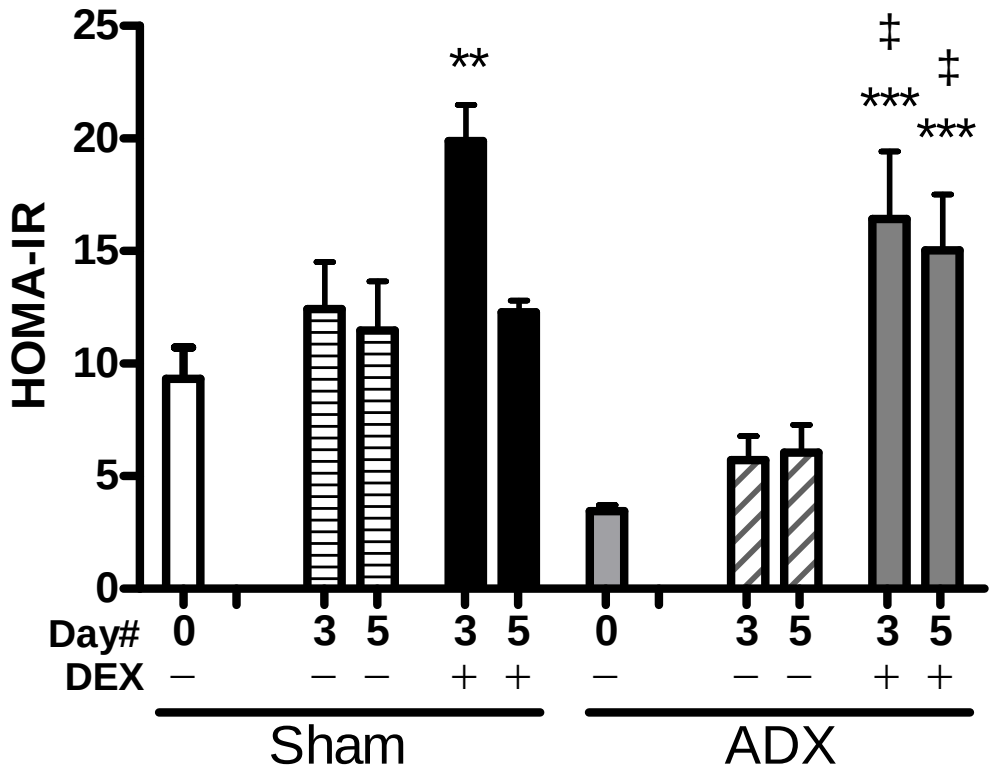


Fig. 21: HOMA-IR in adrenalectomized (ADX) Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Following a recovery period of 1 week, rats were injected either dexamethasone (DEX, 1 mg/kg body weight) or saline once daily for 4 days, as described previously. HOMA-IR using non-fasted blood samples was calculated as:

$$\text{HOMA-IR} = [\text{Insulin } (\mu U/mL) \times \text{Glucose } (mmol/L)] / 22.5$$

Data are expressed as mean ± SEM (n=5 or 6 in each group). ‡ $p < 0.05$ vs. same-day Saline [ADX]; ** $p < 0.01$, *** $p < 0.001$ vs. Day 0 [respective surgical group].

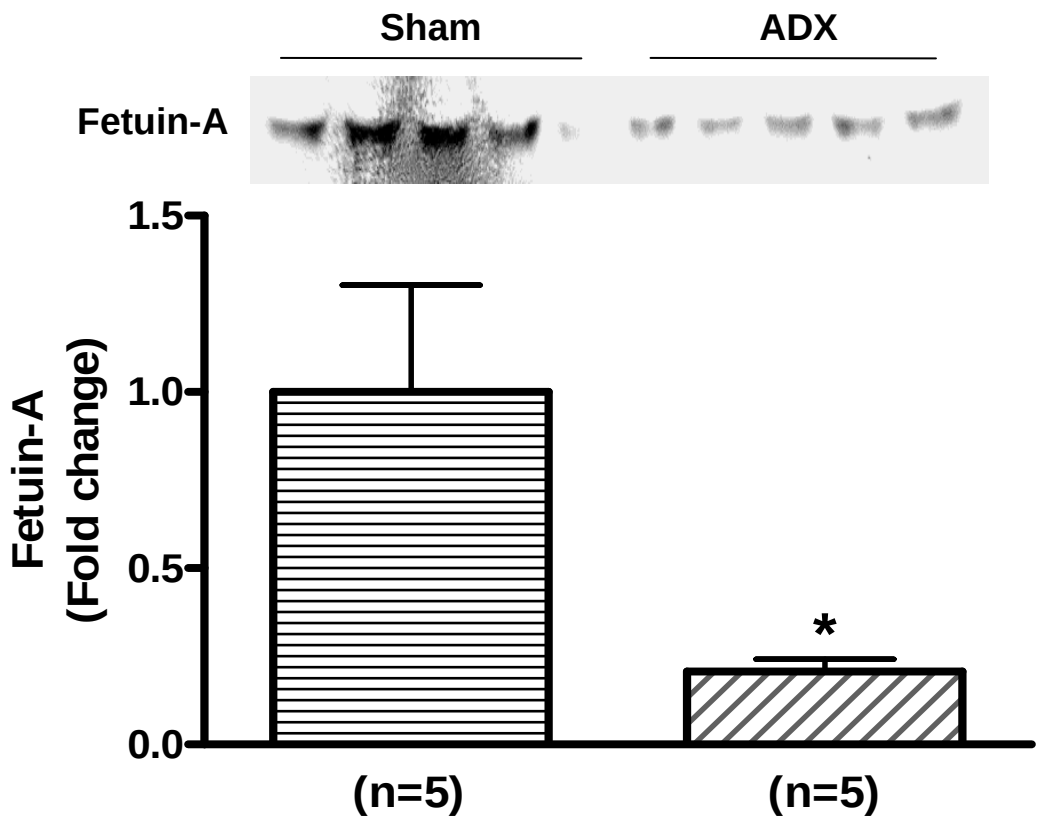


Fig. 22: Fetuin-A levels in adrenalectomized (ADX) Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Following a recovery period of 1 week, rats were injected with saline once daily for 4 days. Data are expressed as mean ± SEM (n=5 per group).
* $p < 0.05$.

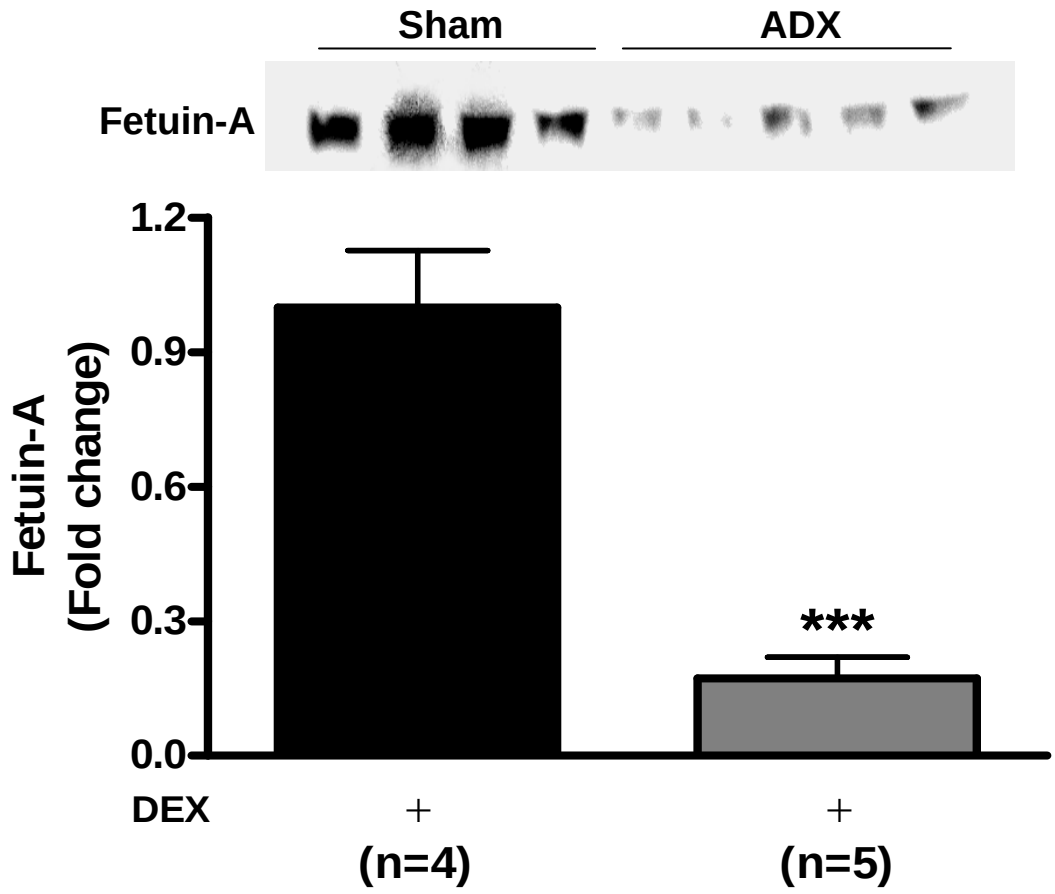


Fig. 23: Fetuin-A levels in DEX-treated Wistar rats following adrenalectomy (ADX). Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Following a recovery period of 1 week, rats were injected either dexamethasone (DEX, 1 mg/kg body weight) or saline, for 4 days, as described previously. Data are expressed as mean ± SEM (n=4 or 5 per group). *** $p < 0.001$.

