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INVESTIGATION OF BIOCHEMICAL MECHANISMS ASSOCIATED WITH INSULIN RESISTANCE IN THE NON-INSULIN-DEPENDENT DIABETES MELLITUS

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

To-Yu Huang

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DEDICATION

This dissertation is dedicated to my grandmother, Mrs. T.-M. Huang, who never hesitated to sacrifice her own happiness for others' and mine.

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First and foremost, I would like to thank my major professor, Dr. John Koontz, for the privilege to work with him. I admire him as my role model not only for his brilliant ideas in science, but also for his exceptional integrity and elite leadership in life. For his personal guidance and extraordinary patience with me over the years, I am forever indebted to him. I also wish to express my admiration and gratitude for my outstanding committee members: Dr. Naima Moustaid-Moussa, Dr. Wesley Wicks, and Dr. Jeffery Becker. Without their inspiring support, my learning experience would have been much less educating and enjoyable. Last and certainly not the least, I want to thank my family, especially my parents, for supporting my career and education by all means. And I certainly could not have finished my degree without the loving support of my wonderful wife who brightens my life everyday.

ABSTRACT

Non-insulin dependent diabetes mellitus (NIDDM) is a heterogeneous disease arising from metabolic defects in insulin secretion and insulin action. The inability of insulin to trigger full metabolic responses results in impaired glucose transport, which elicits compensatory hyperinsulinemia with increased lipogenesis. In order to identify the molecular defects responsible for insulin resistance, we have investigated three different model systems: the ability of sulfonylurea receptor (SUR) ligands to modify insulin regulated events in cultured rodent adipocytes; the ability of agouti and/or agouti related polypeptide (AGRP) to modify insulin-regulated events; and the regulation of the activity and amount of the HNF-1α transcription factor, mutations of which result in a subset of NIDDM, by variations in glucose concentration. Because the results of animal studies are often obscured by the interactions between multiple tissues, we have used model cell lines in culture to investigate these systems.

In obese Zucker rats, diazoxide decreases hyperinsulinemia and lipogenesis while improving the insulin sensitivity of glucose transport in adipose tissue. The opposing effects on glucose transport and lipogenesis

suggest that diazoxide might have extrapancreatic effects that modify insulin signaling in adipose tissues. Accordingly, we have investigated the effect of diazoxide and glibenclamide (another SUR ligand) on glucose transport and insulin signaling in cultured 3T3-L1 adipocytes. Our results suggest that diazoxide has no direct effect on insulin signaling and glucose transport. In contrast, glibenclamide exhibited dual effects on glucose uptake. Acute exposure to glibenclamide resulted in inhibition of glucose uptake, while long-term incubation with glibenclamide enhanced glucose uptake independent of insulin action. Diazoxide was unable to antagonize either effect of glibenclamide, suggesting that the effects of glibenclamide were not mediated by the sulfonylurea receptor.

In the yellow mouse obesity syndromes, ectopic expression of the *Agouti* gene results in late-onset obesity and type II diabetes. Agouti overexpressed in hypothalamus mimics the natural action of AGRP, which antagonizes α-Melanocyte-Stimulating Hormone (α-MSH) at the Melanocortin-4-recptor (MC-4-R) that controls feeding behavior and metabolism. Although this antagonism in the central nervous system is sufficient to cause the agouti phenotype, a calcium-dependent mechanism for agouti's actions in peripheral tissues has been proposed to account for

enhanced lipogenesis and insulin secretion. To better understand the molecular mechanisms utilized by agouti to modify insulin sensitivity, we have investigated the effects of agouti and AGRP on insulin signaling and its biological responses in cultured 3T3-L1 adipocytes. We observed no effects of either agouti or AGRP on glucose uptake or FAS activity. In contrast, insulin stimulates glucose uptake in a dose-dependent manner and induces fatty acid synthase (FAS) activity as previously reported. In addition, the insulin-stimulated protein tyrosine phosphorylation and activation of downstream kinases were not affected by either agouti or AGRP.

Mutations in HNF-1 genes result in MODY3 (a subtype of NIDDM) that are characterized by pancreatic β-cell dysfunction. The diminished insulin storage and secretion are consistent with the reduced HNF-1 activity that regulates the transcription of insulin genes and genes involved in glycolysis and glucose transport. Reduction in HNF-1 activity and protein has been observed in the livers of type I diabetic animals. Restoring euglycemia without affecting insulin levels rescued hepatic HNF-1 activity and protein in these animals. Accordingly, we investigated whether hyperglycemia also suppresses HNF-1 activity in the type II diabetic animals. Examination of hepatonuclear extracts prepared from the obese

Zucker rats revealed that HNF-1 binding activity was reduced. However, the reduction is not due to diminished HNF-1 protein levels as previously reported in the type I diabetic animals. Incubating Fao rat hepatoma cells with high concentrations of glucose resulted in a similar decrease in HNF-1 binding activity, with no changes in HNF-1 protein levels. The results with the hepatoma cells reflect the observation made with the obese Zucker rats and point to nutritional regulation of HNF-1 activity in the liver.

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LIST OF ABBREVIATIONS

α-MSH	α-Melanocyte-Stimulating Hormone
ACTH	Adrenocorticotropic Hormone
AGRP	Agouti-related Protein
CAP	Cbl-associated Protein
FAS	Fatty Acid Synthase
Glut4	Glucose Transporter 4
GS	Glycogen Synthase
GSK-3	Glycogen Synthase Kinase -3
HNF-1	Hepatocyte Nuclear Factor 1
IRS-1	Insulin Receptor Substrate 1
K_{ATP}	ATP-sensitive Potassium Channel
MC4-R	Melanocortin Receptor 4
MODY	Maturity-Onset Diabetes of the Young
NIDDM	Non-Insulin Dependent Diabetes Mellitus
PDK-1	Phosphoinositide-Dependent kinase 1
PI-3K	Phosphatidyl Inositol-3 Kinase
PKB	Protein Kinase B
PP1	Protein Phosphatase 1

SUR	Sulfonylurea Receptor
TNF-α	Tumor Necrosis Factor-α
TZD	Thiozolidinedione

CHAPTER I

INTRODUCTION

Non-insulin-dependent Diabetes Mellitus

Diabetes mellitus is a metabolic disease in which dysregulation of carbohydrate and lipid metabolism by insulin leads to complications including heart disease, stroke, kidney failure, blindness and nerve damage, causing prevalent morbidity and mortality in the human population (43, 74, 127, 128, 141). Based upon distinct pathophysiology, there are two main categories of the disease: type I diabetes and type II diabetes (75, 127). Type I diabetic patients are dependent on exogenous insulin as a result of the autoimmune destruction of the pancreatic β-cells that produce insulin (127). This is sometimes referred to as insulin-dependent diabetes mellitus (IDDM). Loss of insulin results in elevated plasma glucose that results in organ damage over time. Type II diabetes, also known as non-insulin-dependent diabetes mellitus (NIDDM), is the most common form of diabetes in the Western world, accounting for 90-95% of all cases (74, 75). In the United States more than 14 million Americans are estimated to have the disease and one third of these are believed to be undiagnosed, according to the American Diabetes Association (136). Patients who suffer from type II

diabetes display a characteristic combination of defects in insulin secretion and insulin action (Figure 1-1, please see Appendix for all figures) (75).

The inability of insulin to trigger full metabolic responses in its target tissues is referred to as insulin resistance (Figure 1-1). The condition usually occurs in people who are over 45 years of age, overweight and lead a sedentary lifestyle. As fully one third of the American population is now considered obese and obesity in children is escalating dramatically (136), the strong association of obesity with type II diabetes has further increased the risk of the disease in future generations (43). Intensive studies in the past decade has led to much progress in understanding the molecular pathways of insulin action and various aspects of type II diabetes. However, the molecular mechanisms that relate increased adiposity to impaired insulin actions are still not fully understood.

Metabolic Actions of Insulin

Insulin and glucagon are the two major hormones that, acting in an opposing manner, regulate glucose homeostasis. In response to high levels of serum glucose (Figure 1-2), insulin is released by pancreatic β-cells to stimulate glucose uptake, utilization and storage in fat and muscle (Figure 1-

3), while suppressing the hepatic output of glucose. In muscle, glucose is converted to glycogen as an energy storage molecule or oxidized to generate ATP. In adipocytes, glucose is stored after metabolism primarily as lipids. Insulin activates glycogen synthase, glycolytic enzymes such as pyruvate kinase and lipogenic enzymes such as fatty acid synthase (FAS), respectively.

Uptake is the rate-limiting step for glucose utilization and storage. Insulin stimulates the transport of glucose into muscle and fat cells by mobilizing the intracellular pool of glucose transporter 4 (Glut4) so that it is presented on the plasma membrane (Figure 1- 4) (1, 29, 97). Among the family of mammalian glucose transporter proteins, Glut4 is unique in that it is predominantly expressed in muscle and adipose tissues. In resting cells, it is highly sequestered in intracellular compartments. The proportion that is intracellular in unstimulated cells is greater than 95 % of the total. Without this sequestration, insulin would not be able to acutely regulate glucose uptake (57).

The insulin-regulated exocytosis of Glut4 requires phosphatidylinositol-3 kinase (PI-3K) activity (Figure 1-5) (7). Upon insulin

binding to its heterotetrameric ($\alpha_2\beta_2$) receptors, the receptor tyrosine kinase catalyzes intramolecular transphosphorylation on specific tyrosine residues, leading to increased tyrosine kinase activity toward cellular substrates. Subsequent tyrosine phosphorylation on proteins of the insulin receptor substrate family (IRS-1~4)) and other adaptor proteins creates multiple recognition sites for Src Homology 2 (SH2) domain-containing proteins such as the regulatory subunit (p85) of PI-3 Kinase. The association of p85 with IRS proteins activates PI-3 kinase, generating phosphatidylinositol 3,4,5 triphosphate (PIP_3). Increased PIP_3 activates a serine/threonine kinase cascade, including phosphoinositide-dependent kinase 1 (PDK 1) that phosphorylates Akt (PKB) and the atypical protein kinases C ζ and λ (29, 138, 146).

The PI-3K inhibitor wortmannin blocks many of the metabolic actions of insulin, including the effects of the hormone on glucose transport and glycogen synthase (GS) activities (29, 125, 131). Moreover, both the inactivation of glycogen synthase kinase-3 (GSK-3) and the activation of protein phosphatase 1(PP1) are blocked by wortmannin (16, 62). However, the activation of PI-3K alone is not sufficient for insulin action, since other hormones and adhesion molecules can stimulate PI-3K activity without

reproducing the metabolic effects of insulin (52, 150). Furthermore, cell-permeable derivatives of PIP_3 do not fully mimic the effects of insulin on glucose transport (69). Thus, although the PI-3K pathway is necessary, there is at least one additional pathway required for glucose transport regulation by insulin.

Obesity and Insulin Resistance

In patients with type II diabetes, insulin is less capable of stimulating the uptake of glucose into muscle and fat, and of inhibiting the production of glucose by the liver. The pleiotropic actions of insulin are impaired to variable extents, collectively known as insulin resistance (114, 133). The ability of insulin to stimulate glucose uptake is more profoundly affected than its ability to promote lipogenesis (74). Manifesting such discordant actions of insulin, glucose intolerance and obesity are closely associated with the type II diabetes, but the molecular mechanism underlying the differential impairments of insulin signaling remains unclear.

Clinical studies have suggested that obesity precedes insulin resistance in type 2 diabetes (74), indicating that adipose tissue harbors the primary (earliest) cause of insulin resistance (15). Recent studies using

transgenic mice have demonstrated that adipocyte-specific impairment of glucose uptake is sufficient to cause insulin resistance in fat and other insulin-sensitive tissues (1). One potential mechanism that the adipocyte might use to dictate insulin sensitivity in other insulin-sensitive tissues is to secrete hormones such as leptin and tumor necrosis factor-α (TNF-α), which affect insulin signaling through endocrine action (43).

TNF-α levels are elevated in states of obesity, perhaps reflecting increased adiposity (135-137). The adipocyte-derived cytokine exerts catabolic effects on adipocytes, blocking lipid synthesis and lipoprotein lipase expression, while activating lipolysis (136, 137). It can also block or reverse differentiation of fibroblastic precursors into adipocytes (135). Hotamisligil et al have suggested that excessive TNF-α might mediate obesity-induced insulin resistance (58-61). *In vitro* studies demonstrated that TNF-α can impair insulin signaling by stimulating the serine phosphorylation of IRS proteins, leading to decreased tyrosine phosphorylation and PI-3K association (59, 61). However, the kinases that catalyze the inhibitory serine phosphorylation of IRS proteins remain unknown.

Leptin acts primarily in the ventromedial hypothalamus (43, 80). Lesions in this region of the brain are known to result in hyperinsulinemia and insulin resistance, implicating leptin or other centrally acting peptides in the control of insulin sensitivity (37). Moreover, leptin improves insulin sensitivity in normal rodents independent of food intake and profoundly impacts different neuroendocrine pathways that modulate insulin actions (9, 40, 80). However, leptin receptors are also found in many insulin target tissues (41, 80), and the hormone has been reported to increase fatty acid oxidation, suggesting the possibility of a direct effect on the peripheral tissues (43, 90). Therefore, there is precedence that adipocyte-derived hormones may have autocrine or paracrine functions in local tissues while also acting centrally to communicate the metabolic status of peripheral tissues to the brain.

Agouti, AGRP and the Yellow Mouse Obesity Syndrome

The murine *Agouti* gene is normally involved in coat color regulation in wild-type mice. Expression of agouti in the hair follicles during early postnatal development causes a switch from black to yellow pigment synthesis, resulting in a subapical yellow band on otherwise dark hair (104). Agouti is a paracrine-signaling factor that is secreted by dermal papillae

cells, adjacent to melanocytes, and acts within the hair follicle microenvironment to block melanocortin action (35). Melanocortins such as α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) are the natural ligands for a family of G protein-coupled melanocortin receptors (MC-R), of which five isoforms are presently known (46, 47, 63, 102, 103). MC1-R is the melanocyte-stimulating hormone receptor expressed in melanocytes and has a physiological role in pigmentation (103). MC4-R is expressed mainly in the brain and has been implicated in the regulation of feeding behavior and metabolism (46, 47, 63). Binding of α-MSH to the receptor triggers elevation of cAMP levels and activation of tyrosinase, the rate-limiting enzyme of melanogenesis resulting in eumelanin production (35). In the presence of agouti the opposite is true; eumelanin synthesis is shut down and the default pathway that has phaeomelanin as the final product is activated. Therefore, agouti expression controls the relative amount and distribution of eumelanin (brown/black) and phaeomelanin (red/yellow) pigments in the mammalian coat (104).

Aberrant expression of *Agouti* in mice results in obesity and peripheral insulin resistance similar to the conditions found in human type II diabetes (104). Dominant mutations at the *Agouti* locus, such as the lethal

yellow (A^y/a) and viable yellow (A^{vy}/a) result in the yellow obesity syndrome, characterized by yellow coat color as well as late onset obesity associated with hyperphagia, increased linear growth, non-insulin-dependent diabetes and an increased propensity for developing tumors (161). Genetic analysis has shown that A^y is in fact the result of a chromosomal rearrangement in which the promoter and the first non-coding exon of a closely linked gene, Raly, get spliced to exons of the wild-type agouti gene (98). The *Agouti* gene, now under the control of the relaxed Raly promoter and devoid of temporal and regional restrictions, becomes ectopically expressed. Overexpression of Agouti in multiple tissues is therefore the cause of the A^y phenotype. This conclusion is further supported by the ability of agouti, under the control of a β-actin promoter, to recapitulate the A^y phenotype in transgenic animals (82). The agouti protein is a potent melanocortin antagonist (78, 93, 151) (Figure 1-6). Pharmacological studies of murine agouti protein conclude that its mechanism of action is a classical competitive antagonism of melanocortin receptors (78, 93, 151). Structurally, agouti displays the characteristics of a secreted protein having a hydrophobic signal peptide and lacking any transmembrane domains. Within the encoded 131 amino acids, the major structural features of the mature protein are a highly basic N-terminal region, a Proline-rich central domain, and a C-

terminal domain rich in Cysteine residues (19) (Figure 1-7A). Biochemical analysis of the agouti protein shows that it is highly glycosylated and very stable to thermal denaturation (151). The spacing pattern of the 10 Cysteine residues present in the C-terminus is similar to the ordering of conserved Cysteine residues in invertebrate neurotoxins, suggesting a conserved three-dimensional motif (35) (Figure 1-7B). Based on this structural similarity it has been postulated that all Cysteine residues of the Agouti protein are engaged in disulfide bonds (151). In addition, a truncated version of Agouti, residues 83–131, is known to be as potent an antagonist as the full-length protein (151). The Cysteine-rich C terminus is, therefore, sufficient for effective antagonism of melanocortin *in vitro* as well *as in vivo* (113, 151).

Agouti was the first obesity gene to be cloned (104). The agouti-related polypeptide (AGRP) was found recently to share striking similarities in structure and function but to have a completely different expression pattern (35). Although the obesity appears to be caused by agouti's effect in the hypothalamus that controls feeding behavior, the molecular mechanism linking obesity to peripheral insulin resistance is still not fully understood.

Pancreatic Action of Sulfonylureas and Diazoxide

Sulfonylureas such as glibenclamide are widely used as oral hypoglycemic drugs in the treatment of type 2 diabetes (87). Glibenclamide increases insulin secretion by directly closing the ATP-sensitive K^+ channels in pancreatic β-cells (Figure 1-2) (55, 143) causing membrane depolarization and opening of the voltage-dependent Ca^{2+} channels (VDCCs). This allows Ca^{2+} influx and the resultant rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) triggers insulin secretion (8, 25). Diazoxide, in contrast, opens the K^+ channels and consequently inhibits the secretion of insulin by the pancreas (143). In addition to reducing circulating insulin, diazoxide treatments have been shown to result in changes in insulin sensitivity in peripheral tissues of human and rodent models of type II diabetes (2-5). Although the therapeutic effects of diazoxide were attributed to its action in the pancreas, several lines of evidence suggest that an extrapancreatic action of diazoxide on insulin signaling is likely to exist.

Anti-obesity Effect of Diazoxide

Hyperinsulinism and insulin resistance are characteristic findings in obese subjects (5, 74). Obesity in both humans and experimental animals is associated with a reduced number of insulin receptors and a decreased

insulin-mediated glucose disposal, whereas sensitivity to insulin's antilipolytic action is unaltered (3).

Alemzadeh e*t al* reported that diazoxide exerts an anti-obesity effect in hyperinsulinemic obese Zucker rats and human adults (3-5). Specifically, diazoxide treatment results in increased insulin receptor binding and glucose transport while it reduces insulin secretion and insulin sensitivity to the antilipolytic action of insulin (3, 5).

Although the reduction in plasma insulin levels by diazoxide most likely leads to the recovery of the number of insulin receptors, the consequent increase in insulin signaling at the receptor level should result in increases in both glucose transport and lipid storage. On the contrary, diazoxide-treated subjects gain less weight while consuming more calories (3). Therefore, the pancreatic action of diazoxide alone cannot explain the concurrent but opposite regulation of glucose transport and lipogenesis.

Consistent with the implication of an extrapancreatic action of diazoxide, a recent study by Shi *et al* demonstrated that human adipocytes also express sulfonylurea receptor 1 (SUR1) (132), the pharmacological

target of sulfonylurea and diazoxide action in the pancreas. Furthermore, glibenclamide treatment of isolated human adipocytes causes an increase in intracellular calcium and lipogenic enzyme activities (132), consistent with the presence of a sulfonylurea receptor in these cells. As expected, diazoxide inhibits such increases in lipogenesis, but the direct effect of diazoxide on glucose transport remains unclear. Thus, the action of diazoxide has not been reported in rodent adipocytes.

Maturity-onset Diabetes of the Young (MODY)

Maturity-onset diabetes of the young (MODY) is a genetically heterogeneous monogenic form of non-insulin-dependent diabetes mellitus (NIDDM), characterized by early onset, usually before 25 years of age and often in adolescence or childhood, and by autosomal dominant inheritance (158). Clinical studies have shown that prediabetic MODY subjects have normal insulin sensitivity but suffer from a defect in glucose-stimulated insulin secretion, suggesting that pancreatic β-cell dysfunction rather than insulin resistance is the primary defect in this disorder. Linkage studies have localized the genes that are mutated in MODY to human chromosomes 20 (MODY1), 7 (MODY2) and 12 (MODY3) (127). MODY2 and MODY3 are allelic with the genes encoding glucokinase, a key regulator of insulin

secretion, and hepatocyte nuclear factor-1α (HNF-1α), a transcription factor involved in tissue-specific regulation of liver genes but also expressed in pancreatic islets, insulinoma cell lines and other tissues (20).

Patients with the MODY3 form of NIDDM have mutations in the gene encoding HNF-1α (159). HNF-1α is a transcription factor that regulates the tissue-specific expression of several liver genes and is also expressed in pancreatic islets and other tissues (20). It is not clear how mutations in the HNF-1α gene can cause diabetes when present on a single allele. It is possible that a partial deficiency of HNF-1α could lead to β-cell dysfunction and diabetes. Alternatively, mutations in HNF-1α may cause diabetes by a dominant-negative mechanism by interfering with the function of wild type HNF-1α and other proteins that act together with HNF-1α to regulate transcription. Many HNF-1α gene mutations identified would result in the synthesis of a mutant protein impaired in DNA-binding or transactivation. These mutant proteins could form non-productive dimers with the product of the normal HNF-1α allele or with other proteins such as HNF-1β and impair the normal function of HNF-1α.

