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Regulatory Effects of Leptin on Insulin Secretion Target Phospholipase C-Protein Kinase C, But Not Protein Kinase A, Signal Transduction in Islets from Neonatal Mice

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REGULATORY EFFECTS OF LEPTIN ON INSULIN SECRETION TARGET PHOSPHOLIPASE C-PROTEIN KINASE C, BUT NOT PROTEIN KINASE A, SIGNAL TRANSDUCTION IN ISLETS FROM NEONATAL MICE

By

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ABSTRACT

REGULATORY EFFECTS OF LEPTIN ON INSULIN SECRETION TARGET PHOSPHOLIPASE C-PROTEIN KINASE C, BUT NOT PROTEIN KINASE A, SIGNAL TRANSDUCTION IN ISLETS FROM NEONATAL MICE

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Leptin-deficient Lep^{ob}/Lep^{ob} mice develop hyperinsulinemia early in life, before they begin to overeat or develop insulin resistance. Leptin directly acts within pancreatic islets to inhibit insulin secretion. This leptin function provides a potential mechanism to prevent hyperinsulinemia. The present studies were undertaken to better understand the cause of the early-onset hyperinsulinemia in Lep^{ob}/Lep^{ob} mice, and the role of leptin in preventing hyperinsulinemia.

I determined when leptin-deficient mice first hypersecrete insulin in response to acetylcholine. The relative hypersecretion of insulin from islets of leptin-deficient mice first occurred between 1 and 2 weeks of age. Addition of leptin to islets isolated from 4 day, 2 week, and 4-week-old leptin-deficient mice rapidly (i.e., within 30 min) suppressed acetylcholine-induced insulin secretion at each stage of development, while islets from 4 day, 2 week and 4-week-old leptin-sufficient mice became progressively less responsive to leptin with development.

Leptin has been reported to activate phosphatidylinositol 3-kinase (PI 3-K) and subsequently phosphodiesterase (PDE) to impair protein kinase A (PKA)-induced insulin secretion from cultured islets of neonatal rats. I thus determined if PKA-induced insulin secretion was altered in islets from Lep^{ob}/Lep^{ob} mice, and if leptin affected this

pathway in islets from these mice. Incubating islets with GLP-1, forskolin (an activator of adenylyl cyclase), or IBMX (an inhibitor of PDE) did not cause hypersecretion of insulin from islets of young Lep^{ob}/Lep^{ob} mice, and GLP-1-induced insulin secretion was not inhibited by leptin. I conclude that PKA-induced insulin secretion is not altered in young Lep^{ob}/Lep^{ob} mice.

I also determined the possible role for PI 3-K in leptin-induced control of acetylcholine-induced insulin secretion. Preincubation of islets with wortmannin, an inhibitor of PI 3-K, blocked the ability of leptin to constrain acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice. I conclude that leptin may activate PI 3-K to constrain PLC-PKC-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice.

Finally, I determined the effects of chronic administration of leptin to Lep^{ob}/Lep^{ob} mice on the regulation of insulin secretion from their isolated islets. Administration of leptin to young adult Lep^{ob}/Lep^{ob} mice for 8 days normalized their food intake, plasma insulin concentration, and *in vitro* rates of insulin secretion from islets in response to glucose, acetylcholine, and leptin. Food restriction lowered, but did not normalize, plasma insulin concentrations in Lep^{ob}/Lep^{ob} mice. These mice continued to hyperrespond to glucose, but not to acetylcholine or leptin, as occurs in *ad libitum* fed Lep^{ob}/Lep^{ob} mice. Leptin thus functions via food intake dependent and independent mechanisms to regulate insulin secretion

In conclusion, leptin activates a PI 3-K dependent pathway to specifically regulate PLC-PKC-induced insulin secretion. This action of leptin is required to prevent development of hyperinsulinemia early in life of leptin-deficient Lep^{ob}/Lep^{ob} mice.

Dedicated to my mother, Seokjoo Kang Seunim, and my mentor, Dr. Dale Romsos with my deepest respect and admiration from whole my heart

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LIST OF ABBREVIATIONS

AC..... Adenylyl cyclase

ACH.....Acetylcholine

ATP.....Adenosine triphosphate

cAMP.....3', 5'-cyclic adenosine monophosphate

CCK.....Cholecystokinin

CRH.....Corticotropin releasing hormone

DAG..... Diacylglycerol

FA-CoA.....Fatty acyl-CoA

G.....G-protein

GLP-1..... Glucagon like peptide-1

R.....Receptor

INS-R.....Insulin receptor

IBMX.....3-isobutyl-1-methylxanthine

IP.....Intraperitoneal injection

IV.....Intravenous injection

IP_3.....Inositol triphosphate

IRS-1/-2..... Insulin receptor substrate-1/-2

JAK.....Janus kinase

K^+_{ATP}.....ATP-sensitive potassium channel

MAPK.....Mitogen activated protein kinase

NPY.....Neuropeptide Y

LSD.....Least significant difference

OB-R.....Leptin receptor

P.....Phosphate

PDE.....Phosphodiesterase

PI 3-K.....Phosphatidylinositol 3-kinase

PIP_2.....Phosphatidylinositol 4, 5, bisphosphate

PKC.....Protein kinase C

PLC.....Phospholipase C

PMA.....Phorbol-12-myristrate-13-acetate

SOCS-3.....Suppressors of cytokine signaling

STAT.....Signal transducers and activators of transcription

Y.....Tyorosine

CHAPTER I. INTRODUCTION

The increasing incidence of chronic diseases, including diabetic mellitus and heart disease in humans, illustrates the importance of more research to better understand the major causes of these diseases, to suggest approaches to prevent these diseases, and to provide proper medications and appropriate public health guidance for reducing the morbidity and mortality of these diseases. Since it has been suggested that obesity is a risk factor for these chronic diseases, understanding the causes of obesity should contribute to improved public health.

Obesity causes multiple physiological alterations including changes in the pancreatic endocrine system. Malfunctions in homeostasis of this endocrine system lead to abnormal physiological phenotypes, such as hyperinsulinemia, which disturb the control of body fuel metabolism. Hyperinsulinemia, which precedes insulin resistance (Dubuc, 1976), has been suggested as a common early-onset abnormal metabolic reaction in obesity (Chen and Romsos, 1995; Chen and Romsos, 1997). The cause of hyperinsulinemia in obesity is not clear, but it is assumed that hypersecretion of insulin is involved.

Islets function as a fuel sensor to integrate signals for many nutrients as well as a modulator of insulin secretion according to the demand of organisms. The insulin secretory responses from islets are finely controlled by nutritional and endocrine factors, and by neurotransmitters (Zawalich and Rasmussen, 1990) although the specific mechanisms are not totally understood. Glucose is a main nutritional secretogogue

capable of stimulating insulin release alone (Fig. 1). Glucose metabolism increases intracellular ATP, which leads to closing the ATP-sensitive potassium channel (K^+_{ATP}) in the β-cells. Closure of the K^+_{ATP} channel produces cellular depolarization leading to activation of the voltage-dependent calcium channels (VDCCs), which subsequently increases intracellular Ca^{2+} concentration and triggers exocytosis of insulin (Prentki and Matshinsky, 1987; Zawalich and Rasmussen, 1990).

Gastrointestinal peptides such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), and neurotransmitters such as acetylcholine (Ach) and cholecystokinin (CCK) potentiate glucose-induced insulin secretion (Fig. 1). These endocrine compounds normally do not induce insulin secretion from pancreatic islets when glucose is absent (Zawalich and Rasmussen, 1990). Binding of these hormones and neurotransmitters to their specific receptors on β-cells mediates the stimulatory effects of insulin secretion via post-receptor pathways linked to the protein kinase A (PKA) (Holz and Habener, 1992; Holz et al, 1995) or the phospholipase C-protein kinase C (PLC-PKC) signal transduction systems (Zawalich et al, 1995; Zawalich and Zawalich, 1996).

The recent critical discovery of "leptin", a peptide hormone produced and secreted by mature white adiposites, provide obesity researchers new directions to explore. It is now clear that obese syndromes in ob/ob mice are caused by a deficiency in leptin synthesis, which is due to a nonsense mutation (substitution of T for C) at the coding region of the ob gene. This nonsense mutation results in change of the codon for arginine (Arg 105) to a stop codon (Zhang et al, 1994). The leptin receptor, which

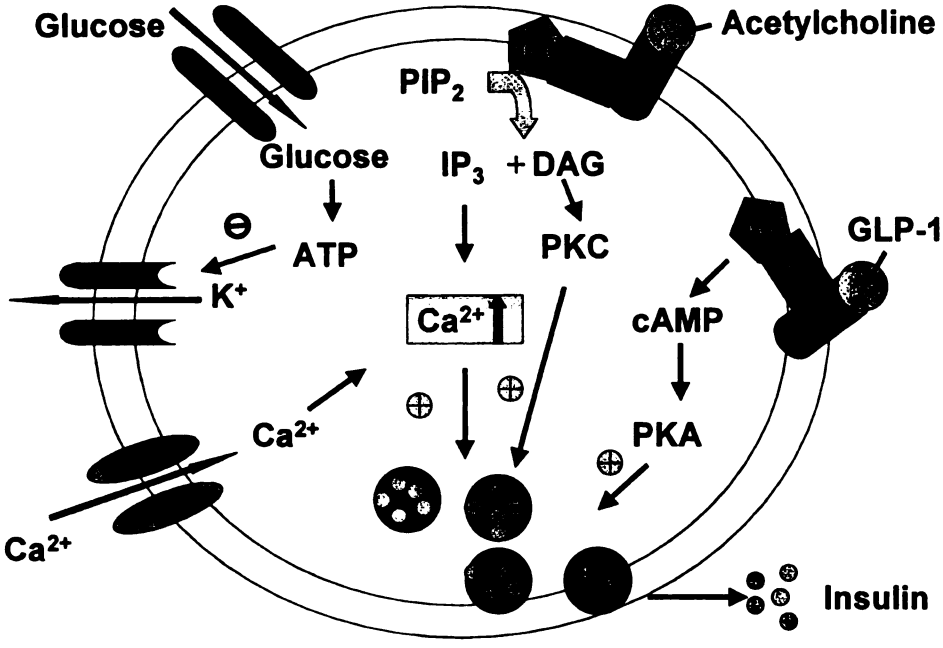

Figure 1. Regulation of insulin secretion. Insulin secretory response from pancreatic islets is finely modulated by nutrients (glucose), hormones (GLP-1: glucagon-like peptide-1), and neurotransmitters (acetylcholine). It is governed by second messengers including Ca^{2+}, cAMP:3'-5'- cyclic adenosine monophosphate, IP_3:inositol triphosphate and DAG:diacylglycerol.

belongs to the cytokine class 1 receptor family, was first cloned from the diabetes (db) gene (Tartaglia et al, 1995). Since this discovery, in addition to a role as a satiety factor, leptin has been noted to have multiple physiological functions including stimulating or maintaining energy expenditure (Pelleymounter et al, 1995; Halaas et al, 1995), functioning as a signal to the reproductive system (Chehab et al, 1996; Cunningham et al, 1999), and as a metabolic hormone influencing insulin action (Barziralli et al, 1997; Rossetti et al, 1997; Aiston and Agius, 1999).

Further, direct effects of leptin on the pancreas have been suggested to modulate insulin secretion (Chen et al, 1997; Kieffer et al 1997; Poitout et al, 1998; Zhao et al, 1998). Although controversial results have been reported, most studies show that leptin inhibits insulin secretion. Exploration of this leptin effect in the pancreas may help to understand the cause of the early-onset metabolic alterations. Islets from pre-obese (Lep^{ob}/Lep^{ob}) mice exhibited an enhanced Ach potentiation of glucose-induced insulin secretion, suggesting that alterations of this signaling pathway may be a potential cause of hypersecretion of insulin from Lep^{ob}/Lep^{ob} mice (Chen and Romsos, 1995; Chen and Romsos, 1997). But it is still unclear when this early-onset Ach potentiation of hypersecretion of insulin from islets of pre-obese mice first appears, and whether alterations in response to other hormones and neurotransmitters might exist in neonatal pre-obese mice. It is assumed that absence of leptin in Lep^{ob}/Lep^{ob} mice disturbes development of regulatory systems, or developmentally influences the endocrine pancreas, leading to altered insulin secretory response, and ultimately to hyerinsulinemia.

Unlike Lep^{ob}/Lep^{ob} mice which have a defect of leptin synthesis, obesity in humans is usually associated with leptin resistance (Maffei, et al, 1995b; Considine et al, 1996a; Segal et al, 1996). The cause of leptin resistance is unclear, but chronic exposure to elevated leptin may be one factor. Adminstered leptin to Lep^{ob}/Lep^{ob} mice exerted more remarkable effect in reducing food intake compared with lean mice (Halaas et al, 1995), probably because Lep^{ob}/Lep^{ob} mice are more sensitive to leptin than lean mice. Neonatal lean mice may be more sensitive to leptin than adult mice because they would not yet have experienced long time exposure to leptin. Comparisons of neonatal lean and Lep^{ob}/Lep^{ob} mice may provide important insights into how leptin resistance might develop.

To assess the cause of the early-onset hyperinsulinemia in obesity, and the role of leptin for this, first, I determined how islets from neonatal preobese Lep^{ob}/Lep^{ob} mice responded to Ach potentiation of glucose-induced insulin secretion. Also, I determined whether heterozygous neonatal lean ($Lep^{+/ob}$) mice have the same characteristics, including body weight, body fat and hypersecretion of insulin, as homozygous lean (+/+) mice. Second, I determined if leptin is involved in aspects of regulation of insulin secretion in neonatal mice. Third, I determined whether leptin resistance can be induced in pancreatic islets of mice. The specific objectives of my study were:

1. Examine whether insulin secretory responses from islets of 1-week-old homozygous (+/+) and heterozygous($Lep^{+/ob}$) lean mice and Lep^{ob}/Lep^{ob} mice to acetylcholine and glucagon-like peptide-1 differ from those of 2-week-old mice.

2. Examine whether leptin will constrain the potentiation of insulin secretion by either acetylcholine or glucagon-like peptide-1 or both in neonatal mice, and to investigate the mechanism responsible for leptin-induced inhibition of insulin secretion.
3. Examine whether chronic admistration of leptin to Lep^{ob}/Lep^{ob} mice would correct their hypersecretion of insulin.