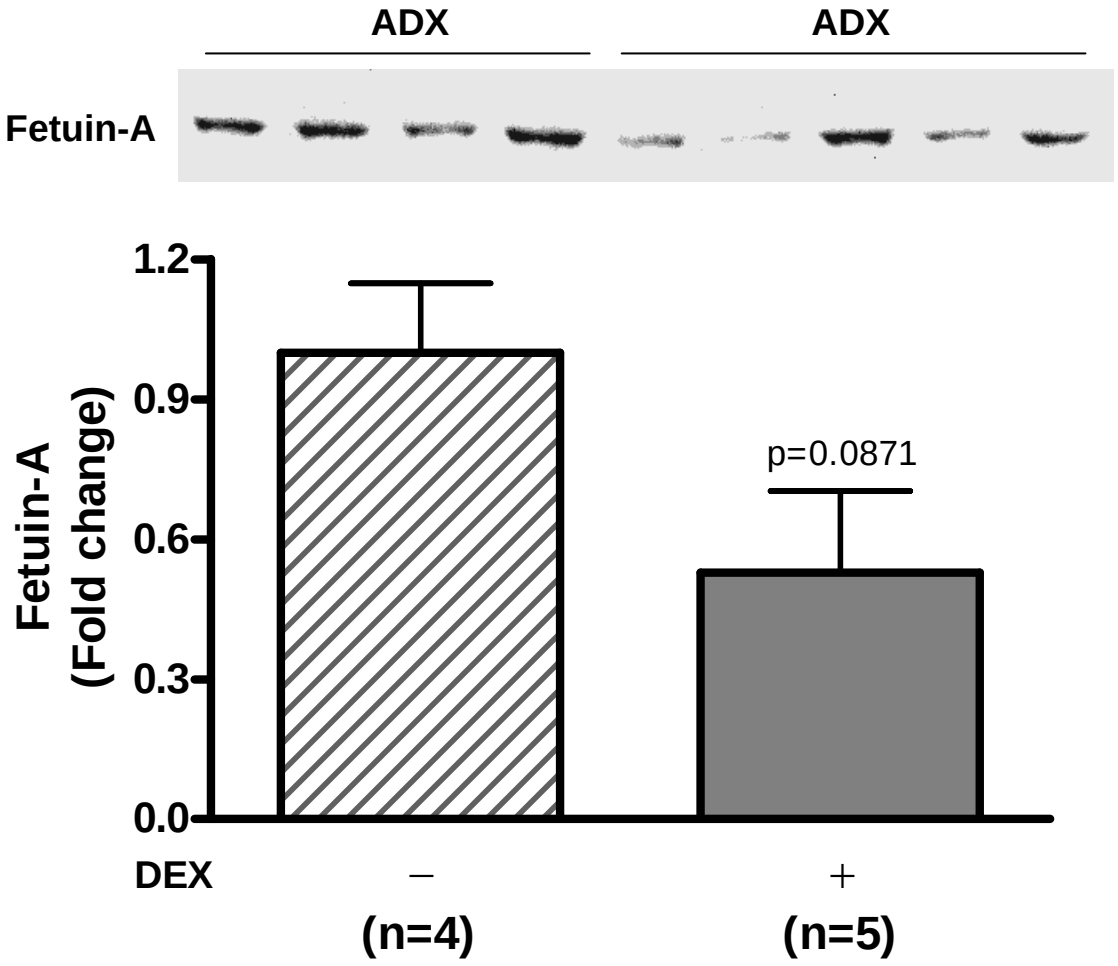


Fig. 24: Fetuin-A levels in DEX-treated adrenalectomized (ADX) Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). Following a recovery period of 1 week, rats were injected either dexamethasone (DEX, 1 mg/kg body weight) or saline, for 4 days, as described previously. Data are expressed as mean ± SEM (n=4 or 5 per group) and are not statistically significant.

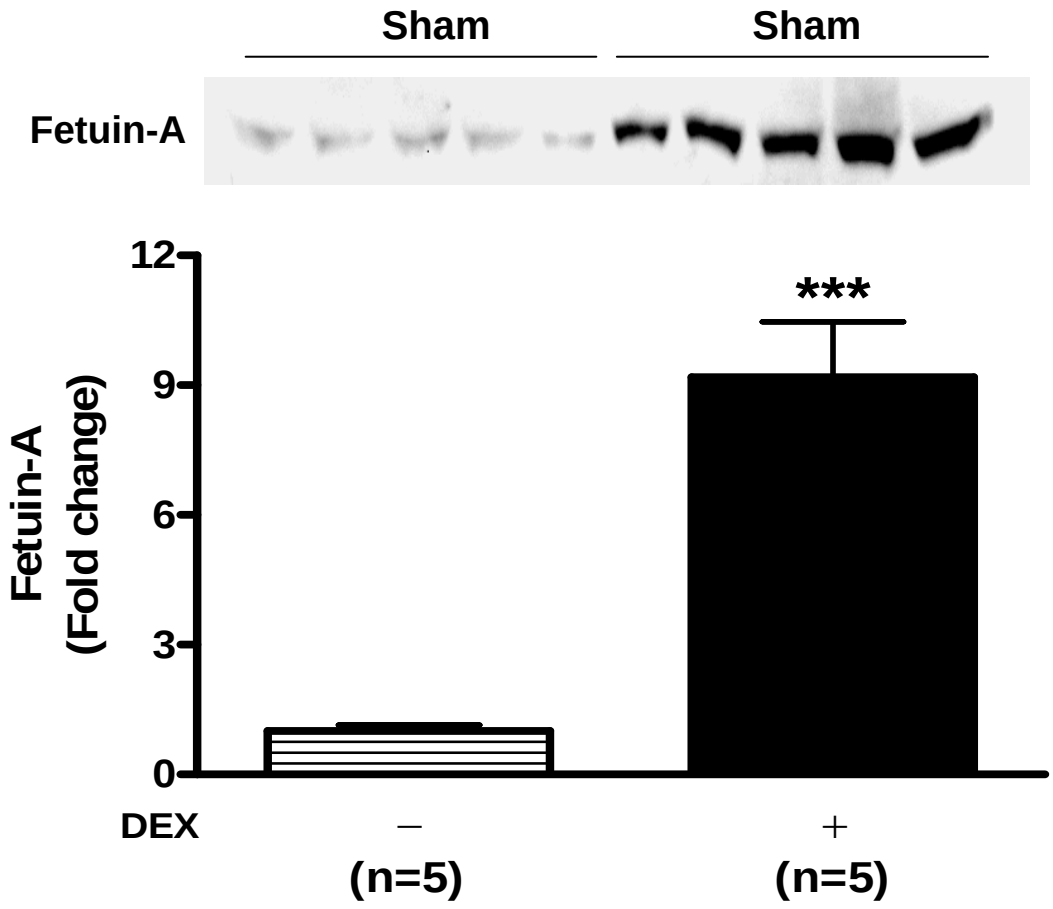


Fig. 25: Fetuin-A levels in DEX-treated sham-operated Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Following a recovery period of 1 week, rats were injected either dexamethasone (DEX, 1 mg/kg body weight) or saline, for 4 days, as described previously. Data are expressed as mean ± SEM (n=5 per group). *** $p < 0.001$.

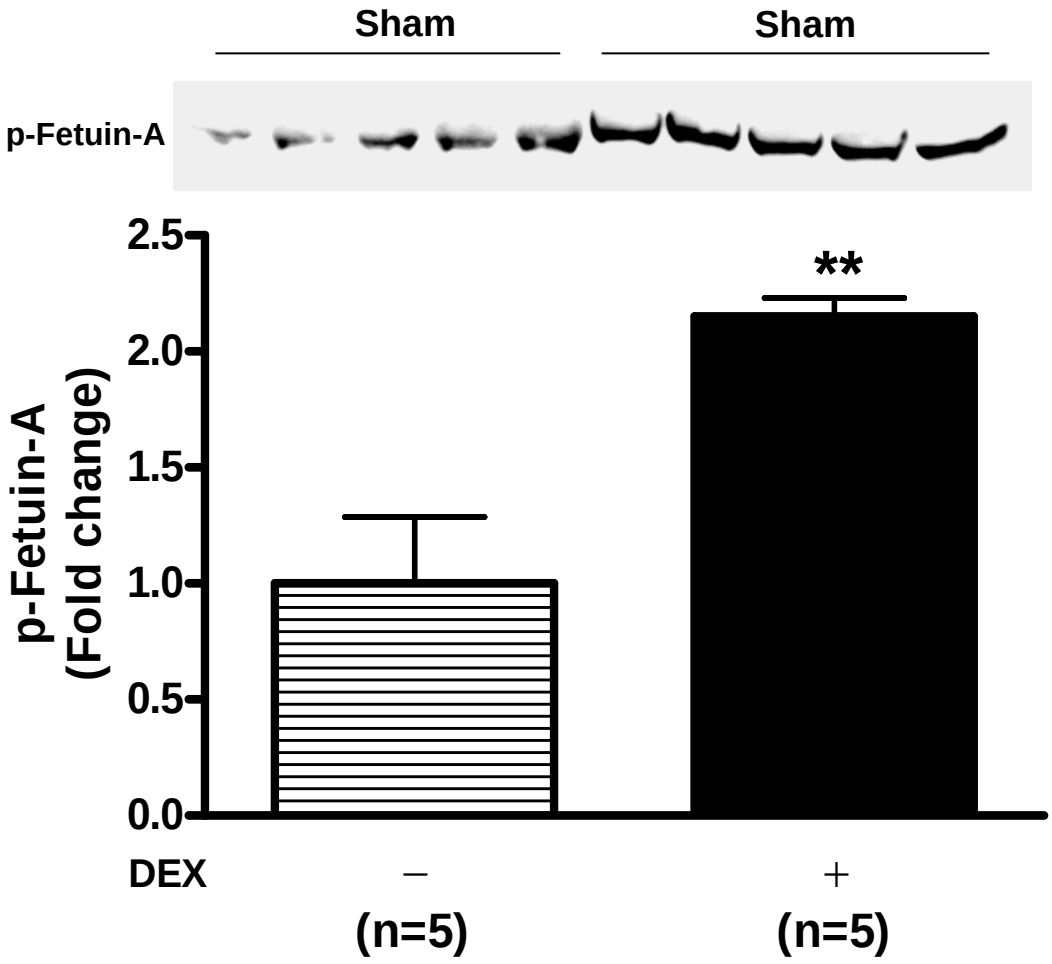


Fig. 26: Phosphorylated fetuin-A (p-Fetuin-A) levels in DEX-treated sham-operated Wistar rats. Adrenal glands were surgically removed from 4-week old, male, Wistar rats (ADX, n=11). A sham group of 10 rats were subjected to surgical procedures, except that the adrenal glands were not excised from these animals. Following a recovery period of 1 week, rats were injected either dexamethasone (DEX, 1 mg/kg body weight) or saline, for 4 days, as described previously. Data are expressed as mean ± SEM (n=5 per group). *** $p < 0.001$.