Transgenic mice that lack HNF-1/α have been generated (117). Pontoglio et al. compared insulin secretory function in HNF-1α+/-, and HNF-1α-/- mice with wild type HNF-1α+/+ mice. Blood glucose concentrations were similar in HNF-1α+/+ and HNF-1α+/- mice, but were significantly higher in HNF-1α-/- mice. Insulin secretory responses to glucose in the pancreas from HNF-1α-/- mice were <15% of the values in the other two groups and were associated with a similar reduction in the intracellular Ca^{2+} response (115, 118, 119). These defects were not due to a decrease in glucokinase or insulin gene transcription. Therefore, the mutation in the HNF-1a gene in mice probably leads to diabetes due to alterations in the pathways that regulate β-cell responses to secretagogues including glucose. Dukes et al. showed that in the HNF-1/α $^{-/-}$ mice the ATP-sensitive K^+ current (K_{ATP}) in β-cells is insensitive to suppression by glucose but normally sensitive to ATP (36). In addition, NAD(P)H production from glucose oxidation was substantially reduced, and an inhibitor of glycolytic NADH generation reproduced the mutant phenotype in normal islets. Therefore, MODY3 seems to result from defective β–cell glycolytic signaling.

Research Project Overview

This dissertation is divided into three parts. Each part addresses a question associated with understanding the biochemical mechanisms underlying insulin resistance in type II diabetes.

In the first part, we investigated the effects of diazoxide (a K_{ATP} channel opener) and glibenclamide (a K_{ATP} channel blocker) on insulin actions in cultured adipocytes. Several lines of evidence suggest that an extrapancreatic effect of diazoxide on glucose transport likely exists. Although the therapeutic effects of diazoxide have been established, the molecular mechanism underlying the differential modifications of insulin signaling is still unclear. Second, the effects of diazoxide on post-receptor insulin signaling are unknown. Because the PI-3 kinase pathway is known to mediate the metabolic actions of insulin, if the actions of diazoxide on adipose tissue directly contribute to the improved insulin sensitivity, activation of molecules within the PI-3 kinase pathway will likely suggest a molecular mechanism linking insulin signaling to diazoxide action. Therefore, the purpose of this study was to determine whether the beneficial effects of diazoxide treatment *in vivo* can be reproduced in cultured adipocytes. Also, we sought to determine which molecule (s) within the PI-3

kinase pathway, if any, might be affected by diazoxide treatment. Accordingly, our studies were designed to explore the possibilities that: 1) The effects of diazoxide on glucose transport in adipose tissue are extrapancreatic; and 2) diazoxide modifies insulin sensitivity by activation of the PI-3 kinase pathway.

In the second part, we investigated the ability of the Agouti protein or the agouti-related protein (AGRP) to effect insulin signaling and insulin action. Although obesity appears to be caused by agouti's effect in the hypothalamus that controls feeding behavior, the molecular mechanism linking obesity to peripheral insulin resistance is still not fully understood. Recently, it was reported that, in cultured adipocytes, agouti stimulates the transcription of the fatty acid synthase (FAS) gene that is normally regulated by insulin and that it acts synergistically with insulin to regulate this gene. This observation raised the possibility that agouti may interact with a component involved in insulin signaling. Insulin stimulates FAS transcription through a cascade of kinase activities including PI-3 kinase (PI-3K) and its downstream effector, Akt or protein kinase B (PKB). Interestingly, both PI-3K and PKB activity are required for the insulin-stimulated translocation of glucose transporter, which leads to increased

glucose uptake by the peripheral tissues. Therefore, to investigate whether agouti interacts and alters insulin-initiated signal transduction, we set out to test if glucose uptake and PKB activation are affected by agouti treatment.

In the third part, we investigated the effects of hyperglycemia on HNF-1 expression and DNA-binding activity in livers of obese and lean Zucker rats treated with diazoxide or left untreated. It has been reported that hyperglycemia in animals with type I diabetes results in suppression of hepatic HNF-1 expression and its ability to activate the transcription of the albumin gene. Given that obese Zucker rats are both hyperglycemic and hyperinsulinemic, we wondered whether the expression or the activity of hepatic HNF-1 is affected in the type II diabetic animals. Accordingly, we sought to examine the expression levels of HNF-1 and albumin in the obese livers. In addition, we also assessed the DNA-binding activity of HNF-1 in hepatonuclear extracts for comparison with the data on protein expression.

In order to isolate the effects of hyperglycemia from those of hyperinsulinemia in the obese Zucker rats, we examined the effects of diazoxide that significantly attenuates insulin secretion in these animals. Moreover, we employed cultured hepatoma cells as a model system to

address the direct effects of glucose concentrations on HNF-1 expression and activity.

CHAPTER II

The Effects of Sulfonylurea Receptor Ligands on Insulin Signaling in 3T3-L1 Adipocytes

Introduction

Studies on twins and different ethnic groups have indicated that insulin resistance is genetically predisposed in NIDDM patients and is induced later in life by environmental factors (75). However, due to the heterogeneous nature of the disease, it has proven difficult to identify the susceptibility genes by the methods of molecular genetics (72, 128). Targeted disruptions of the candidate genes involved in insulin signaling, glucose metabolism and adipocyte differentiation have all yielded associations with diabetes, but mutations in these genes only account for 2-5% of the total NIDDM population (127). Likewise, positional cloning has identified several rare mutations in the MODY genes only because a distinctive defect in insulin secretion is associated with these uncommon forms of type II diabetes (54, 112). Therefore, it is unlikely that one or two major genes are responsible for the metabolic defects presented in the majority of NIDDM. Rather, the impaired glucose metabolism probably arises from a number of genes whose expression and protein activities are modified under

appropriate environmental conditions. In order to identify the naturally occurring molecular modifications leading to NIDDM in a defined genetic background, we have taken the approach that investigates the actions of an anti-diabetes drug, which specifically reverses insulin resistance in a monogenic model of NIDDM.

Diazoxide, a sulfonylurea antagonist acting on the pancreatic K_{ATP} channels, has been shown to modify insulin sensitivity in obese Zucker rats (3, 5). Insulin resistance and compensatory hyperinsulinemia are characteristic findings in obese subjects. To better understand the relationships between insulin resistance, hyperinsulinemia and obesity, Alemzadeh *et al* ameliorated the hyperinsulinemia in obese Zucker rats and human adults by treatments with diazoxide, which is known to block insulin secretion by the pancreas (3-5). As a result, the diazoxide treatments produced increased insulin receptor binding and glucose transport while reducing insulin secretion and sensitivity to the antilipolytic action of insulin in adipocytes.

However, not all of these effects are consistent with the pancreatic action of diazoxide. Because prolonged exposure to high levels of insulin

results in the internalization of the insulin receptor (76), the reduction in plasma insulin levels by diazoxide most likely leads to the recovery of the insulin receptor number on the cell surface and hence the observed increase in insulin binding. The consequent increase in insulin signaling (at the receptor levels) should result in increases in both glucose transport and lipogenesis. Instead, the diazoxide-treated subjects gained less weight while consuming more calories (3, 5). Therefore, the pancreatic action of diazoxide alone cannot explain the concurrent but opposing regulation of glucose transport and lipogenesis.

Consistent with the implication of an extrapancreatic action of diazoxide, previous studies on the sulfonylurea action also suggested an insulin-independent effect of glibenclamide on glucose utilization (6, 17, 32, 39, 65-67, 95, 105, 106, 142, 144, 164). Sulfonylureas are widely used to control hyperglycemia in type II diabetes (18, 99, 106). The sulfonylurea receptor (SUR) is an integral component of the pancreatic K_{ATP} channels that regulate insulin secretion (18, 99). The therapeutic effect of sulfonylureas is based upon their ability to augment insulin release by blocking the K_{ATP} channel. However, several studies have reported that the hypoglycemic effect of sulfonylureas is still present in patients whose pancreas has completely lost

the ability to secrete insulin (56, 84, 88, 96, 120). Therefore, a number of *in vitro* studies have examined the possibility that sulfonylureas may have extrapancreatic effects on glucose utilization. These results confirmed the existence of a stimulatory effect of sulfonylureas on glucose utilization in peripheral tissues, but the molecular mechanism mediating such stimulation is still a matter of debate.

The expression of SUR1, the pharmacological target of sulfonylureas and diazoxide in the pancreas, was recently demonstrated in isolated human adipocytes (132). Furthermore, the sulfonylurea glibenclamide causes an increase in intracellular calcium content and lipogenic enzyme activities in the human adipocytes (132, 140). As expected, diazoxide inhibits the stimulation of lipogenesis by glibenclamide in these cells. Therefore, it appears that glibenclamide and diazoxide may directly influence lipid metabolism through SUR1 in human adipocytes. But it remains unclear whether glucose transport is also subject to the same kind of regulation by SUR ligands in adipocytes.

The multiple effects of sulfonylureas on glucose utilization *in vitro* suggest potential regulation of insulin signaling by SUR ligands. Insulin

stimulates various aspects of glucose utilization through the PI-3K pathway (146). In particular, glucose transport is the rate-limiting process for glycogen synthesis and lipogenesis (83). The regulation of lipogenesis by SUR ligands in human adipocytes suggests the possibility that glucose transport in adipocyte is also subject to calcium-dependent modification. One potential mechanism for sulfonylureas to stimulate glucose transport is by interacting with the PI-3K pathway. Accordingly, to elucidate the extrapancreatic actions of SUR ligands on glucose transport and insulin signaling, we examined the effects of glibenclamide and diazoxide in cultured 3T3-L1 adipocytes.

Materials and Methods

Materials

Human insulin was purchased from Eli Lily (Indianapolis, IN). [1,2-^{3}H] 2-deoxyglucose was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA) with a specific activity of 30 Ci/mmol and a concentration of 1 mCi/ml. Monoclonal Anti-phosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-IR and anti-IRS-1 antiserum were gifts from Dr. Bentley Cheatham (Joslin Diabetes Center, Boston, MA). Anti-p85 was purchased from Sigma (Saint Louis,

Missouri). Protein A-Sepharose was purchased from Pharmacia Biothch (Peapack, NJ). Assay kit for Akt Serine 473 phosphorylation was purchased from New England Biolabs, Inc. (Beverly, MA). For adipocyte differentiation, 1-methyl-3-isobutylxanthine (IBMX) and dexamethasone were purchased from Sigma (St. Louis, MO).

Cell Culture

3T3-L1 fibroblast cells (CCL 92.1; American Type Culture Collection, Rockville, MD) were grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose) supplemented with 10% (v/v) calf serum and 1% (v/v) antibiotic solution (10,000 units/ml penicillin and 10 mg/ml streptomycin) in an atmosphere of 7.5% CO_2 at 37 °C. Confluent cultures were induced to differentiate into adipocytes by incubating the cells in 0.5 mM 1-methyl-3-isobutylxanthine, 0.25 mM dexamethasone and 10 mg/ml insulin as described previously (28). Cells were used for experiments 7-10 days after initiation of differentiation. At this time, greater than 90% of the cells were filled with lipid droplets. These cells showed large increases in glucose uptake and in phosphorylation of signaling molecules in response to nanomolar concentrations of insulin as described below.

Determination of 2-deoxyglucose Uptake

3T3-L1 adipocytes cultured in 12-well plates were incubated in serum-free DMEM for 2 hours before experimental manipulations. 2-Deoxyglucose uptake measurements were carried out as described previously (106). Briefly, following incubations with K_{ATP} channel effectors as indicated, cell monolayers were washed 3 times with Krebs-Ringer phosphate (KRP) buffer (140 mM NaCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 4.7 mM KCl, 5 mM sodium phosphate, pH 7.4) and any remaining liquid was aspirated. Cells were then incubated for 15 minutes in KRP buffer in the absence or presence of the ion channel effectors and/or insulin. 2-deoxyglucose uptake was initiated by the addition of 10 µl 2 mM 2-[1,2-^{3}H] deoxyglucose to a final concentration of 33 nM 2-[1,2-^{3}H] deoxyglucose at a specific activity of 30 Ci/mmol. Preliminary studies demonstrated that 2-deoxyglucose uptake was linear under these condition. For all the uptake experiment presented, the reaction was terminated after 5 min by rapidly washing cells three times with ice-cold phosphate-buffered saline (PBS) containing 10 mM glucose. Cell associated radioactivity was determined by lysing the cells with 0.05 N NaOH, followed by liquid scintillation counting. Total cellular protein was determined by the Bradford method (32). To correct for radioactivity non-specifically associated with the cells or

entrapped in extracellular spaces, non-specific uptake was determined in the presence of 10µM cytochalasin B (106). This value was subtracted from the uptake in the absence of cytochlasin to obtain specific glucose transport.

Immunoprecipitation

Following treatments with insulin or K_{ATP} channel effectors as indicated, the cells were lysed in 1 ml of lysis buffer (137 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 µM Na_3VO_4, 2 mM PMSF, 10% glycerol (v/v), and 1% Nonidet P-40 (v/v)). Cell lysates were then centrifuged for 5 min at 10,000 X g to remove cell debris and nuclei. The supernatant fractions were pre-cleared by incubation with Protein A-Sepharose. Anti-phosphotyrosine antibody (2 µg/ tube) was then added for overnight incubation with shaking at 4 °C, followed by addition of 30 µl (10% w/v) of protein A-Sepharose for 1 hour. Following centrifugation, the pellets were washed three times with lysis buffer, solubilized in 30 µl of 2 X SDS-PAGE sample buffer, boiled for 5 minutes, and separated by 10 % SDS-PAGE. Following electrophoretic transfer of the proteins to a polyvinylidene difluoride (PVDF) membrane, an appropriate dilution of primary antibody was added to the membrane, followed by appropriate

secondary antibody conjugated to horseradish peroxidase (1:5000 dilution). Protein was visualized by the enhanced chemiluminescence method (ICN Biomedicals, Inc., Costa Mesa, CA).

Image Analysis

Densitometry analysis of autoradiograms was performed by scanning the films electronically using an UMAX Astra 610S scanner connected to a PowerMacintosh computer. The resultant images were then analyzed by the NIH Image (v. 1.59) software that measures the area and mean density values of the bands of interest after substracting the background.

Statistics

All data are expressed as means ± standard deviation. Statistical analysis of subgroups was performed by independent sample Student's *t* test with significant differences between means determined at a P-value < 0.05.

Results

Effects on glucose uptake

Interpretation of the effects of diazoxide and sulfonylureas on glucose transport is complicated in animal studies. Any effects observed could well be indirect, caused by alteration in pancreatic release of insulin into the circulation. In order to isolate the extrapancreatic effects of SUR ligands, we employed cellular assays using cultured 3T3-L1 adipocytes to assess possible direct effects of these drugs on glucose transport and insulin signaling. Under our cell culture conditions, virtually all of the 3T3-L1 fibroblasts differentiated into mature adipocytes that exhibit morphological and biochemical features of adipocytes *in vivo* (Figure 2-1).

Under our assay conditions, 3T3-L1 adipocytes exhibit a dose-dependent increase in glucose uptake in response to varying concentrations of insulin stimulation (Fig.2-2). The maximum increase in glucose uptake was about 4 fold and reached a plateau at an insulin concentration of 30 nM. In contrast, KRC-7 hepatoma cells, which do not express Glut4 showed virtually no change in glucose uptake.

Previous studies with isolated rat adipocytes have suggested that sulfonylureas bind to cell membranes and potentiate glucose uptake maximally after a 24-hour exposure (105, 106). We observed that incubating 3T3-L1 adipocytes with 100 μM glibenclamide for 24 hours causes a consistent 50% increase in basal glucose uptake and a much more modest increase in insulin-stimulated glucose uptake (Fig.2-3). The increase by glibenclamide in the absence of insulin appears to be additive with the effect of insulin alone. In contrast, diazoxide alone (100 μM) does not affect basal or insulin-stimulated glucose uptake after 24 hours of incubation with the adipocytes (Figure 2-3).

Because the effect of glibenclamide on insulin release is very rapid, we also examined the effect of acute exposure to glibenclamide, i.e. with the drug present during the 15-minute incubation with insulin. At low concentrations, there was no effect on basal rates of glucose uptake (Figure 2-4). However, at higher concentrations up to 100 μM, we observed a significant inhibition of basal glucose uptake (Figure 2-4). In the experiments presented in Figure 2-3, we did not observe this inhibition of basal uptake. However, although incubation with glibenclamide was for 24 hours, the drug was not present during the last 15 minutes, the period during

which cells were incubated with insulin to measure the insulin stimulated uptake. Therefore, we repeated the experiment and included the drug during the 15 minutes of incubation with insulin and prior to addition of the radiolabelled glucose. These experiments demonstrated that with the 24-hour exposure to the drug, we do observe an increase in basal uptake and that this is observed regardless of whether the drug is removed from the culture medium for the final 15 minutes prior to incubation with the radiolabelled sugar (Figure 2-4).

The results presented in Figure 2-3 demonstrating that the effect of glibenclamide on basal uptake was additive with insulin stimulated uptake were obtained by incubating the cells with glibenclamide for 24 hours, washing the cells to remove the drug, and then incubating an additional 15 minutes with or without insulin. That is, the glibenclamide was not present during the final 15 minutes of the incubation prior to addition of radiolabelled sugar. We repeated that experiment measuring the effect of acute or long-term exposure to glibenclamide on insulin stimulated glucose uptake and kept the glibenclamide present up to and during the period of incubation with the labeled deoxyglucose. The 15 min exposure to higher concentrations of glibenclamide causes an inhibition of insulin-stimulated

deoxyglucose uptake (Figure 2-5) but does not cause any increase over the effect of insulin alone. The 24 hr plus 15 min exposure to glibenclamide causes an inhibition of uptake at higher concentrations but causes an increase over insulin alone at low concentrations (Figure 2-5).

Diazoxide alone (100 μM) does not affect basal or insulin-stimulated glucose uptake after 24 hours of incubation with the adipocytes (Fig.2-3). Given that diazoxide is reported to antagonize the effects of the sulfonylureas, we evaluated the ability of diazoxide to alter the effects of glibenclamide on insulin stimulated glucose uptake. The potentiation of insulin-stimulated glucose uptake by 100 μM glibenclamide is not inhibited by up to 200 μM of diazoxide after 24 hours incubation (Fig. 2-6). Acute exposure to glibenclamide in the presence of diazoxide does not prevent the cells from losing more than 50% of insulin-stimulated glucose uptake (Fig. 2-6). The inability of diazoxide to antagonize the actions of glibenclamide suggests that the K_{ATP} channel does not mediate the potentiation effect of glibenclamide on glucose uptake. Whether it involves changes in intracellular calcium in adipocytes remains unsettled.

Effects on Insulin Signaling

Our evidence suggests that the effect of glibenclamide is independent of the SUR. The possibility exists that glibenclamide might act through an insulin-signaling pathway independent of the SUR. Accordingly, we have investigated the effects of glibenclamide and/or diazoxide treatment on tyrosine phosphorylation of the insulin receptor (IR) and insulin receptor substrate-1 (IRS1). The association of p85 with tyrosine-phosphorylated IRS-1 was also examined as an index for PI-3 kinase activation.

To evaluate whether the immunocomplex assay we planned to use is capable of detecting varying degrees of tyrosine phosphorylation initiated by insulin, the tyrosine phosphorylation status of IR and IRS1, as well as the association of p85 with IRS1, were examined in differentiated 3T3-L1 adipocytes with undifferentiated fibroblasts as a control. Undifferentiated fibroblasts express little, if any, insulin receptor while mature adipocytes express a significant quantity. The results (Fig. 2-7A and 2-7B) indicate that IR and IRS1 are heavily phosphorylated at tyrosine residues after exposure to 100 nM insulin for 10 minutes, in contrast to a much lower level of tyrosine phosphorylation in the absence of insulin. This response to insulin

stimulation is absent in undifferentiated fibroblasts, consistent with the lack of expression of insulin receptor in these control cells.

As further control experiments, the dynamics of insulin signaling was examined to show the time-dependent changes in tyrosine phosphorylation of IR and IRS1 (Fig. 2-8). IR becomes phosphorylated as quickly as 1 minute after 100 nM insulin stimulation and stays heavily phosphorylated even after 10 minutes. A similarly rapid response of IRS1 is consistent with its action directly downstream of IR. But after 8 minutes, the IRS1 is getting dephosphorylated. The decrease in IRS1 phosphorylation is independently reproducible by monitoring p85 association with IRS1 (Fig. 2-9). The amount of p85 binding to IRS1 was assessed by immunoprecipitation using a polyclonal antibody against IRS1. An insulin-stimulated increase of p85 binding to IRS-1 reached maximum after 8 minutes and then attenuated, consistent with the timing assessment made using the anti-phosphotyrosine antibody (Fig. 2-8). Therefore, under our assay conditions, the changes observed in tyrosine phosphorylation status are indicative of insulin signaling *in vivo.*

Because the regulatory effects of sulfonylureas and diazoxide on pancreatic insulin release are virtually instantaneous and the inhibitory actions seen with glucose uptake are rapid, we have only examined the acute effects of sulfonylurea and diazoxide on insulin signaling. Glibenclamide and diazoxide appeared to have little effect on tyrosine phosphorylation of IR and IRS1 (a representative experiment is presented in Figure 2-10). Similarly, glibenclamide and diazoxide caused virtually no change in the association of p85 (or a SH2 domain–containing molecule) with IRS1 (Figure 2-11).

Discussion

Clinical studies in animal and human subjects of NIDDM have suggested that diazoxide enhances the insulin sensitivity of adipose tissues in *vivo* (3-5). Although the improved glucose tolerance in diazoxide-treated subjects was attributed to the ameliorated hyperinsulinemia, previous studies in our laboratory and others have also indicated that the anti-diabetic effects of diazoxide cannot be adequately explained by its pancreatic action alone (132, 140). An extrapancreatic effect of diazoxide has been documented in isolated human adipocyte in terms of reversing the effect of glibenclamide on lipogenesis (132). Glibenclamide has been shown to increase glucose

utilization by peripheral tissues *in vitro* and *in vivo* (6, 32, 39, 65-67). Moreover, the pharmacological target of sulfonylureas and diazoxide in pancreas, SUR1, was recently shown to be present in isolated human adipocytes (132). Therefore, it is possible that diazoxide antagonizes the multiple effects of sulfonylureas on glucose utilization in adipocytes through SUR1. In fact, diazoxide was shown to prevent the stimulation of lipogenic enzyme activities as well as the inhibition of lipolysis by glibenclamide in isolated human adipocytes. However, if diazoxide antagonizes the stimulatory effect of glibenclamide on glucose transport as well, one would predict it to inhibit glucose transport, which is contrary to the observation made in clinical studies. To investigate this discrepancy and potential interactions between diazoxide and insulin signaling in adipocytes, the present study examined the effects of diazoxide on glucose transport and insulin signaling in cultured 3T3-L1 adipocytes.