CHAPTER II. REVIEW OF LITERATURE

A. Ob/ob mouse model

Several animal models of obesity including Lep^{ob}/Lep^{ob} mice are extensively used to better understand causes of obesity. Lep^{ob}/Lep^{ob} mice were discovered in 1950. These mice contain a single gene mutation residing on mouse chromosome six locus. They are sterile and as adults weigh three times more than normal mice, have over 50% body fat, and have multiple metabolic alterations, including hyperphagia, decreased thermogenesis, hypercorticosteronemia and hyperinsulinemia (Leibel et al, 1997). Hyperinsulinemia is an early-onset metabolic alteration. It precedes the onset of overeating or overweight in Lep^{ob}/Lep^{ob} mice (Lin et al, 1977; Rath and Thenen, 1979). This hyperinsulinemia may be caused by lack of a direct inhibitory control of insulin secretion in these mice.

B. Regulation of insulin secretion

The insulin secretory response from pancreatic islets is finely controlled by nutrients, hormones, and neurotransmitters at multiple steps, i.e., insulin synthesis, translocation, docking, and priming of secretory granules, followed by exocytosis. Insulin secretion is governed by second messenger systems including Ca^{2+}, cyclic nucleotides (cAMP), and the phospholipid metabolites inositol triphosphate (IP_3) and diacylglycerol (DAG) (Zawalich and Rasmussen, 1990) (Fig. 1).

1. Nutrient secretogogues

Glucose functions as a physiological stimulus for insulin secretion and biosynthesis in pancreatic β-cells. Glucose is the only nutrient to stimulate insulin release alone. Recognition of blood glucose concentration is required for initiation of insulin secretion. Increased blood glucose is sensed and transported into islets by facilitated diffusion via Glut 2, a glucose transporter on the plasma membrane of pancreatic β-cells. Glucokinase phosphorylates glucose, a first rate-limiting step for glucose utilization. Glucose phosphorylation is thus the prime step to sense plasma glucose concentration, and it plays a role in generating a signal for the initiation of glucose-induced insulin secretion in pancreatic β-cells (Lenzen and Panten, 1988). Phosphorylated glucose is metabolized further to produce ATP, a primary messenger for triggering insulin secretion from β-cells (Zawalich and Rasmussen, 1990). Increasing intracellular ATP concentrations are observed when blood glucose is 10 mM or higher. A rising intracellular ATP concentration closes the ATP-sensitive potassium channel (K^+_{ATP}) in plasma membrane of β-cells. Closure of the K^+_{ATP} channel inhibits K^+ efflux through this channel and depolarizes β-cells. Subsequently, voltage-dependent calcium channels (VDCCs) are opened, and influx of Ca^{2+} occurs to increase intracellular Ca^{2+}, a second messenger of signal transduction generated in relation to increases in blood glucose concentration. Increased intracellular Ca^{2+} triggers exocytosis of insulin (Prentki and Mastchinsky, 1987; Zawalich and Rasmussen, 1990).

High concentration of glucose induces a biphasic pattern of the insulin release from rat and human islets. It is characterized by an initial rapid rise of the first phase insulin secretion followed by the second phase of insulin secretion which progressively

increases to a sustained plateau (Zwalich et al, 1995; Zawalich and Zawalich, 1996; 1997). On the other hand, mouse islets show a first phase of insulin secretion comparable with rat or human, whereas they show markedly different second phase of insulin secretion. Islets from mice exhibit a flat and only slightly greater prestimulatory release rates (Zawalich et al, 1995; Zawalich and Zawalich, 1996; 1997). The flat second phase in mice is caused by less increment of phospholipase C (PLC)-mediated phosphoinositide (PI) hydrolysis in response to high glucose stimulus in mice than in rats because of species differences in the expression and activation of specific PLC isoforms in response to glucose (Zawalich et al, 1995; Zawalich and Zawalich, 1996; 1997).

Nutrients other than glucose including fructose, some amino acids and fatty acids also influence insulin secretion. Fructose (Zawalich et al, 1977) and arginine (Thams and Capito, 1999), a positively charged amino acid, alone do not stimulate insulin secretion, but both augment insulin secretion in the presence of glucose. Leucine, a branched chain amino acid, and its metabolite 2-ketoisocapric acid stimulate insulin secretion by enhancing the ATP content in β-cells during metabolism through the TCA cycle (Zawalich, 1988).

Long chain fatty acids (Vara et al, 1988) and malonyl CoA (Corkey et al, 1989) cannot initiate insulin secretion by themselves, they cause potentiation of glucose-induced insulin secretion *in vitro* (Prenki et al, 1992; Stein et al, 1996) as well as *in vivo* (Stein et al, 1996). The insulinotropic effect of fatty acids depends on the chain length and degree of saturation (Stein et al, 1997). Palmitate ($C_{16:0}$) potentiates insulin secretion from perifused islets in the presence of suprathreshold glucose concentration

(> 3mM) (Warnotte et al, 1994; Alcázar et al, 1997) (Fig. 2). This insulinotropic effect of palmitate can not be ascribed to impedance of the K^+_{ATP} channel. Palmitate might interact with Ca^{2+} channels, resulting in increased intracellular Ca^{2+} concentration if the glucose concentration is high enough (Warnotte, et al, 1994). Glucose concentration governs the metabolic fate of long chain fatty acids in islets toward either oxidation or esterification. Intracellular concentration of glucose-derived malonyl-CoA inhibits carnitine palmitoyl transferase (CPT), resulting in restriction of the rate limiting step of long chain fatty acids oxidation in mitochondria (Chen et al, 1994). Consequently, fatty acid esterification is increased. Thus, high glucose concentration accelerates fatty acid esterification leading to formation of complex lipids, whereas oxidation of fatty acids is increased by low glucose concentration (Corkey et al, 1989; Vara et al, 1986). In islet cells (Liang et al, 1991) as well as HIT cell (Corkey et al, 1989), glucose raises malonyl-CoA concentration, subsequently, increased long chain fatty acyl-CoA and synthesis of complex lipids including DAG and PI which might serve as messengers in the signaling pathways of insulin secretion (Farese et al, 1986; Vara et al, 1988). In the presence of high glucose concentration (20 mM), but not 3 mM glucose, palmitate stimulates PKC translocation from cytoplasma to plasma membrane, a process activated by lipid messengers including DAG or phosphatidylserine (PS) (Alcázar et al, 1997). Possibly this potentiation of insulin secretion by long chain fatty acids, including palmitate, is mediated by increased formation of complex lipids, including DAG or PS.

Chronic elevation of long chain free fatty acids decreases the potentiation of the glucose-induced insulin secretion from pancreatic β-cells, presumably due to loss of

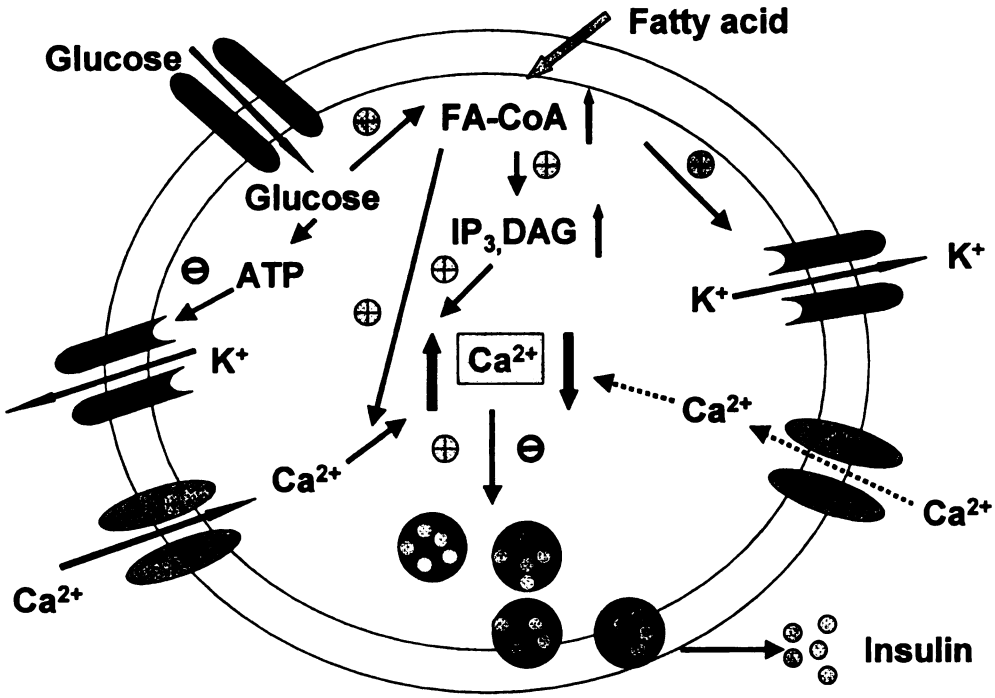

Figure 2. Potentiation of glucose-induced insulin secretion by fatty acids. Long chain fatty acids potentiate insulin secretion, but they are not able to initiate insulin secretion by themselves. Acute exposure to fatty acids exerts an insulinotrophic effect by increasing intracellular Ca^{2+} through direct interaction with calcium channels or increased synthesis of complex lipids served messengers in the signaling pathways of insulin secretion. But, chronic elevation of long chain fatty acids diminish insulin secretion presumably by direct activation of K^+ channels resulting in repolarization of membrane, consequently decreased intracellular Ca^{2+} and diminution of insulin secretion from the β-cells. Fatty acyl-CoA, FA-CoA; IP_3, insoitol triphosphate; DAG, diacylglycerol.

glucose sensitivity of β-cells (Larsson et al, 1996a). The mechanism for this is not clear, but it has been demonstrated that long chain fatty acyl-CoA bind to the pore-forming Kir6.2 subunit of the K^+_{ATP} channel, and this induces a conformational change of the channel protein leading to direct activation of the Kir6.2 subunit of the channel. This opens the channel and repolarizes the membrane in β-cells (Bränström et al, 1998). Thus, accumulation of long chain fatty acyl-CoA diminishes insulin secretion by inducing prolonged open state of the β-cell K^+_{ATP} channel (Larsson et al, 1996; Bränström et al, 1997; 1998) whereas acute exposure to fatty acids in the presence of glucose potentiate insulin secretion.

2. Hormonal secretogogues

Glucose given orally induces greater insulin secretion than that produced by glucose infused intravenously, suggesting that gastrointestinal hormones, including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) released from the gut by oral glucose stimulus, have an insulinotropic effect. GLP-1 and GIP normally do not trigger insulin secretion from pancreatic islets when glucose is absent (Zawalich and Rasmussen, 1990). Binding of GLP-1 and GIP to specific G protein-coupled receptors on the plasma membrane of β-cells mediates stimulatory effects of these hormones on adenylyl cyclase (AC) to catalyze production of cAMP from ATP (Fig. 1). Increasing intracellular cAMP activates protein kinase A (PKA). PKA mediates phosphorylation of proteins that increases Ca^{2+} influx (i.e., VDCCs) or that directly interact with the exocytotic machinery to facilitate exocytosis of insulin-containing granules (Holz and Habener et al, 1992; Holz et al, 1995).

3. Neurotransmitter secretogogues

Neurotransmitters, including acetylcholine (Ach) and cholesystokinin (CCK), also potentiate glucose-induced insulin secretion (Zawalich et al, 1989). Ach is released from vagal nerve or autonomic nerve fibers innervating the pancreas. CCK is secreted by endocrine cells of the gut after eating as well as from nerve fibers innervating islets. Ach and CCK stimulate insulin secretion via phospholipase C-protein kinase C (PLC-PKC) signal transduction linked to their specific receptors (Muscarinic receptor or G protein receptor, respectively) on the plasma membrane of β-cells. PLC stimulation by Ach and CCK causes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers, IP_3 and DAG. A rise in intracellular IP_3 increases free Ca^{2+} by mobilization of intracellular Ca^{2+} from the endoplasmic reticulum (ER). DAG activates PKC to regulate downstream proteins involved in insulin secretion (Fig. 1). These Ach- and CCK-stimulated cellular signaling pathways in β-cells are important for second phase of insulin secretion (Prentki and Matshinsky, 1987; Zawalich et al, 1995; Zawalich and Zawalich, 1996).

C. Hyperinsulinemia in obesity

Hyperinsulinemia in Lep^{ob}/Lep^{ob} mice is detectable as early as 6 days of age (Dubuc, 1981) and precedes an increase body fat, overeating, and insulin resistance (Boissonneault et al, 1978; Dubuc 1976; Lin et al, 1977). This early-onset hyperinsulinemia might be an important factor for the development of obesity, and is likely due to hypersecretion of insulin from pancreatic islets rather than to reduced rates

of insulin clearance. However, the metabolic and/or hormonal signals that contribute to the development of obesity-associated early-onset hyperinsulinemia are not clear.

It is well established that pancreatic islets from adult *Lep^{ob}/Lep^{ob}* mice (Tassava et al, 1992; Chen et al, 1993) are larger than islets from lean counterparts, and show enhanced sensitivity and responsiveness to glucose-induced insulin secretion. In young *Lep^{ob}/Lep^{ob}* mice, however, normal insulin secretion in response to glucose was reported (Chen and Romsos, 1995). This suggests that hypersensitivity and hyperresponsiveness of islets from adult *Lep^{ob}/Lep^{ob}* mice to glucose could be caused by the secondary effects of prolonged insulin secretion during development of obesity. The potentiation effects of Ach on glucose-induced insulin secretion were much greater in islets from adult *Lep^{ob}/Lep^{ob}* mice than in islets of lean counterparts (Tassava et al, 1992; Chen et al, 1993). This abnormality is also present in islets from 2-week-old *Lep^{ob}/Lep^{ob}* mice (Chen and Romsos, 1995; Chen and Romsos, 1997). At 2 weeks of age, islets from *Lep^{ob}/Lep^{ob}* mice also hypersecreted insulin in response to CCK which shares a common postreceptor signaling pathway with Ach (Chen and Romsos, 1995). This suggests that the PLC-PKC post-receptor signaling system may function as a primary mechanism in the development of hyperinsulinemia in pre-obese 2-week-old *Lep^{ob}/Lep^{ob}* mice. I will determine if altered insulin secretion is present in islets from 1-week-old *Lep^{ob}/Lep^{ob}* mice.