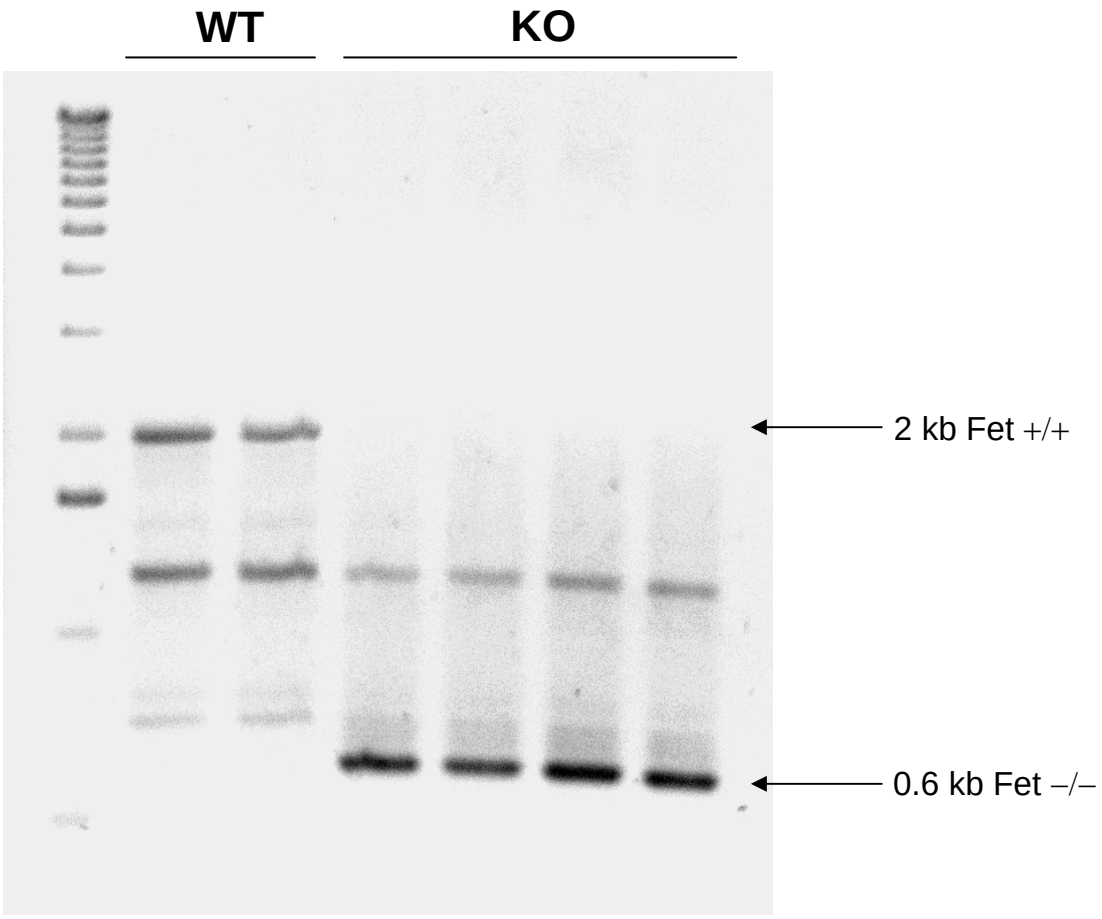


Fig. 27: Wild-type and fetuin-A knockout DNA genotyping. Mouse tail DNA from wild-type and fetuin-A knockout mice were amplified through Polymerase Chain Reaction, then loaded onto a 1% agarose gel. Staining and visualization were carried out using ethidium bromide and UV light. Wild-type and knockout animals exhibited 2.0 kb and 0.6 kb reference amplicons, respectively.

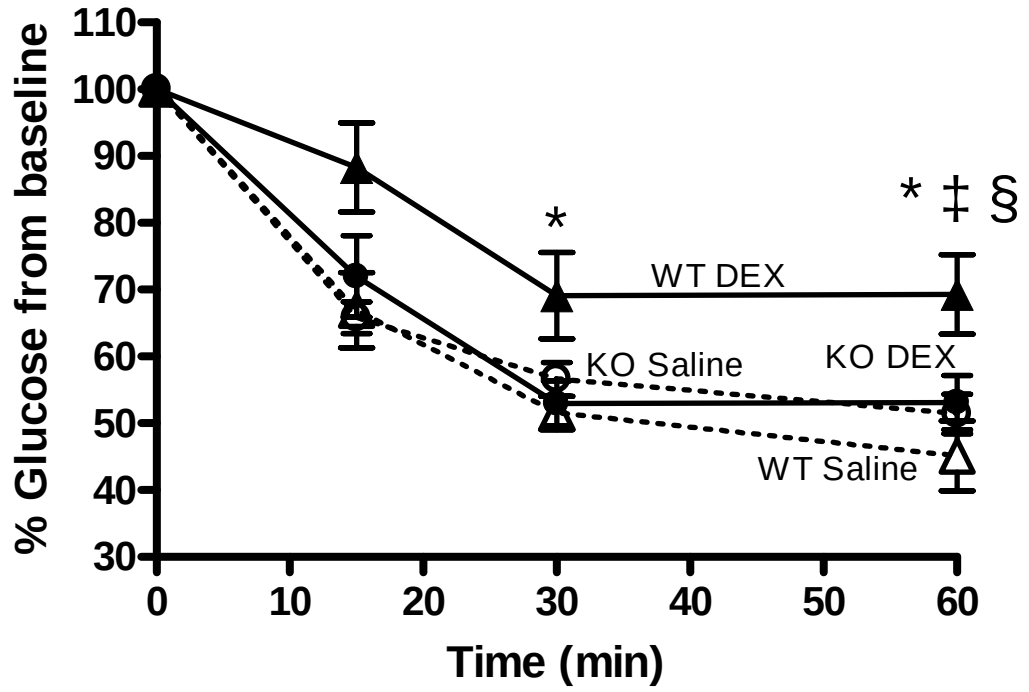


Fig. 28: Insulin tolerance test in wild-type and fetuin-A knockout mice treated with either DEX or saline. Wild-type and fetuin-A knockout C57Bl/6 mice were injected either DEX (1 mg/kg body weight) or saline, for 4 days, as described previously. Food was removed 4 hours prior to the insulin tolerance test. An intraperitoneal injection of human insulin (0.5 U/kg body weight) was given to each animal, and blood was taken from the tail vein at 0, 15, 30, and 60 minute time points for blood glucose analysis. Data are expressed as mean ± SEM (n=4 to 7 per group). * $p < 0.05$ WT DEX vs. KO DEX; ‡ $p < 0.05$ WT DEX vs. WT Saline; § $p < 0.05$ WT DEX vs. KO Saline.

REFERENCES

1. Facts about overweight and obesity. In *Obesity and Overweight*, World Health Organization, 2007
2. Chronic diseases: The leading causes of death in alabama. National Center for Chronic Disease Prevention and Health Promotion, 2005
3. Bray GA: Medical consequences of obesity. *J Clin Endocrinol Metab* 89:2583-2589, 2004
4. Diabetes facts & figures. Brussels, International Diabetes Federation, 2007
5. Colditz GA, Willett WC, Rotnitzky A, Manson JE: Weight gain as a risk factor for clinical diabetes mellitus in women. *Ann Intern Med* 122:481-486, 1995
6. Chan JM, Rimm EB, Colditz GA, Stampfer MJ, Willett WC: Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. *Diabetes Care* 17:961-969, 1994
7. National diabetes statistics. Clearinghouse NDI, Ed. Bethesda, MD, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 2007
8. Greenberg AS, McDaniel ML: Identifying the links between obesity, insulin resistance and beta-cell function: Potential role of adipocyte-derived cytokines in the pathogenesis of type 2 diabetes. *European Journal of Clinical Investigation* 32:24-34, 2002
9. Campbell PJ, Gerich JE: Impact of obesity on insulin action in volunteers with normal glucose tolerance: Demonstration of a threshold for the adverse effect of obesity. *J Clin Endocrinol Metab* 70:1114-1118, 1990
10. Hotamisligil GS, Budavari A, Murray D, Spiegelman BM: Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-alpha. *J Clin Invest* 94:1543-1549, 1994
11. Boden G: Role of fatty acids in the pathogenesis of insulin resistance and niddm. *Diabetes* 46:3-10, 1997
12. Patton JS, Shepard HM, Wilking H, Lewis G, Aggarwal BB, Eessalu TE, Gavin LA, Grunfeld C: Interferons and tumor necrosis factors have similar catabolic effects on 3t3 l1 cells. *Proc Natl Acad Sci USA* 83:8313-8317, 1986
13. Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-{alpha}, in vivo. *J Clin Endocrinol Metab* 82:4196-4200, 1997
14. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G: Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 280:E745-751, 2001
15. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409-2415, 1995

16. Bastard J-P, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, Vidal H, Hainque B: Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab* 85:3338-3342, 2000
17. Belfort R, Mandarino L, Kashyap S, Wirfel K, Pratipanawatr T, Berria R, DeFronzo RA, Cusi K: Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* 54:1640-1648, 2005
18. Petersen KF, Oral EA, Dufour S, Befroy D, Ariyan C, Yu C, Cline GW, DePaoli AM, Taylor SI, Gorden P: Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. *Journal of Clinical Investigation* 109:1345-1350, 2002
19. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA: Hypoadiponectinemia in obesity and type 2 diabetes: Close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 86:1930-1935, 2001
20. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA: The hormone resistin links obesity to diabetes. *Nature* 409:307-312, 2001
21. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB: Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 436:356-362, 2005
22. Le Cam A, Le Cam G: A new negatively regulated acute-phase phosphoprotein synthesized by rat hepatocytes. *Biochem J* 230:603-607, 1985
23. Haasemann M, Nawratil P, Muller-Esterl W: Rat tyrosine kinase inhibitor shows sequence similarity to human alpha 2-hs glycoprotein and bovine fetuin. *Biochem J* 274 (Pt 3):899-902, 1991
24. Srinivas PR, Wagner AS, Reddy LV, Deutsch DD, Leon MA, Goustin AS, Grunberger G: Serum alpha 2-hs-glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. *Mol Endocrinol* 7:1445-1455, 1993
25. Auberger P, Falquerho L, Contreres JO, Pages G, Cam GL, Rossi B, Cam AL: Characterization of a natural inhibitor of the insulin receptor tyrosine kinase: Cdna cloning, purification, and anti-mitogenic activity. *Cell* 58:631-640, 1989
26. Srinivas PR, Deutsch DD, Mathews ST, Goustin AS, Leon MA, Grunberger G: Recombinant human [alpha]2-hs glycoprotein inhibits insulin-stimulated mitogenic pathway without affecting metabolic signalling in chinese hamster ovary cells overexpressing the human insulin receptor. *Cellular Signalling* 8:567-573, 1996
27. Mathews ST, Chellam N, Srinivas PR, Cintron VJ, Leon MA, Goustin AS, Grunberger G: [alpha]2-hsg, a specific inhibitor of insulin receptor autophosphorylation, interacts with the insulin receptor. *Molecular and Cellular Endocrinology* 164:87-98, 2000
28. Kissebah AH, Sonnenberg GE, Myklebust J, Goldstein M, Broman K, James RG, Marks JA, Krakower GR, Jacob HJ, Weber J, Martin L, Blangero J, Comuzzie AG: Quantitative trait loci on chromosomes 3 and 17 influence phenotypes of the metabolic syndrome. *Proc Natl Acad Sci USA* 97:14478-14483, 2000
29. Ix JH, Shlipak MG, Brandenburg VM, Ali S, Ketteler M, Whooley MA: Association between human fetuin-a and the metabolic syndrome: Data from the heart and soul study. *Circulation* 113:1760-1767, 2006