We have demonstrated that diazoxide has no direct effect on the basal or insulin-stimulated glucose uptake in 3T3-L1 adipocytes (Figure 2-3). The lack of response was specific to the diazoxide treatments as insulin was able to stimulate glucose uptake in a dose-dependent manner (Figure 2-2). The maximal stimulation by insulin was up to 450% of the basal uptake, presumably due to the mobilization of the Glut4 by insulin signaling . Consistent with this assumption, insulin stimulated glucose uptake to a much lesser extent in the KRC-7 hepatoma cells (Figure 2-2), which do not express the Glut4 (49, 50), but otherwise respond similarly to insulin in terms of protein tyrosine phosphorylations (data not shown). To further confirm the action of diazoxide in the adipocytes, we included in our experiments the use of glibenclamide whose action on lipogenesis was reversed by diazoxide (132).

Glibenclamide enhanced the basal glucose uptake after long-term incubation. The increase in the basal uptake by glibenclamide was additive to the insulin-stimulated glucose uptake and only seen after a 24-hour exposure to the drug. Stimulation of glucose transport by sulfonylureas has been documented by a number of studies (26, 31, 32, 39, 65-67, 105, 106, 123, 139, 142, 144, 148, 164). The potentiation by glibenclamide demonstrated in the

present study is independent of and additive to the acute stimulation by insulin, suggesting that glibenclamide activates glucose transport independently of the insulin signal transduction cascade. In addition, the lack of acute stimulation by glibenclamide in the present study implies a mechanism that requires protein synthesis to mediate the direct effect of glibenclamide.

The long-term incubation required for the potentiation by sulfonylureas has been described by a number of studies, most of which attributed the increase in glucose transport to enhanced gene expression and protein translocation of the glucose transporters (66, 67, 105, 106, 142, 148). Therefore, glibenclamide may increase glucose uptake by activating the synthesis of glucose transporters. In support of this possibility and consistent with our observation, glibenclamide and diazoxide have recently been shown to modify gene expression of key lipogenic enzymes in isolated human adipocytes (132, 140).

Alternatively, glibenclamide has been shown to decrease the cytosolic cAMP levels and protein kinase A (PKA) activity in rodent adipocytes and muscle cells (106). Together with the insulin signaling pathway, cAMP is the

major intracellular signal that regulates glucose utilization. Sulfonylureas have been shown to decrease cytosolic cAMP levels and protein kinase A (PKA) activities in isolated rat adipocytes (17, 106, 153). The decrease in cAMP-dependent phosphorylation of Glut4 results in a state of the transporter protein that is competent for translocation to and/or residence in the plasma membrane (13, 122). In fact, diminished glucose transport activity is associated with elevated levels of phosphorylated Glut4 in insulin-resistant rat adipocytes (13, 122). There is also some evidence for Ser/Thr phosphorylation of Glut4 by PKA during induction of insulin resistance (68, 81, 86).

Since insulin mobilizes the Glut4 in an acute manner, we also examined the acute effect of glibenclamide on glucose transport in order to differentiate the two possibilities described above. Indeed, when the cells were exposed to glibenclamide for only 15 minutes, the basal and the insulin-stimulated glucose uptake were not enhanced by glibenclamide at low concentrations and even suffered from the presence of glibenclamide in higher (> 10 mM) concentrations (Figure 2-4 and 2-5). Furthermore, when the cells were exposed to glibenclamide for 24 hours plus an additional 15 minutes in the presence or absence of insulin, the long-term potentiation by

glibenclamide and the acute insulin stimulation of glucose uptake were still clearly visible at low concentrations of glibenclamide (< 10 mM). Therefore, the acute inhibition of glucose uptake by glibenclamide appears to be dose-dependent and mediated by a mechanism not specific to either the basal or the insulin-stimulated glucose uptake. There has been discussion about whether the effective concentrations of sulfonylureas in vivo were significantly higher than the expected ones according to in vitro binding experiments [25]. It remains unclear whether our observation of the acute inhibition by glibenclamide indicates a similar mechanism or if the high concentrations of glibenclamide simply have a detrimental effect on the cell membrane that is unfavorable to the sugar transport process.

Because glibenclamide acutely inhibited the insulin-stimulated glucose uptake, we were prompted to ask if glibenclamide modifies the insulin signaling responsible for mobilizing the Glut4. We observed no modification of the insulin signaling leading to enhanced mobilization of the Glut4 after acute exposure to glibenclamide (Figure 2-10). Consistent with the data on glucose uptake experiments, neither glibenclamide nor diazoxide enhance or inhibit insulin signaling in 3T3-L1 adipocytes in spite of the demonstration that they improve glucose tolerance *in vivo*.

To our surprise, diazoxide was unable to reverse any of the effects of glibenclamide under the same experimental conditions (Figure 2-6). The long-term potentiation of basal uptake by glibenclamide in addition to the acute insulin stimulation was unaffected in the presence of up to 200 mM diazoxide (Figure 2-6). Similarly, the short-term inhibitory effect of 100 mM on acute insulin stimulation of glucose uptake was not reversed by the presence of up to 200 mM diazoxide (Figure 2-6). The lack of antagonism of the glibenclamide action by diazoxide suggests that the extrapancreatic effects of glibenclamide on glucose transport in adipocytes are probably not mediated by SUR1, the pancreatic sulfonylurea receptor. SUR1 is normally expressed in the pancreas, while isoforms SUR2A and SUR2B are expressed in other electrically excitable cells (18, 99). There have been both positive and negative reports on the presence of high-affinity binding sites for sulfonylureas on the adipocyte membrane (48, 111). However, the expression of SUR1 was recently demonstrated in isolated human adipocytes and suggested to mediate the direct effects of glibenclamide and diazoxide on lipid metabolism (132). In contrast, our data suggest that SUR1-mediated direct effects of diazoxide and glibenclamide on glucose transport were unlikely. Considering that glucose transport is the rate-limiting process for lipogenesis, it remains unclear what is the reason for this discrepancy.

CHAPTER III

The Effects of Agouti and Agouti-Related Polypeptide on Insulin Signaling in 3T3-L1 Adipocytes

Introduction

Agouti was the first obesity gene to be cloned (19). Aberrant expression of the agouti gene results in late-onset obesity and type II diabetes (104, 161). Several lines of evidence strongly suggest that the metabolic anomalies induced by mis-expressed agouti stem from its effects on the central nervous system (35, 38, 64). The recent discovery of AGRP (Agouti-related polypeptide), which shares striking similarities with agouti in structure and in biochemical properties (77, 93, 151), indicates that ectopic agouti mimics the normal biological actions of AGRP. AGRP is expressed primarily in the hypothalamus, adrenal medulla, and at low levels in testis, lung, and kidney (109, 134). Both the agouti and AGRP proteins are highly specific antagonists for the melanocortin receptor MC4-R. MC4-R is also expressed mainly in the brain [Gantz, 1993 #711] [Mountjoy, 1994 #712]. Central administration of synthetic MC4-R antagonists stimulates feeding behavior

and metabolism (38), indicating a functional role for melanocortin antagonists *in vivo* (64). Therefore, the identification of a hypothalamic melanocortin receptor, MC4-R, together with AGRP as central components of feeding behavior and metabolism has lent support for a central mechanism of agouti-induced obesity.

However, a significant number of studies have also suggested that agouti has additional effects on insulin-sensitive peripheral tissues such as adipose tissue and the pancreas (24, 71, 79, 155, 156, 163). Based on the structural similarity between agouti and the invertebrate neurotoxins which are known to block calcium channels, Zemel et al have proposed that agouti is able to regulate lipogenesis and insulin release via a calcium-dependent mechanism (162). In support of the peripheral actions of agouti, Xue et al. have shown that recombinant agouti protein exhibits potent lipogenic and antilipolytic effects in human adipocytes via a calcium-dependent mechanism (71, 79, 155), thereby promoting lipid storage in adipose tissue. Subsequent data from the same laboratory demonstrated that agouti protein stimulates calcium signaling and serves as a potent insulin secretagogue in both a pancreatic ß-cell line and human pancreatic islets (154). Accordingly, they have proposed that this effect of agouti on insulin release, combined

with its effect on adipocyte lipid metabolism, may represent an alternative mechanism for agouti in the development of obesity.

The fundamental similarity between insulin secretion and glucose transport raise the possibility that if agouti is able to stimulate the exocytosis of insulin, it may be able to stimulate Glut4 translocation in adipocytes, another calcium-affected exocytotic event. Since agouti is a paracrine factor (104), it may directly stimulate glucose transport into adipose tissue, or it may do that indirectly via increased plasma insulin levels. Furthermore, although the obesity appears to be caused by agouti's effect on feeding behavior that is controlled by the hypothalamus (35, 74), the molecular mechanism which links obesity to peripheral insulin resistance is still not fully understood. The current understanding of agouti action does not explain the molecular mechanism by which the increased adiposity causes insulin resistance. However, it was recently reported that in cultured 3T3-L1 adipocytes, agouti stimulates the transcription of the fatty acid synthase (FAS) gene that is normally regulated by insulin (24). Insulin stimulates FAS transcription through a cascade of kinase activity including PI-3 kinase (PI-3K) and its downstream effector, Akt or protein kinase B (PKB) (147). This observation raises the possibility that agouti may interact with insulin

signaling. Perhaps the induction of FAS activity by agouti involves activation of the insulin signaling pathway. If so, then this persistent activation of the pathway by ectopically expressed agouti could lead to down-regulation (desensitization) of the pathway.

Whether the action of agouti is central or peripheral, the proposed mechanisms for agouti stimulation of lipogenesis and insulin secretion has significant implications for our understanding the molecular mechanism underlying insulin resistance in type II diabetes. We are particularly interested in the PI-3K pathway because of its essential role in the process of insulin-stimulated glucose transport and lipogenesis. In order to assess the potential interaction between agouti protein and insulin signaling, we examined agouti's effects on the insulin-stimulated glucose transport and activation of PI-3K pathway in 3T3-L1 adipocytes.

Materials and Methods

Materials

Partially purified recombinant agouti was a gift from Dr. Naima Moustaid-Moussa and its preparation has been described elsewhere (24, 93). Synthetic C-terminal peptide (residues 83-131) of AGRP was purchased

from Phoenix Pharmaceuticals, Inc. (Phoenix, AZ). The C-terminus of AGRP has been shown to retain the biological activity of the full-length protein *in vivo* and *in vitro* (124, 160). Reagents and antiserums for 2-deoxyglucose uptake and immunoprecipitation experiments have been described in "Materials and Methods " of chapter II. For FAS enzyme activity assay, NADPH, malonyl CoA and acetyl CoA were purchased from Sigma (St. Louis, MO).

Cell Culture

3T3-L1 fibroblast cells (CCL 92.1; American Type Culture Collection, Rockville, MD) were grown and induced into differentiated adipocytes as previously described in "Materials and Methods " of chapter II.

Determination of 2-deoxyglucose Uptake

Deoxyglucose uptake measurements were carried out according to Muller et al. (106) and has been described in "Materials and Methods " of chapter II.

Immunoprecipitation and western blot analysis

Immunoprecipitation with monoclonal anti-phosphotyrosine antiserum and western blot analysis was performed as previously described in “Materials and Methods “ of chapter II.

FAS enzyme activity assay

FAS enzyme activity was measured spectrophotometrically in crude cytosolic extracts of 3T3-L1 adipocytes by monitoring the oxidation rate of NADPH at 340 nM. The enzyme activity was normalized to per min and per mg protein as previously described (24, 71).

Statistics

All data are expressed as means ± standard deviation. Statistical significance was assessed by independent sample Student’s *t* test. A *P*-value < 0.05 was considered significant.

Results

Effect of agouti and AGRP on Glucose Transport

As shown in Figure 3-1 and 3-2, 2-deoxyglucose uptake was measured in 3T3-L1 adipocytes in the presence of increasing concentrations of agouti protein. Exposure of 3T3-L1 adipocytes to agouti protein concentrations ranging from 1.45 nM to 745 nM has very little effect on basal 2-deoxy glucose uptake (Figure 3-2) or on the 100 nM insulin-stimulated uptake (Figure 3-1). One hundred nm insulin alone gave a 5-fold stimulation, the maximal observed response. The lack of response to agouti protein was further confirmed in another experiment using 3 nM insulin, a more physiological concentration (Figure 3-2). Because this concentration does not give maximal induction, it allows for the possibility of additional effects of agouti that might be manifest as an increase in activity. It also allows for the possibility that agouti might inhibit the insulin stimulated glucose uptake in the presence of an insulin concentration closer to physiological.

Effect of agouti and AGRP on insulin signaling

To assess the potential effects of agouti or AGRP on insulin signaling, we examined the insulin-stimulated tyrosine phosphorylation of IR and IRS-1

as well as the activation of Akt/PKB in the presence of 100 nM agouti or AGRP. As shown in figure 3-3, 100 nM agouti has no detectable effect on tyrosine phosphorylation of IR. Similarly, tyrosine phosphorylation of IR and IRS-1 was not affected by 100 nM AGRP (Figure 3-4). Examination of Akt /PKB activation by insulin also revealed that phosphorylation at Ser473 was not affected by the presence of 100 nM AGRP (Figure 3-5).

Effect of agouti and AGRP on FAS enzyme activity

Pharmacological studies of murine agouti have concluded that its mechanism of action is a classical competitive antagonism of melanocortin receptors (78, 93, 113, 151). In addition, *In vitro* studies using recombinant mouse agouti protein have shown that agouti is a potent melanocortin antagonist (nanomolar range) at MC-R subtypes 1 ($K_{I(app)}$ = 3 ± 1 nM) and 4 ($K_{I(app)}$ = 54 ± 18 nM), a relatively weak antagonist at MC3-R ($K_{I(app)}$ = 190 ± 74 nM), and a very weak antagonist at MC5-R ($K_{I(app)}$ = 12,000 ± 340 nM) (78, 93, 151).

As we observed virtually no stimulation or inhibition of glucose transport by agouti protein at concentrations encompassing its potency to antagonize MC1-R, MC3-R and MC4-R, one potential explanation that

could account for the lack of an effect is that our agouti protein was not biologically active. Seeking to eliminate this possibility, we measured the effects of agouti and human AGRP on Fatty Acid Synthase (FAS) activity in 3T3-L1 adipocytes. A previous study by Claycombe *et al.* (24) has demonstrated that agouti and insulin independently and additively regulate lipogenesis by inducing FAS activity in 3T3-L1 and primary human adipocytes. The mechanism of FAS activity induction by insulin and agouti is through similarly positive regulation of FAS mRNA content.

Consistent with the previously reported value, we observed a 2-3 fold increase in FAS activity by 100 nM insulin treatment (Figure 3-8). However, there is no stimulation of FAS activity by agouti or AGRP either in the absence or presence of insulin. Interestingly, the ability of insulin to significantly induce FAS activity in 3T3-L1 adipocytes appears to be limited to the first week after the induction of differentiation. In fact, measurements of FAS activity using 3T3-L1 adipocytes 10 days into differentiation showed a much higher basal activity (~ 12 fold) and a 25 % decrease in FAS activity after a 48-hour exposure to 100 nM insulin (Figure 3-6).

Discussion

Previous studies have indicated that agouti protein enhances lipogenesis *in vivo* and *in vitro* (24, 71, 79, 155). Of particular interest, the experiments *in vitro* using cultured 3T3-L1 adipocytes and isolated human adipocytes demonstrated that agouti increased the transcription of FAS gene (24), which is also regulated by insulin through activation of the PI-3K pathway (147). Therefore, it appeared that agouti may have direct effects on the activation of PI-3K and subsequently the glucose transport and lipogenesis. However, in the present study, we demonstrated that in cultured 3T3 –L1 adipocytes, agouti protein has no effects on insulin stimulation of glucose transport and PI-3K activation.

The lack of response to agouti was observed under the same experimental conditions where insulin was able to stimulate glucose uptake to different degrees (Figure 3-1 and 3-2). The response to insulin stimulation indicates that the glucose transport process was not impaired in any way and so the absence of effects by agouti is specific to this reagent. Agouti at various concentrations not only failed to enhance glucose uptake beyond the maximal induction by insulin, they were also unsuccessful in stimulating glucose uptake even when the stimulation was submaximal (i.e.,

with 3 nM insulin). Therefore, agouti protein does not stimulate glucose uptake independent of insulin or synergistically with insulin. Similarly, the basal uptake was not affected by incubating with various concentrations of agouti protein that encompass the effective concentrations documented in the literature regarding its antagonism at the MC-R's (78, 93, 113, 151). Thus, the results on glucose uptake suggest that agouti may stimulate glucose uptake through an alternative mechanism, or the agouti reagent we used may be biologically inactive.

In order to confirm the biological activity of our agouti reagent, we examined its documented effects on the FAS gene expression in the 3T3-L1 adipocytes (24). This experiment also served to assess the possibility that the effects of agouti may require a longer time frame to manifest. Compared to the 15 min incubation in the glucose uptake experiment, cells were incubated with agouti protein for 48 hr as previously described. As an additional control, we also included in our experiment the C-terminal peptide derived from the human AGRP. Despite this, no induction of the FAS enzyme activity by agouti or AGRP was detected, although insulin produced a 3-fold induction in FAS enzyme activity under certain conditions (Figure 3-8). Therefore, in contrast to previous studies demonstrating a

peripheral effect of agouti on lipogenesis, our results suggest that agouti and AGRP have no effect on the FAS gene expression. Although we cannot completely exclude the possibility that both agouti and AGRP were biologically inactive in our experiments, it is unlikely given that the peptide has been shown to be biological equivalent to the full-length protein *in vivo* as well as *in vitro* (124, 160).

Interestingly, the induction by insulin we observed in the FAS enzyme assay was present only when adipocytes early into differentiation were used (Figure 3-8). When cells more than one week into differentiation were used, the basal activity of FAS enzyme became much higher and the induction by insulin decreased (Figure 3-7). After 10 days into differentiation, basal FAS activity rose to a level at least 10 fold higher than that previously reported and no response to insulin stimulation was detectable (Figure 3-6). The results suggest that the insulin induction of FAS activity previously reported may very well arise from the hormone's ability to promote adipocyte differentiation. Whether agouti or AGRP enhances lipogenesis through direct effects on adipocyte differentiation remain unclear at this point, but our data suggest that regardless of the extent of differentiation, agouti and AGRP have no effect on FAS enzyme activity

CHAPTER IV

The Effects of Hyperglycemia on Hepatic HNF-1α Protein Abundance and Binding Activity

Introduction

Hepatocyte Nuclear factor-1α (HNF-1α) is a homeodomain-containing transcription factor (23, 45, 108), well studied for its roles in the development and regulation of liver functions (20). Mice deficient in HNF-1α developed phenylketonuria and died from acute wasting in one month (116). In adult liver, HNF-1α regulates the tissue-specific expression of a number of genes involved in the synthesis of serum proteins, regulation of carbohydrate metabolism, and detoxification (22, 27, 53). The liver-specific expression of these genes is regulated at the level of transcriptional initiation (22). HNF-lα was first identified as the transcriptional activator interacting with an essential DNA sequence that confers liver-specific transcription on the albumin, α-fibrinogen, and α1-antitrypsin promoters (22, 27, 53). HNF-1α binding sites have since been found in the promoters of more than 30 liver-specific genes (20). In cultured hepatoma cell lines, the expression of HNF1 strictly parallels the differentiated phenotype of the hepatocytes (12, 21, 22,

85). Dedifferentiated hepatocytes have no detectable HNF-1α transcripts (20) instead expressing HNF-1β, but selection for reversion to the hepatic phenotype was accompanied by re-expression of HNF-1α (12, 21). However, the recent discovery that mutations in the HNF-1α genes are associated with Maturity-onset Diabetes of the Young (MODY) implies that pancreatic HNF-1α may have an additional role in the regulation of carbohydrate metabolism (54, 112, 127).

Although HNF-1α is a transcription factor enriched in liver and involved in tissue-specific regulation of hepatic genes, it is also expressed in pancreatic islets, insulinoma cells and other tissues (115). In cultured insulinoma cells, dominant-negative suppression of HNF-1α activity results in pronounced decreases in transcription of the insulin gene as well as in the expression of genes involved in glucose transport (Glut 2) and glucose metabolism (pyruvate kinase) (152). Dominant mutations in the human HNF-1α genes result in MODY3, a monogenic form of NIDDM (159). In clinical studies, prediabetic patients of MODY exhibit normal insulin sensitivity but have severe defects in glucose-induced insulin secretion, suggesting that pancreatic dysfunction is the primary disorder in the disease. Diminished

insulin storage and exocytosis are consistent not only with MODY3, but also with MODY1, which is caused by mutations in the HNF-4 genes whose products regulate the expression of HNF-1α (158). Therefore, diminished HNF-1α activity in the pancreas is sufficient to initiate the development of type II diabetes.

Interestingly, diminished HNF-1α activity in the liver of experimental animals has also been documented in studies of type I diabetes (10, 11). Albumin transcripts are reduced in type I diabetic livers (10) and albumin expression in the liver is predominantly controlled by the activity of HNF-1α. In fact, only fully differentiated hepatocyte cell lines that express HNF-1α at high levels secret albumin at appreciable levels (20). Consistent with down-regulated albumin transcription, the abundance and DNA-binding activities of HNF-1α were also decreased in the livers of streptozotocin-treated diabetic rats (10). They exhibit hypoinsulinemia and severe hyperglycemia. Subsequent experiments using vanadate and phlorizin to normalize the hyperglycemia without changing circulating insulin levels, not only restored the levels and DNA-binding activities of HNF-1α but also the albumin mRNA levels (11). Examination of various deletion mutants of the

albumin promoter suggested that the sequence to which HNF-1α binds mediates the suppressive effects of hyperglycemia (10). Therefore, the transactivational activity of hepatic HNF-1α appears subject to regulation by plasma glucose.