It has been reported that insulin binds to insulin receptors on β-cell, triggering activation of receptor tyrosine kinase, phosphorylation of insulin receptor substrates and activation of phosphatidylinositol 3-kinase (PI 3-K) (Rothenberg et al, 1995). Thereby insulin regulates insulin gene transcription (Leibiger et al, 1998) as well as its own

secretion via negative feed back (Argoud et al, 1987; Draznin et al, 1986). Specifically, the fact that inhibition of PI 3-K augmented insulin secretion suggested an important role for PI 3-K activity to negatively regulate insulin secretion (Eto et al, 2002; Hagiwara et al, 1995; Nunoi et al, 2000; Zawalich and Zawalich, 2000). But other evidence conflicts with these reports of insulin secretion regulating via a PI 3-K-dependent pathway (Aspinwall et al, 1999; 2000). In obese Zucker *fatty* rats, which are characterized by hyperinsulinemia and insulin resistance, reduced PI 3-K activity in islets has been suggested to lead to hypersecretion of insulin (Zawalich and Zawalich, 2000). This is consistent with the altered PI 3-K expression and activity that occurred in peripheral tissues of insulin resistant animals (Anai et al, 1998; Kerouz et al, 2000; Heydrick et al, 1995). This evidence suggests that reduced activity of a PI 3-K-dependent pathway controlling insulin secretion might be linked to hyperinsulinemia in obese animals. Further, it raises the possibility that PI 3-K may interact with PLC-PKC signaling system to prevent hyperinsulinemia, because enhanced sensitivity of PLC-PKC signaling pathway has been suggested as a primary mechanism for development of hyperinsulinemia in Lep^{ob}/Lep^{ob} mice (Chen and Romsos, 1995). I will investigate this possibility.

D. Leptin

1. Ob gene, ob gene protein and ob gene mutation

The phenotypic characteristics and pathophysiology of Lep^{ob}/Lep^{ob} mice have been intensively studied. Since the autosomal recessive mutation of the ob gene results in such profound metabolic changes including increased food intake and body fat

deposition, hyperinsulinemia, and hypothermia (Leibel et al, 1997) the ob gene product may function to regulate obesity-associated syndromes. Primary causes for the metabolic defects in Lep^{ob}/Lep^{ob} mice, however, were not revealed until the critical discovery of the ob gene product.

The Lep^{ob} gene was identified by positional cloning (Zhang et al, 1994). The Lep^{ob} gene encodes 4.5 kbs mRNA primarily expressed in mature adipose tissues into a 18 kDa protein containing a 2 kDa signal sequence which is cleaved when it is secreted. A 16 kDa monomer, now named leptin from the Greek word *leptos* meaning "thin" is released from adipose tissues into the circulation (Zhang et al, 1994). Leptin is considered as an "adipostatic hormone" which maintains adiposity of the body via communication with the central nervous system to control food behavior, neurotransmitter response and body energy balance (Campfield et al, 1995; 1996). Leptin circulates in the blood in association with binding proteins (Houseknecht et al, 1996), enters the brain (Banks et al, 1996), binds to its receptor in the hypothalamus and functions as an afferent satiety signal to regulate body weight, food intake and energy expenditure (Campfield et al, 1995; Schwartz et al, 1996b).

Lep^{ob} gene expression and secretion of leptin into the circulation are correlated positively with body-fat mass in rodents and humans (Maffei et al, 1995b; Considine et al, 1996a). Elevated leptin concentrations in the circulation result from accelerated leptin release from the adipose tissues caused by increased Lep^{ob} gene expression (Lönnqvist et al, 1997). Because Lep^{ob} gene expression and plasma leptin concentrations are associated with size of adipose tissue mass, regulation of Lep^{ob} gene expression and plasma leptin concentrations are influenced by factors modulating size

of the adipose tissues, including feeding and multi-hormonal controls (Saladin et al, 1995; Ostlund et al, 1996; Mizuno et al, 1996; Halleux et al, 1998).

In mice, *Lep*^{ob} mRNA declined after either 7 h or prolonged 72 h fasting, and was elevated 30 min after intraperitoneal injection of glucose (2 mg/g body weight) (Mizno et al, 1996). Another study showed that overnight fasting (16 h) remarkably decreases ob mRNA in adipose tissues of rats, and refeeding for 4 h returns *Lep*^{ob} mRNA to normal levels (MacDougald et al, 1995). It has been suggested that increased sympathetic activity and decreased insulin secretion may be a responsible for decreased *Lep*^{ob} mRNA during the fasting (Trayhurn et al, 1999). Leptin in the circulation decreases after 12 h fasting (Kolaczynski et al, 1996a) and increases with either short-term (12 h) overfeeding or prolonged (2 week) overfeeding sufficient to obtain 10 % weight gain in humans (Kolaczynski et al, 1996b). However, others reported that plasma leptin concentrations did not change acutely for 3 h after food administration in humans (Korbonits et al, 1997).

Glucocorticoids consistently increase *Lep*^{ob} gene expression and leptin secretion in both humans and rats *in vivo* (De Vos et al, 1995; Larsson et al, 1996b; Rao et al, 1997) as well as *in vitro* (Halleux et al, 1998; Russell et al, 1998; Bradley and Cheatham, 1999). The ability of insulin to regulate *Lep*^{ob} gene expression and leptin secretion is controversial. Numerous reports show stimulatory effects of prolonged insulin exposure on leptin secretion, whereas short-term exposure of adipose tissues to insulin does not fully stimulate leptin secretion (Russell et al, 1998; Bradley and Cheatham, 1999). Exposure of adipocytes to 1-3 nM insulin for 24 h increased the level of ob mRNA as well as the amount of secreted leptin (Leory et al, 1996). The

stimulatory effect of insulin on *Lep*^{ob} mRNA varied in specific adipose depot sites (Zheng et al, 1996). Infusion of insulin to rats increased *Lep*^{ob} mRNA in epididymal and perirenal fat pads, but not in the subcutaneous fat depots (Zheng et al, 1996). These hormone effects are reversible and mediated by a complex set of signals (Bradley and Cheatham, 1999). The underlying molecular mechanism to explain these effects needs further elucidation. Others have reported that leptin secretion in humans is primarily regulated by glucose metabolism rather than by insulin, and that insulin only plays a permissive role to facilitate glucose uptake (Wellhoener et al, 2000).

In addition, growth hormone increases leptin release from rat adipose tissues (Fain and Bahouth, 2000). Prostaglanidin E_2 (PGE_2), which inhibits lipolysis, stimulates leptin release by mouse adipose tissue cultured in medium containing 25 nM dexamethasone, whereas blocking formation of PGE_2 with NS-398, a selective cyclooxygenase-2 inhibitor, lowered leptin release (Fain et al, 2000a). A similar effect of PGE_2 was observed in adipose tissues of rats (Fain et al, 2000b). The effect of PGE_2 on leptin release may be mediated by either its interaction with a nuclear receptor, or by regulating lipolysis via interaction with EP_3 receptors on the surface of adipocytes.

In contrast, long chain fatty acids (Rentsch et al, 1996), catecholamines, (Trayhurn et al, 1995; Kosaki et al, 1996; Gettys et al, 1996; Mantzoros et al, 1996; Li et al, 1997), and cAMP (Kosaki et al, 1996) acutely suppress *Lep*^{ob} gene expression. Activation of the signaling pathways mediated by β_3-adrenergic receptors completely blocks ob gene expression by insulin in isolated rat adipocytes (Gettys et al, 1996). However, another study showed that suppressed leptin release by catecholamines is mediated by β_1-, β_2-, not β_3-adrenergic receptor in subcutaneous human adipoctyes

(Scriba et al, 2000). Thyroid hormone (T_3) also suppressed leptin secretion by rat adipose tissues (Fain and Bahouth, 1998).

White adipose tissue is the major site for leptin synthesis, although brown adipose tissues (Buyse et al, 2001) and some nonadipose tissues produce leptin. For instance, rodent fetal tissues including placenta, cartilage and hair follicles (Hoggard et al, 1997) as well as human placenta synthesize leptin (Señaris et al, 1997; Masuzaki et al, 1997). Leptin is detected in human cord blood (Sivan et al, 1997; Schubring et al, 1997), and cord blood leptin is positively associated with body weight at birth (Koistinen et al, 1997; Ong et al, 1999).

Lep^{ob}/*Lep*^{ob} mice are deficient in leptin because these mice have a genetic defect resulting in a non-sense mutation (i.e., a single base pair substitution of T for C) in the *Lep*^{ob} gene coding region. This substitution changes the codon for arginine (Arg 105) to a stop codon in leptin mRNA chain, resulting in production of a truncated leptin that is degraded in adipose tissues (Zhang et al, 1994). As a consequence of this defect in leptin, *Lep*^{ob}/*Lep*^{ob} mice exhibit a failure in the feed back loop for regulation of multi-metabolic reactions including the control of energy balance, which ultimately leads to the obese phenotype. Several other models of genetic obesity including db/db mice (Zhang et al, 1994; Maffei et al, 1995a; Maffei et al, 1995b), Zucker fatty rats (Ogawa et al, 1995; Maffei et al, 1995b), yellow agouti mice (Maffei et al, 1995b; Mizno et al, 1996), animals made obese by lesioning the ventromedial region of the hypothalamus (VMH) (Funahashi et al, 1995; Maffei et al, 1995a; Suga et al, 1999), or by ablation of brown adipose tissues (BAT) (Fredreich et al, 1995) or by infusion with gold thioglucose (Maffei et al, 1995a; Bryson et al, 1999) or monoglutamate (Fredreich et al,

1995) all revealed increased *Lep*^{ob} mRNA level in adipocytes, and increased circulating leptin concentrations. These animals all express leptin in proportion to mature adipose tissue mass, but appear to have some resistance to endogenous leptin.

The human *Lep*^{ob} gene has been cloned and identified. The *Lep*^{ob} gene is approximately 20 kb containing 3 exons inserted by 2 introns and resides on chromosome 7q 31.3. (Isse et al, 1995). Unlike *Lep*^{ob}/*Lep*^{ob} mice which have a defect in leptin synthesis, the obese gene defects are rare in human obesity (Maffei et al, 1996; Carlsson et al, 1997). Only a few humans have been identified with an impairment in leptin synthesis (Montague et al, 1997). Rather, obese humans commonly have increased *Lep*^{ob} gene expression (Considine et al, 1995) as well as elevated plasma leptin concentrations similar to db/db mice and Zucker fatty rats (Maffei, et al, 1995b; Considine et al, 1996a; Segal et al, 1996). *Lep*^{ob} gene expression and plasma leptin concentrations in humans are correlated with body fat content (Considine et al, 1995; Considine et al, 1996a). Obese subjects have higher leptin levels in plasma than lean subjects (Considine et al, 1996a; Segal et al, 1996).

Leptin in the circulation diffuses into the brain via cerebrospinal fluid (CSF) to regulate food intake and body weight by acting on its receptor in specific regions of the hypothalamus. The concentration of leptin in CSF of obese subjects is low or similar to lean subjects, even though obese subjects showed high plasma leptin concentrations compared with lean subjects (Schwartz et al, 1996a; Caro et al, 1996). This indicates that obese subjects may have a defect in uptake of leptin into brain, which may explain their leptin resistance.

2. Leptin receptor and signaling

2.1. Leptin receptor structure

The leptin receptor was first identified from the mouse choroid plexus by positional cloning. The gene encoding OB-R was localized to mouse chromosome 4 of db locus (Tartaglia et al, 1995). Leptin is similar in protein structure to cytokine-like proteins (Madej et al, 1995), and this implied that the leptin receptor is a member of the cytokine receptor family. Consistent with this prediction, sequencing comparisons of cDNAs revealed leptin receptor homology to a single membrane-spanning receptor of the cytokine class 1 receptor superfamily including interleukin (IL)-6, gp 130, granulocyte colony stimulating factor (G-CSF), and leukemia inhibitory factor (LIF) (Tartaglia et al, 1995). Several variants of the leptin receptor, i.e. OB-Ra, b, c, d, and e, differing in the length of the intracellular domain, caused by alternative RNA splicing at C-terminal coding exon, were subsequently cloned (Chen et al, 1996b; Lee et al, 1996; Wang et al, 1996). The long form of the leptin receptor (OB-Rb) consists of a large extracellular domain (816 amino acid), and an intracellular domain of about 303 amino acid. The short form of the leptin receptor has a short intracellular domain (34 amino acid), which lacks the sequence motifs necessary for intracellular signal transduction. This suggests that the short form of the leptin receptor may not mediate intracellular signal transduction (Chen et al, 1996b; Lee et al, 1996; Tartaglia et al, 1995).

Various isoforms of OB-R are widely expressed in tissues (Löllmann et al, 1997). The long form of the OB-Rb is predominantly expressed in the hypothalamus, a major site implicated in the control of food intake and body weight (Chen et al, 1996b), but it has also been identified in other peripheral tissues including pancreas, liver and

heart (Chen et al, 1996b; Emilsson et al, 1997). The short forms of OB-R are expressed ubiquitously although their functions remain unclear. Possibly the short form of OB-R may participate in transport of leptin from blood to CSF and also possibly in clearance of leptin (Uotani et al, 1999). But also, it has been demonstrated that short form OB-R in some of peripheral tissues including myocytes (Keller et al, 1997) and hepatocyes (Zhao et al, 2000) which are not abundant in long form of OB-R compared to hypothalamus, conduct physiological function of leptin.

Early parabiosis studies suggested that db/db mice may not be able to respond to a satiety factor (Coleman, 1973; 1978). This suggestion has now been confirmed with the identification of a mutation in the leptin receptor in these mice. Sequencing comparisons of the db gene from normal and db/db mice showed that db/db mice have a point mutation (G to T transversion) in the OB-R gene, resulting in a new splice donor site and production of a intracellular truncated OB-R domain, which corresponds to a short form of OB-R (Chen et al, 1996b; Lee et al, 1996; Chua et al, 1996a). This isoform of the OB-R does not respond to leptin. The activation of STAT 3 by leptin through OB-R was only observed in the hypothalamus of wild type mice, but not db/db mice (Vaisse et al, 1996). Consistent with this, administration of exogenous leptin to db/db mice did not reduce food intake or decrease body weight (Pelleymounter et al, 1995; Halaas et al, 1995; Campfield et al, 1995).