30. Fiore CE, Celotta G, Politi GG, Di Pino L, Castelli Z, Mangiafico RA, Signorelli SS, Pennisi P: Association of high alpha2-heremans-schmid glycoprotein/fetuin concentration in serum and intima-media thickness in patients with atherosclerotic vascular disease and low bone mass. *Atherosclerosis* 195:110-115, 2007
31. Stefan N, Hennige AM, Staiger H, Machann J, Schick F, Krober SM, Machicao F, Fritsche A, Haring H-U: {alpha}2-heremans-schmid glycoprotein/ fetuin-a is associated with insulin resistance and fat accumulation in the liver in humans. *Diabetes Care* 29:853-857, 2006
32. Mathews ST, Singh GP, Ranalletta M, Cintron VJ, Qiang X, Goustin AS, Jen K-LC, Charron MJ, Jahnen-Dechent W, Grunberger G: Improved insulin sensitivity and resistance to weight gain in mice null for the ahsg gene. *Diabetes* 51:2450-2458, 2002
33. Mathews ST, Rakhade S, Zhou X, Parker GC, Coscina DV, Grunberger G: Fetuin-null mice are protected against obesity and insulin resistance associated with aging. *Biochemical and Biophysical Research Communications* 350:437-443, 2006
34. Schacke H, Docke W-D, Asadullah K: Mechanisms involved in the side effects of glucocorticoids. *Pharmacology & Therapeutics* 96:23-43, 2002
35. Gallant C, Kenny P: Oral glucocorticoids and their complications. A review. *J Am Acad Dermatol* 14:161-177, 1986
36. Girod JP, Brotman DJ: Does altered glucocorticoid homeostasis increase cardiovascular risk? *Cardiovascular Research* 64:217-226, 2004
37. Arnaldi G, Angeli A, Atkinson AB, Bertagna X, Cavagnini F, Chrousos GP, Fava GA, Findling JW, Gaillard RC, Grossman AB, Kola B, Lacroix A, Mancini T, Mantero F, Newell-Price J, Nieman LK, Sonino N, Vance ML, Giustina A, Boscaro M: Diagnosis and complications of cushing's syndrome: A consensus statement. *J Clin Endocrinol Metab* 88:5593-5602, 2003
38. Nosadini R, Del Prato S, Tiengo A, Valerio A, Muggeo M, Opocher G, Mantero F, Duner E, Marescotti C, Mollo F, Belloni F: Insulin resistance in cushing's syndrome. *J Clin Endocrinol Metab* 57:529-536, 1983
39. Ruzzin J, Wagman A, Jensen J: Glucocorticoid-induced insulin resistance in skeletal muscles: Defects in insulin signalling and the effects of a selective glycogen synthase kinase-3 inhibitor. *Diabetologia* 48:2119-2130, 2005
40. Binnert C, Ruchat S, Nicod N, Tappy L: Dexamethasone-induced insulin resistance shows no gender difference in healthy humans. *Diabetes & Metabolism* 30:321-326, 2004
41. Tappy L, Randin D, Vollenweider P, Vollenweider L, Paquot N, Scherrer U, Schneiter P, Nicod P, Jequier E: Mechanisms of dexamethasone-induced insulin resistance in healthy humans. *J Clin Endocrinol Metab* 79:1063-1069, 1994
42. Qi D, Rodrigues B: Glucocorticoids produce whole body insulin resistance with changes in cardiac metabolism. *Am J Physiol Endocrinol Metab* 292:E654-667, 2007
43. Gounarides JS, Korach-Andre M, Killary K, Argentieri G, Turner O, Laurent D: Effect of dexamethasone on glucose tolerance and fat metabolism in a diet-induced obesity mouse model. *Endocrinology*:en.2007-1214, 2007
44. Zhang XP, Chen L, Hu QF, Tian H, Xu RJ, Wang ZW, Wang KY, Cheng QH, Yan W, Li Y, Li QY, He Q, Wang F: Effects of large dose of dexamethasone on inflammatory

mediators and pancreatic cell apoptosis of rats with severe acute pancreatitis. *World J Gastroenterol* 13:5506-5511, 2007
45. Henzi I, Walder B, Tramer MR: Dexamethasone for the prevention of postoperative nausea and vomiting: A quantitative systematic review. *Anesth Analg* 90:186-, 2000
46. Baxendale BR, Vater M, Lavery KM: Dexamethasone reduces pain and swelling following extraction of third molar teeth. *Anaesthesia* 48:961-964, 1993
47. Weinstein SP, Paquin T, Pritsker A, Haber RS: Glucocorticoid-induced insulin resistance: Dexamethasone inhibits the activation of glucose transport in rat skeletal muscle by both insulin- and non-insulin-related stimuli. *Diabetes* 44:441-445, 1995
48. Buren J, Liu HX, Jensen J, Eriksson JW: Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase b in primary cultured rat adipocytes. *Eur J Endocrinol* 146:419-429, 2002
49. Qi D, Pulinilkunnil T, An D, Ghosh S, Abrahani A, Pospisilik JA, Brownsey R, Wambolt R, Allard M, Rodrigues B: Single-dose dexamethasone induces whole-body insulin resistance and alters both cardiac fatty acid and carbohydrate metabolism. *Diabetes* 53:1790-1797, 2004
50. Woltje M, Tschoke B, von Bulow V, Westenfeld R, Denecke B, Graber S, Jahnen-Dechent W: Ccaat enhancer binding protein {beta} and hepatocyte nuclear factor 3{beta} are necessary and sufficient to mediate dexamethasone-induced up-regulation of alpha2hs-glycoprotein/fetuin-a gene expression. *J Mol Endocrinol* 36:261-277, 2006
51. Statistics related to overweight and obesity. Bathesda, MD, National Institute of Diabetes and Digestive and Kidney Diseases, 2007
52. Gesta S, Tseng YH, Kahn CR: Developmental origin of fat: Tracking obesity to its source. *Cell* 131:242-256, 2007
53. Hill JO, Wyatt HR, Reed GW, Peters JC: Obesity and the environment: Where do we go from here? *Science* 299:853-855, 2003
54. Weiss R: Fat distribution and storage: How much, where, and how? *Eur J Endocrinol* 157:S39-45, 2007
55. Goralski KB, Sinal CJ: Type 2 diabetes and cardiovascular disease: Getting to the fat of the matter. *Can J Physiol Pharmacol* 85:113-132, 2007
56. Marchesini G, Brizi M, Morselli-Labate AM, Bianchi G, Bugianesi E, McCullough AJ, Forlani G, Melchionda N: Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 107:450-455, 1999
57. Angulo P, Lindor KD: Non-alcoholic fatty liver disease. *Journal of Gastroenterology and Hepatology* 17:S186-S190, 2002
58. Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H: Insulin-stimulated akt kinase activity is reduced in skeletal muscle from niddm subjects. *Diabetes* 47:1281-1286, 1998
59. Bjornholm M, Kawano Y, Lehtihet M, Zierath JR: Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from niddm subjects after in vivo insulin stimulation. *Diabetes* 46:524-527, 1997
60. Timothy Garvey W, Maianu L, Zhu JH, Brechtel-Hook G, Wallace P, Baron AD: Evidence for defects in the trafficking and translocation of glut4 glucose transporters in

skeletal muscle as a cause of human insulin resistance. *Journal of Clinical Investigation* 101:2377-2386, 1998
61. Vogt B, Mühlbacher C, Carrascosa J, Obermaier-Kusser B, Seffer E, Mushack J, Pongratz D, Häring HU: Subcellular distribution of glut 4 in the skeletal muscle of lean type 2 (non-insulin-dependent) diabetic patients in the basal state. *Diabetologia* 35:456-463, 1992
62. American Diabetes A: Diagnosis and classification of diabetes mellitus. *Diabetes Care* 31:S55-60, 2008
63. All about diabetes. American Diabetes Association, 2007
64. Peiris AN, Mueller RA, Smith GA, Struve MF, Kissebah AH: Splanchnic insulin metabolism in obesity. Influence of body fat distribution. *J Clin Invest* 78:1648-1657, 1986
65. Faber OK, Christensen K, Kehlet H, Madsbad S, Binder C: Decreased insulin removal contributes to hyperinsulinemia in obesity. *J Clin Endocrinol Metab* 53:618-621, 1981
66. Rossell R, Gomis R, Casamitjana R, Segura R, Vilardell E, Rivera F: Reduced hepatic insulin extraction in obesity: Relationship with plasma insulin levels. *J Clin Endocrinol Metab* 56:608-611, 1983
67. Kissebah AH, Vydelingum N, Murray R, Evans DJ, Hartz AJ, Kalkhoff RK, Adams PW: Relation of body fat distribution to metabolic complications of obesity. *J Clin Endocrinol Metab* 54:254-260, 1982
68. Meistas MT, Rendell M, Margolis S, Kowarski AA: Estimation of the secretion rate of insulin from the urinary excretion rate of c-peptide. Study in obese and diabetic subjects. *Diabetes* 31:449-453, 1982
69. Prentki M, Nolan CJ: Islet {beta} cell failure in type 2 diabetes. *Journal of Clinical Investigation* 116:1802, 2006
70. Polonsky KS, Given BD, Hirsch L, Shapiro ET, Tillil H, Beebe C, Galloway JA, Frank BH, Karrison T, Van Cauter E: Quantitative study of insulin secretion and clearance in normal and obese subjects. *J Clin Invest* 81:435-441, 1988
71. Reaven GM: Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes* 37:1595-1607, 1988
72. Taniguchi CM, Emanuelli B, Kahn CR: Critical nodes in signalling pathways: Insights into insulin action. *Nat Rev Mol Cell Biol* 7:85-96, 2006
73. Youngren J: Regulation of insulin receptor function. *Cellular and Molecular Life Sciences (CMLS)* 64:873-891, 2007
74. De Camilli P, Emr SD, McPherson PS, Novick P: Phosphoinositides as regulators in membrane traffic. *Science* 271:1533-1539, 1996
75. van der Kaay J, Batty IH, Cross DAE, Watt PW, Downes CP: A novel, rapid, and highly sensitive mass assay for phosphatidylinositol 3,4,5-trisphosphate (ptdins(3,4,5)p3) and its application to measure insulin-stimulated ptdins(3,4,5)p3 production in rat skeletal muscle in vivo. *J. Biol. Chem.* 272:5477-5481, 1997
76. Alessi DR, Cohen P: Mechanism of activation and function of protein kinase b. *Current Opinion in Genetics & Development* 8:55-62, 1998

77. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA: Mechanism of activation of protein kinase b by insulin and igf-1. *Embo J* 15:6541-6551, 1996
78. Hill MM, Andjelkovic M, Brazil DP, Ferrari S, Fabbro D, Hemmings BA: Insulin-stimulated protein kinase b phosphorylation on ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase. *J. Biol. Chem.* 276:25643-25646, 2001
79. Chang PY, Goodyear LJ, Benecke H, Markuns JS, Moller DE: Impaired insulin signaling in skeletal muscles from transgenic mice expressing kinase-deficient insulin receptors. *J Biol Chem* 270:12593-12600, 1995
80. Chang PY, Benecke H, Le Marchand-Brustel Y, Lawitts J, Moller DE: Expression of a dominant-negative mutant human insulin receptor in the muscle of transgenic mice. *J Biol Chem* 269:16034-16040, 1994
81. Pessin JE, Thurmond DC, Elmendorf JS, Coker KJ, Okada S: Molecular basis of insulin-stimulated glut4 vesicle trafficking. Location! Location! Location! *J Biol Chem* 274:2593-2596, 1999
82. Welsh GI, Wilson C, Proud CG: Gsk3: A shaggy frog story. *Trends in Cell Biology* 6:274-279, 1996
83. Deprez J, Vertommen D, Alessi DR, Hue L, Rider MH: Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase b and other protein kinases of the insulin signaling cascades. *J Biol Chem* 272:17269-17275, 1997
84. Skolnik EY, Batzer A, Li N, Lee CH, Lowenstein E, Mohammadi M, Margolis B, Schlessinger J: The function of grb2 in linking the insulin receptor to ras signaling pathways. *Science* 260:1953-1955, 1993
85. Widegren U, Ryder JW, Zierath JR: Mitogen-activated protein kinase signal transduction in skeletal muscle: Effects of exercise and muscle contraction. *Acta Physiologica Scandinavica* 172:227-238, 2001
86. Zheng CF, Guan KL: Properties of meks, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. *J. Biol. Chem.* 268:23933-23939, 1993
87. Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799-806, 2001
88. Sutherland C, O'Brien RM, Granner DK, Saklatvala J, Cohen P, White MF: New connections in the regulation of pepck gene expression by insulin [and discussion]. *Philosophical Transactions: Biological Sciences* 351:191-199, 1996
89. Anthonsen MW, Ronnstrand L, Wernstedt C, Degerman E, Holm C: Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. *J. Biol. Chem.* 273:215-221, 1998
90. Lamounier-Zepter V, Ehrhart-Bornstein M, Bornstein SR: Insulin resistance in hypertension and cardiovascular disease. *Best Practice & Research Clinical Endocrinology & Metabolism* 20:355-367, 2006
91. Ferrannini E, Camastra S: Relationship between impaired glucose tolerance, non-insulin-dependent diabetes mellitus and obesity. *Eur J Clin Invest* 28 Suppl 2:3-6; discussion 6-7, 1998

92. Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD: Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with niddm. *Diabetes* 37:1020-1024, 1988
93. Frayn KN: Insulin resistance and lipid metabolism. Lipid metabolism. *Current Opinion in Lipidology* 4:197-204, 1993
94. Boden G, Chen X, Ruiz J, White JV, Rossetti L: Mechanisms of fatty acid-induced inhibition of glucose uptake. *J Clin Invest* 93:2438-2446, 1994
95. Perseghin G, Ghosh S, Gerow K, Shulman GI: Metabolic defects in lean nondiabetic offspring of niddm parents: A cross-sectional study. *Diabetes* 46:1001-1009, 1997
96. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. *Science* 259:87-91, 1993
97. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425-432, 1994
98. Fried SK, Bunkin DA, Greenberg AS: Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: Depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab* 83:847-850, 1998
99. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for jnk in obesity and insulin resistance. *Nature* 420:333-336, 2002
100. Aguirre V, Uchida T, Yenush L, Davis R, White MF: The c-jun nh2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of ser307. *J. Biol. Chem.* 275:9047-9054, 2000
101. Lee YH, Giraud J, Davis RJ, White MF: C-jun n-terminal kinase (jnk) mediates feedback inhibition of the insulin signaling cascade. *J. Biol. Chem.* 278:2896-2902, 2003
102. Yin M-J, Yamamoto Y, Gaynor RB: The anti-inflammatory agents aspirin and salicylate inhibit the activity of i[kappa]b kinase-[beta]. *Nature* 396:77-80, 1998
103. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J: Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa b kinase complex. *J. Biol. Chem.* 277:48115-48121, 2002
104. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li Z-W, Karin M, Shoelson SE: Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of ikkbeta. *Science* 293:1673-1677, 2001
105. Stumvoll M, Haring H: Resistin and adiponectin--of mice and men. *Obesity Res* 10:1197-1199, 2002
106. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y: Diet-induced insulin resistance in mice lacking adiponectin/acrp30. *Nat Med* 8:731-737, 2002
107. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE: The adipocyte-secreted protein acrp30 enhances hepatic insulin action. *Nat Med* 7:947-953, 2001
108. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7:941-946, 2001

109. Moon B, Kwan JJ-M, Duddy N, Sweeney G, Begum N: Resistin inhibits glucose uptake in l6 cells independently of changes in insulin signaling and glut4 translocation. *Am J Physiol Endocrinol Metab* 285:E106-115, 2003
110. Pravenec M, Kazdova L, Landa V, Zidek V, Mlejnek P, Jansa P, Wang J, Qi N, Kurtz TW: Transgenic and recombinant resistin impair skeletal muscle glucose metabolism in the spontaneously hypertensive rat. *J. Biol. Chem.* 278:45209-45215, 2003
111. Rajala MW, Obici S, Scherer PE, Rossetti L: Adipose-derived resistin and gut-derived resistin-like molecule-{beta} selectively impair insulin action on glucose production. *J. Clin. Invest.* 111:225-230, 2003
112. Banerjee RR, Rangwala SM, Shapiro JS, Rich AS, Rhoades B, Qi Y, Wang J, Rajala MW, Pocai A, Scherer PE, Steppan CM, Ahima RS, Obici S, Rossetti L, Lazar MA: Regulation of fasted blood glucose by resistin. *Science* 303:1195-1198, 2004
113. McGarry JD: Banting lecture 2001: Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* 51:7-18, 2002
114. Moller DE: New drug targets for type 2 diabetes and the metabolic syndrome. *Nature* 414:821-827, 2001
115. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI: Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (irs-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J. Biol. Chem.* 277:50230-50236, 2002
116. Ahmad F, Azevedo JL, Jr., Cortright R, Dohm GL, Goldstein BJ: Alterations in skeletal muscle protein-tyrosine phosphatase activity and expression in insulin-resistant human obesity and diabetes. *J. Clin. Invest.* 100:449-458, 1997
117. Chen H, Wertheimer SJ, Lin CH, Katz SL, Amrein KE, Burn P, Quon MJ: Protein-tyrosine phosphatases ptp1b and syp are modulators of insulin-stimulated translocation of glut4 in transfected rat adipose cells. *J. Biol. Chem.* 272:8026-8031, 1997
118. Kenner KA, Anyanwu E, Olefsky JM, Kusari J: Protein-tyrosine phosphatase 1b is a negative regulator of insulin- and insulin-like growth factor-i-stimulated signaling. *J. Biol. Chem.* 271:19810-19816, 1996
119. Zabolotny JM, Kim Y-B, Peroni OD, Kim JK, Pani MA, Boss O, Klaman LD, Kamatkar S, Shulman GI, Kahn BB, Neel BG: Overexpression of the lar (leukocyte antigen-related) protein-tyrosine phosphatase in muscle causes insulin resistance. *Proc Natl Acad Sci USA* 98:071050398, 2001
120. Starr R, Willson TA, Viney EM, Murray LJL, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, Hilton DJ: A family of cytokine-inducible inhibitors of signalling. *Nature* 387:917-921, 1997
121. Emanuelli B, Peraldi P, Filloux C, Sawka-Verhelle D, Hilton D, Van Obberghen E: Socs-3 is an insulin-induced negative regulator of insulin signaling. *J. Biol. Chem.* 275:15985-15991, 2000
122. Mooney RA, Senn J, Cameron S, Inamdar N, Boivin LM, Shang Y, Furlanetto RW: Suppressors of cytokine signaling-1 and -6 associate with and inhibit the insulin receptor. A potential mechanism for cytokine-mediated insulin resistance. *J. Biol. Chem.* 276:25889-25893, 2001

123. Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E: Socs-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. *J. Biol. Chem.* 276:47944-47949, 2001
124. Senn JJ, Klover PJ, Nowak IA, Zimmers TA, Koniaris LG, Furlanetto RW, Mooney RA: Suppressor of cytokine signaling-3 (socs-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. *J. Biol. Chem.* 278:13740-13746, 2003
125. Ueki K, Kondo T, Tseng Y-H, Kahn CR: Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. *Proc Natl Acad Sci USA* 101:10422-10427, 2004
126. Goldfine ID, Maddux BA, Youngren JF, Reaven G, Accili D, Trischitta V, Vigneri R, Frittitta L: The role of membrane glycoprotein pc-1/enpp1 in the pathogenesis of insulin resistance and related abnormalities. *Endocr Rev*:er.2007-0004, 2008
127. Maddux BA, Chang Y-N, Accili D, McGuinness OP, Youngren JF, Goldfine ID: Overexpression of the insulin receptor inhibitor pc-1/enpp1 induces insulin resistance and hyperglycemia. *Am J Physiol Endocrinol Metab* 290:E746-749, 2006
128. Goldfine ID, Maddux BA, Youngren JF, Trischitta V, Frittitta L: Role of pc-1 in the etiology of insulin resistance. *Ann NY Acad Sci* 892:204-222, 1999
129. Goldfine ID, Maddux BA, Youngren JF, Frittitta L, Trischitta V, Dohm GL: Membrane glycoprotein pc-1 and insulin resistance. *Molecular and Cellular Biochemistry* 182:177-184, 1998
130. Maddux BA, Goldfine ID: Membrane glycoprotein pc-1 inhibition of insulin receptor function occurs via direct interaction with the receptor alpha-subunit. *Diabetes* 49:13-19, 2000
131. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW: Negative regulation of pkb/akt-dependent cell survival by the tumor suppressor pten. *Cell* 95:29-39, 1998
132. Weng L-P, Smith WM, Brown JL, Eng C: Pten inhibits insulin-stimulated mek/mapk activation and cell growth by blocking irs-1 phosphorylation and irs-1/grb-2/sos complex formation in a breast cancer model. *Hum. Mol. Genet.* 10:605-616, 2001
133. Ishihara H, Sasaoka T, Hori H, Wada T, Hirai H, Haruta T, Langlois WJ, Kobayashi M: Molecular cloning of rat sh2-containing inositol phosphatase 2 (ship2) and its role in the regulation of insulin signaling. *Biochemical and Biophysical Research Communications* 260:265-272, 1999
134. Tang X, Powelka AM, Soriano NA, Czech MP, Guilherme A: Pten, but not ship2, suppresses insulin signaling through the phosphatidylinositol 3-kinase/akt pathway in 3t3-l1 adipocytes. *J. Biol. Chem.* 280:22523-22529, 2005
135. Olivier S, Risler, Smih, Schneider, Lochner, Jouzeau, Fey and Salier: Fetuin-b, a second member of the fetuin family in mammals. *Biochem J* 350:589-597, 1999
136. Mathews ST, Srinivas PR, Leon MA, Grunberger G: Bovine fetuin is an inhibitor of insulin receptor tyrosine kinase. *Life Sciences* 61:1583-1592, 1997
137. Rankinen T, Zuberi A, Chagnon YC, Weisnagel SJ, Argyropoulos G, Walts B, Perusse L, Bouchard C: The human obesity gene map: The 2005 update. *Obesity* 14:529-644, 2006

138. Vionnet N, Hani EH, Dupont S, Gallina S, Francke S, Dotte S, De Matos F, Durand E, Lepretre F, Lecoeur C: Genomewide search for type 2 diabetes–susceptibility genes in french whites: Evidence for a novel susceptibility locus for early-onset diabetes on chromosome 3 q 27-qter and independent replication of a type 2–diabetes locus on chromosome 1 q 21–q 24. *The American Journal of Human Genetics* 67:1470-1480, 2000
139. Francke S, Manraj M, Lacquemant C, Lecoeur C, Lepretre F, Passa P, Hebe A, Corset L, Yan SLK, Lahmidi S, Jankee S, Gunness TK, Ramjuttun US, Balgobin V, Dina C, Froguel P: A genome-wide scan for coronary heart disease suggests in indo-mauritians a susceptibility locus on chromosome 16p13 and replicates linkage with the metabolic syndrome on 3q27. *Hum. Mol. Genet.* 10:2751-2765, 2001
140. Siddiq A, Lepretre F, Hercberg S, Froguel P, Gibson F: A synonymous coding polymorphism in the {alpha} 2-heremans-schmid glycoprotein gene is associated with type 2 diabetes in french caucasians. *Diabetes* 54:2477, 2005
141. Lavebratt C, Wahlqvist S, Nordfors L, Hoffstedt J, Arner P: Ahsg gene variant is associated with leanness among swedish men. *Hum Genet* 117:54-60, 2005
142. Lehtinen AB, Burdon KP, Lewis JP, Langefeld CD, Ziegler JT, Rich SS, Register TC, Carr JJ, Freedman BI, Bowden DW: Association of {alpha}2-heremans-schmid glycoprotein polymorphisms with subclinical atherosclerosis. *J Clin Endocrinol Metab* 92:345-352, 2007
143. Andersen G, Burgdorf KS, Sparso T, Borch-Johnsen K, Jorgensen T, Hansen T, Pedersen O: Ahsg tagsnps associate with type 2 diabetes and dyslipidemia: Studies of metabolic traits in 7,683 danish whites. *Diabetes*:db07-0558, 2008
144. Mori K, Emoto M, Araki T, Yokoyama H, Teramura M, Lee E, Motoyama K, Koyama H, Shoji T, Inaba M, Nishizawa Y: Association of serum fetuin-a with carotid arterial stiffness. *Clinical Endocrinology* 66:246-250, 2007
145. Haglund AC, Ek, Bo, and Ek, Pia: Phosphorylation of human plasma a2-heremans-schmid glycoprotein (human fetuin) *in vivo*. *Biochem J* 357:437-445, 2001
146. Hill JO, Melanson EL, Wyatt HT: Dietary fat intake and regulation of energy balance: Implications for obesity. *J. Nutr.* 130:284-, 2000
147. Lin X, Braymer HD, G.A. B, D.A. Y: Differential expression of insulin receptor tyrosine kinase inhibitor (fetuin) gene in a model of diet-induced obesity. *Life Sciences* 63:9, 1998
148. Walker BR, Campbell JC, Fraser R, Stewart PM, Edwards CR: Mineralocorticoid excess and inhibition of 11 beta-hydroxysteroid dehydrogenase in patients with ectopic acth syndrome. *Clin Endocrinol (Oxf)* 37:483-492, 1992
149. Guyton AC, Hall JE: *Textbook of medical physiology*. Jackson, Elsevier Saunders, 2006
150. Guillaume-Gentil C, Assimacopoulos-Jeannet F, Jeanrenaud B: Involvement of non-esterified fatty acid oxidation in glucocorticoid-induced peripheral insulin resistance in vivo in rats. *Diabetologia* 36:899-906, 1993
151. Tsigos C, Chrousos GP: Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *Journal of psychosomatic research* 53:865-871, 2002
152. deKloet E: Brain corticosteroid receptor balance and homeostatic control. *Frontiers in Neuroendocrinology* 12:95-164, 1991

153. Leonard BE: The hpa and immune axes in stress: The involvement of the serotonergic system. *European Psychiatry* 20:S302-S306, 2005
154. Sapolsky RM, Plotsky PM: Hypercortisolism and its possible neural bases. *Biological psychiatry* 27:937-952, 1990
155. White BD, Dean RG, Edwards GL, Martin RJ: Type ii corticosteroid receptor stimulation increases npy gene expression in basomedial hypothalamus of rats. *Am J Physiol Regul Integr Comp Physiol* 266:R1523-1529, 1994
156. White BD, Edwards GL, Martin RJ: Interaction of type i and type ii corticosteroid receptor stimulation on carcass energy and carcass water. *Am J Physiol Regul Integr Comp Physiol* 270:R1099-1108, 1996
157. Bamberger CM, Schulte HM, Chrousos GP: Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocr Rev* 17:245-261, 1996
158. Lee W, Mitchell P, Tjian R: Purified transcription factor ap-1 interacts with tpa-inducible enhancer elements. *Cell* 49:741-752, 1987
159. Yang-Yen H-F, Chambard J-C, Sun Y-L, Smeal T, Schmidt TJ, Drouin J, Karin M: Transcriptional interference between c-jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* 62:1205-1215, 1990
160. Seckl JR, Morton NM, Chapman KE, Walker BR: Glucocorticoids and 11beta-hydroxysteroid dehydrogenase in adipose tissue. *Recent Prog Horm Res* 59:359-393, 2004
161. Liu Y, Nakagawa Y, Wang Y, Sakurai R, Tripathi PV, Lutfy K, Friedman TC: Increased glucocorticoid receptor and 11{beta}-hydroxysteroid dehydrogenase type 1 expression in hepatocytes may contribute to the phenotype of type 2 diabetes in db/db mice. *Diabetes* 54:32-40, 2005
162. Alberts P, Engblom L, Edling N, Forsgren M, Klingström G, Larsson C, Rönquist-Nii Y, Öhman B, Abrahmsén L: Selective inhibition of 11ß-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice. *Diabetologia* 45:1528-1532, 2002
163. Liu Y, Nakagawa Y, Wang Y, Li R, Li X, Ohzeki T, Friedman TC: Leptin activation of corticosterone production in hepatocytes may contribute to the reversal of obesity and hyperglycemia in leptin-deficient ob/ob mice. *Diabetes* 52:1409-1416, 2003
164. Rask E, Olsson T, Soderberg S, Andrew R, Livingstone DEW, Johnson O, Walker BR: Tissue-specific dysregulation of cortisol metabolism in human obesity. *J Clin Endocrinol Metab* 86:1418-1421, 2001
165. Stewart PM, Boulton A, Kumar S, Clark PMS, Shackleton CHL: Cortisol metabolism in human obesity: Impaired cortisone->cortisol conversion in subjects with central adiposity. *J Clin Endocrinol Metab* 84:1022-1027, 1999
166. Rask E, Walker BR, Soderberg S, Livingstone DEW, Eliasson M, Johnson O, Andrew R, Olsson T: Tissue-specific changes in peripheral cortisol metabolism in obese women: Increased adipose 11{beta}-hydroxysteroid dehydrogenase type 1 activity. *J Clin Endocrinol Metab* 87:3330-3336, 2002
167. Walker BR, Connacher AA, Lindsay RM, Webb DJ, Edwards CR: Carbenoxolone increases hepatic insulin sensitivity in man: A novel role for 11-oxosteroid reductase in