Taken together, the down-regulation of hepatic HNF-1α activity by hyperglycemia in type I diabetes suggests a mechanism that potentially mediates the development of type II diabetes through hyperglycemia-induced decreases in pancreatic HNF-1α activity. Preliminary studies in our laboratory have indicated that hepatic HNF-1α DNA-binding activity is significantly reduced in the obese Zucker rats (Figure 4-1). This activity is restored in animals treated with diazoxide. It is possible that the reduction observed is due to the hyperglycemia observed in these animals, as found in the liver in type I diabetes. This may well contribute to the alterations in hepatic glucose metabolism observed in the type II diabetic animal.

In order to confirm our speculation that hyperglycemia results in diminished hepatic HNF-1α activities and/or protein levels in type II diabetes, we followed up the preliminary studies performed with the obese

Zucker rats by examining the amount of HNF-1α protein expressed in the hepatonuclear extracts from these animals and in cultured Fao rat hepatoma cells exposed to various concentrations of glucose.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was from GIBCO, Grand Island N.Y. [α]-32P-dCTP was from ICN (Irvine, CA). Iron-supplemented bovine serum was obtained from Hy-Clone (Logan, UT). Pepstatin A, leupeptin, PMSF, and poly dI-dC were purchased from Boehringer Mannheim (Indianapolis, IN). Aprotinin was purchased from Sigma (St. Louis, MO). Oligonucleotides were purchased from Oligo etc. (Wilsonville, OR). The Fao cell lines were obtained from Mary Weiss. Anti-HNF-1α antiserums are kind gifts from

Animals procedures

Seven-week-old female Zucker fat (fa/fa) rats and female Zucker lean (Fa/Fa) rats were used in this study. The animals were maintained as previously described by Alemzadeh et al (3). Diazoxide (150 mg/kg/day)

was administered in two doses daily by gavage using Proglycem pediatric suspension (Baker-Norton Pharmaceuticals, Inc.). The animals in control group were treated with equivalent volume of placebo suspension twice daily. Treatments with diazoxide proceeded for a period of 6 weeks. Rat livers were removed immediately after death, frozen in liquid nitrogen and stored in -80°C until experiments. The animal procedures were reviewed and approved by the University of Tennessee Medical Center animal care committee.

Cell Culture

Fao rat hepatoma cell lines were grown in DMEM containing 10% iron-supplemented bovine serum in a humidified atmosphere of 10% CO2, 90% air. Cells to be used for experiments were grown to about 70% confluency and then incubated overnight in serum-free medium. Medium containing glucose and xylose of different concentrations were then added and incubations continued for the times indicated in individual experiments.

Nuclear Extract

Nuclear extracts were prepared according to the established protocol in our laboratory. Unless specified otherwise, each step was performed at

4°C and in the presence of protease inhibitors (100 μg/ml PMSF, 0.5 μg/ml leupeptin, 1μg/ml pepstatin A, and 2 μg/ml aprotinin). Briefly, rat liver or Fao hepatoma cells were homogenized in the homogenization buffer, HB (10 mM HEPES, pH 7.5, 0.5mM spermidine, 0.15 mM spermine, 5 mM EDTA, 0.25 mM EGTA, 50 mM NaF and 7 mM 2-mercaptoethanol). The homogenate was then centrifuged at 7,500 X g for 15 min, and the pellet was resuspended in 4 ml of solution #2 (1X HB, 0.5 mM sucrose, and 0.05% NP-40). After centrifuged at 10,000 X g for 15 min, the resultant pellet was resuspended in 5 ml of solution #3 (1X HB, 0.35 mM sucrose). The centrifugation and washing was repeated one more time with 2.5 ml of solution #3, and the nuclei-containing pellet was resuspended in 4 ml of solution #4 (1X HB, 10% glycerol, and 50 mM NaCl). Low-salt extraction was performed for 15 min by mixing on a rocking platform. After centrifugation at 10,000 X g for 20 min, the remaining pellet was resuspended in solution #5 (1X HB, 10% glycerol, and 300 mM NaCl). High salt extraction was performed for 60 min with mixing by agitation followed by centrifugation at 3,000 X g for 30 min. Solid ammonium sulfate (0.326 g/ml) was then added to the supernatant fraction containing nuclear proteins and mixed for 30 min by agitation. The mixture was centrifuged at 30,000 X g for 30 min, and the pellet resuspended in 1 ml of solution #6 (1X HB, 10%

glycerol, 50 mM NaCl, and 2mM $MgCl_2$). The nuclear extract was then dialyzed overnight against 300 ml of solution #6. After measuring protein concentration the nuclear extracts were stored as small aliquots at – 80 ^{o}C until further use.

Electrophoretic mobility shift assay

Two complementary single strands of oligonucleotides containing the HNF-1α binding site were purchased form Oligo's etc (Wilsonville, OR), annealed and [^{32}P]-labeled by the Klenow fill-in reaction for use as a probe:

5' - GATCCGTGGTTAATGATCTACAGTTAT
GCACCAATTACTAGATGTCAATAGATC - 5'

Nuclear extract was incubated together with about 50,000 cpm of the probe in a reaction mixture containing gel shift buffer (20 mM HEPES, pH 7.9, 40 M KCl, 2 mM $MgCl_2$, 1 mM DTT, and 4% Ficoll), spermidine (2.5 mM), poly(dIdC) (1μg), sonicated salmon sperm DNA (1μg), and pGEM3Z (Promega) (1μg). After incubation at room temperature for 30 min, 20 μl of the reaction mixture was resolved by electrophoresis in a 5% nondenaturing polyacrylamide gel in TBE buffer at 150 V for 2 hr. Where indicated, 1 μl of anti-HNF-1α antiserum was added to the reaction mixture prior to the

addition of the probe. After electrophoresis, the gel was exposed to Kodak XAR-5 film at –80 ^{o}C before development.

Results

In order to determine whether a modification of HNF-1α exists in the liver of type II diabetes, previous studies in our laboratory examined the promoter-binding activity of liver nuclear extracts prepared from the obese Zucker rats, an animal model for type II diabetes (Benninghoff, unpublished data). The promoter-binding activity of liver nuclear extracts was assessed by the electrophoretic mobility shift assay (EMSA) using a synthetic probe derived from the target sequence of HNF-1α in the albumin promoter. As shown in Figure 4-1, DNA binding was decreased more than 80% in the obese liver nuclear extracts when compared to that of the lean controls. Furthermore, this decrease is almost fully reversible by diazoxide treatment in the obese group. In contrast, diazoxide caused a 70% reduction, instead of an increase, in DNA binding in the liver extracts of lean animals.

We also examined the protein levels of HNF-1α in the liver extracts of the Zucker rat because the abundance of this transcription factor was reduced in the type I diabetic animals (11). As determined by western blots

(Figure 4-2), the protein levels of HNF-1α were modestly higher in the livers of obese animals than that in the lean controls. Diazoxide treatment did not significantly affect the abundance of the HNF-1α protein in either genetic group. Interestingly, the abundance of HNF-1α in liver extracts could not account for the differences in promoter-binding activity observed in EMSA assay.

Given that the obese Zucker rats were mildly hyperglycemic, the decreased binding to the promoter sequence we observed in the EMSA is consistent with the notion that hyperglycemia suppresses HNF-1α binding activity. However, our experimental animals were also severely hyperinsulinemic due to the inherent insulin resistance characteristic of type II diabetes. Diazoxide blocks insulin secretion and therefore indirectly alters serum glucose levels as well. Consequently, it is difficult to assess the effect of hyperglycemia or hyperinsulinemia alone in the obese animals. More importantly, there was no significant change in the plasma glucose levels in the diazoxide-treated lean animals (3), indicating that the dramatic reduction in nuclear extract binding that we observed in EMSA was not caused by changes in glycemia in the lean animals.

To confirm that hyperglycemia was indeed the cause of changes in HNF-1α binding activity in the obese animals, we chose to evaluate the binding activity and the amount of HNF-1α protein in the Fao rat hepatoma cell line grown in the presence of different glucose concentrations. The use of a cultured cell line eliminates the inherent complications in trying to manipulate glycemia in whole animals. In addition, this cell line has been demonstrated to reflect the intact liver in terms of albumin expression, regulation of albumin expression and expression of HNF-1α (20, 22, 70).

When nuclear extracts of Fao cells were examined by EMSA, there were multiple protein-DNA complexes being detected (Figure 4-3). To identify the protein-DNA complexes that contain HNF-1α, we utilized antibodies against HNF-1α to alter the formation of the normal complexes. In the presence of the HNF-1α antibodies, the two predominant complexes disappeared and a single band of higher molecular weight and lower intensity appeared. Thus, these two bands affected by the antibodies are most likely due to HNF-1α interaction with the ologonucleotide. This doublet pattern is also consistent with the ability of HNF-1α to dimerize with different partners and further interact with other nuclear proteins to

enhance DNA binding. Similar patterns of DNA-protein complexes have been observed by us and others using cell or liver nuclear extracts (10, 11).

Quantitative densitometric analysis of the two predominant DNA-protein complexes revealed that HNF-1α binding activity was decreased in Fao cells exposed to 15 and 25 mM glucose (Figure 4-3 and 4-4). This reduction was minimal after 24 hr exposure but was increased by longer exposure to high glucose concentrations. Compared to normal (5 mM) glucose concentration, 15 mM glucose resulted in 20% and 30% reductions in HNF-1α binding activity after 36 and 48 hr of incubation. Incubation with 25 mM glucose caused a similar reduction, which was not significantly different from that observed with15 mM glucose. Exposure to glucose for longer than 48 hr involved replacing the culture medium and, therefore, replenishing the glucose levels. As a result, HNF-1α binding activity showed a more variable response after 48 hr (data not shown).

The decrease of HNF-1α binding activity caused by high glucose concentrations in Fao cells raised a number of questions about the molecular mechanism(s) mediating this response. One potential mechanism for glucose to decrease HNF-1α activity is through down-regulation of HNF-4 activity.

HNF-4 is the transcription factor largely responsible for tissue-specific expression of HNF-1α (20). Mutations in the HNF-4 gene result in MODY1, presumably due to detrimental effects on HNF-1α expression and consequently diminished insulin gene expression in the pancreas. Furthermore, the glucose-responsive expression of L-pyruvate kinase (L-PK) in the pancreas is partially mediated by HNF-4 (34, 51, 92, 100, 101, 110, 121, 157), indicating a precedent for glucose to regulate HNF-4 activity. Therefore, we also examined, by EMSA, the effect of high glucose concentration on HNF-4 binding activity in Fao cells. However, the abundance of HNF-4 is much lower than that of HNF-1α, making it difficult to determine whether there is specific reduction due to the high glucose concentrations (data not shown).

Other aspects regarding HNF-1α activity we tried to address were the effects of high glucose concentration on the protein levels of HNF-1α and albumin. Western blots of nuclear extracts prepared from Fao cells suggest that there is no significant change in HNF-1α protein levels due to exposure to different concentrations of glucose (Figure 4-5). Similarly, western blots

of postnuclear supernatants from Fao cells indicate there is no significant change in albumin protein levels either (Figure 4-5).

Discussion

We have demonstrated that HNF-1α binding activity was sharply reduced in the hepatonuclear extracts of type II diabetic animals (Figure 4-1). This reduction is consistent with the previous reports that hyperglycemia suppresses the hepatic HNF-1α activity in animals with type I diabetes (10, 11). Because the suppressed HNF-1 activity can be rescued by restoring euglycemia in type I diabetic rats without altering insulin secretion, it was suggested that hyperglycemia alone induced an insulin-resistant state that resulted in lower HNF-1α activity and decreased albumin expression. However, it is unclear in the previous studies that whether this hyperglycemia-induced insulin resistance can be compensated by high levels of insulin as in type II diabetes.

Both hyperinsulinemia and hyperglycemia were observed in obese Zucker rats (149), reflecting the conditions in type II diabetes. In order to assess the direct effect of hyperinsulinemia, the experimental animals were given diazoxide (3, 5). When hyperinsulinemia was ameliorated by

diazoxide, euglycemia was restored in the obese Zucker rats and the HNF-1α binding activity was rescued and returned to control levels. For comparison, the postabsorptive plasma insulin level in the diazoxide-treated obese animals was 62% lower than that of the obese control, nonetheless still 3-4 times higher than the lean control (5). In contrast, the post-absorptive plasma glucose levels of the diazoxide-treated obese animals were fully returned to the levels of lean controls, as was the HNF-1 binding activity. Therefore, it appears that the hepatic HNF-1α activity is more closely correlated with the plasma glucose level than with the plasma insulin levels in the obese animals.

While attenuating insulin secretion in both genetic strains (5), diazoxide treatment resulted in opposite regulation of HNF-1α binding activity in the obese and in the lean animals. In lean animals, diazoxide caused a 70% loss in HNF-1α binding activity despite no change in the plasma glucose levels. This glycemia-independent reduction is probably due to the subnormal insulin levels that failed to sustain the full HNF-1α activity and albumin expression (10, 11). The obese animals treated with diazoxide did not suffer this reduction since they still have plasma insulin levels several times higher than normal. Therefore, our data suggests that

hyperinsulinemia did not affect the HNF-1α binding activity directly in the type II diabetic rats.

Furthermore, we have shown that the decrease in hepatic HNF-1α binding activity was not associated with a concomitant reduction in the abundance of this transcription factor in the livers of obese Zucker rats. Western blots of hepatonuclear extracts revealed that the obese animals, in spite of having lower HNF-1α activity, in fact have slightly higher HNF-1 protein levels than their lean littermates (Figure 4-2). In addition, diazoxide had no significant effect on HNF-1α protein levels in either the lean or obese animals. Therefore, the changes in HNF-1α binding activity caused by obesity or the diazoxide treatment cannot be explained by the changes in the HNF-1α protein levels. In comparison, previous studies on type I diabetic rats have shown that the DNA-binding activity and protein levels of HNF-1α were both reduced in the diabetic animals (10, 11). It is unclear whether this discrepancy between our observations and others is due to the extent of hyperglycemia or the presence of hyperinsulinemia. Compared to the obese Zucker rats, the streptozotocin-rendered diabetic rats have plasma glucose levels twice as high and much lower insulin levels (10, 11). If

hyperglycemia indeed down-regulated the protein levels of HNF-1α, the glycemia condition in the type II diabetic animals may be too mild to elicit a significant reduction in the presence of strong mitogenic response to the hyperinsulinemia. Alternatively, post-translational modification of HNF-1α may be involved in mediating the effects of hyperglycemia and diazoxide treatment. A second protein which interacts with HNF-1α and mediates its binding to the target sequence may also be subject to the regulation by changes in glycemia, as suggested by the doublet binding pattern in EMSA (Figure 4-3 and 4-4).

To isolate the effects of hyperglycemia on HNF-1α activity *in vitro*, we evaluated the expression of HNF-1α activity in Fao rat hepatoma cells cultured in different concentrations of glucose. The results of these studies confirm that high concentrations of glucose alone suppress HNF-1α activity in Fao rat hepatoma cells. The reduction in HNF-1α binding activity was not due to increased osmotic stress as reported previously because the medium with the lower glucose concentrations were supplemented with appropriate concentration of xylose to equalize the osmolarity in the culture medium. Compared to the animal studies of type I and type II diabetes, the extent of

reduction in HNF-1α activity we observed in Fao cells was far more modest. One possible explanation for this discrepancy is that insulin is required to maintain the full activity of HNF-1α. Even the type I diabetic rats rendered insulin deficient by streptozotocin still have one-third the normal insulin levels (10, 11). Consequently, when the hyperglycemia was ameliorated by vanadate or phlorizin, the residual insulin level was still enough to sustain the full return of HNF-1α activity. In contrast, our Fao cells were depleted of insulin while incubating with different concentrations of glucose. Nonetheless, the difference in HNF-1α activity between 5 mM glucose and higher concentrations suggests that HNF-1α activity was indeed affected by the high glucose concentration. Exposure to 25 mM glucose resulted in a reduction similar in extent to that caused by 15 mM glucose probably due to the fact that, in the absence of insulin, there was not much HNF-1α activity for further reduction.

To address the potential mechanisms accounting for the reduction in HNF-1α activity, we examined the upstream effector and downstream target gene of HNF-1 activity. HNF-4 has been shown to mediate the up-regulation of L-PK by glucose (92). Based on the observation, one would expect that

high glucose concentrations would increase HNF-4 activity and, consequently, HNF-1α activity. In contrast, we observed a significant reduction in HNF-4 activity regardless of the glucose concentration. This glucose-independent reduction probably is due to, again, the absence of insulin.

On the other hand, albumin transcription has been shown to be independent of glucose concentration in the absence of insulin (33). In the presence of insulin, glucose increases the stability of albumin mRNA. Our results with albumin protein levels suggest that the albumin message levels were not affected by the reduction in HNF-1α activity without insulin. Most likely, the half-life of the albumin protein was too long to demonstrate significant reduction during the course of our experiments.

CHAPTER V

Conclusions and Future Directions

Research into the development of type II diabetes and its related syndromes is crucial for improving the treatments for this disease. Previous studies elucidating the metabolic staging of type II diabetes have led to significant advances in the regimen of treatment **(127)**. In addition, novel therapeutic agents such as thiazolidinediones (TZDs) have provided new options for treatment **(129)**. However, it remains unclear whether the current therapeutic approaches truly target the underlying metabolic defects of type II diabetes.

In the present study, we have taken several experimental approaches to better understand the molecular mechanisms underlying the insulin resistance in type II diabetes. First, we examined the direct effects of two anti-diabetic drugs in cultured adipocytes to gain insight into the molecular processes that mediate their therapeutic effects. Second, we attempted to reconstruct the state of insulin resistance *in vitro* with agouti protein and its analogue peptide. Last, we examined the effects of high glucose

concentration on hepatic HNF-1α activity because diminished HNF-1α activity has been linked to both type I and type II diabetes **(10, 11, 159)**.

These approaches were carried out in cultured cell lines in order to isolate the molecular components directly affected without the complications of secondary effects resulting from interacting tissues in studies using whole animals. The results, in parts, address questions about the insulin resistance in type II diabetes. Taken together, they outlline the complex interactions among multiple tissues that lead to the development of type II diabetes.

Extrapancreatic Effects of SUR Ligands

Sulfonylureas are the most widely used hypoglycemic drugs to stimulate insulin secretion in patients with type II diabetes (127). Our results demonstrate that glibenclamide, a sulfonylurea, has extrapancreatic effects on glucose transport in cultured 3T3-L1 adipocytes. The enhancement of glucose uptake after long-term exposure is independent of insulin's acute action, consistent with our observations that SUR ligands have no effect on the insulin signaling events leading to PI-3K activation. Furthermore, acute inhibition of glucose uptake by glibenclamide occurs only at concentrations approaching the limit of solubilization, suggesting that nonspecific damages

to the membrane temporarily impair the transport process and that long-term exposure is necessary for the enhancement of glucose uptake.

The molecular mechanism mediates the stimulatory effects of glibenclamide on glucose utilization is still controversial (106, 132). Our results indicate that its effects on glucose transport are not sensitive to the antagonism by diazoxide. The lack of antagonism and the fact that diazoxide alone has no effect on glucose transport argue against a calcium-dependent mechanism mediated by SUR1 in the human adipocytes (132). Instead, our observation is consistent with a cAMP-dependent mechanism previously demonstrated in cultured 3T3 adipocyte (106). Future experiments in different species that examine the effects of variety of sulfonylureas and their sensitivity to diazoxide antagonism in terms of glucose transport will likely provide more insight into this discrepancy. Alternatively, a potential explanation may reside in the recent discovery that adipocytes derived from different fat depots in vivo exhibit differential sensitivity toward insulin action (74, 136).

Although sulfonylureas are effective in terms of stimulating insulin secretion, they have significant side effects, which include some cardiovascular liabilities associated with hyperinsulinemia, and might

accelerate the progression of the disease by exhausting the pancreatic β-cells (127). The results presented here suggest that treatments with sulfonylureas in patients who have lost the ability to secrete insulin may retain the beneficiary effect of the drugs without the caveat of enhanced lipogenesis.

Agouti /AGRP Effects on Glucose Transport and Insulin Signaling

Our results suggest that agouti or AGRP does not affect glucose transport in cultured 3T3-L1 adipocytes. In addition, neither agouti nor AGRP has any effect on the insulin-stimulated tyrosine phosphorylation of IR, IRS-1 or the activation of the PI-3K pathway. These observations are consistent with the previous study that overexpressed agouti in an adipocyte-specific manner (104, 107). The resultant transgenic animals did not exhibit the yellow mouse obesity syndromes unless given daily insulin injections (107). Therefore, insulin resistance in the agouti mouse most likely requires the interactions of peripheral tissues and perhaps the central nervous system as well.

The recent discovery of novel signaling molecules and pathways provides potential mechanism for agouti to elicit such interactions. For example, resistin was identified as the adipocyte-derived factor whose expression and secretion is up-regulated during adipocyte differentiation but

is down-regulated by TZDs (141). TZDs are a class of anti-diabetic drugs which enhance target-tissue sensitivity to insulin *in vivo*(129). Resistin serum levels are markedly elevated in diet-induced obesity as well as genetic models of obesity and insulin resistance. Administration of recombinant resistin to mice and cultured adipocytes decreased insulin sensitivity. Neutralization with anti-resistin IgG reversed the obesity-induced insulin resistance by potentiating the insulin stimulation of glucose uptake. Therefore, the effects of agouti on the pancreas or central nervous system may serve to regulate the expression of resistin which in turn functions as a signal to decrease insulin-stimulated glucose uptake through an autocrine or paracrine mechanism.