Fatty Zucker rats exhibit an obese phenotype similar to Lep^{ob}/Lep^{ob} mice. Phenotype of fatty rats is due to a single missense mutation in the OB-R fatty allele at position 880 (A to C). This causes an amino acid substitution at position 269 (Gln to Pro) in the C-domain, a conserved domain in the cytokine receptor family, and

production of OB-R containing a short extracellular domain. This mutation impairs OB-R mediated signaling in the fatty rats (Chua et al, 1996b; Phillips et al, 1996; White et al, 1997b).

Human OB-R is highly homologous with mouse OB-R (78% identical amino acid sequence) (Tartaglia et al, 1995). Full-length leptin receptor gene is expressed in the hypothalamus (Considine et al, 1996b). Mutation of human OB-R as observed in db/db mice and fatty rats containing defect of OB-R gene leading to profound early-onset obesity, may be a potential candidate to cause leptin resistance commonly observed in human obesity (Maffei et al, 1995b; Considine et al, 1996a). Rarely few humans have been identified with homozygous mutation (G→A substitution) at splice donor site of exon 16 in human OB-R resulting in truncated leptin receptor of 831 amino acids containing normal extracellular domain (830 amino acid), but lacking transmembrane as well as intracellular domain (Clément et al, 1998). This mutation causes early-onset obesity with elevated plasma leptin concentrations, no pubertal development and decreased growth hormone and thyrotropin secretion (Clément et al, 1998). However, in population study, very few obese humans have been identified with mutations similar to those in db/db mice or fa/fa rats, although several sequence polymorphisms, (i.e., $Phe^{17}Leu$, $Lys^{109}Arg$, $Val^{110}Met$, $Lys^{204}Arg$, $Gln^{223}Arg$, and $Lys^{656}Asn$) in human OB-R have been detected (Considine et al, 1996b; Chung et al, 1997; Echwald et al, 1997a; 1997b; Thompson et al, 1997). These sequence variants in human OB-R are not associated with profound obesity, and do not appear to cause leptin resistance (Considine et al, 1996b; Echwald et al, 1997b; Silver et al, 1997; Thompson et al, 1997). Also, it has been reported that no differences in expression of

hypothalamic OB-R between lean and obese humans were observed (Considine et al, 1996b). Therefore, mutations of OB-R are unlikely explain the cause of leptin resistance in most case of human obesity.

2.2 Leptin receptor and gene regulation

Since the leptin receptor structurally belongs to the cytokine class 1 receptor family (Tartaglia et al, 1995), the mechanism of signal transduction of OB-R would be expected to the similar to this receptor superfamily. Members of this receptor family lack intrinsic tyrosine kinase activity, and are activated by ligand induced homo- or hetero-dimerization of the receptor (Heldin, 1995; Kishimoto et al, 1994; Ihle, 1995; 1996). This dimerization of the receptor causes phosphorylation of receptor-associated janus kinase (JAK). JAK is constitutively associated with the leptin receptor. When this tyrosine kinase is activated it phosphorylates more intracellular tyrosine residues, which are docking places recognized by the Src homology (SH2) domain of DNA binding proteins, signal transducers and activators of transcription (STATs). JAK activates STATs, through tyrosine phosphorylation of STATs. This results in formation of STATs dimers, and translocation to the nucleus to modulate transcription of genes involved in regulation of target cellular function (Heldin, 1995; Kishimoto et al, 1994; Ihle, 1995; 1996) (Fig. 3). Leptin-induced OB-R triggering activates STAT 3 and 5, but activation of STAT 1 and 6 are controversial in COS cells *in vitro,* (Baumann et al, 1996; Ghilardi et al, 1996). Activation of STAT 3 by leptin is also observed in the hypothalamus *in vivo* (Vaisse et al, 1996). Only the long isoform of the OB-R, which contains a long cytoplasmic domain of 302 amino acids containing sequence motifs

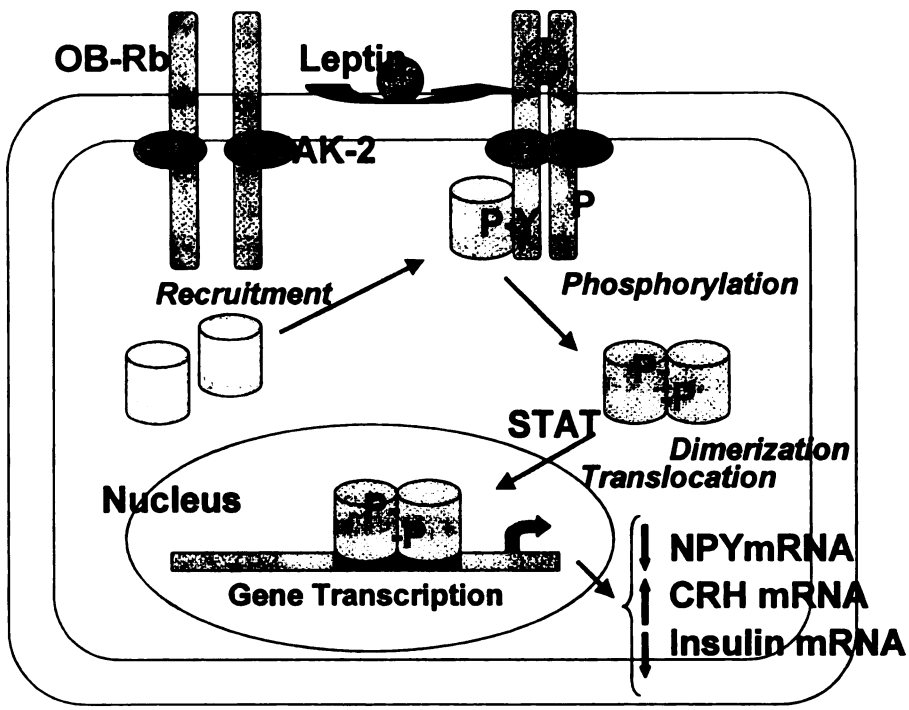

Figure 3. Signal transduction of leptin receptor. Long form of OB-R (OB-Rb) belongs to cytokine class 1 receptor family. OB-Rb mediates external signal of leptin through JAK-STAT signaling pathway, which is shared with the cytokine class 1 receptor family. Physiological roles of leptin including reducing food intake are mediated by modulating of gene expression through OB-Rb-mediated JAK-STAT pathway. OB-Rb, long form of leptin receptor; JAK, janus kinase; STAT, signal transducer and activators of transcription; NPY, neuropeptide Y; CRH, corticotropin releasing hormone.

interacting with JAK-STAT, can mediate this signal transduction (Baumann et al, 1996; Ghilardi et al, 1996). This internal signal mediated by OB-Rb is similar to IL-6 cytokine receptor, but unlike the IL-6 cytokine receptor, OB-R signaling does not require interaction with gp 130, a common signal component of IL-6 cytokine receptor (Baumann et al, 1996). OB-R may be activated by simple homo- or hetero dimerization and so far, two distinct regions of the intracellular domain may be involved in generating intracellular signals (White et al, 1997a).

Thus, it is suggested that antiobesity effects of leptin by regulating food intake and energy expenditure (Pelleymounter et al, 1995; Halaas et al, 1995; Campfield et al, 1995; Weigle et al, 1995) may be accomplished by modulation of gene expression associated with food behavior or energy balance through activation of the JAK-STAT signaling pathway in the hypothalamus, a major target of leptin. Neuropeptides, including neuropeptide Y (NPY) that stimulates food intake and corticotropin-releasing hormone (CRH) that acts opposite to NPY, are involved in regulation of food intake. *Lep*^{ob}*/Lep*^{ob} mice exhibited higher NPY versus lower CRH concentration in arcuate nucleus than lean mice (Jang and Romsos, 1998). ICV administration of leptin decreases mRNA of NPY (Stephens et al, 1995) and increases CRH mRNA in the hypothalamus (Schwartz et al, 1996b). These regulations occurred between 6 h and 5 day after administration of leptin. Leptin also decreases expression of preproinsulin mRNA in islets from *Lep*^{ob}*/Lep*^{ob} mice and in an INS-1 β-cell line by decreasing promoter transcriptional activity as well as by altering binding of proteins including STAT5b, to upstream sequences of the 5'-promoter region in the rat insulin gene (Seufert et al, 1999).

2.3. Rapid-onset actions of leptin

Several lines of evidence suggest that some of the physiological roles of leptin are rapid in onset. For instance, inhibitory effects of leptin on food intake occurs within 30 min (Campfield et al, 1995; Mistry et al, 1997), and leptin rapidly modulates synaptic current in the arcuate nucleus, a region of the hypothalamus affecting regulation of food intake (Glaum et al, 1996). In adrenalectomized mice, leptin acutely regulates neuropeptides secretion (i.e., inhibition of NPY or stimulation of CRH), which might be a possible mechanism for rapidly reducing food intake (Jang et al, 2000). Within 30 min leptin suppresses insulin secretion from islets *in vitro* (Chen et al, 1997; Zhao et al, 1998). These acute effects of leptin may be mediated by mechanisms that do not require modulation of gene expression, which often requires longer time.

The mechanisms responsible for rapid-onset actions of leptin are not clear. Recently, it has been shown that leptin shares some of components, i.e., IRS-1, IRS-2, PI 3-K, and mitogen-activated protein kinase (MAPK; ERK-1 and -2), of insulin signaling cascade. In fact, ERK-1 activity in COS cells, as well as phosphorylation of endogenous ERK2 in Chinese hamster ovary (CHO) cells, is stimulated by leptin (Bjørbæk et al, 1997), and leptin increased MAPK phosphorylation in adipose tissues (3.2 fold) and liver (3.8 fold) within 3 min after intravenous injection of leptin (1 mg/kg) (Kim et al, 2000).

In MDCK kidney epithelial cells, leptin maximizes PI 3-K activation in JAK-2 immunoprecitates within 3 min after adding leptin to cells (Attoub et al, 2000). Leptin also stimulates the tyrosine phosphorylation of JAK-2 and IRS-2 as well as activates the PI 3-K pathway to generate insulin-like effects in C_2C_{12} myotubes (Kellerer et al, 1997)

(Fig. 4). This may be mediated by cross-talk between the leptin and insulin signaling pathways, namely, that leptin-stimulated JAK-2 induces tyrosine phosphorylation of IRS-2 leading to activation of PI 3-K (Kellerer et al, 1997). This is supported by a report that activated JAK-1 and JAK-2 interact with the insulin receptor and phosphorylate tyrosine residues of IRS-1 and IRS-2 (Gual et al, 1998). In insulin-sensitive tissues (i.e., liver, adipose tissues and muscle), Kim et al demonstrated that leptin increased IRS-2-associated PI 3-K activity in liver (1.7 fold) and decreased activity in muscle (-28 %), and that leptin also increased IRS-1-associated PI 3-K activity in adipose tissues (2 fold), liver (1.3 fold) and muscle (1.5 fold), within 3 min after leptin injection (Kim et al, 2000). Another study showed that leptin increased activity of IRS-1-, but not IRS-2-, related PI 3-K in HepG2 human hepatoma cells (Cohen et al, 1997). But in hepatoma Fao cells, leptin alone was not effective in activating PI 3-K via an IRS-associated pathway, but rather leptin enhanced insulin-induced tyrosine phosphorylation of IRS-1 and subsequent binding of PI 3-K to IRS-1, as well as conversely decreased PI 3-K associated with IRS-2 (Szanto and Kahn, 2000). Together, these reports suggest that tissue-specific interactions between leptin and insulin are quite complex and diverse, and that leptin-induced signal transduction pathways might overlap with those mediated by insulin.

Leptin-mediated activation of PI 3-K has been defined as a pivotal component of signaling that reduces cytoplasmic cAMP concentrations. In hepatocytes, leptin, at a physiological concentration, stimulates binding of PI 3-K to IRS-2 and subsequently PI 3-K-dependent activation of PDE results in suppression of glucagons-induced cAMP

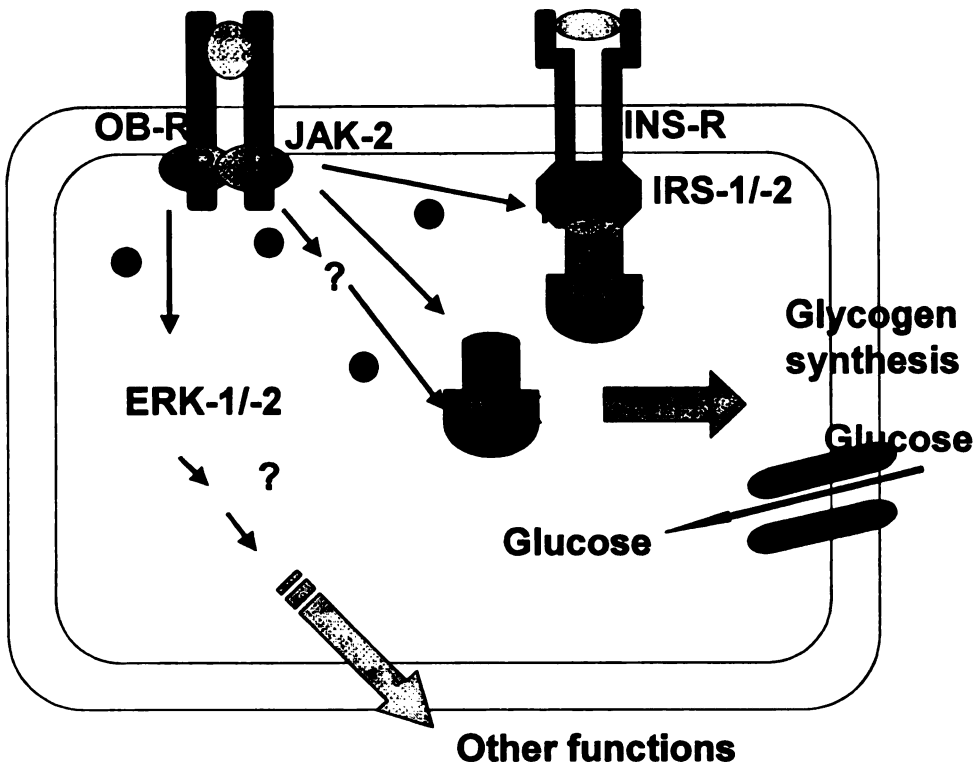

Figure 4. Possible mechanisms for rapid-onset action of leptin in peripheral tissues. Leptin may regulate insulin-like effects by stimulating PI 3-K or MAPKinase (ie, ERK-1/-2) signaling pathways like cytokine class 1 receptors. JAK-2, janus kinase-2; PI 3-K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; INS-R, insulin receptor; IRS-1/-2, insulin receptor substrate-1/-2.

elevation (Zhao et al, 2000). Leptin also suppressed cytoplasmic cAMP concentration in pancreatic islets of rats by activating PDE 3B via the activation of PI 3-K (Zhao et al, 1998) (Fig. 5). This results in suppression of the potentiation of GLP-1 on glucose-induced insulin secretion from pancreatic islets of neonates. However, others have reported that the acute inhibitory effect of leptin on glucose-induced insulin secretion was not mediated by activation of the PDE 3B pathway (Cases et al, 2001). In other studies leptin only very modestly increased cAMP concentration in chromaffin cells (Takekoshi et al, 1999). Thus it is not possible, based on these data, to conclude whether the leptin-PI 3-K-PDE 3 B pathway plays a novel in regulation of insulin secretion from islets of Lep^{ob}/Lep^{ob} mice. I will investigate this pathway.