enhancing glucocorticoid receptor activation. *J Clin Endocrinol Metab* 80:3155-3159, 1995
168. Andrews RC, Rooyackers O, Walker BR: Effects of the 11{beta}-hydroxysteroid dehydrogenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes. *J Clin Endocrinol Metab* 88:285-291, 2003
169. Walker BR: Cortisol - cause and cure for metabolic syndrome? *Diabetic Medicine* 23:1281-1288, 2006
170. Alberts P, Nilsson C, Selen G, Engblom LOM, Edling NHM, Norling S, Klingstrom G, Larsson C, Forsgren M, Ashkzari M, Nilsson CE, Fiedler M, Bergqvist E, Ohman B, Bjorkstrand E, Abrahmsen LB: Selective inhibition of 11{beta}-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. *Endocrinology* 144:4755-4762, 2003
171. Hermanowski-Vosatka A, Balkovec JM, Cheng K, Chen HY, Hernandez M, Koo GC, Le Grand CB, Li Z, Metzger JM, Mundt SS, Noonan H, Nunes CN, Olson SH, Pikounis B, Ren N, Robertson N, Schaeffer JM, Shah K, Springer MS, Strack AM, Strowski M, Wu K, Wu T, Xiao J, Zhang BB, Wright SD, Thieringer R: 11{beta}-hsd1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. *J. Exp. Med.* 202:517-527, 2005
172. Morton NM, Paterson JM, Masuzaki H, Holmes MC, Staels B, Fievet C, Walker BR, Flier JS, Mullins JJ, Seckl JR: Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 {beta}-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes* 53:931, 2004
173. Morton NM, Holmes MC, Fievet C, Staels B, Tailleux A, Mullins JJ, Seckl JR: Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11beta -hydroxysteroid dehydrogenase type 1 null mice. *J. Biol. Chem.* 276:41293-41300, 2001
174. Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CRW, Seckl JR: 11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc Natl Acad Sci USA* 94:14924, 1997
175. Wang M: The role of glucocorticoid action in the pathophysiology of the metabolic syndrome. *Nutr Metab* 2:1-14, 2005
176. Fisher JE, Smith RS, Lagrandeur R, Lorenz RP: Gestational diabetes mellitus in women receiving beta-adrenergics and corticosteroids for threatened preterm delivery. *Obstet Gynecol* 90:880-883, 1997
177. Biering H, Knappe G, Gerl H, Lochs H: [prevalence of diabetes in acromegaly and cushing syndrome]. *Acta Med Austriaca* 27:27-31, 2000
178. Onwubalili JK, Obineche EN: High incidence of post-transplant diabetes mellitus in a single-centre study. *Nephrol. Dial. Transplant.* 7:346-349, 1992
179. Prelovsek O, Mars T, Jevsek M, Podbregar M, Grubic Z: High dexamethasone concentration prevents stimulatory effects of tnf-{alpha} and lps on il-6 secretion from the precursors of human muscle regeneration. *Am J Physiol Regul Integr Comp Physiol* 291:R1651-1656, 2006

180. Asensio C, Muzzin P, Rohner-Jeanrenaud F: Role of glucocorticoids in the physiopathology of excessive fat deposition and insulin resistance. *Int J Obes Relat Metab Disord* 28 Suppl 4:S45-52, 2004
181. Korach-Andre M, Gao J, Gounarides JS, Deacon R, Islam A, Laurent D: Relationship between visceral adiposity and intramyocellular lipid content in two rat models of insulin resistance. *Am J Physiol Endocrinol Metab* 288:E106-116, 2005
182. Besse C, Nicod N, Tappy L: Changes in insulin secretion and glucose metabolism induced by dexamethasone in lean and obese females. *Obesity Res* 13:306-311, 2005
183. Gremlich S, Roduit R, Thorens B: Dexamethasone induces posttranslational degradation of glut2 and inhibition of insulin secretion in isolated pancreatic beta cells. Comparison with the effects of fatty acids. *J Biol Chem* 272:3216-3222, 1997
184. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: Insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28:412-419, 1985
185. Jahnen-Dechent W, Trindl A, Godovac-Zimmermann J, Muller-Esterl W: Posttranslational processing of human alpha2-hs glycoprotein (human fetuin). Evidence for the production of a phosphorylated single-chain form by hepatoma cells. *European Journal of Biochemistry* 226:59-69, 1994
186. Lee C, Bowman B, Yang F: Human alpha 2-hs-glycoprotein: The a and b chains with a connecting sequence are encoded by a single mrna transcript. *Proc Natl Acad Sci USA* 84:4403-4407, 1987
187. Akhoundi C, Amiot M, Auberger P, Le Cam A, Rossi B: Insulin and interleukin-1 differentially regulate pp63, an acute phase phosphoprotein in hepatoma cell line. *J. Biol. Chem.* 269:15925-15930, 1994
188. Hiron M, Daveau M, Arnaud P, Bauer J, Lebreton JP: The human hepatoma hep3b cell line as an experimental model in the study of the long-term regulation of acute-phase proteins by cytokines. *Biochem J* 287 (Pt 1):255-259, 1992
189. Southorn BG, Palmer RM, Garlick PJ: Acute effects of corticosterone on tissue protein synthesis and insulin-sensitivity in rats in vivo. *Biochem J* 272:187-191, 1990
190. Coleman DL, Burkart DL: Plasma corticosterone concentrations in diabetic (db) mice. *Diabetologia* 13:25-26, 1977
191. Kalabay L, Cseh K, Pajor A, Baranyi E, Csakany GM, Melczer Z, Speer G, Kovacs M, Siller G, Karadi I, Winkler G: Correlation of maternal serum fetuin/alpha2-hs-glycoprotein concentration with maternal insulin resistance and anthropometric parameters of neonates in normal pregnancy and gestational diabetes. *Eur J Endocrinol* 147:243-248, 2002
192. Johanssen S, Allolio B: Mifepristone (ru 486) in cushing's syndrome. *Eur J Endocrinol* 157:561-569, 2007
193. Picard F, Wanatabe M, Schoonjans K, Lydon J, O'Malley BW, Auwerx J: Progesterone receptor knockout mice have an improved glucose homeostasis secondary to beta -cell proliferation. *Proceedings of the National Academy of Sciences* 99:15644-15648, 2002
194. Shoelson SE, Herrero L, Naaz A: Obesity, inflammation, and insulin resistance. *Gastroenterology* 132:2169-2180, 2007

195. Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI: Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest* 97:2859-2865, 1996
196. Dandona P, Aljada A, Bandyopadhyay A: Inflammation: The link between insulin resistance, obesity and diabetes. *Trends in Immunology* 25:4-7, 2004
197. Lazar MA: The humoral side of insulin resistance. *Nat Med* 12:43-44, 2006
198. Goldstein BJ, Ahmad F, Ding W, Li P-M, Zhang W-R: Regulation of the insulin signalling pathway by cellular protein-tyrosine phosphatases. *Molecular and Cellular Biochemistry* 182:91-99, 1998
199. Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP: Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1b gene. *Science* 283:1544-1548, 1999
200. Klaman LD, Boss O, Peroni OD, Kim JK, Martino JL, Zabolotny JM, Moghal N, Lubkin M, Kim Y-B, Sharpe AH, Stricker-Krongrad A, Shulman GI, Neel BG, Kahn BB: Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1b-deficient mice. *Mol. Cell. Biol.* 20:5479-5489, 2000
201. Frittitta L, Camastra S, Baratta R, Costanzo BV, D'Adamo M, Graci S, Spampinato D, Maddux BA, Vigneri R, Ferrannini E, Trischitta V: A soluble pc-1 circulates in human plasma: Relationship with insulin resistance and associated abnormalities. *J Clin Endocrinol Metab* 84:3620-3625, 1999
202. Maddux BA, Sbraccia P, Kumakura S, Sasson S, Youngren J, Fisher A, Spencer S, Grupe A, Henzel W, Stewart TA, Reaven GM, Goldfine ID: Membrane glycoprotein pc-1 and insulin resistance in non-insulin-dependent diabetes mellitus. *Nature* 373:448-451, 1995
203. Frittitta L, Youngren J, Vigneri R, Maddux B, Trischitta V, Goldfine I: Pc-1 content in skeletal muscle of non-obese, non-diabetic subjects: Relationship to insulin receptor tyrosine kinase and whole body insulin sensitivity. *Diabetologia* 39:1190-1195, 1996
204. Teno S, Kanno H, Oga S, Kumakura S, Kanamuro R, Iwamoto Y: Increased activity of membrane glycoprotein pc-1 in the fibroblasts from non-insulin-dependent diabetes mellitus patients with insulin resistance. *Diabetes Research and Clinical Practice* 45:25-30, 1999
205. Pagano G, Cavallo-Perin P, Cassader M, Bruno A, Ozzello A, Masciola P, Dall'omo AM, Imbimbo B: An in vivo and in vitro study of the mechanism of prednisone-induced insulin resistance in healthy subjects. *J Clin Invest* 72:1814-1820, 1983
206. Rushakoff RJ, Kalkhoff RK: Relative effects of pregnancy and corticosterone administration on skeletal muscle metabolism in the rat. *Endocrinology* 113:43-47, 1983
207. Rooney DP, Neely RD, Cullen C, Ennis CN, Sheridan B, Atkinson AB, Trimble ER, Bell PM: The effect of cortisol on glucose/glucose-6-phosphate cycle activity and insulin action. *J Clin Endocrinol Metab* 77:1180-1183, 1993
208. Rizza RA, Mandarino LJ, Gerich JE: Cortisol-induced insulin resistance in man: Impaired suppression of glucose production and stimulation of glucose utilization due to a postreceptor detect of insulin action. *J Clin Endocrinol Metab* 54:131-138, 1982
209. Perley M, Kipnis DM: Effect of glucocorticoids on plasma insulin. *N Engl J Med* 274:1237-1241, 1966

210. Xing L, Quinn PG: Involvement of 3',5'-cyclic adenosine monophosphate regulatory element binding protein (creb) in both basal and hormone-mediated expression of the phosphoenolpyruvate carboxykinase (pepck) gene. *Mol Endocrinol* 7:1484-1494, 1993
211. Granner D, Andreone T, Sasaki K, Beale E: Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin. *Nature* 305:549-551, 1983
212. Schmidt RE, Dorsey DA, Beaudet LN, Peterson RG: Analysis of the zucker diabetic fatty (zdf) type 2 diabetic rat model suggests a neurotrophic role for insulin/igf-i in diabetic autonomic neuropathy. *Am J Pathol* 163:21-28, 2003
213. Cavaghan MK, Ehrmann DA, Polonsky KS: Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. *J Clin Invest* 106:329-333, 2000
214. Ogawa A, Johnson JH, Ohneda M, McAllister CT, Inman L, Alam T, Unger RH: Roles of insulin resistance and beta-cell dysfunction in dexamethasone-induced diabetes. *J Clin Invest* 90:497-504, 1992
215. Severino C, Brizzi P, Solinas A, Secchi G, Maioli M, Tonolo G: Low-dose dexamethasone in the rat: A model to study insulin resistance. *Am J Physiol Endocrinol Metab* 283:E367-373, 2002
216. Kalimi M: Role of antiglucocorticoid ru 486 on dexamethasone-induced hypertension in rats. *Am J Physiol* 256:E682-685, 1989
217. Ma K, Mallidis C, Bhasin S, Mahabadi V, Artaza J, Gonzalez-Cadavid N, Arias J, Salehian B: Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. *Am J Physiol Endocrinol Metab* 285:E363-371, 2003
218. DeFronzo RA, Tobin JD, Andres R: Glucose clamp technique: A method for quantifying insulin secretion and resistance. *Am J Physiol Endocrinol Metab* 237:E214-223, 1979
219. Gutt M, Davis CL, Spitzer SB, Llabre MM, Kumar M, Czarnecki EM, Schneiderman N, Skyler JS, Marks JB: Validation of the insulin sensitivity index (isi0,120): Comparison with other measures. *Diabetes Research and Clinical Practice* 47:177-184, 2000
220. Uwaifo GI, Fallon EM, Chin J, Elberg J, Parikh SJ, Yanovski JA: Indices of insulin action, disposal, and secretion derived from fasting samples and clamps in normal glucose-tolerant black and white children. *Diabetes Care* 25:2081-2087, 2002
221. Legro RS, Finegood D, Dunaif A: A fasting glucose to insulin ratio is a useful measure of insulin sensitivity in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 83:2694-2698, 1998
222. Conwell LS, Trost SG, Brown WJ, Batch JA: Indexes of insulin resistance and secretion in obese children and adolescents: A validation study. *Diabetes Care* 27:314-319, 2004
223. Angulo P, Alba LM, Petrovic LM, Adams LA, Lindor KD, Jensen MD: Leptin, insulin resistance, and liver fibrosis in human nonalcoholic fatty liver disease. *Journal of Hepatology* 41:943-949, 2004
224. Kitagawa Y, Bujo H, Takahashi K, Shibasaki M, Ishikawa K, Yagui K, Hashimoto N, Noda K, Nakamura T, Yano S, Saito Y: Impaired glucose tolerance is accompanied by