HNF-1α Activity in Type II Diabetes

Our results strongly suggest that the hepatic HNF-1α activity is affected by the hyperglycemia in type II diabetes. Although the suppression of HNF-1α activity did not result in changes in the abundance of albumin proteins, the decreased transactivation by HNF-1α could elicit different responses in the expression of other target genes. Of particular interest is the expression of insulin gene in the pancreas. Because HNF-1α is also expressed in the pancreas and responsible for the activation of insulin gene

transcription (20, 145), our observations about the hepatic HNF-1α activity indicate a potential mechanism for the hyperglycemia to cause defective insulin secretion in the type II diabetes. To further investigate this possibility, we propose the following future experiments to address the various aspects of glucose regulation of HNF-1α activity in the type II diabetes:

1) To confirm that hyperglycemia could results in decreased HNF-1α activity in the pancreas of type II diabetic animals, we will examine HNF-1α mRNA/protein levels and DNA binding activity in β-cells isolated from the pancreas of obese Zucker rats. We expect the results to be positive, in consistence with our observation on the liver. Furthermore, the results in HNF-1α message/protein abundance will provide additional clues as to the mechanism HNF-1α is modified in response to hyperglycemia.

2) To establish the direct effect(s) of hyperglycemia, we will examine, in hepatoma and/or insulinoma cell lines, the effects of glucose on HNF-1α regulated gene expression in the presence of various concentrations of insulin. Specifically, we will focus on the transcription of pyruvate kinase

and insulin genes initially. Both genes are regulated by glucose and by HNF-1α (14, 28, 34, 42, 73, 91, 94, 130), indicating that HNF-1α potentially mediating the effect of glucose. It will be interesting to see how their promoter activities respond to different combination of glucose and insulin levels, especially compared to that of the albumin promoter. By assessing the promoter activity through a reporter gene construct, we hope to eliminate the interference from the long half-life of mRNA or protein. Alternatively, we will also attempt long-term exposure of the cells to high glucose, to simulate more closely the conditions and effects *in vivo*. Needless to say, any changes in target gene expression will be correlated with HNF-1α binding activity determined in the same conditions.

3) For potential post-translational modifications of HNF-1α suggested by our present results, we will follow up on the gene identified by the afore-mentioned experiments that are affected by hyperglycemia. Mutation or deletion study of the promoter will be done to see whether additional DNA sequence other than the HNF-1α binding site is required for glucose regulation. If no additional protein factor is implicated, expression of mutant HNF-1α will be attempted to identify the region that interacts with other protein factor to facilitate binding to DNA and confers the sensitivity to

glucose. Alternatively, we will investigate the possibility that HNF-1α is regulated by AMP-activated protein kinase (AMPK) whose activity is reduced by elevated glucose concentration (30, 126). This prospect is supported by the fact that glucose regulation of L-PK in pancreas is mediated by AMPK (30, 44, 89, 126), although it remains to be seen that whether HNF-1 is required for this response. We will seek to confirm the involvement of HNF-1α by expression of dominant-negative HNF-1α mutants in addition to microinjection of antibodies to the AMPK.

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APPENDIX

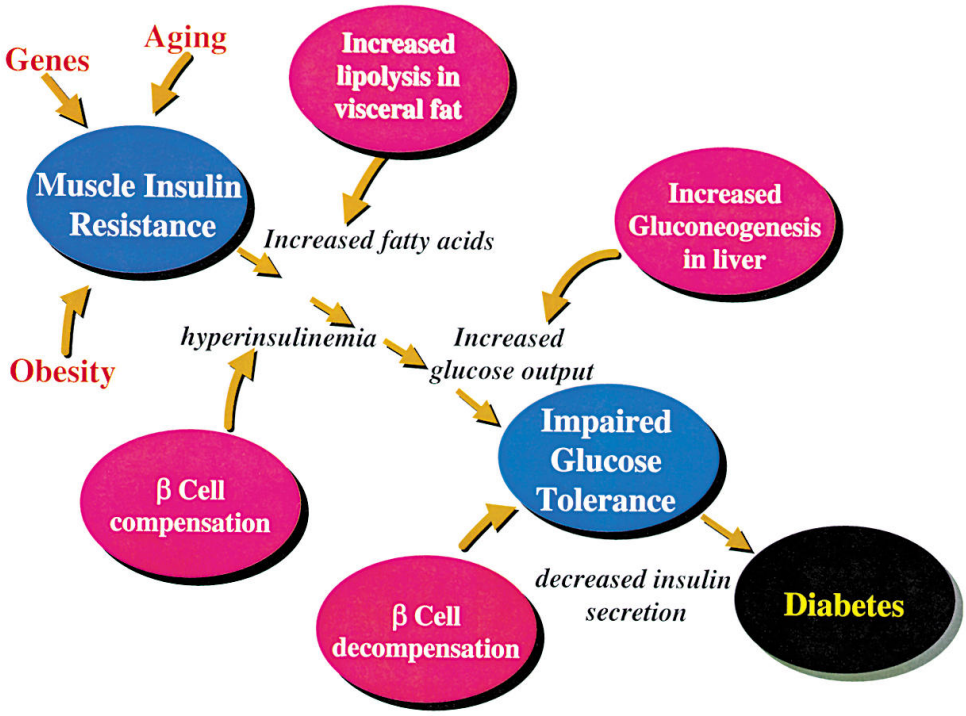

Figure 1-1. Metabolic staging of type II diabetes. Interactions among genetic background, aging, and obesity initiate the peripheral insulin resistance in type II diabetes, followed by pancreatic β-cell dysfunction. Seconday metabolic changes, including increased fatty acid production and compensatory hyperinsulinemia, exacerbates insulin resistance in muscle and liver. The progressive loss of insulin sensitivity eventually results in the failure of insulin secretion, perhaps mediated by hyperglycemia. As insulin secretion can no longer compensate for the insulin resistance, impaired glucose tolerance ensues, leading to full manifestation of diabetes.

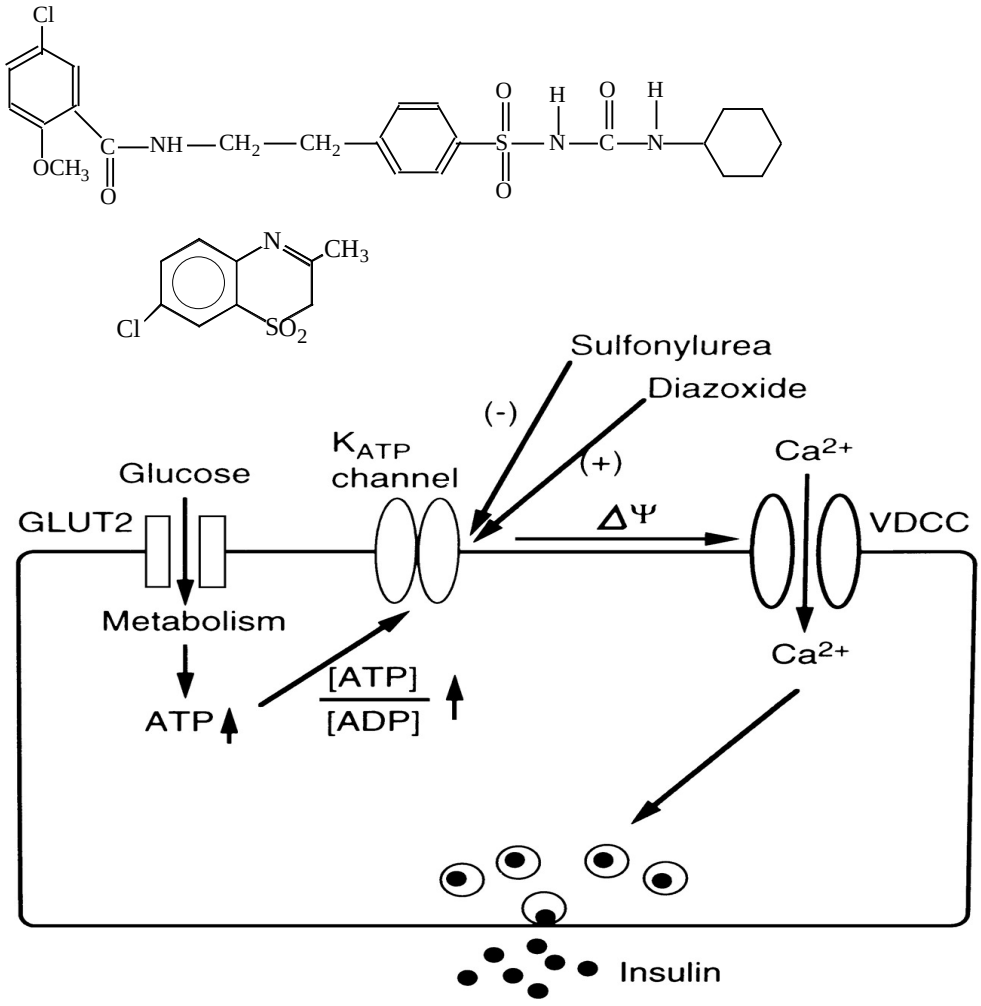

Figure 1-2. The structures and pancreatic actions of Glibenclamide and Diazoxide. Sulfonylureas such as Glibenclamid (Top structure) bind and block the pacreactic K_{ATP} channels which set the resting membrane potential of β-cells, leading to membrane depolarization and the opening of voltage-gated calcium channels (VDCC). The resultant influx of intracellular calcium triggers the exocytosis of insulin. Diazoxide (bottom structure) antagonizes the effect of sulfonyurea by opeening the K_{ATP} channels.

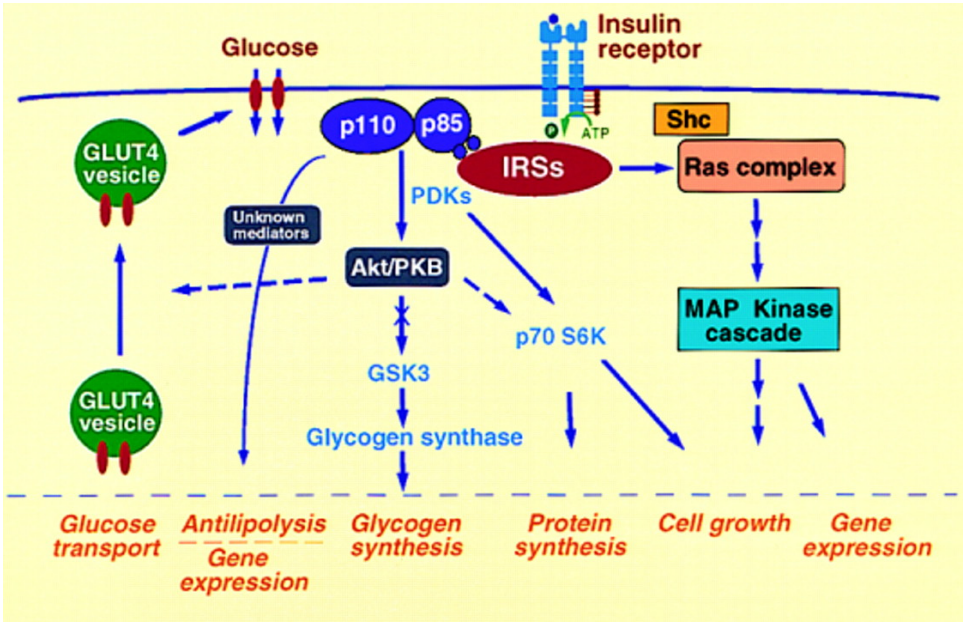

Figure 1-3. Pleiotropic actions of Insulin Signaling. Most metabolic responses of insulin, including stimulation of glucose transport, are mediated by the PI-3K pathway. Specific tyrosine phosphorylation of IRS-1 triggered by insulin binding to its receptors recruit the SH2-domain-containing p85 subunit of PI-3K to the vicinity of plasma membrane. The lipid kinase activity of PI-3K generates IP_3 that activates downstream serine/threonine kinases which in turn elicits specific metabolic responses such as glycogen synthesis and lipogenesis. But the specific signal leading to increased glucose transport is still unknown.

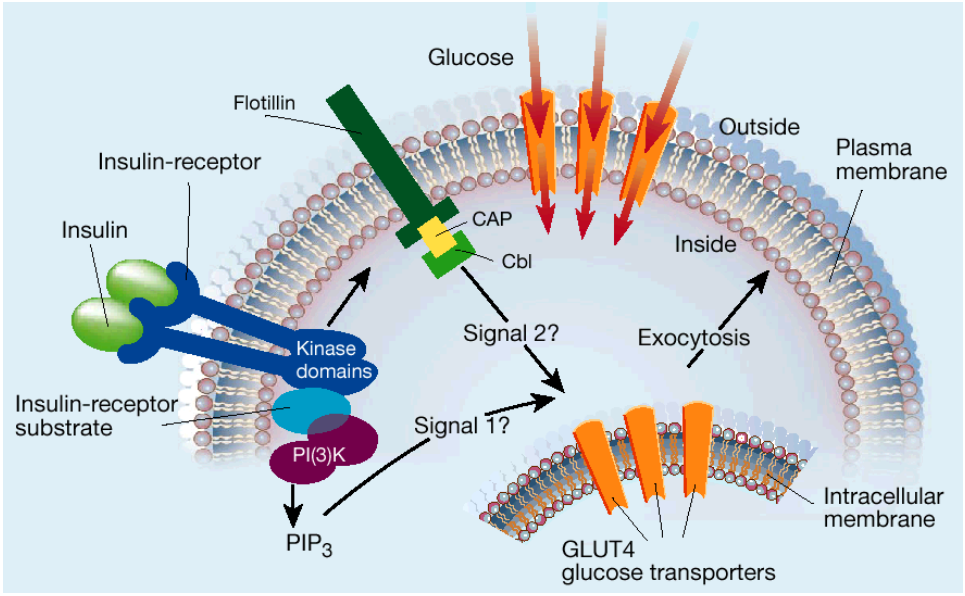

Figure 1-4. Insulin-stimulated glucose transport. Insulin increases glucose transport by stimulating the exocytosis of glut4-containing vesicles from their intracellular sites of sequestration to the plasma membrane. Glut4 is only expressed in skeletal muscle and adipose tissue, tissues that exhibit insulin-stimulated glucose transport.

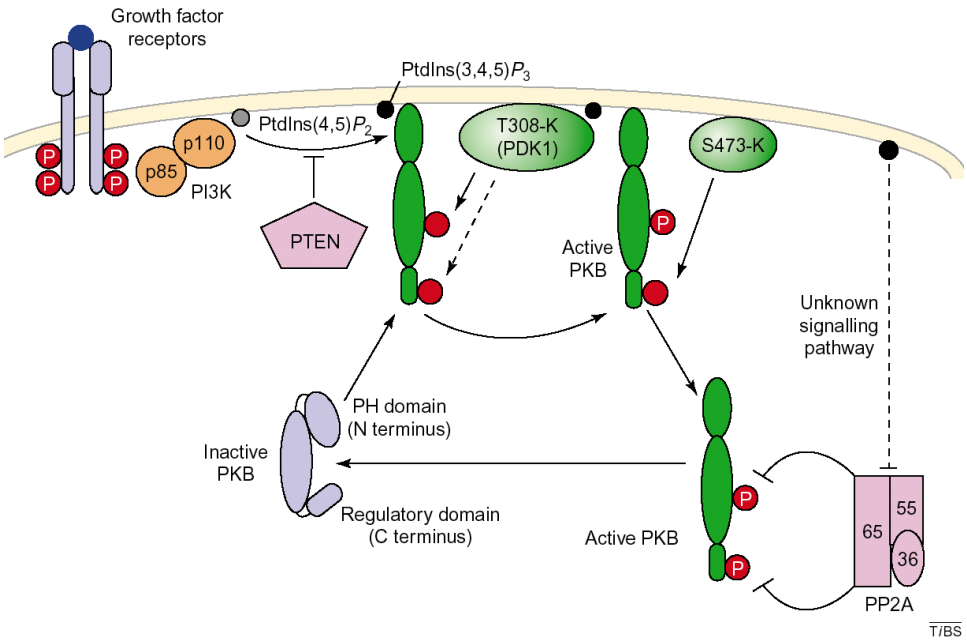

Figure 1-5. Activation of Akt/PKB by Insulin signaling. Phosphorylation at both Thr308 and Ser473 is essential to the activation of Akt/PKB. Insulin activates PI-3K, which generates IP_3 that binds to the pleckstrin homology domain of Akt/PKB and recruits it to the membrane where it can be phosphorylated by PDK1. This lipid binding also results in structural changes that expose the Thr308 and Ser473 to phosphorylation. Although Thr308 is known to be phosphorylated by PDK1, the kinase that phosphorylates Ser473 is still unknown.

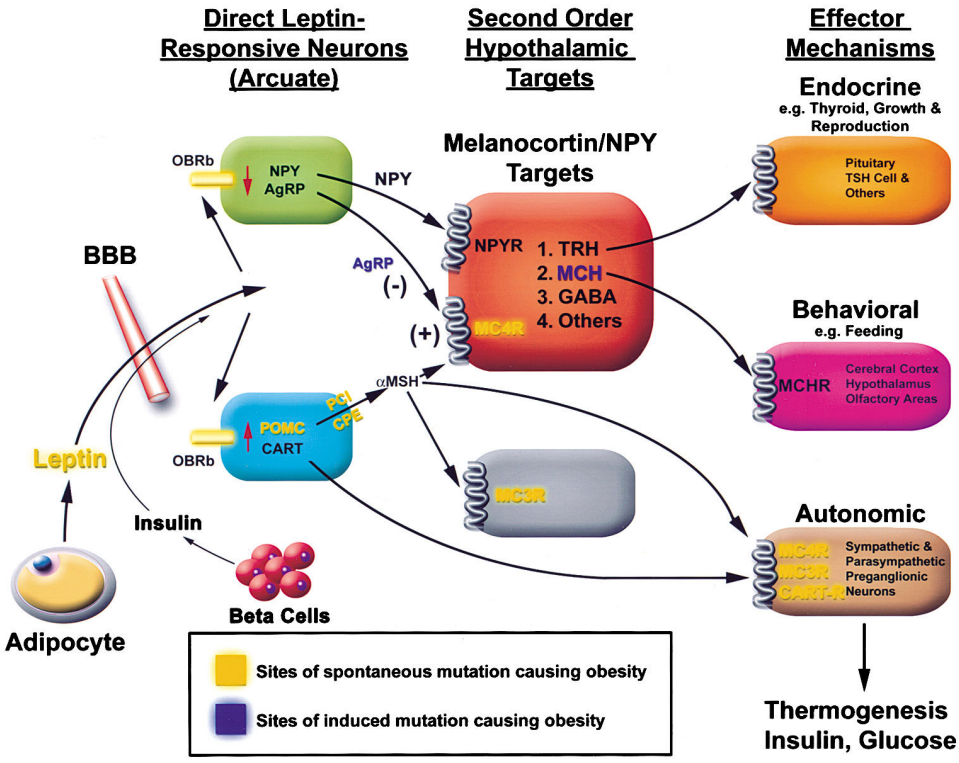

Figure 1-6. Mechanism for the central action of agouti or AGRP.

Ectopic expression of agouti in the hypothalamus mimics the natural action of AGRP. AGRP antagonizes the effects of α-MSH on the centrally expressed MC3R and MC4R, leading to increased feeding behavior and decreased metabolism.

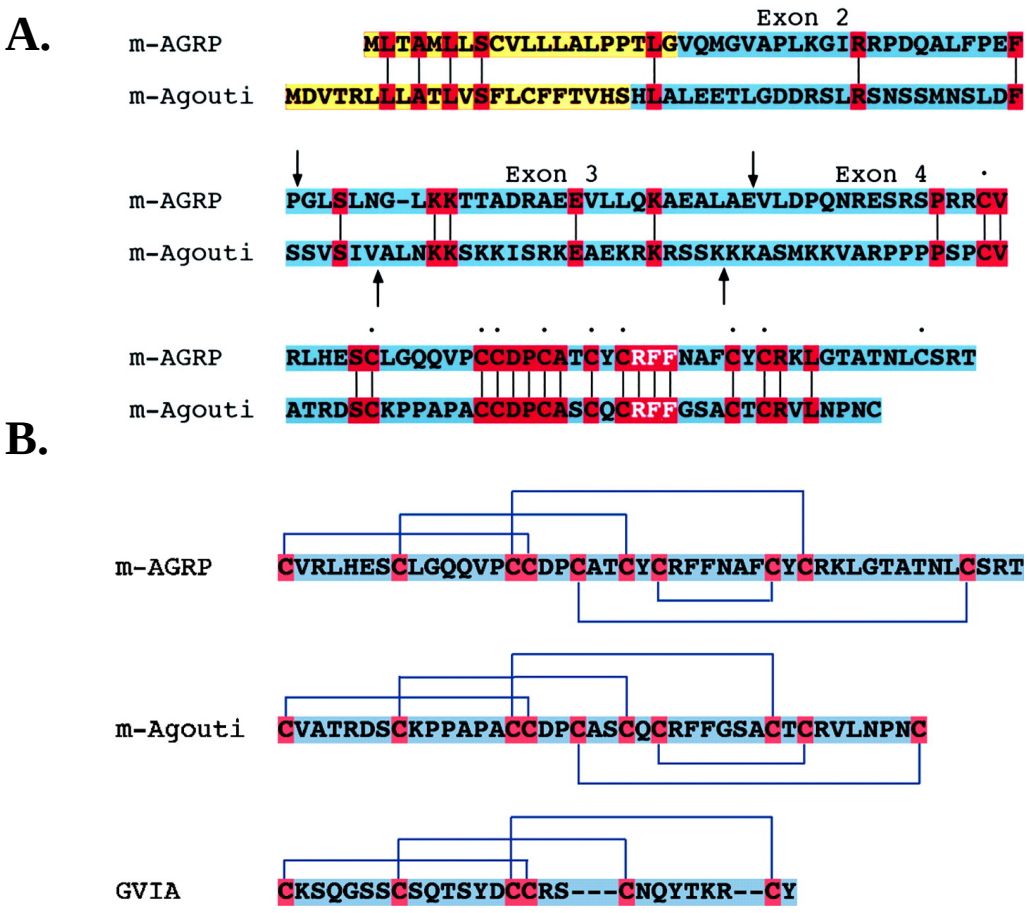

Figure 1-7. Sequence alignment of murine agouti protein and AGRP. A, Agouti and AGRP show 25% identity in amino acid sequence, but the C-terminals of the two proteins exhibits much higher identity. The C-terminal peptide (amino acids 81-131) of AGRP is fully functional as the full length AGRP in terms of antagonizing α-MSH action at the MC4R. **B,** the spatial arrangement of 10 Cys residues in the C-terminals of agouti and AGRP is similar to that in the invertebrate neurotoxin (GVIA), which acts on the calcium channels.