In addition, PI 3-K has been associated with activation of K^{+}_{ATP} channels by leptin in a rat insulinoma cell line, which results in diminution of insulin secretion through this pathway (Harvey and Ashford, 1998; Harvey et al, 2000). The observation that JAK-2 is involved in regulation of prolactin secretion by phosphorylating tyrosine residues in K^{+}_{ATP} channel (Prevarskaya et al, 1995) supports another potential target and mechanism where leptin acts rapidly to conduct its function (Fig. 6).

Another role for leptin-stimulated PI-3 K activity has been demonstrated in colonic and kidney epithelial cells via activation of multiple downstream signaling components including, PKB, p70 S6, PKC and Rho-like G-proteins (Attoub et al, 2000). Also, leptin activates hormone sensitive lipase in J774.2 macrophage cells via PI-3 K dependent mechanism (O'Rourke et al, 2001). All these studies suggested that leptin may regulate a number of signal transduction pathway via control of PI 3-K. I will further investigate this.

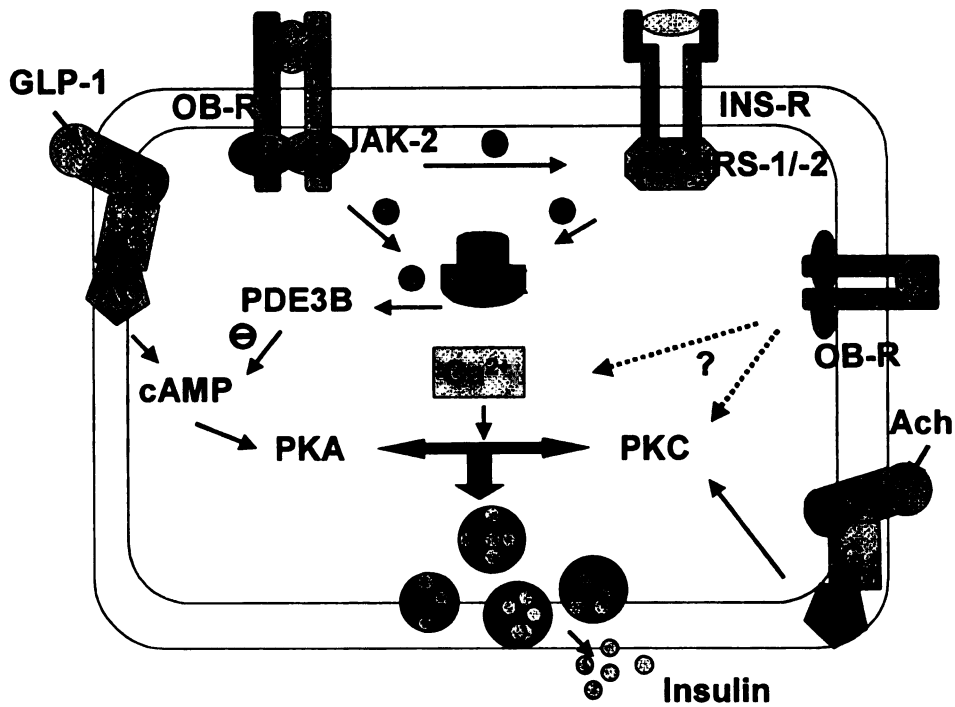

Figure 5. Possible mechanisms for rapid-onset action of leptin on insulin secretory response in pancreatic β-cells. Leptin may modulate insulin secretion through direct or indirect via IRS-1/-2 activation of PI 3-K which activates PDE 3B resulting in diminution of insulin secretion. Leptin may interact other signaling pathways responsible for insulin secretion to regulate insulin release. JAK-2, janus kinase-2; PI 3-K, phosphatidylinositol 3-kinase; INS-R, insulin receptor; IRS-1/-2, insulin receptor substrate-1/-2; PDE 3B, phosphodiesterase B; PLC, phospholipase C; PKC, protein kinase C; PKA, protein kinase A; cAMP, adenosine 3'-5'-cyclic monophosphate

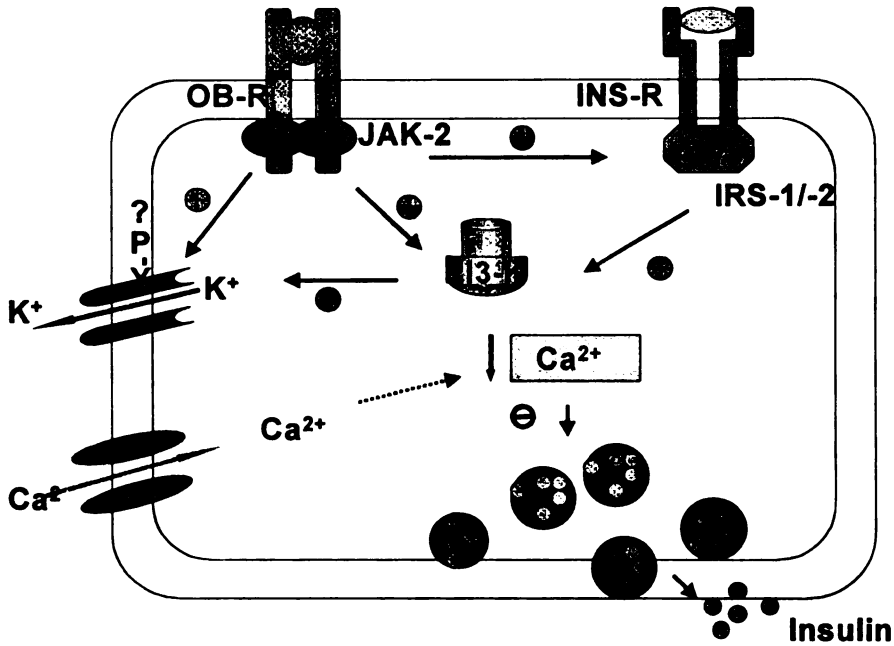

Figure 6. Possible rapid-onset action of leptin on K^+_{ATP} channels in pancreatic β-cells. Leptin may modulate insulin secretion through OB-R by direct or indirect via IRS-1/-2 activation of PI 3-K involved in activation of K^+_{ATP} channels resulting in diminution of insulin secretion. Leptin may be interacting directly with channels to regulate secretory response. JAK-2, janus kinase-2; PI 3-K, phosphatidylinositol 3-kinase; INS-R, insulin receptor; IRS-1/-2, insulin receptor substrate-1/-2; K^+_{ATP}, ATP-sensitive potassium channel.

3. Inhibition of leptin signaling

As a classic negative regulator to switch off cytokines (including leptin) signaling pathway, new family of cytokine-inducible inhibitors of signaling including suppressors of cytokine signaling (SOCSs) and cytokine-inducible SH_2 proteins (CISs) has been identified (Endo et al, 1997; Naka et al, 1997; Starr et al, 1997). Several kinds of cytokines including interleukine-6, erythropoietin, leukemia-inhibitory factor induce expression of these proteins (Endo et al, 1997; Naka et al, 1997; Starr et al, 1997). Leptin, which belongs to the cytokine family, also induces SOCS-3 in hypothalamic nuclei (Bjørbæk et al, 1998). It has shown that the accumulation of SOCS-3 mRNA is mediated by the recruitment of STAT 3 to Tyr^{1138} in the intracellular domain of leptin receptor by using chimeric (erythropoietin receptor/OB-R_L) construct (Banks et al, 2000). Those proteins that are cytokine-inducible inhibitors of signaling contain a SH_2 domain, which can interact with phosphorylated tyrosine residue of JAK (Endo et al, 1997). Interaction of these proteins with JAK constitutively associated with cytokine receptors suppressed cytokine-induced signaling (Haque et al, 2000; Sasaki et al, 2000). SOCS-3 has been shown to block leptin-induced signaling via a negative feedback loop (Starr et al, 1997; Bjørbæk et al, 1998) (Fig. 7).

It has been shown that obese yellow mice, which exhibit hyperleptinemia and leptin resistance have increased expression of SOCS-3 in their hypothalamic nuclei, compare to normal animals (Bjørbæk et al, 1998). It has been suggested that inhibition of leptin-induced signaling is caused by excess SOCS-3 activity, and that this may be a potential mechanism for leptin resistance in human obesity and some animal-models of obesity.

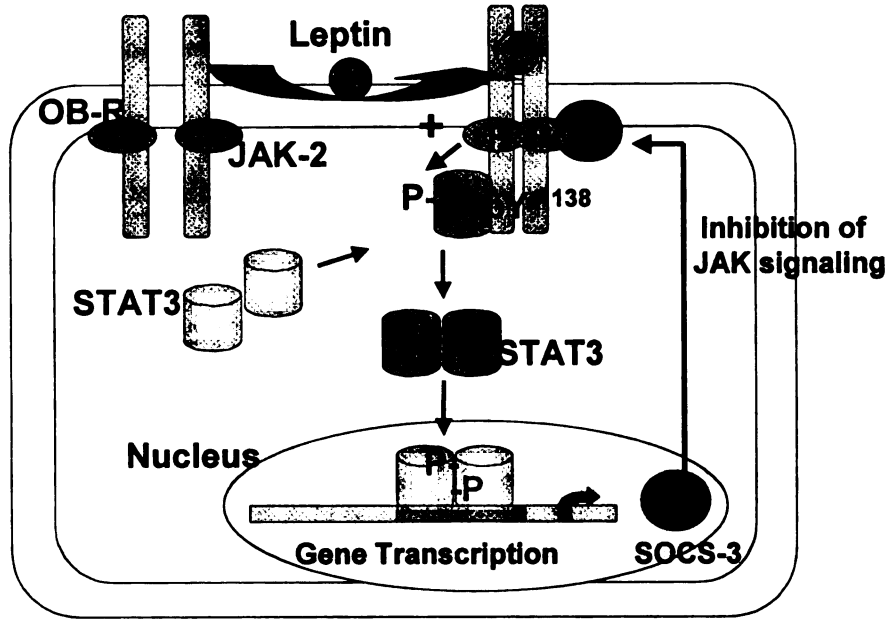

Figure 7. Model of inhibition of leptin signaling via long form of OB-R. OB-R belongs to cytokine class 1 receptor family. Leptin-induced transcription of SOCS-3 via JAK-STAT pathway feed back on and inhibits JAK/OB-Rb signaling. OB-Rb, long form of leptin receptor; JAK, janus kinase; STAT, signal transducers and activators of transcription; SOCS-3, suppressors of cytokine signaling.

4. Physiological functions of the leptin

4.1. Regulation of food intake, body weight and energy balance

Leptin functions as a satiety factor to reduce food intake and decrease body weight. Daily intraperitoneal (IP) injection of leptin to Lep^{ob}/Lep^{ob} mice lowered their body weight, body fat and food intake as well as increased metabolic rate, body temperature and activity level (Pelleymounter et al, 1995; Halaas et al, 1995; Weigle et al, 1995). Single intracerebroventricular (ICV) injection of leptin reduced food intake and elevated metabolic rates in lean and Lep^{ob}/Lep^{ob} mice (Mistry et al, 1997). These functions of leptin were less pronounced in wild-type mice administered mouse leptin than in Lep^{ob}/Lep^{ob} mice (Halaas et al, 1995). A direct effect of leptin on neural networks to control feeding and energy balance was suggested. Evidence for this was obtained by demonstrating that central administration of leptin reduced body weight of Lep^{ob}/Lep^{ob} mice and diet-induced obese mice more effectively than did peripheral administration of leptin (Campfield et al, 1995). Hyperleptinemia in normal rats induced by infusing a recombinant adenovirus containing the rat leptin cDNA caused a 30-50 % reduction in food intake, and these animals gained only about 10% as much body weight as normoleptinemic controls that received saline or β-galactosidase gene. Body fat was completely absent in hyperleptinemic rats, but not in pair-fed normaleptinemic rats (Chen et al, 1996a). This suggested that leptin not only reduced food intake, but also had a thermogenic effect.

These functions of leptin may not be fully developed in neonates. Neither IP nor ICV administration of leptin affected milk intake and oxygen consumption in neonatal (7-10 day old) lean and Lep^{ob}/Lep^{ob} pups (Mistry et al, 1999). Probably, the

critical physiological requirement for growth as well as thermoregulation during neonatal development influence the development of leptin actions on food intake and energy expenditure.

4.2. Regulation of reproductive system

The adequacy of nutrition and the state of metabolic reserves influence the reproductive axis. Undernutrition during adulthood can lead to infertility through reduction of gonadotropin releasing hormone (GnRH) secretion (Brown, 1994). The failure of ovulation in extremely thin women and the delayed onset of puberty in abnormally thin young women suggested that some signal from adipose tissue may be involved in regulation of the reproduction system. It is feasible that the adipose-derived hormone leptin, which reflects the state of nutrition, as described previously, may have a physiological function in this system. In fact, leptin is involved in regulation of the hypothalamus-pituitary-gonadal axis, which controls the secretion of sexual hormones including GnRH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Cunningham et al, 1999). Leptin has a direct stimulatory action of GnRH release in cultured hypothalamus (Woller et al, 2001). Decreased circulating levels of leptin in food restricted animals are associated with markedly reduced release of GnRH, LH and FSH (Cunningham et al, 1999). Identification of OB-R within hypothalamic GnRH neurons (Finn et al, 1998) suggests that leptin serves as a metabolic signal for neural activation of GnRH. Male and female leptin-deficient Lep^{ob}/Lep^{ob} mice are infertile. The treatment of Lep^{ob}/Lep^{ob} mice with exogenous leptin stimulates the activity of the

reproductive endocrine system and induces fertility (Cunningham et al, 1999; Chehab et al, 1996; Ewart-Toland et al, 1999).