decreased insulin sensitivity in tissues of mice implanted with cells that overexpress resistin. *Diabetologia* 47:1847-1853, 2004
225. Wang Y, Wang P-Y, Qin L-Q, Davaasambuu G, Kaneko T, Xu J, Shin-ichi M, Katoh R, Sato A: The development of diabetes mellitus in wistar rats kept on a high-fat/low-carbohydrate diet for long periods. *Endocrine* 22:85-92, 2003
226. Hirose H, Kawai T, Yamamoto Y, Taniyama M, Tomita M, Matsubara K, Okazaki Y, Ishii T, Oguma Y, Takei I, Saruta T: Effects of pioglitazone on metabolic parameters, body fat distribution, and serum adiponectin levels in japanese male patients with type 2 diabetes. *Metabolism* 51:314-317, 2002
227. Brown P, Badal S, Morrison S, Ragoobirsingh D: Acute impairment of insulin signalling by dexamethasone in primary cultured rat skeletal myocytes. *Molecular and Cellular Biochemistry* 297:171-177, 2007
228. Weinstein SP, Wilson CM, Pritsker A, Cushman SW: Dexamethasone inhibits insulin-stimulated recruitment of glut4 to the cell surface in rat skeletal muscle. *Metabolism* 47:3-6, 1998
229. Zhang B, Tavare JM, Ellis L, Roth RA: The regulatory role of known tyrosine autophosphorylation sites of the insulin receptor kinase domain. An assessment by replacement with neutral and negatively charged amino acids. *J. Biol. Chem.* 266:990-996, 1991
230. White MF, Shoelson SE, Keutmann H, Kahn CR: A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. *J. Biol. Chem.* 263:2969-2980, 1988
231. Long W, Barrett EJ, Wei L, Liu Z: Adrenalectomy enhances the insulin sensitivity of muscle protein synthesis. *Am J Physiol Endocrinol Metab* 284:E102-109, 2003
232. Bray GA, Stern JS, Castonguay TW: Effect of adrenalectomy and high-fat diet on the fatty zucker rat. *Am J Physiol Endocrinol Metab* 262:E32-39, 1992
233. Chavez M, Seeley RJ, Green PK, Wilkinson CW, Schwartz MW, Woods SC: Adrenalectomy increases sensitivity to central insulin. *Physiology & Behavior* 62:631-634, 1997
234. Naeser P: Effects of adrenalectomy on the obese-hyperglycemic syndrome in mice (gene symbolob). *Diabetologia* 9:376-379, 1973
235. Solomon J, Mayer J: The effect of adrenalectomy on the development of the obese-hyperglycemic syndrome in ob-ob mice. *Endocrinology* 93:510-512, 1973
236. Wisialowski T, Parker R, Preston E, Sainsbury A, Kraegen E, Herzog H, Cooney G: Adrenalectomy reduces neuropeptide y-induced insulin release and npy receptor expression in the rat ventromedial hypothalamus. *Journal of Clinical Investigation* 105:1253-1259, 2000
237. Bray GA: Regulation of energy balance: Studies on genetic, hypothalamic and dietary obesity. *Proc Nutr Soc* 41:95-108, 1982
238. Quan ZY, Walser M, Hill GS: Adrenalectomy ameliorates ablative nephropathy in the rat independently of corticosterone maintenance level. *Kidney Int* 41:326-333, 1992
239. Goeders NE, Guerin GF: Effects of surgical and pharmacological adrenalectomy on the initiation and maintenance of intravenous cocaine self-administration in rats. *Brain Research* 722:145-152, 1996

240. Solomon J, Bradwin G, Cocchia MA, Coffey D, Condon T, Garrity W, Grieco W: Effects of adrenalectomy on body weight and hyperglycemia in five months old ob/ob mice. *Horm Metab Res* 9:152-156, 1977
241. Yukimura Y, Bray GA: Effects of adrenalectomy on thyroid function and insulin levels in obese (ob/ob) mice. *Proc Soc Exp Biol Med* 159:364-367, 1978
242. Bray GA: Autonomic and endocrine factors in the regulation of food intake. *Brain Res Bull* 14:505-510, 1985
243. Ohshima K, Shargill NS, Chan TM, Bray GA: Adrenalectomy reverses insulin resistance in muscle from obese (ob/ob) mice. *Am J Physiol Endocrinol Metab* 246:E193-197, 1984
244. Freedman MR, Horwitz BA, Stern JS: Effect of adrenalectomy and glucocorticoid replacement on development of obesity. *Am J Physiol Regul Integr Comp Physiol* 250:R595-607, 1986
245. Shimomura Y, Bray GA, Lee M: Adrenalectomy and steroid treatment in obese (ob/ob) and diabetic (db/db) mice. *Horm Metab Res* 19:295-299, 1987
246. Jahnen-Dechent W, Schinke T, Trindl A, Muller-Esterl W, Sablitzky F, Kaiser S, Blessing M: Cloning and targeted deletion of the mouse fetuin gene. *Journal of Biological Chemistry* 272:31496-31503, 1997
247. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS: Protection from obesity-induced insulin resistance in mice lacking tnf-[alpha] function. *Nature* 389:610-614, 1997
248. Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR: A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of niddm without altering glucose tolerance. *Molecular Cell* 2:559-569, 1998
249. Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C, for the Conference P: Definition of metabolic syndrome: Report of the national heart, lung, and blood institute/american heart association conference on scientific issues related to definition. *Circulation* 109:433-438, 2004
250. Schulze MB, Shai I, Rimm EB, Li T, Rifai N, Hu FB: Adiponectin and future coronary heart disease events among men with type 2 diabetes. *Diabetes* 54:534-539, 2005
251. Hennige AM, Staiger H, Wicke C, Machicao F, Fritsche A, HÃ¤ring H-U, Stefan N: Fetuin-a induces cytokine expression and suppresses adiponectin production. *PLoS ONE* 3:e1765, 2008
252. Bjorntorp P, Holm G, Rosmond R: Hypothalamic arousal, insulin resistance and type 2 diabetes mellitus. *Diabetic Medicine* 16:373-383, 1999
253. Walker BR, Phillips DIW, Noon JP, Panarelli M, Andrew R, Edwards HV, Holton DW, Seckl JR, Webb DJ, Watt GCM: Increased glucocorticoid activity in men with cardiovascular risk factors. *Hypertension* 31:891-895, 1998
254. Pasquali R: The hypothalamic-pituitary-adrenal axis in obese women with different patterns of body fat distribution. *Journal of Clinical Endocrinology & Metabolism* 77:341-346, 1993
255. Moyer AE, Rodin J, Grilo CM, Cummings N, Larson LM, Rebuffe-Scrive M: Stress-induced cortisol response and fat distribution in women. *Obes Res* 2:255-262, 1994

256. Ricketts ML, Verhaeg JM, Bujalska I, Howie AJ, Rainey WE, Stewart PM: Immunohistochemical localization of type 1 11{beta}-hydroxysteroid dehydrogenase in human tissues. *J Clin Endocrinol Metab* 83:1325-1335, 1998
257. Livingstone DEW, Jones GC, Smith K, Jamieson PM, Andrew R, Kenyon CJ, Walker BR: Understanding the role of glucocorticoids in obesity: Tissue-specific alterations of corticosterone metabolism in obese zucker rats 1. *Endocrinology* 141:560-563, 2000
258. Whorwood CB, Donovan SJ, Flanagan D, Phillips DIW, Byrne CD: Increased glucocorticoid receptor expression in human skeletal muscle cells may contribute to the pathogenesis of the metabolic syndrome. *Diabetes* 51:1066, 2002
259. Valsamakis G, Anwar A, Tomlinson JW, Shackleton CHL, McTernan PG, Chetty R, Wood PJ, Banerjee AK, Holder G, Barnett AH, Stewart PM, Kumar S: 11{beta}-hydroxysteroid dehydrogenase type 1 activity in lean and obese males with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 89:4755-4761, 2004
260. Paterson JM: Metabolic syndrome without obesity: Hepatic overexpression of 11-hydroxysteroid dehydrogenase type 1 in transgenic mice. *Proceedings of the National Academy of Sciences* 101:7088-7093, 2004
261. Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, Flier JS: A transgenic model of visceral obesity and the metabolic syndrome. *Science* 294:2166-2170, 2001
262. Kershaw EE, Morton NM, Dhillon H, Ramage L, Seckl JR, Flier JS: Adipocyte-specific glucocorticoid inactivation protects against diet-induced obesity. *Diabetes* 54:1023-1031, 2005
263. Holland WL, Brozinick JT, Wang L-P, Hawkins ED, Sargent KM, Liu Y, Narra K, Hoehn KL, Knotts TA, Siesky A, Nelson DH, Karathanasis SK, Fontenot GK, Birnbaum MJ, Summers SA: Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metabolism* 5:167-179, 2007
264. Mori K, Emoto M, Yokoyama H, Araki T, Teramura M, Koyama H, Shoji T, Inaba M, Nishizawa Y: Association of serum fetuin-a with insulin resistance in type 2 diabetic and nondiabetic subjects. *Diabetes Care* 29:468-, 2006
265. Lavebratt C, Wahlqvist S, Nordfors L, Hoffstedt J, Arner P: Ahsg gene variant is associated with leanness among swedish men. *Human Genetics* 117:54-60, 2005