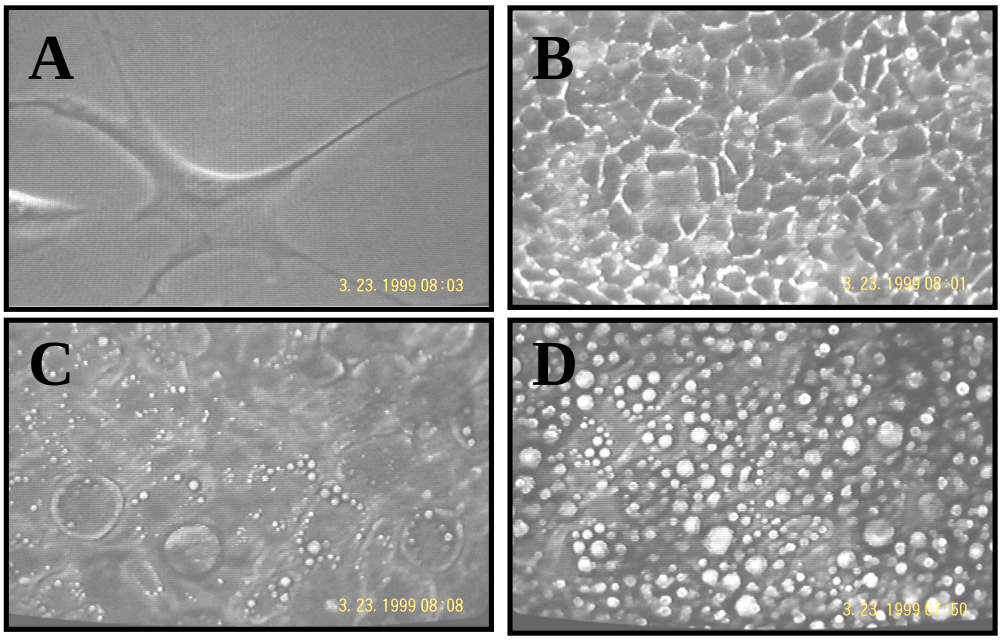

Figure 2-1. Morphology of 3T3-L1 adipocytes during different stages of differentiation. Panel A, Undifferentiated preadipocytes adopt an irregular shape in culture. Panel B, Morphology of KRC7 rat hepatoma cells presented for comparison. Panel C, 4 days after the induction of differentiation, differentiated 3T3-L1 adipocytes adopt a unicentric morphology and begin to accumulate lipid droplets. Panel D, 14 days after induction of differentiation, most 3T3-L1 adipocytes becomes filled with lipid droplets.

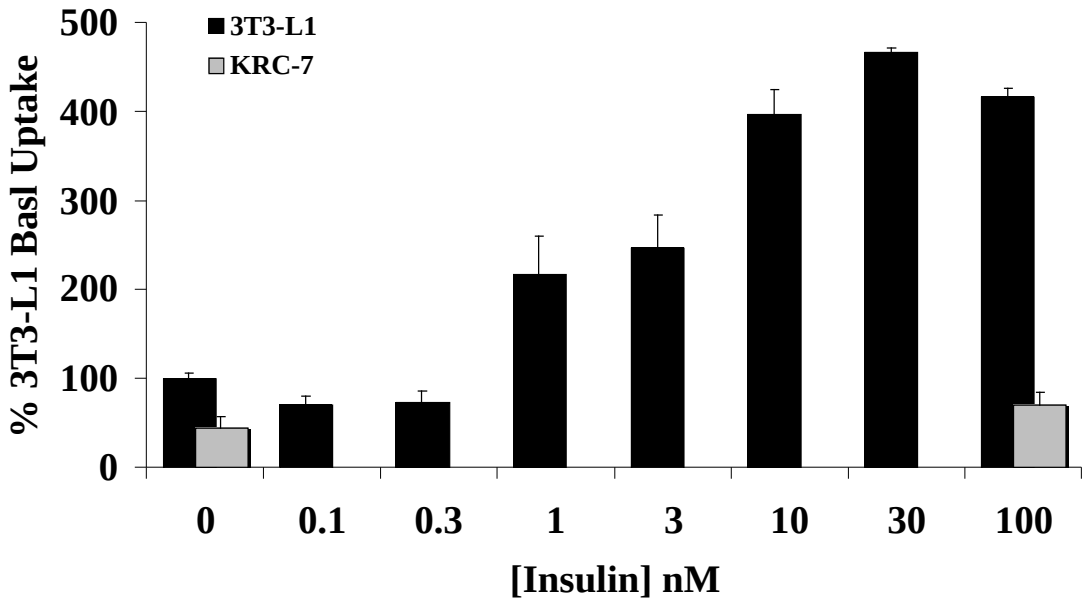

Figure 2-2. 2-deoxyglucose uptake into 3T3-L1 adipocytes and KRC7 hepatoma cells. 2-deoxyglucose transport into 3T3-L1 adipocytes (solid bars) and KRC-7 hepatoma cells (hatched bars) was assayed following a 15-minute exposure to the indicated concentrations of insulin. The cell-associated ^{3}H-radioactivity measured was corrected for non-specific binding (incubation in the presence of 10 μM cytochalasin B) and normalized to the amount of total cellular proteins. The basal uptake of 3T3-L1 adipocytes was set at 100%. Each point represents the mean (± SD) of at least four independent measurements.

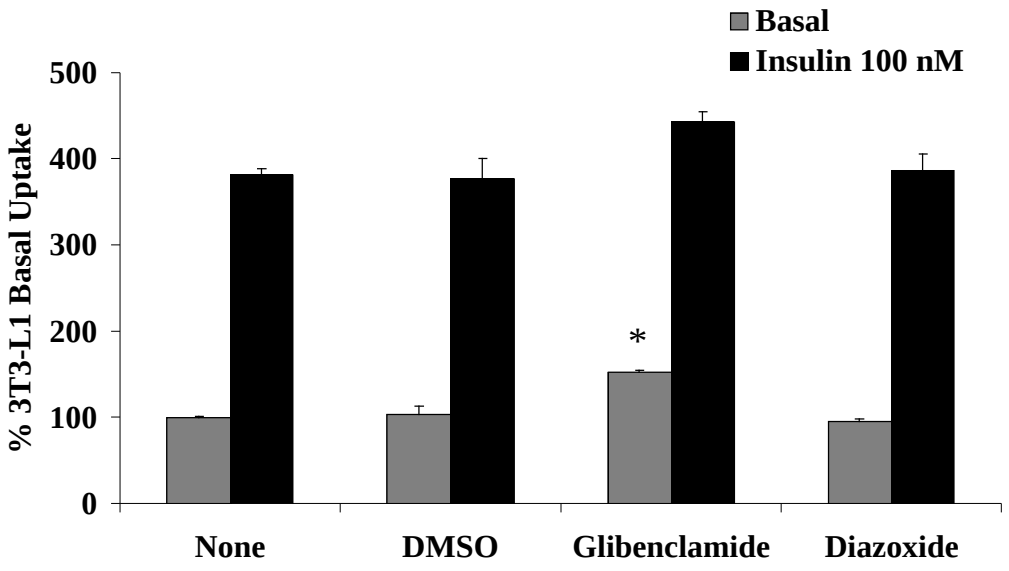

Figure 2-3. Effect of SUR ligands on glucose transport in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated in serum-free medium with the vehicle (0.1% v/v DMSO, the solvent in which the drugs are dissolved), 100 μM glibenclamide, or 100 μM diazoxide for 24 hours. Cells were then washed to remove the drugs. Cell-associated ^{3}H-radioactivity was measured in the Krebs-Ringer-phosphate buffer after another 15 minutes incubation with (solid bar) or without (hatched bar) 100 nM insulin. The results were corrected for non-specific binding and normalized to the total amount of cellular proteins.

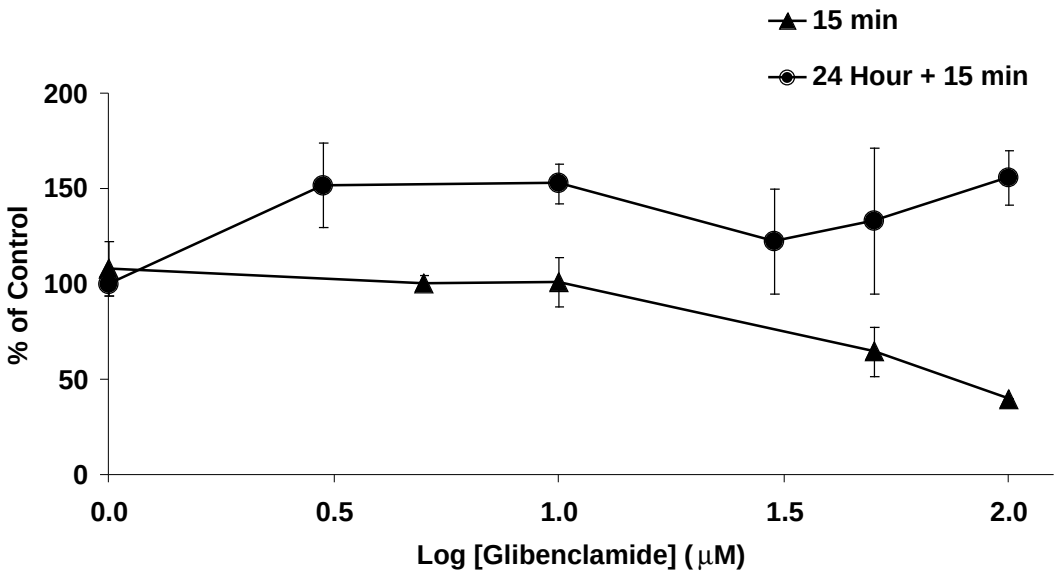

Figure 2-4. The acute and long-term effects of glibenclamide on basal glucose transport into 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with vehicle or the indicated concentration of glibenclamide for 24 hr in serum-free medium before assayed for 2-deoxyglucose uptake. The cell-associated ^{3}H-radioactivity was measured after another 15 min incubation with glibenclamide in the Krebs-Ringer-phosphate buffer. Results were corrected for non-specific binding and normalized to the amount of total cellular proteins. Each point represents the mean (±SD) of at least four independent measurements.

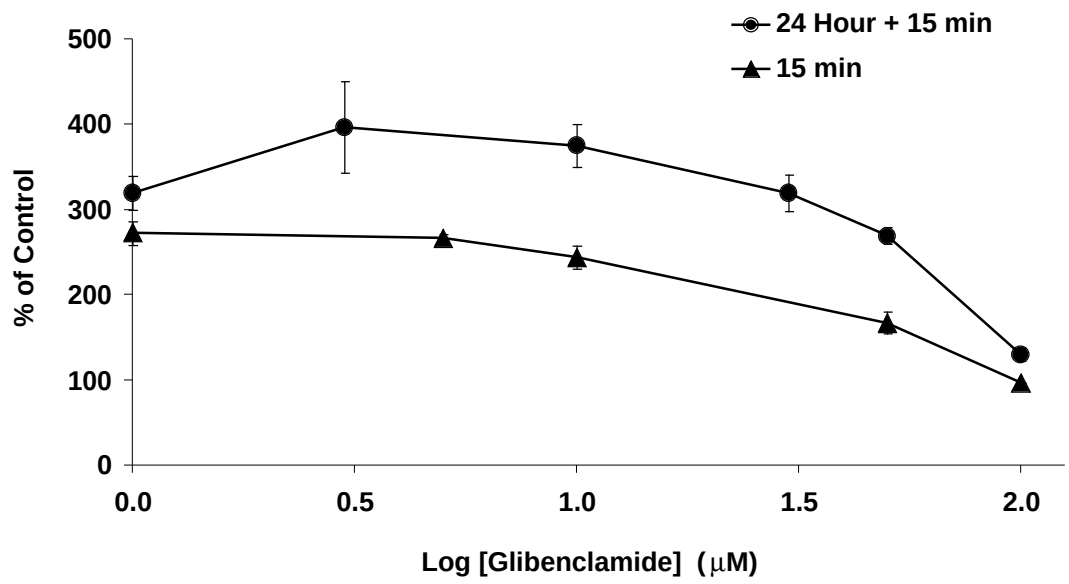

Figure 2-5. The acute and long-term effects of Glibenclamide on insulin stimulated glucose uptake. 3T3-L1 adipocytes were incubated in serum-free medium with the vehicle (DMSO) or glibenclamide at the indicated concentrations for 24 hours prior to the 2-deoxyglucose uptake measurements. Cell-associated ^{3}H-radioactivity was measured in the Krebs-Ringer-phosphate buffer after another 15 minutes incubation with glibenclamide at various concentrations and 100 nM insulin. The results were corrected for non-specific binding and normalized to the total amount of cellular proteins.

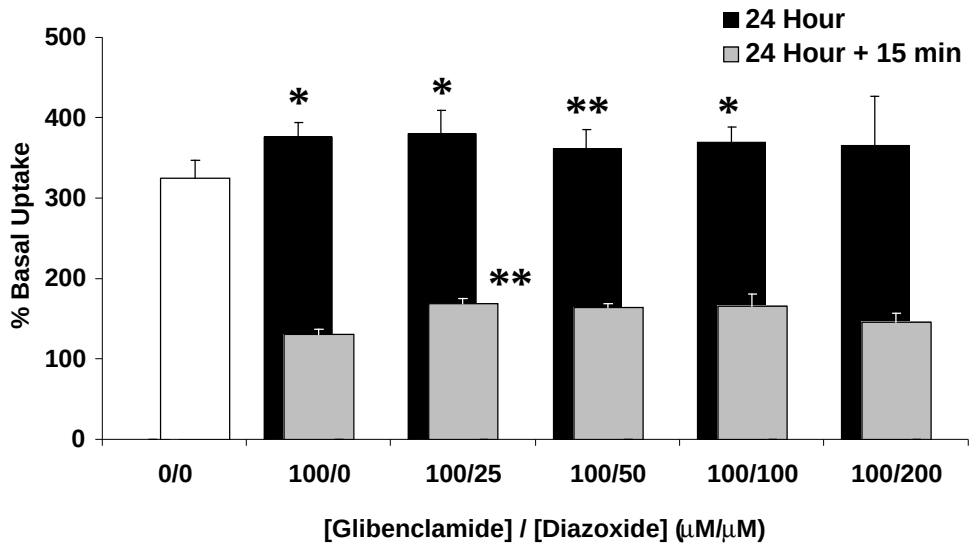

Figure 2-6. Diazoxide does not antagonize the effect of acute or long-term exposure to glibenclamide on insulin-stimulated glucose transport in 3T3-L1 adipocyte. 3T3-L1 adipocytes were incubated with 100 μM glibenclamide and indicated concentrations of diazoxide in serum-free medium for 24 hr. Prior to the 2-deoxyglucose uptake measurements, cells were stimulated with 100 nM insulin for 15 min in the absence of the drugs (24 hr) or in the presence of the drugs (24 hr/ 15 min). Each point represents the mean ($\pm$ SD) of at least four independent measurements.

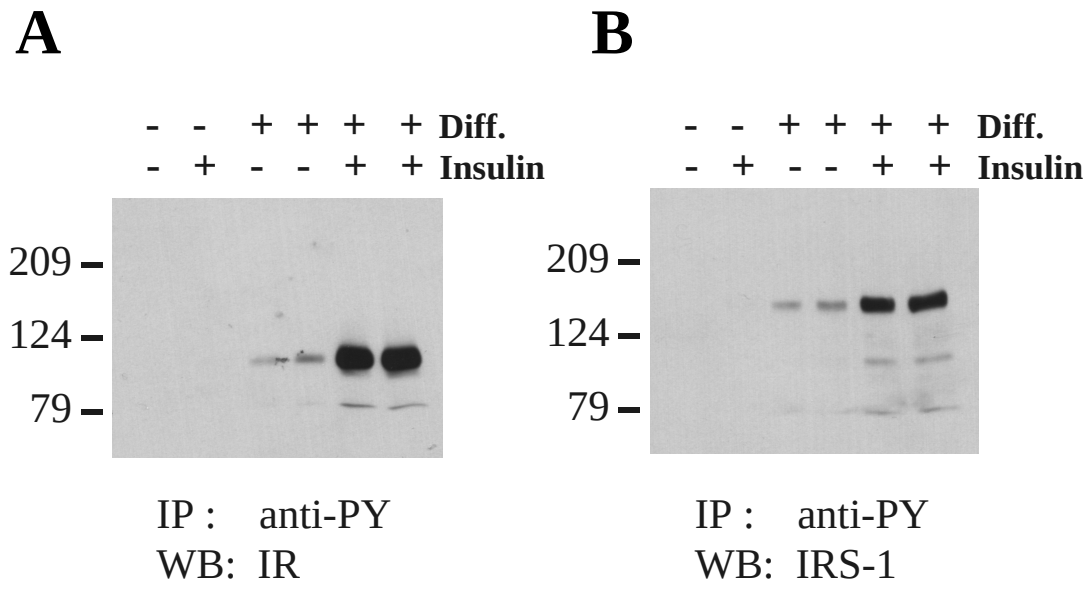

Figure 2-7. Insulin-stimulated tyrosine phosphorylation of IR and IRS-1 in 3T3-L1 fibroblasts and adipocytes. 3T3-L1 fibroblasts (- diff.) and adipocytes (+ diff.) were incubated with or without 100 nM insulin for 10 minutes. Cells were harvested and subjected to immunoprecipitation (IP) with monoclonal anti-phosphotyrosine (anti-PY) antibodies as described in Material and Methods. Immune complexes derived from an equal amount of cell extract protein were resolved by SDS-PAGE and transferred to a PVDF membrane as described in Material and Methods. Panel A, The membrane was probed with anti-IR to detect the intracellular β-subunit of IR in the immunocomplex. Panel *B*, The membrane was probed with anti- IRS-1 to detect the IR receptor substrate in the immunocomplex.

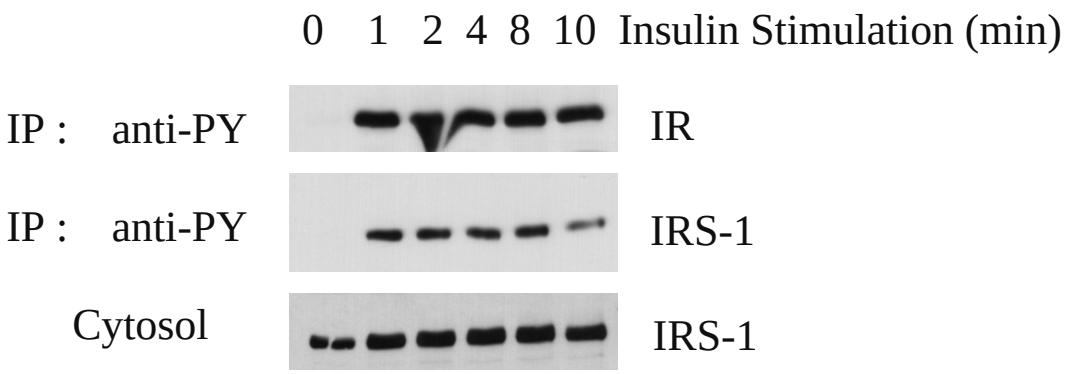

Figure 2-8. Time-course of insulin-stimulated tyrosine phosphorylation on IR and IRS-1. 3T3-L1 adipocytes were serum-starved overnight before incubation with 100 nM insulin for the indicated period of time. Cells were then harvested and subjected to immunoprecipitation (IP) with monoclonal anti-phosphotyrosine (anti-PY) antibodies as described under "Materials and Methods". Western blots were perfomed to detect the phosphotyrosine-containing IR and IRS-1 in the immunocomplexes (upper and middle panels, respectively). In the lower panel, equal amounts of cell extract protein were assessed by western blot to demonstrate the amount of IRS-1 in the cell extract before immunoprecipitation.

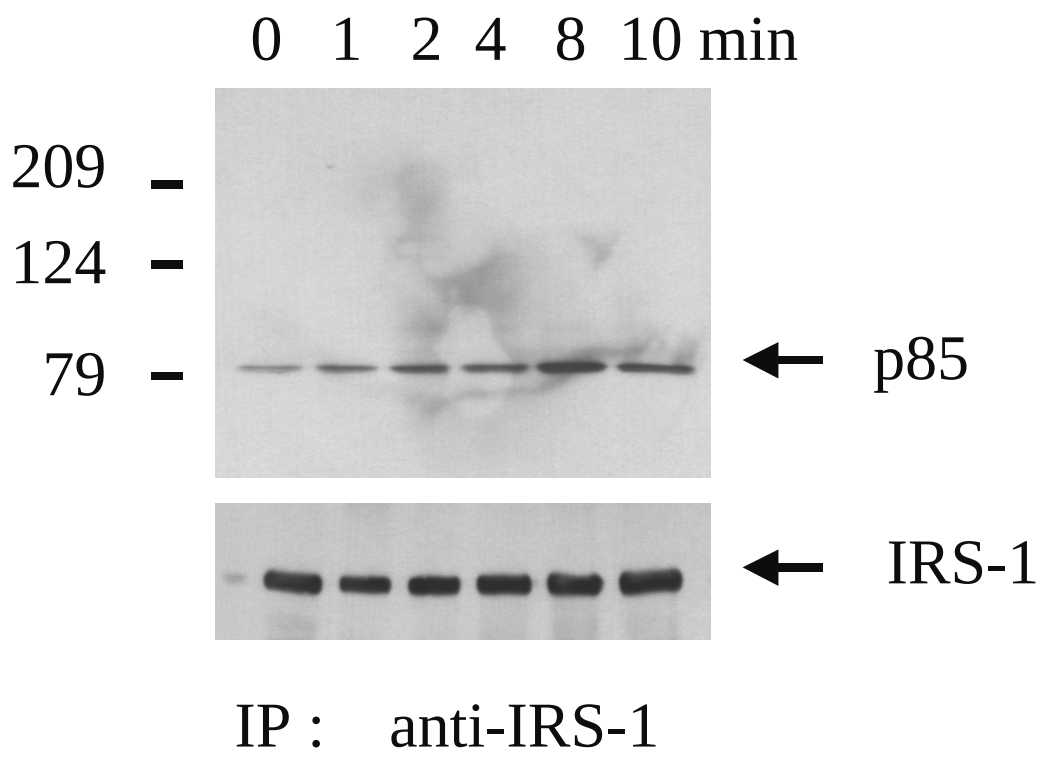

Figure 2-9. Time-course of insulin-induced p85 association with IRS-1. 3T3-L1 adipocytes were serum-starved overnight before incubation with 100 nM insulin for the indicated period of time. Cells were then harvested and subjected to immunoprecipitation (IP) with anti-IRS-1 antiserum as described under “Materials and Methods”. The immunocomplexes derived from equal amounts of cell extract protein were resolved by SDS-PAGE and the p85 subunits of PI-3K were detected by western blots as described in the “Materials and Methods”. In the lower panel, a duplicate western blot of the immunocomplexes probed with anti-IRS-1 antibody demonstrates that there are similar amounts of IRS-1 in the immunocomplexes.