The role of leptin in timing the onset of puberty has been also demonstrated in normal mice, but the exact role for leptin as the primary metabolic signal for initiating the onset of puberty is unclear. Female mice treated with leptin showed an acceleration in maturation of the reproductive tract and exhibit an earlier onset of puberty (i.e., vaginal opening, estrus, cycling) (Chehab et al, 1997; Ahima et al, 1997). Also, leptin reverses the delayed sexual maturation caused by food restriction. These effects suggested that leptin is the signal communicating with brain on the availability of energy stores to support the high-energy demands of reproduction. Adequate levels of leptin in the circulation indicate that adipose tissue stores are sufficient to support reproduction and are essential for triggering puberty. Reduced leptin levels in the circulation act as metabolic cue to inhibit activity of the neuroendocrine reproductive axis.

4.3. Other functions

Leptin regulates a variety of metabolic pathways beyond food intake and body weight. Daily intraperitoneal injection of leptin to Lep^{ob}/Lep^{ob} mice normalizes blood glucose concentrations as well as reduces plasma insulin level. This change might be as a consequence of reduced food intake (Pelleymounter et al, 1995). But, in pair-feeding studies, administration of leptin decreased plasma insulin concentration to a greater extent than that produced by an equivalent reduction in food intake (Levin et al, 1996). Hyperleptinemic animals exhibited markedly lower plasma insulin and triglyceride

levels than in pair-fed animals or controls (Chen et al, 1996a; Koyama et al, 1997). These reports indicated that change of glycemia and insulin sensitivity in Lep^{ob}/Lep^{ob} mice given leptin might be not only a simple consequence of the reduced obesity by leptin action within hypothalamus, but also possibly a direct action leptin in the extrahypothalamic target tissues including liver, muscle, adipose tissues and endocrine pancreas. In fact, independent of reducing food intake, leptin regulates insulin sensitivity and glucose disposal in mice with the feature congenital generalized lipodystrophy characterized by severe insulin resistance, hyperglycemia and enlarged fatty liver (Shimomura et al, 1999).

Leptin has been implicated in insulin action to regulate lipid and carbohydrate metabolism (Barzilai et al, 1997; Rossetti et al, 1997; Kamohara et al, 1997). Intravenous (IV) injection of leptin to rats during physiologic hyperinsulinemia (~ 65 uU/kg·min) acutely enhanced insulin inhibition of hepatic glucose production by suppressing glycogenolysis (Barzilai et al, 1997; Rossetti et al, 1997). But leptin did not affect insulin action in peripheral tissues including muscle and adipose tissues (Rossetti et al, 1997). Leptin increased glycogen synthesis by inhibition of phosphorylase *a*, and this effect was more pronounced with insulin (Aiston and Agius, 1999). Moderate hyperleptinemia induced by AdCMV-leptin in the normal rat spared liver glycogen stores during the transition between fed and fasting state, which might be associated with decreased degradation of liver glycogen (O'Doherty et al, 1999). Chronic (7 days) intraperitoneal infusion of leptin to mice, but not short-term infusion, increased insulin-stimulated muscle and liver glycogen synthesis and inhibited insulin release in response to oral glucose test, *in vivo* as well as addition of leptin for 2 h

improved the insulin-like effects in muscle and adipocytes *in vitro* (Harris, 1998). Possibly these effects of leptin are a part of a compensatory mechanism for leptin-induced inhibition of glucose-induced insulin secretion (Harris, 1998). In contrast, partially fasted mice receiving ICV or IV leptin decreased liver glycogen levels (Kamohara et al, 1997).

These studies suggest differential effects of leptin on insulin action in extra-hypothalamic tissues. Since these metabolic alterations are linked to the regulation of insulin secretion it will be important to identify the metabolic regulatory role of leptin not only on insulin sensitive tissues but also on the endocrine pancreas.

5. Leptin and modulation of insulin secretion

In addition to its anorectic actions, leptin may also directly control insulin secretion from pancreatic islets. This effect may be accomplished as a part of an adipose-insular axis feed back mechanism. The potential importance of this interaction between insulin and leptin is suggested by several studies demonstrating that administration of insulin increases the expression of leptin and plasma leptin concentration (Russell et al, 1998; Bradley and Cheatham, 1999). Further, insulin resistance is positively related to increased plasma leptin levels independent of body fat (Segal et al, 1996). The ability of the long form of the leptin receptor (Kieffer et al, 1997; Emilsson et al, 1997) to activate STAT3 in the pancreas (Morton et al, 1999) suggests a possible direct action of leptin on insulin secretion. However, the effects of leptin on insulin secretion have been contradictory, depending on species, concentration of leptin administered, and type of insulin secretory response measured. In Lep^{ob}/Lep^{ob}

mice, 1-100 nM leptin in a dose dependent manner inhibited the stimulatory effect of 16.7 mM glucose on the insulin secretion from islets (Emilsson et al, 1997). This inhibitory effect was not observed in db/db mice and fa/fa rats, confirming a role for the long form OB-R in the pancreas to modulate insulin release (Emilsson et al, 1997). In another study, leptin (6.2 nM) suppressed basal (5.5 mM) as well as glucose (11.1 mM)-stimulated insulin secretion from pancreatic islets of *Lep^{ob}/Lep^{ob}* mice (Kieffer et al, 1997). They also showed that β-cells exposed to 6.2 nM leptin exert increased membrane conductance with the activation of the ATP-sensitive potassium channels (K^+_{ATP}) and hyperpolarization accompanied by decreased intracellular Ca^{2+} concentrations (Kieffer et al, 1997). This suggested that the suppression effect of leptin may due to decrease intracellular Ca^{2+} concentrations in *Lep^{ob}/Lep^{ob}* β-cells by activation of K^+_{ATP} channels (Kieffer et al, 1997). But, this is not in accord with the suggestion that leptin lacks effect on KCl-stimulated insulin secretion (Zhao et al, 1998). Since islets from *Lep^{ob}/Lep^{ob}* mice show hypersensitivity to hormones and altered the regulation of insulin secretion, these mice might be good models to use to determine the role for leptin in insulin secretion.

In normal rats, 1.5-3 nM range of leptin suppressed insulin secretion from islets incubated statically with 16.7 mM glucose (Ookuma et al, 1998). Also, leptin inhibited 20 mM glucose-induced insulin secretion from normal rat islets, but in a U-shaped dose response manner with significant effects exhibited at 1 and 10 nM of leptin, but not at 0.1 nM and 100 nM (Pallett et al, 1997). In HIT-15 pancreatic cells incubated with 10, 50, or 100 ng/ml of leptin for 40 h, 16.7 mM glucose-induced insulin secretion was dose-dependently reduced, but only leptin at 100 ng/ml effectively suppressed basal (7

mM) insulin secretion (Tsiotra et al, 2001). In another study, leptin (0.1 nM) acutely inhibited 8 mM glucose-induced insulin secretion from perifused islets of normal rats (Kulkarni et al, 1997). Basal (5.5 mM glucose) insulin secretion from normal rat islets (Ishida et al, 1997) and cultured βTC6 cell line (Kulkarni et al, 1997) were also inhibited by 80 nM and 0.1 nM-1 nM leptin, receptively. These differential effects may be derived from leptin action at different backgrounds of glucose stimulus. In a human study, this aspect was also observed. Leptin (10 and 100 nM) significantly constrained 20 mM glucose-induced insulin secretion from human islets, without effects at lower glucose concentrations (2.8 and 10 mM) (Fehmann et al, 1997a). But in another study, leptin (0.1 nM-1 nM) dose-dependently suppressed insulin secretion from human pancreatic islets stimulated by 5.5 mM glucose (Kulkarni et al, 1997).

Inconsistent with the inhibitory effect of leptin on glucose-stimulated insulin secretion reported above, leptin (1 nM) has been reported to acutely stimulate basal insulin secretion from islets of normal rats and β-cell line MIN6 (Tanizawa et al, 1997). In HIT-T 15 cells leptin (0.06 nM-6 nM) dose-dependently, also, stimulated 7 mM glucose-induced insulin secretion for 24 h (Shimizu et al, 1997). In other studies, leptin failed to alter either basal or glucose-induced insulin secretion from the rats (Leclercq-Meyer et al, 1997; Leclercq-Meyer and Malaisse, 1997; Poitout et al, 1998), and in either lean (Poitout et al, 1998) or Lep^{ob}/Lep^{ob} mice (Chen et al, 1997).

Chronic feeding of high glycemic index (GI) diet causes hypersecretion of insulin in response to acute glucose challenge. Leptin (100 ng/ml) reduced plasma insulin concentrations in response to glucose load (1g/kg) via IV as well as basal (5

mM) and glucose-induced insulin secretion (20 mM) in high GI diet-fed rats, but not in low GI diet (i.e., mostly amylose)-fed rats (Widdup et al, 2000).

Glucose-stimulated insulin secretory responses in rats and humans occur in a biphasic pattern, with the second phase characterized by sustained rising secretion, (Zawalich, 1996). Identifying the inhibitory pattern of leptin during the two glucose-induced bihasic patterns of insulin secretion may help us understanding how leptin acts to modulate insulin secretion. But, contradictory inhibitory patterns of insulin secretion by leptin were reported. Leptin (3 nM) suppressed significantly the 2nd phase, but not the 1st phase, of 16.7 mM glucose-induced insulin secretion from perifused rat islets (Ookuma et al, 1998). This was again revealed by another report from the same laboratory that showed that 3 nM leptin fail to suppress insulin secretion stimulated by glibenclamide (1 and 5 uM), a stimulator of the 1st phase of insulin secretion (Ookuma et al, 1998). A report from another laboratory showed that leptin (1 and 10 nM) inhibited the 1st phase of the insulin release from perifused rat islets stimulated by either 2.8 mM or 10 mM glucose (Fehmann et al, 1997c). In yet another study, leptin suppressed both 1st and 2nd phase of glucose (16.7 mM)-induced insulin secretion from normal rats, without effect in fa/fa rats, in only when rat islets preexposed to10 or 100 nM leptin (Roduit and Thorens, 1997). Thus, it is very difficult to define the exact conditions where leptin would be expected to consistently affect glucose-induced insulin secretion.

Glucose-induced insulin secretion is potentiated by factors that elevate the PKA or the PLC-PKC pathways. Effects of leptin on these pathways of insulin secretion have been examined, again with controversial results reported. Leptin suppressed

glucose (16.7 mM)-induced insulin secretion from normal rat islets potentiated by 0.1 mM IBMX, an antagonist of phosphodiesterase (PDE) which raise intracellular concentration of cAMP that involves the insulinotropic effect by activating PKA (Poitout et al, 1998). This inhibitory effect of leptin was again supported by the study showing that leptin markedly inhibited the incretin effect of 0.1 nM GLP-1 on the glucose (11.1 mM)-induced insulin secretion from islets of neonatal rats by activation of PDE 3B via PI 3-K dependent mechanism (Zhao et al, 1998). Selective inhibitors of PDE 3B and PI 3-K completely impeded the inhibitory effect of leptin on the insulin secretion and cAMP accumulation, suggesting a role for PDE 3B as a mediator of the physiological function of leptin in the pancreas (Zhao et al, 1998). Further, the inhibition of GLP-1 insulinotropic effects by leptin may be partly mediated by leptin action on the non-selective cation channel activated by cAMP (Fehmann et al, 1997b). This suggestion was derived from the observations that in perifused rat islets, GLP-1 (1 nM) insulinotropic effects on both phases of glucose (10 mM)-stimulated insulin secretion were inhibited by leptin (1 and 10 nM), and by the observation that leptin (10 mM) attenuated GLP-1 (10 nM)- or forskolin, a activator of adenylate cyclase (0.1 and 1 μM)-induced intracellular Ca^{2+} concentration in INS-1 cells (Fehmann et al, 1997b). However, other reports indicated that a physiological concentration leptin (3 nM) failed to suppress insulin secretion from rat islets stimulated by 1 μM forskolin (Ookuma et al, 1998) and 20 nM leptin failed to constrain glucose (10 mM)-stimulated insulin release potentiated by 10 nM GLP-1 in either lean or *Lep^{ob}/Lep^{ob}* mice (Chen et al, 1997). In addition, leptin (6.2 nM) did not affect GLP-1 (10 nM) potentiation of 11.1 mM glucose-induced insulin secretion from islets of *Lep^{ob}/Lep^{ob}* mice (Kieffer et al, 1997).

A specific inhibition by leptin on insulin secretion potentiated by PLC-PKC signaling pathways has been demonstrated. In Lep^{ob}/Lep^{ob} mice, but not in lean mice, 20 nM leptin constrained Ach potentiation of 10 mM glucose-induced insulin secretion (Chen et al, 1997). Another study demonstrated that this effect of leptin in normal rat islets (Ookuma et al, 1998). Leptin (3 nM) significantly inhibited insulin secretion stimulated by 30 nM phorbol 12-myristrarte 13-acetate (PMA), an activatior of PKC, only during the 2^{nd} phase in the presence of Ca^{2+}, suggesting leptin may constrain insulin secretion during the 2^{nd} phase by reducing Ca^{2+}-dependent PKC isoform activity, independently of influence on PKA mediated insulin secretion (Ookuma et al, 1998).

In addition to direct effects on insulin secretion, leptin inhibits insulin biosynthesis in pancreatic β-cells at the level of preproinsulin gene transcription, which would indirectly limit insulin secretion. Leptin reduced expression of preproinsulin mRNA in islets from ob/ob mice and INS-1 β-cell line (Seufert et al, 1999) and insulin mRNA in rat islets and βTC6 cells (Kulkarni et al, 1997). This effect of leptin was mediated by decreased promoter transcriptional activity of the rat insulin I gene, as well as by altered binding of proteins, including STAT 5b complexes, to upstream sequences within the 5'-promoter region of the rat insulin gene (Seufert et al, 1999). In HIT-T15 pancreatic cells, leptin (10, 50, and100 ng/ml) significantly reduced glucose (16.7 mM)-induced expression of preproinsulin mRNA in dose-dependent manner, but only 100 ng/ml leptin significantly suppressed preproinsulin mRNA expression in cells incubated with 7 mM glucose (Tsiotra et al, 2001).