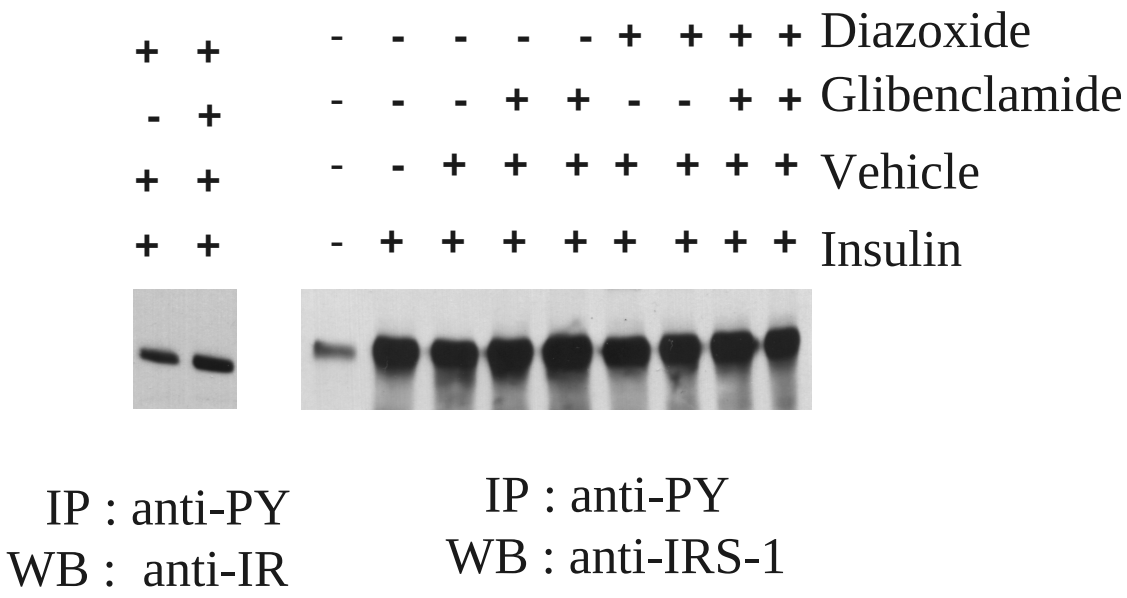

Figure 2-10. Glibenclamide and diazoxide have no effect on insulin-stimulated tyrosine phosphorylations on IR and IRS-1. 3T3-L1 adipocytes were treated with vehicle or 100 μM glibemclamide, or 100 μM diazoxide, or both SUR ligands for 24 hours in the serum-free medium. Cells were incubated for an additional 10 minutes with or without 100 nM insulin. Cells were harvested and equal amounts of cell extract protein were subjected to immunoprecipitation with monoclonal anti-phosphotyrosine (anti-PY) antibodies, followed by western blots (WB) to detect the phosphtyrosine-containing IR (left) or IRS-1 (right).

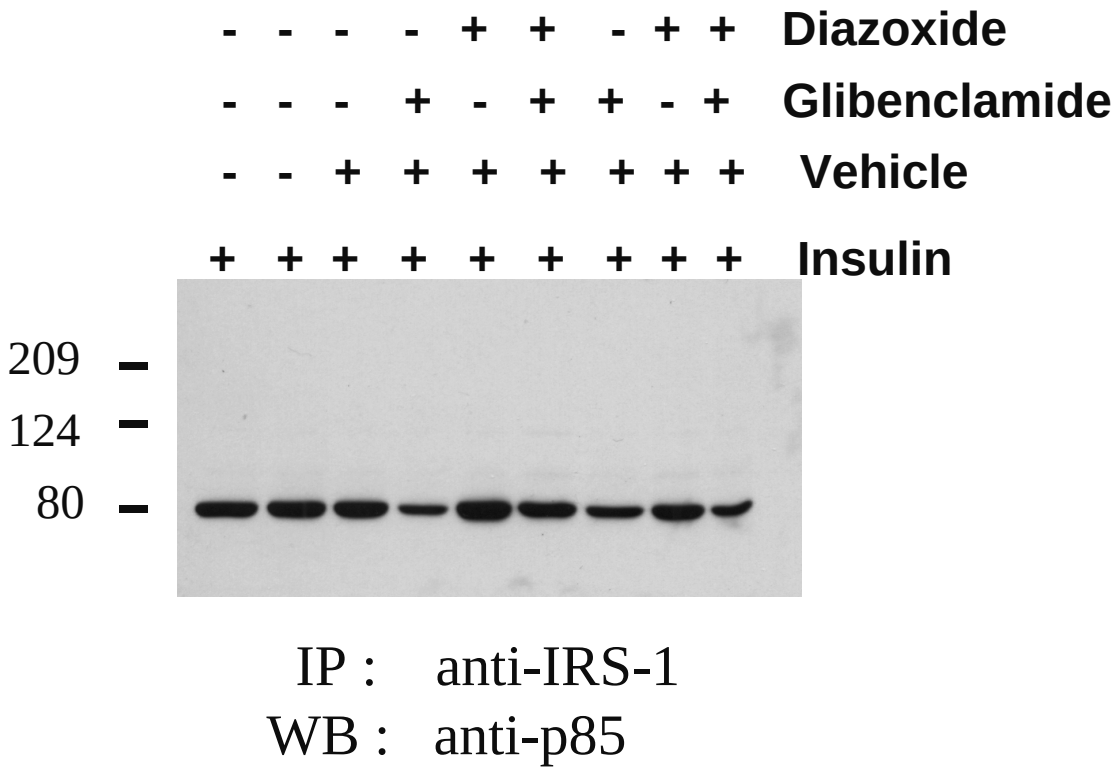

Figure 2-11. The effects of glibenclamide and diazoxide on the insulin-stimulated p85 association with IRS-1. 3T3-L1 adipocytes were incubated with 100 nM insulin for 10 minutes in the presence of vehicle or 100 μM glibemclamide, or 100 μM diazoxide, or both SUR ligands. Cells were harvested and equal amounts of cell extract protein were subjected to immunoprecipitation with anti-IRS-1 antiserum, followed by western blots (WB) to detect the p85 subunits of PI-3K that were associated with the IRS-1.

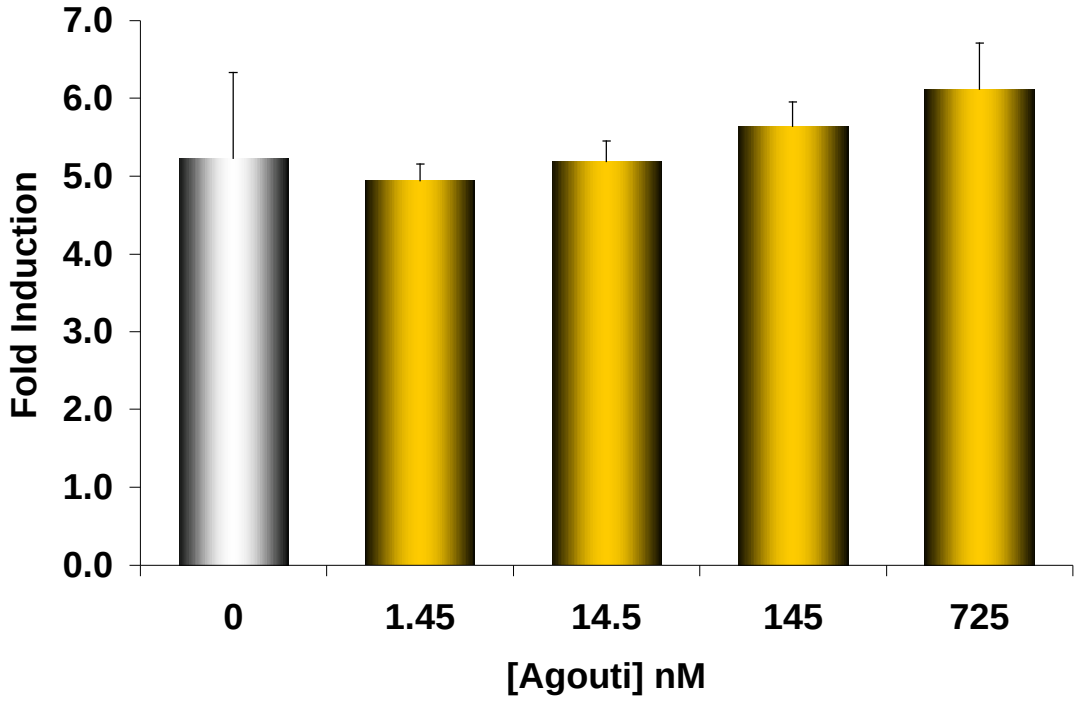

Figure 3-1. The effect of agouti on insulin-stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with 100 nM insulin for 10 min in the presence of various concentrations of agouti protein as indicated above. 2-deoxyglucose uptake was measured and expressed as fold induction over basal uptake. Each treatment represents the average of triplicates (+/- SD) and the experiment was repeated twice. Statistical significance was determined by Student's *t*-test at a confidence level of $P < 0.05$.

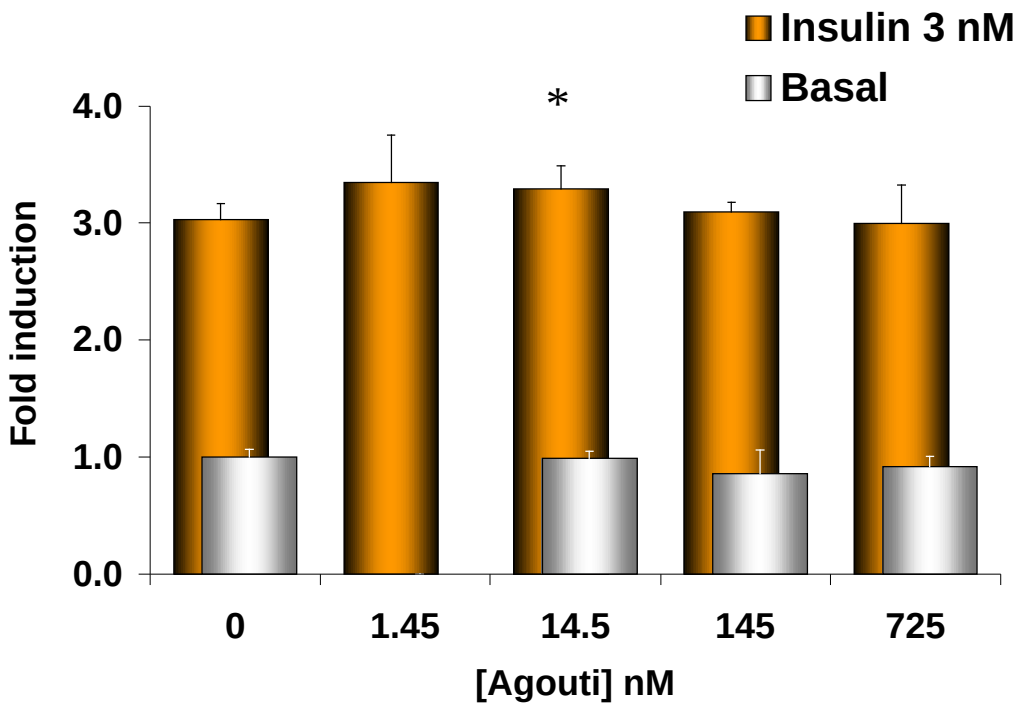

Figure 3-2. Agouti was unable to increase 2-deoxyglucose uptake beyond the submaximal stimulated uptake by 3 nM insulin. 3T3-L1 adipocytes were incubated with or without 3 nM insulin for 10 min in the presence of various concentrations of agouti protein as indicated in the figure above. 2-deoxyglucose uptake was measured and expressed as fold induction over basal uptake. Each treatment represents the average of triplicates (+/- SD) and the experiment was repeated twice. Statistical significance was determined by Student's *t*-test at a confidence level of $P < 0.05$. * ($P < 0.05$).

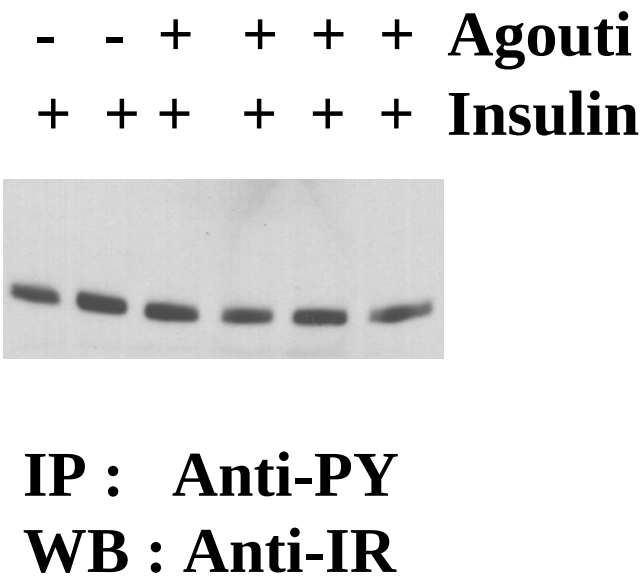

Figure 3-3. Agouti has no effect on the insulin-stimulated tyrosine phosphorylation of IR. 3T3-L1 adipocytes were serum-starved overnight and incubated with 100 nM insulin for 10 min in the presence or absence of 100 nM agouti protein. Cells were harvested and equal amounts of cell extract protein were subjected to immunoprecipitation (IP) with monoclonal anti-phosphotyrosine (anti-PY) antibodies as described in "Materials and Methods". Immunocomplexes were resolved by SDS-PAGE and transferred to a PVDF membrane and probed with polyclonal anti-IR antiserum. The detected IR represents the phosphotyrosine-containing IR.

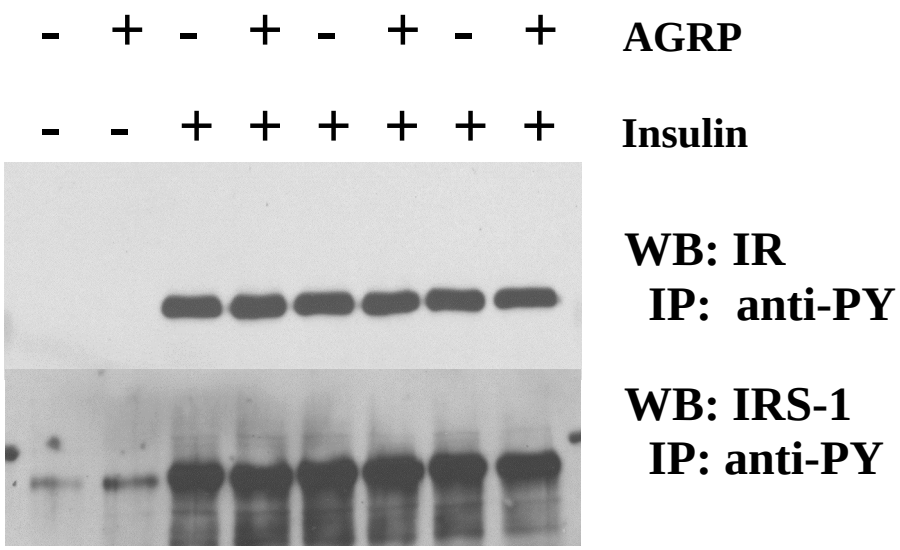

Figure 3-4. AGRP has no effect on the insulin-stimulated tyrosine phosphorylation of IR and IRS-1. 3T3-L1 adipocytes were serum-starved overnight and incubated with or without 100 nM insulin for 10 min in the presence or absence of 100 nM AGRP. Cells were harvested and equal amounts of cell extract proteins were subjected to immunoprecipitation (IP) with monoclonal anti-phosphotyrosine (anti-PY) antibodies as described in "Materials and Methods". Immunocomplexes were resolved by SDS-PAGE and transferred to a PVDF membrane and probed with polyclonal anti-IR (top) or anti-IRS-1 (bottom) antiserum. The detected IR and IRS-1 represents the phosphotyrosine-containing IR and IRS-1.

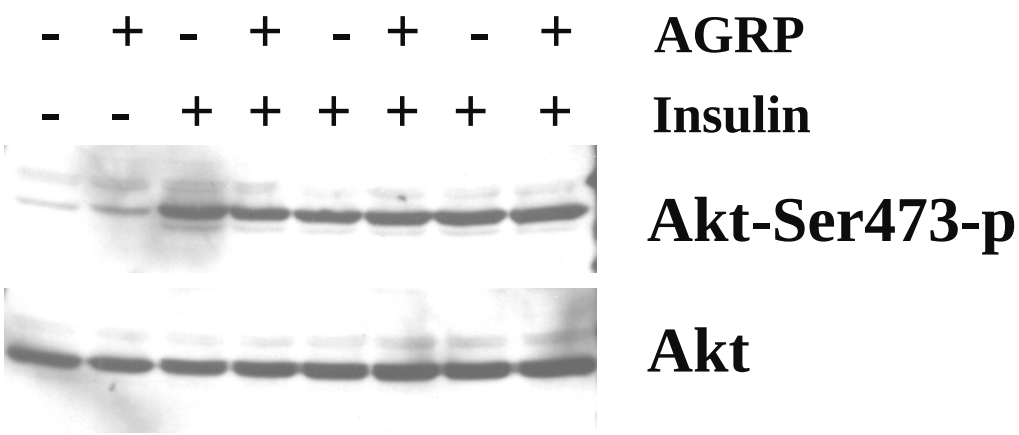

Figure 3-5. Agouti has no effect on the insulin-stimulated activation of Akt/PKB. 3T3-L1 adipocytes were serum-starved overnight and incubated with or without 100 nM insulin for 10 min in the presence or absence of 100 nM AGRP. Cells were harvested and equal amounts of cell extract proteins were resolved by SDS-PAGE and transferred to a PVDF membrane and probed with monoclonal antibodies specific for the Akt/PKB phosphorylated at Ser473 (top) or polyclonal anti-Akt/PKB antiserum (bottom).

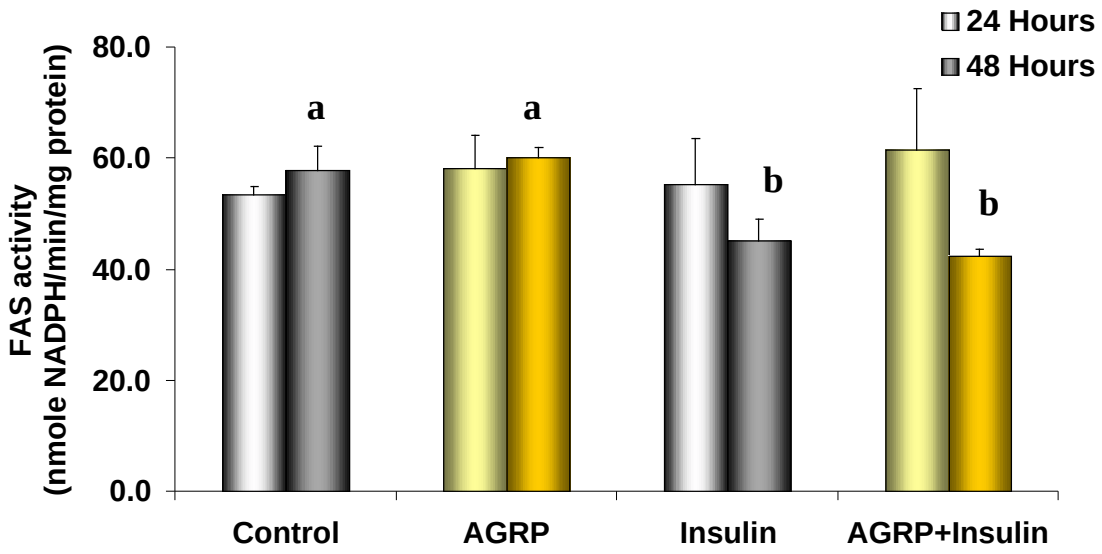

Figure 3-6. The effects of AGRP and insulin on fatty acide synthase (FAS) activity in 3T3-L1 adipocytes (Day 10). 3T3-L1 adipocytes were maintained in culture until 10 days after the induction of differentiation. Cells were then deprived of serum overnight and incubated with or without 100 nM insulin in the presence or absence of 100 nM AGRP for 24 hrs (light colors) or 48 hrs (heavy colors). FAS enzyme activity was assayed as described in "Materials and Methods", and data are expressed as mean (+SD). The figure is representative of two independent experiments, with a minimum of triplicates for each treatment. ^{a,b} $P < 0.05$, groups with different symbols are statistically different from each other.

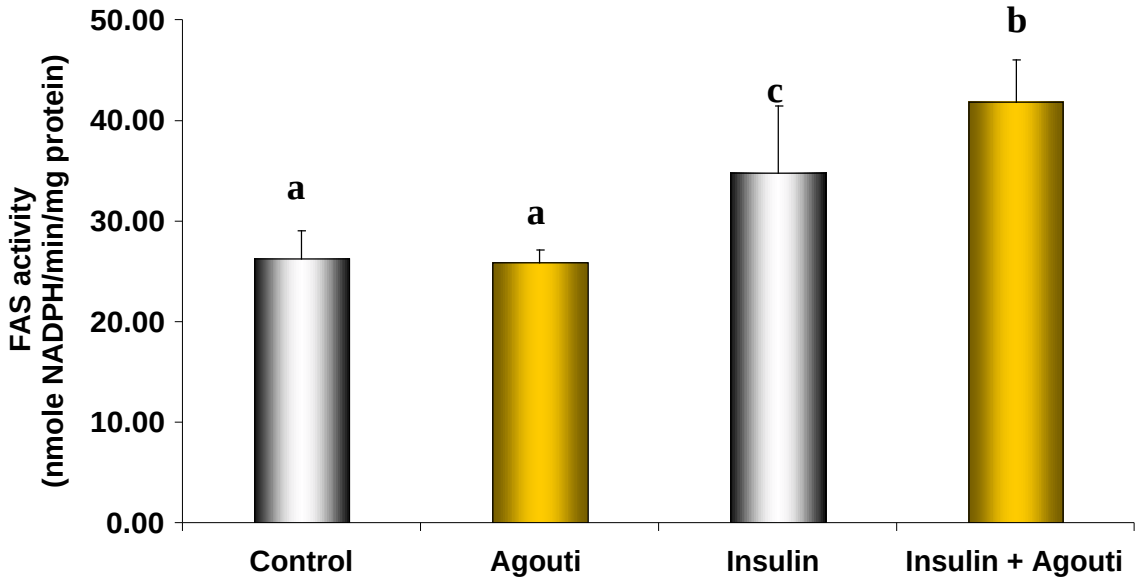

Figure 3-7. The effects of agouti and insulin on fatty acid synthase (FAS) activity in 3T3-L1 adipocytes (Day 8). 3T3-L1 adipocytes were maintained in culture for 8 days after the induction of differentiation. Cells were then deprived of serum overnight and incubated with or without 100 nM insulin in the presence or absence of 100 nM agouti (yellow) for 48 hrs. FAS enzyme activity were assayed as described in "Materials and Methods", and data were expressed as mean (± SD). The figure is representative of two independent experiments, with a minimum of triplicates for each treatment. Groups with different symbols are statistically different from each other. ^{a,b} $P < 0.01$, ^{b,c} $P < 0.05$, $0.05 < {}^{a,c} P < 0.1$.

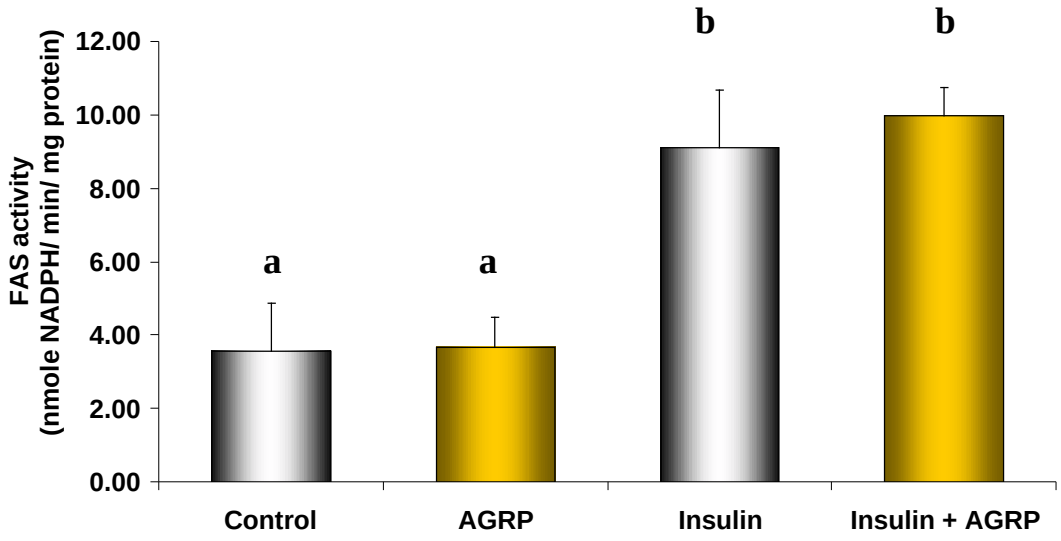

Figure 3-8. The effects of AGRP and insulin on fatty acide synthase (FAS) activity in 3T3-L1 adipocytes (Day 6). 3T3-L1 adipocytes were maintined in culture until 6 days after the induction of differentiation. Cells were then deprived of serum overnight and incubated with or without 100 nM insulin in the presence or absence of 100 nM AGRP (yellow) for 48 hrs. FAS enzyme activity was assayed as described in "Materials and Methods", and data were expressed as mean (± SD). The result is representative of two independent experiments, with a minimum of triplicates for each treatment. [a,b] $P < 0.05$, groups with different symbols are statistically different from each other.

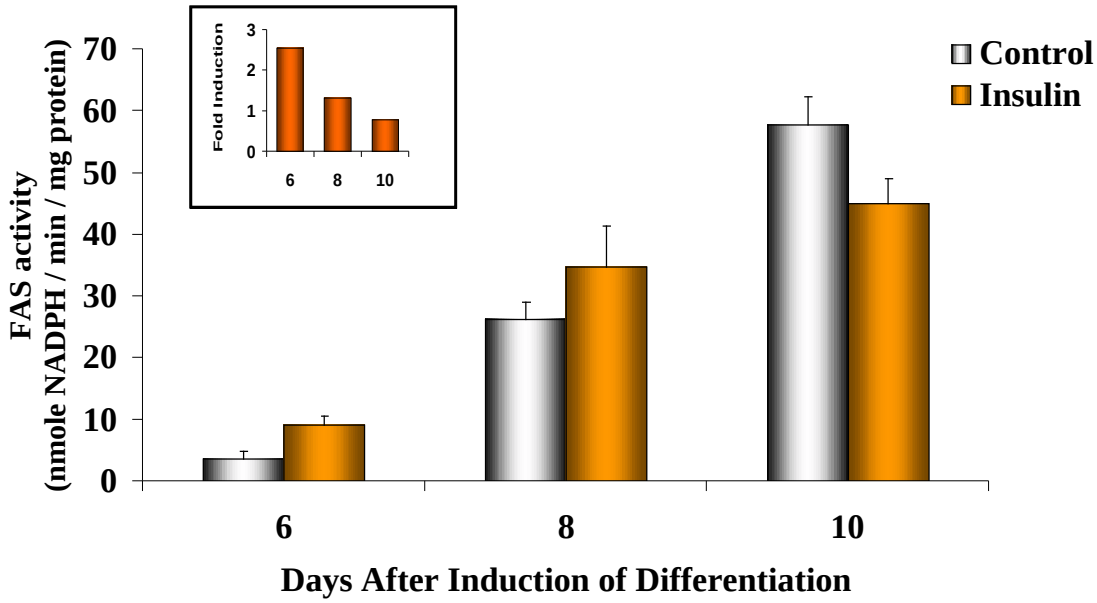

Figure 3-9. The relationship between adipocyte differentiation and insulin induction of FAS activity. The basal and insulin-stimulated FAS activities in 3T3-L1 adipocytes presented in Figures 3-6, 3-7 and 3-8 were plotted against the length of time that cells stayed in culture after the induction of differentiation. Insert, fold induction by insulin was calculated and plotted against the length of time that cells stayed in culture after the induction of differentiation.

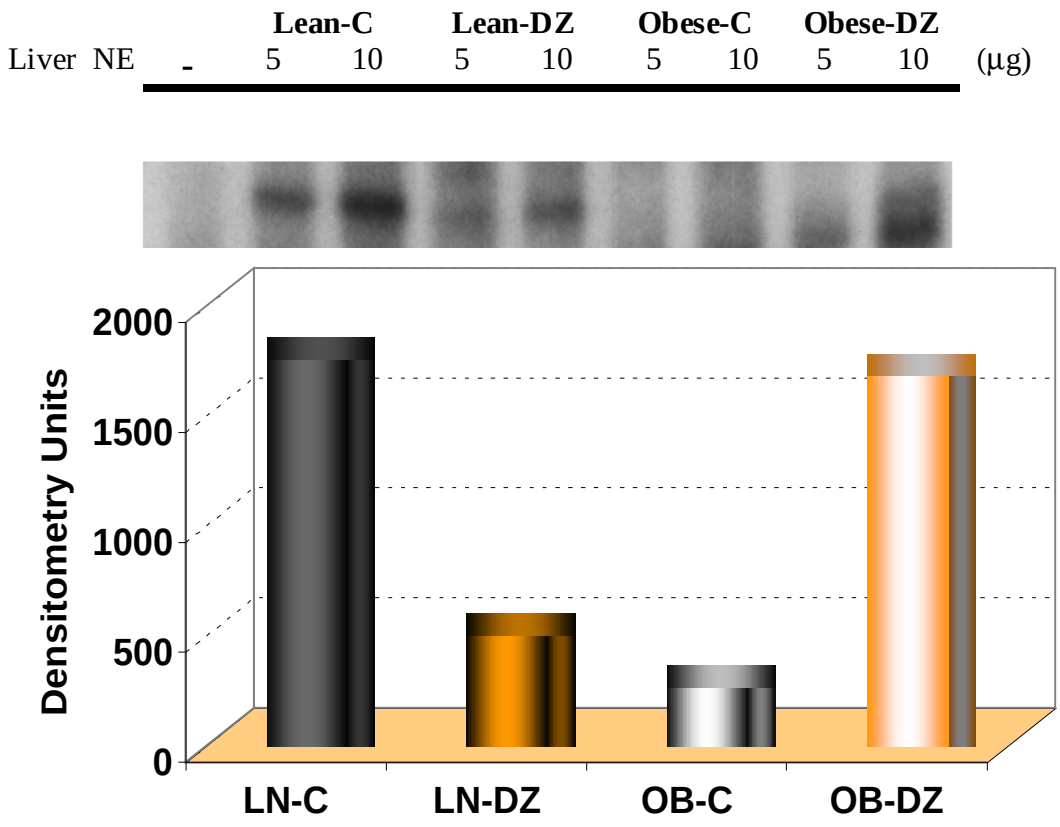

Figure 4-1. HNF-1 binding activity was sharply reduced in the hepatonuclear extracts of Obese Zucker rats. Top, a representative autoradiogram of electrophoretic mobility shift assays (EMSA) obtained with nuclear extract protein prepared from livers of lean (LN) and obese (OB) Zucker rats treated or untreated with diazoxide (DZ). Bottom, densitometry analysis of the autoradiogram shown above. Preparation of nuclear extracts, EMSA, and densitometry analysis were performed as describes in “Materials and Methods”.

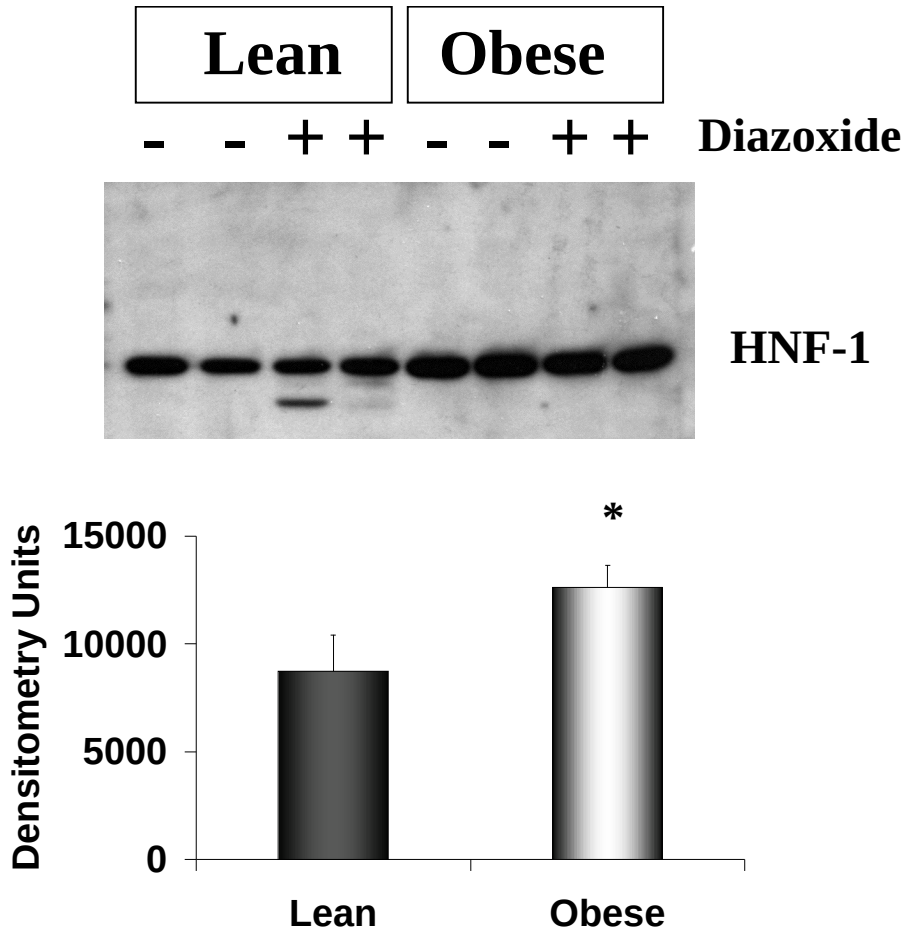

Figure 4-2. HNF-1 protein levels in livers of lean and obese Zucker rats. Levels of HNF-1 proteins in the heptonuclear extracts prepared from the lean and obese animals were compared by western blot analysis using anti-HNF-1 antiserum. Top, equal amounts of nuclear extracts from four lean and four obese animals were resolved by SDS-PAGE, transferred to a PVDF membrane and probed with anti-HNF-1 antiserum. Bottom, densitometry analysis of the autoradiogram shown above. Western blot and video densitometry were performed as described in "Materials and Methods". * $P < 0.05$.

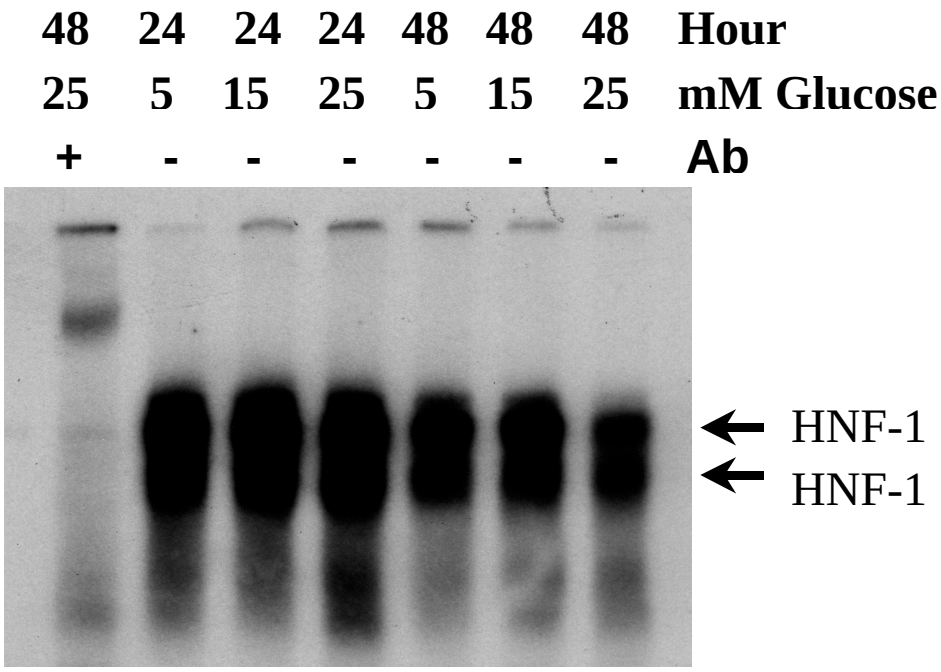

Figure 4-3. Supershift of HNF-1-containing DNA-protein complexes with anti-HNF-1 antiserum. Fao rat hepatoma cells were deprived of serum overnight and incubated in the media containing 5-25 mM glucose for the indicated period of time. Nuclear extracts were prepared and incubated with a radiolabeled probe containing the HNF-1 binding sites. After 1 hr incubation at room temperature, anti-HNF-1 antiserum (Ab) was added to the binding reaction, followed by EMSA as described in "Materials and Methods".

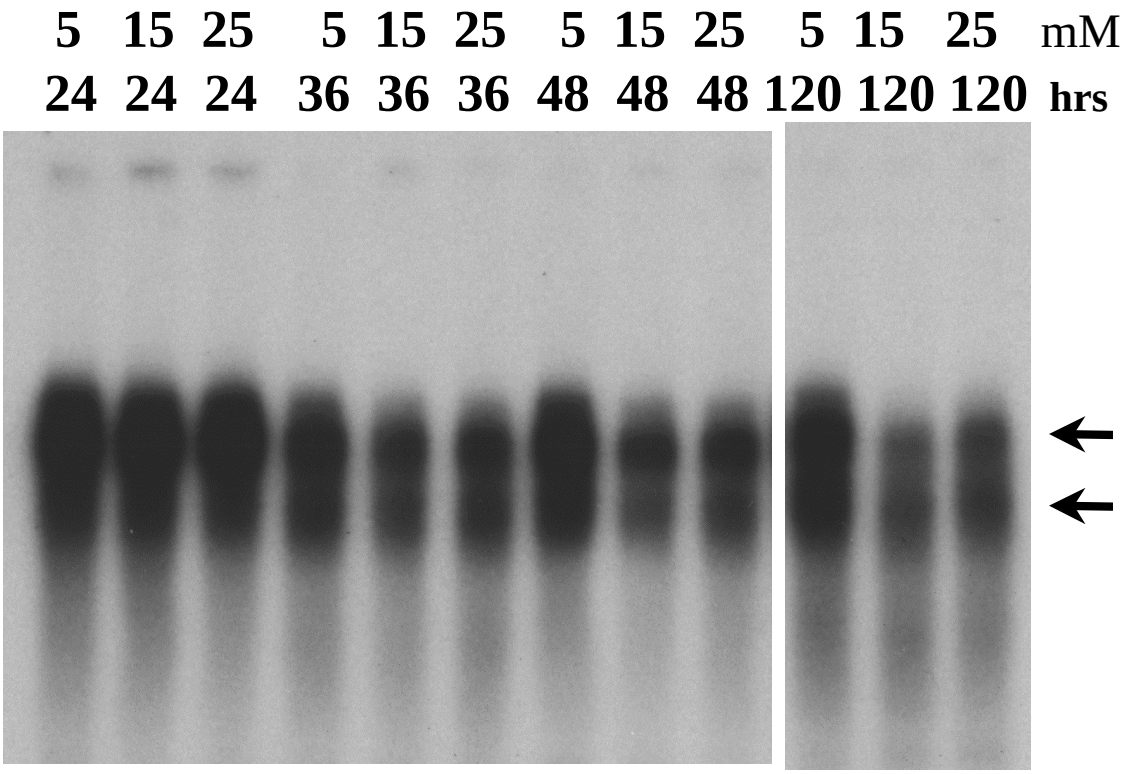

Figure 4-4. Effect of glucose on HNF-1 binding activity in Fao rat hepatoma cells. Fao cells were grown to about 80% confluency and deprived of serum overnight. Cells were then incubated for 24-120 hrs with DMEM containing glucose of indicated concentrations and appropriate concentrations of xylose to equalize osmolarity. Nuclear extracts were prepared and subjected to EMSA as described in "Materials and Methods". A representative autoradiogram of EMSA is shown with protein-DNA complexes containing HNF-1 are indicated by the arrowheads.

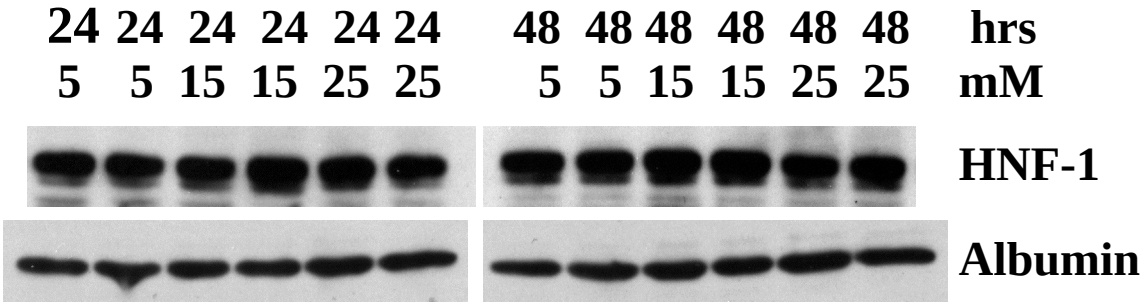

Figure 4-5. HNF-1 and albumin protein levels were not affected by glucose. Fao cells were grown to about 80% confluency and deprived of serum overnight. Cells were then incubated for 24-48 hrs with DMEM containing glucose of indicated concentrations and appropriate concentrations of xylose to equalize osmolarity. Cytosolic proteins and nuclear extracts were prepared and subjected to western blot analysis as described in "Materials and Methods". Equal amounts of nuclear protein (top) or cytosolic protein (bottom) were loaded on each lane and resolved by SDS-PAGE, followed by transfer to a PVDF membrane and probed with antiserum against HNF-1 and albumin, respectively.

VITA

To-Yu Huang was born in Lotung, Taiwan in September 7, 1969. He lived with his family and attended local elementary and high schools until going to college in 1987. In 1991, he earned his bachelor degree in science with a major in Chemistry and a minor in Foreign Language and Literature from the National Sun-Yat-Sen University in Kaoshiung, Taiwan. He then served as a reserve officer in the Marine Corp. for two years before he came to the United States in 1993 to continue graduate study as biochemistry major in the University of Tennessee at Knoxville. He later earned his master degree in science in 1997, and was awarded the doctor of philosophy degree with a major in Biochemistry, Cellular and Molecular Biology in 2002.