Most of the studies reported to date suggest that leptin is a potent inhibitor of insulin secretion from pancreatic islets, although a considerable number of inconsistencies have been reported. This inhibition of insulin secretion by leptin may represent one way for leptin to function as an integrated modulator of energy balance in the body. More studies are required to elucidate the exact role for leptin as an inhibitor of insulin secretion, and the mechanisms by which leptin directly regulates insulin secretion from pancreatic islets. This is the focus of my dissertation.

CHAPTER III. LEPTIN-DEFICIENT MICE COMMENCE HYPERSECRETING INSULIN IN RESPONSE TO ACETYLCHOLINE BETWEEN 1 AND 2 WEEKS OF AGE

A. ABSTRACT

Leptin-deficient Lep^{ob}/Lep^{ob} mice develop hyperinsulinemia early in life, before they begin to overeat or develop insulin resistance. Pancreatic islets from these young mice do not yet hypersecrete insulin in response to glucose, but hyperrespond to acetylcholine. Islets from 4 day, 1, 2 and 4-week-old mice were used in the present study to determine when leptin-deficient mice first hypersecrete insulin in response to acetylcholine. This relative hypersecretion of insulin from islets of leptin-deficient mice occurred between 1 and 2 weeks of age. The divergence in insulin secretion occurred at this time because islets from lean, leptin-sufficient mice became relatively less responsive to acetylcholine between 1 and 2 weeks of age whereas islets from leptin-deficient mice maintained a high responsiveness to acetylcholine during development. Leptin addition to islets isolated from 4 day, 2 week, and 4-week-old leptin-deficient mice rapidly (i.e., within 30 min) suppressed acetylcholine-induced insulin secretion at each stage of development. In contrast, islets from 4 day, 2 week and 4-week-old leptin-sufficient mice became progressively less responsive to leptin with development. Leptin targets pancreatic islets early in development to specifically constrain the overall capacity for acetylcholine-induced insulin secretion, and to acutely modulate this secretion.

B. INTRODUCTION

Hyperinsulinemia is detectable early in development of Lep^{ob}/Lep^{ob} mice, before they begin to overeat or develop insulin resistance and observable obesity (Dubuc, 1976). Pancreatic islets from 2-week-old Lep^{ob}/Lep^{ob} mice, the earliest age examined, do not yet hypersecrete insulin in response to glucose, but are already hypersensitive and hyperresponsive to acetylcholine potentiation of glucose-induced insulin secretion (Chen and Romsos, 1997). Presumably this acetylcholine-potentiated hypersecretion of insulin contributes to the early-onset hyperinsulinemia characteristic of these Lep^{ob}/Lep^{ob} mice.

Since an inability of Lep^{ob}/Lep^{ob} mice to synthesize the adipose tissue-derived polypeptide leptin is now known to be the primary cause of obesity in these animals (Zhang et al, 1994), it has been speculated that leptin acts within pancreatic islets to inhibit insulin secretion (Chen et al, 1997; Emilsson et al, 1997; Kieffer et al, 1997; Kulkarni et al, 1997; Ookuma et al, 1998; Pallet et al, 1997; Poitout et al, 1998; Zhao et al, 1998). Leptin receptors are present in pancreatic islets and in insulin-secreting cell lines (Chen et al, 1997). Longer-term exposure of islets or insulin-secreting cell lines to exogenous leptin lowers insulin mRNA abundance and insulin synthesis (Kulkarni et al, 1997; Seufert et al, 1997). This action of leptin provides one potential mechanism to prevent hyperinsulinemia. Other studies have examined more acute effects of leptin on insulin secretion *per se*. Leptin has been shown to inhibit insulin secretion in some studies (Chen et al, 1997; Emilsson et al, 1997; Kieffer et al, 1997; Kulkarni et al, 1997; Ookuma et al, 1998; Pallet et al, 1997; Poitout et al, 1998; Zhao et al, 1998), but not in others (Leclercq-Meyer et al, 1996; Leclercq-Meyer and Malaisse, 1997). Since leptin-

deficient *Lep*^{ob}/*Lep*^{ob} mice as early as 2 weeks of age exhibit a specific enhancement in acetylcholine-induced insulin secretion (Chen and Romsos, 1995; Chen and Romsos, 1997) this pathway might be a target for leptin action. Indeed, addition of leptin to islets from 4-wk-old *Lep*^{ob}/*Lep*^{ob} mice rapidly abolishes their enhanced acetylcholine potentiation of insulin secretion (Chen et al, 1997).

A characterization of the temporal relationship between the initial development of enhanced acetylcholine-potentiated insulin secretion from islets of neonatal *Lep*^{ob}/*Lep*^{ob} mice and of the effects of leptin on this pathway should add to our understanding of how hyperinsulinemia develops in these mice. The present study was thus conducted to first determine when the enhanced insulin secretion response to acetylcholine initially appears in *Lep*^{ob}/*Lep*^{ob} mice by examining mice younger than 2 weeks of age. Comparisons of insulin secretion from islets of +/+ versus *Lep*^{ob}/+ mice were included to determine if a single copy of the mutated *Lep*^{ob} gene would enhance acetylcholine-induced insulin secretion. The second aim of this study was to examine the role of leptin to modulate acetylcholine-induced insulin secretion from islets of young *Lep*^{ob}/*Lep*^{ob} and lean mice. Neonatal (4-day-old), 2-week-old, and 4-week-old mice were used to determine if the insulin-secretion response of islets to leptin changes as neonatal mice develop.

C. MATERIALS AND METHODS

Animals

Lep^{ob}/*Lep*^{ob} mice and lean (*Lep*^{ob}/+ and/or +/+) mice were obtained from our breeding colony (C57BL/6J-*Lep*^{ob}/+). The Guide for the Care and Use of Laboratory

Animals (National Research Council, 1985) and local institutional guidelines were followed for the care and treatment of the mice. They were housed in solid-bottom cages with wood shaving for bedding in a room maintained at 25°C with a 12:12-hr light-dark cycle (lights on at 0700). Mice were fed a nonpurified diet (Teklad Rodent Diet 8640; Harlan, Bartonville, IL). Litters were adjusted to 6 pups per litter within a few days after birth. Mice were weaned at 3 weeks of age. Approximately equal numbers of male and female mice were assigned to each group. Mice were used at 4 days, 1 week, 2 weeks and 4-5 weeks of age as noted in the experimental design. Littermate mice were compared in selected trials as noted in the table footnotes and figure legends.

Experimental design

Experiment 1. Glucose and acetylcholine-potentiated insulin secretion from islets of neonatal mice.

Islets from 1- and 2-week-old +/+, *Lep*^{ob}/+, and *Lep*^{ob}/*Lep*^{ob} mice were incubated for three consecutive 30 min periods in Krebs-Ringer bicarbonate buffer (KRB, pH, 7.4) with 0.1% bovine serum albumin (BSA, Amoresco, Solon, OH), and containing 0.5 m*M* glucose during the first 30 min period, then 20 m*M* glucose during the second period, and finally 20 m*M* glucose + 10 μ*M* acetylcholine (Sigma Chemical, St. Louis, MO) during the last period. Liver samples were obtained to genotype each pup to retrospectively separate pups into groups, i.e., +/+, *Lep*^{ob}/+, and *Lep*^{ob}/*Lep*^{ob}. Body weights and abdominal body fat pads were measured.

Experiment 2. Leptin effects on acetylcholine- potentiated insulin secretion.

The role of leptin (murine leptin, a generous gift from Pfizer Central Research, Groton, CT) to regulate insulin secretion potentiated by the acetylcholine signaling pathway was examined. Islets from lean (+/+ or Lep^{ob}/+) and Lep^{ob}/Lep^{ob} mice at 4 days, 2 weeks, and 4-5 weeks of age were incubated for three, 30-min periods in 37°C KRB with 0.1% BSA, and containing 0.5 m*M* glucose, then 10 m*M* glucose, and finally 10 m*M* glucose + 10 μ*M* acetylcholine ± 20 n*M* leptin during the last 30-min period, respectively. This dose of leptin was previously shown to maximally inhibit acethylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} (Chen et al, 1997). Lean and Lep^{ob}/Lep^{ob} mice at 4 days and 2 weeks of age were genotyped for identification. They were identified visually at 4 weeks of age.

Islet isolation

Pancreatic islets were isolated by collagenase type V (Sigma Chemical, St. Louis, MO) digestion (Lacy and Kostianovosky, 1967). Pancreases from 4 day, 1 and 2-week-old, and from 4 to 5-week-old mice were injected in multiple sites with a total of 3 ml of 37°C KRB (pH 7.4) containing 0.5 m*M* glucose, 0.01% BSA, and 0.5, 1, and 2.5 mg collagenase/ml, respectively. Each pancreas was then quickly dissected and transferred into a small tube containing 0.5 ml of 37°C KRB and 0.5 mg collagenase/ml, and incubated at 37°C with gentle shaking for about 2-3 min. Ice-cold KRB was then added to stop the digestion. After washing 2-3 times with ice-cold KRB to remove digested acinar tissue and collagenase, isolated islets were selected with the aid of a pipette under a stereoscopic microscope.

Insulin secretion and measurement of insulin

Similar-sized islets (7-10 islets/mouse) at each age were selected and distributed into small black-bottom petri dishes. Islets were preincubated at 37°C for 30 min under a 95% O_2 - 5% CO_2 atmosphere in 1 ml KRB containing 0.5 m*M* glucose and 0.1% BSA. This 30 min preincubation was followed by 30 min consecutive incubations in KRB containing various treatments.

In an earlier study from our lab, insulin secretion from islets of 2-week-old mice remained constant for 1 h when islets were exposed to 20 mM glucose (Chen and Romsos, 1995). I confirmed this observation is in the present study. Islets from 2-week-old lean mice secreted 0.6 ± 0.1 fmole insulin · $islet^{-1}$ · min^{-1} when maintained in 0.5 mM glucose for 30 min, and then 2.4 ± 0.2 and 2.2 ± 0.4 fmole insulin · $islet^{-1}$ · min^{-1} in the next two, 30-min, consecutive periods, respectively (n=5 mice). To measure insulin secretion from islets stimulated by various secretogogues, 0.5 ml of incubation media was collected. Islets secreting more than 2 fmol insulin · $islet^{-1}$ · min^{-1} in 0.5 mM glucose were considered damaged during isolation. Data from these islets were excluded.

Insulin was quantified by an enzyme-linked-immunosorbent assay (Kekow et al, 1988). Rabbit anti-guinea pig immunoglobulin G and guinea pig anti-rat insulin were purchased from EY Lab, SanMedeo, CA and Linco Research, St. Louis, MO, respectively. Rat insulin standard was purchased from Crystal Chemical, Chicago, IL. Peroxidase-labeled insulin was obtained from Sigma Chemical, St. Louis, MO.

Genotyping

DNA was extracted from livers of mice (4 day, 1 and 2-week-old mice) by a modified phenol extraction method (Sambrook et al, 1989) and used to distinguish the Lep^{ob}/Lep^{ob}, $Lep^{ob}/+$, and +/+ mice. Two different sense primers (i.e., the wild and mutant types) paired with same antisense primer were used (Namae et al, 1998). Polymerase chain reaction (PCR) products were electrophoresed on 3.5% Nuseive 3:1 agarose gel (FMC Bioproducts, Rockland, ME) and stained with ethidium bromide (Sigma Chemical, St. Louis, MO). DNA from known $Lep^{ob}/+$ mice was used as a control. Homozygous lean and Lep^{ob}/Lep^{ob} mice exhibit 100-base-pair bands amplified in the presence of wild type and mutant type primers, respectively. Heterozygous mice exhibit a 100-base-pair band amplified in the presence of both wild and mutant type primers.

Satistical analysis

Data were presented as means ± SE. Data in experiment 1 for 1-week-old and 2-week-old Lep^{ob}/Lep^{ob} versus lean littermates, and for 1-week-old +/+ versus $Lep^{ob}/+$ littermates were analyzed by the Student's paired *t*-test. Comparisons of 2-week-old +/+ versus $Lep^{ob}/+$ mice were analyzed by the Student's unpaired *t*-test because littermates were not always available. Effects of phenotype, leptin, and phenotype-leptin interactions on insulin secretion in experiment 2 were analyzed by two-way ANOVA in conjunction with LSD adjustment. Differences were considered statistically significant at $P < 0.05$.

D. RESULTS

A single copy of the mutated *Lep*^{ob} gene did not influence body weight and fat pad weights of 1- and 2-week-old pups (+/+ versus *Lep*^{ob}/+ pups) (Table 1). Although 1-and 2-week-old *Lep*^{ob}/*Lep*^{ob} and lean (+/+ or *Lep*^{ob}/+) littermates had similar body weights, abdominal fat pads of *Lep*^{ob}/*Lep*^{ob} mice were already enlarged (Table 1).

Gucose and acetylcholine- potentiated insulin secretion from neonatal mice

Islets from 1-week-old +/+ and *Lep*^{ob}/+ lean mice secreted similar amounts of insulin in response to 20 m*M* glucose, as well as in response to 20 m*M* glucose + 10 μ*M* acetylcholine (Fig. 8). At 2 weeks of age, insulin secretion also remained unaffected by a single copy of the mutated *Lep*^{ob} gene. In subsequent trials, data from +/+ and *Lep*^{ob}/+ mice (i.e., lean mice) were combined for comparisons of lean versus *Lep*^{ob}/*Lep*^{ob} mice.

Enhanced insulin secretion in response to acetylcholine has been reported in 2-week-old *Lep*^{ob}/*Lep*^{ob} mice (Chen and Romsos, 1995; Chen and Romsos, 1997). To determine whether this alteration in insulin secretion occurs in islets from younger *Lep*^{ob}/*Lep*^{ob} mice, 1-week-old mice were examined. Islets from *Lep*^{ob}/*Lep*^{ob} mice and their lean littermates secreted similar amounts of insulin in response to 20 m*M* glucose at either 1 or 2 weeks of age (Fig. 9). Addition of 10 μ*M* acetylcholine similarly increased insulin secretion from islets of 1-week-old *Lep*^{ob}/*Lep*^{ob} mice and lean littermates. But as observed previously (Chen and Romsos, 1995; Chen and Romsos, 1997), islets from 2-week-old *Lep*^{ob}/*Lep*^{ob} mice secreted more insulin in the presence of acetylcholine than did islets from lean littermates (Fig. 9). Thus, the enhanced

Table 1. Comparisons of body and abdominal fat pad weights of +/+, *Lep*^{ob}/+ and *Lep*^{ob}/*Lep*^{ob} mice

	1-week-old		2-week-old	
	+/+ (7)	*Lep*^{ob}/+ (7)	+/+ (6)	*Lep*^{ob}/+ (6)
Body weight - g	4.0 ± 0.2	4.0 ± 0.2	7.3 ± 0.2	7.0 ± 0.2
Fat pad - mg	47 ± 5	54 ± 4	94 ± 9	94 ± 12
	lean (6)	*Lep*^{ob}/*Lep*^{ob} (6)	lean (5)	*Lep*^{ob}/*Lep*^{ob} (5)
Body weight - g	3.7 ± 0.1	4.1 ± 0.1	7.0 ± 0.2	7.6 ± 0.3
Fat pad - mg	48 ± 3	81 ± 7*	84 ± 9	235 ± 33*

Values are means ± SE; numbers of animals are indicated in parentheses. Data were analyzed by the Student's paired *t*-test utilizing littermate lean (+/+ or *Lep*^{ob}/+) and *Lep*^{ob}/*Lep*^{ob} pairs, and +/+ and *Lep*^{ob}/+ pairs, and except for the comparison between 2-week-old +/+ and *Lep*^{ob}/+ mice which was made with the Student's unpaired *t*-test.
* Indicates significant differences ($P < 0.05$)

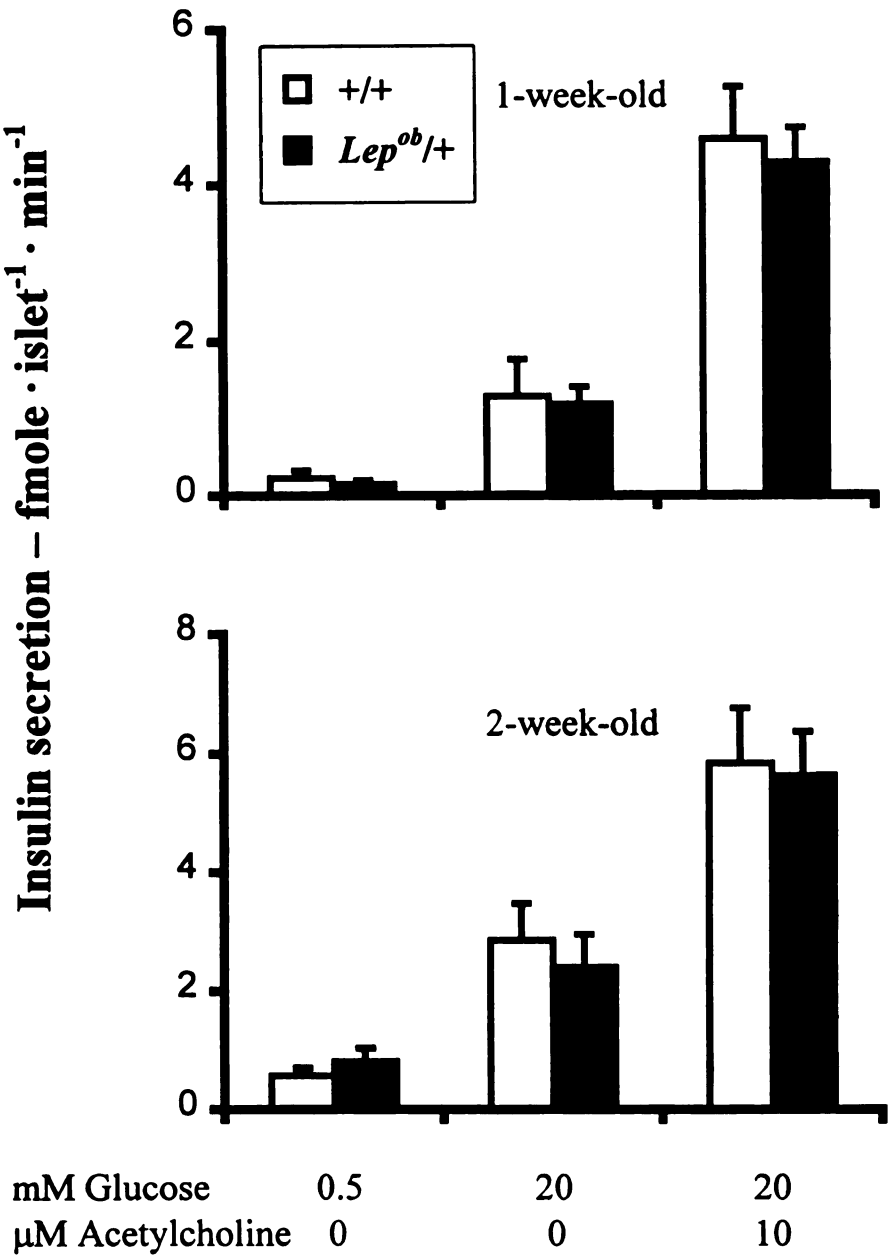

Figure 8. Insulin secretion from islets of homozygote (+/+) and heterozygote (*Lep*^{ob}/+) lean mice. Islets from 1-week- (n=7) and 2-week-old (n=6) mice were incubated for 30 min in 0.5 m*M* glucose, then in 20 m*M* glucose for 30 min, and finally in 20 m*M* glucose + 10 μ*M* acetylcholine for 30 min. Heterozygosity did not influence glucose or acetycholine-potentiated insulin secretion at either age as determined by Student's *t*-test ($P>0.05$).

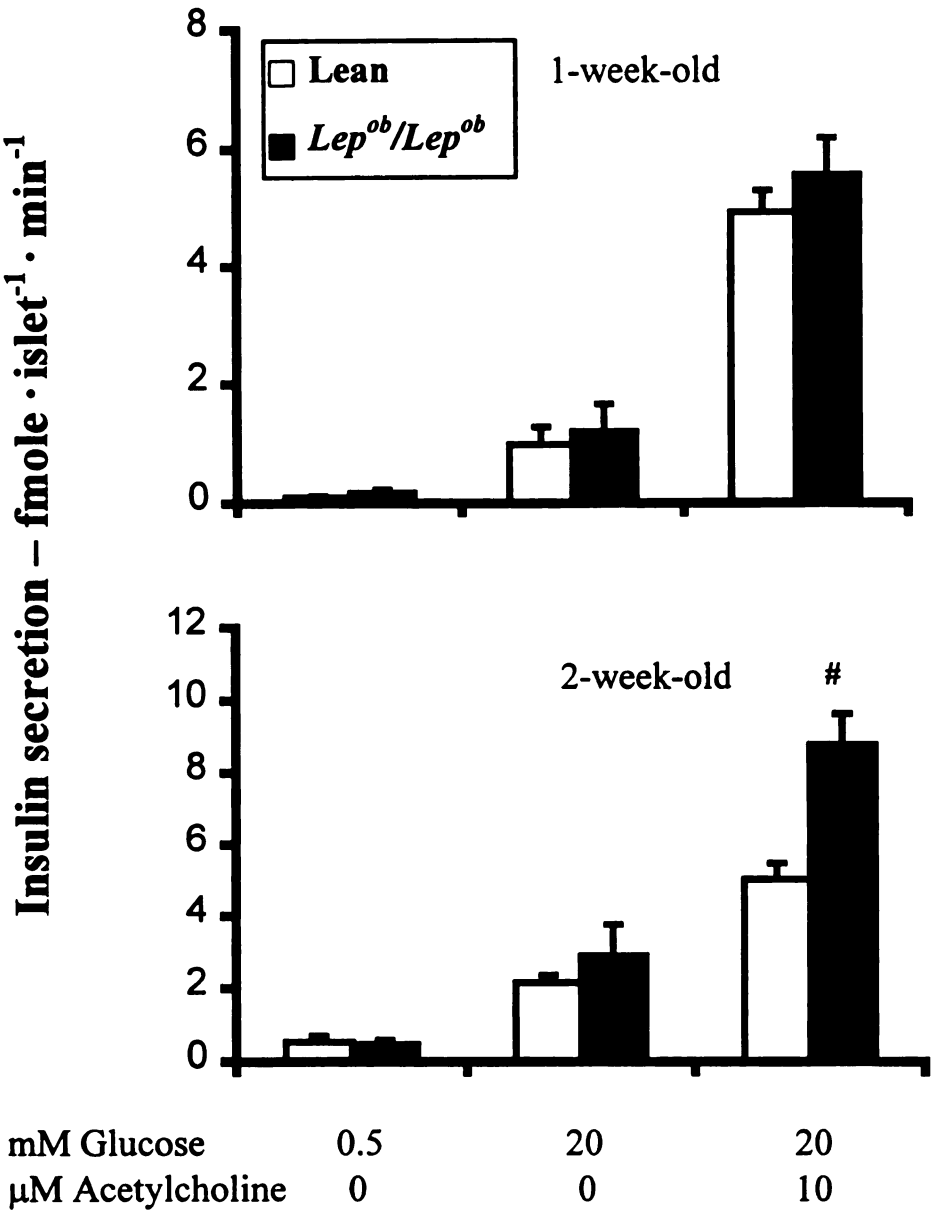

Figure 9. Insulin secretion from pancreatic islets of 1 and 2-week-old mice. Islets from 1-week-old (n=6) and 2-week-old (n=5) *Lep^{ob}/Lep^{ob}* and lean littermate mice were incubated in 0.5 mM glucose for 30 min, and then in 20 mM glucose for 30 min, followed by 20 mM glucose + 10 μM acetylcholine for 30 min. Diameters of the islets from 1-week-old *Lep^{ob}/Lep^{ob}* and lean littermates averaged 0.098 ± 0.004 and 0.095 ± 0.002 mm, respectively. Diameters of the islets from 2-week-old *Lep^{ob}/Lep^{ob}* and lean littermates averaged 0.111 ± 0.002 and 0.107 ± 0.001 mm, respectively. Data represent means ± SE. Phenotype effects on insulin secretion stimulated by 20 mM glucose and by 20 mM glucose plus 10 μM acetylcholine were determined by Student's paired *t*-test. A significant (#, $P < 0.05$) phenotype effect on insulin secretion was observed in the presence of acetylcholine at 2 weeks of age.

acetylcholine-induced insulin secretion in *Lep*^{ob}/*Lep*^{ob} pups first occurs between 1 and 2 weeks of age.

Effects of leptin on acetylcholine- potentiated insulin secretion

At 4 days of age, acetylcholine-potentiated insulin secretion equally from islets of lean and *Lep*^{ob}/*Lep*^{ob} mice (Fig. 10, upper panel), consistent with observations in 1-week-old mice (Fig. 9). At this age, leptin suppressed acetylcholine-potentiated insulin secretion equally from islets of lean and *Lep*^{ob}/*Lep*^{ob} pups (Fig. 10, upper panel). At 2 weeks of age, *Lep*^{ob}/*Lep*^{ob} mice begin to hypersecrete insulin in response to acetylcholine (Fig. 9, and Fig. 10, middle panel). At this age, islets from both lean and *Lep*^{ob}/*Lep*^{ob} mice respond to leptin with lowered acetylcholine-induced insulin secretion (Fig. 10, middle panel). Leptin no longer inhibited *in vitro* acetylcholine-potentiated insulin secretion from islets of young adult (4-week-old) lean mice, but leptin continued to suppress acetylcholine-induced insulin secretion from islets of adult leptin-deficient *Lep*^{ob}/*Lep*^{ob} mice (Fig. 10, lower panel).

E. DISCUSSION

The two major findings from the present study are: 1) hypersecretion of insulin in response to acetylcholine first appears in islets from *Lep*^{ob}/*Lep*^{ob} mice between 1 and 2 weeks of age; and 2) the effectiveness of leptin to constrain acetylcholine-induced insulin secretion persists in islets of *Lep*^{ob}/*Lep*^{ob} mice, but moderates in islets from lean mice, between 4 days of age and 4 weeks of age.

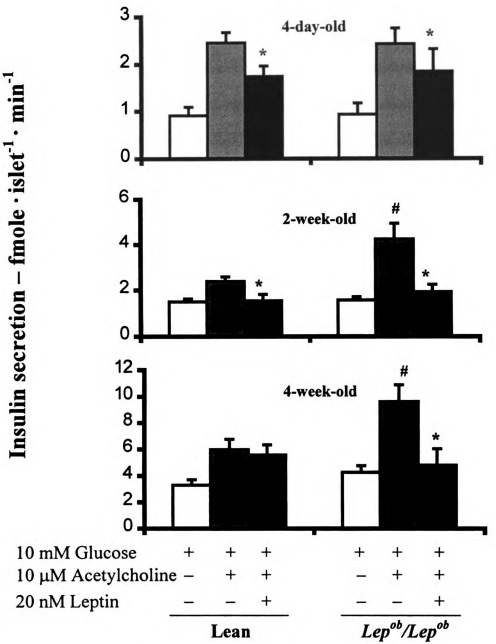

Figure 10. Effects of leptin on acetylcholine potentiation of glucose-induced insulin secretion. Islets from 4 day, 2 and 4-week-old *Lep^{ob}/Lep^{ob}* and lean counterparts (n=11-23, 9-11, and 9-12 mice at 4 days, 2 and 4 weeks of age, respectively) were incubated in 0.5 mM glucose for 30 min, and then in 10 mM glucose for 30 min, followed by 10 mM glucose + 10 μM acetylcholine ± 20 nM leptin for 30 min. Data represent means ± SE. Significant ($P < 0.05$) effects of leptin on acetylcholine potentiation of insulin secretion as analyzed by two-way ANOVA in conjunction with LSD adjustment, are indicated by the asterisks. # Indicates a significant effect of phenotype on acetylcholine-induced insulin secretion.

Comparisons of adult lean *Lep*^{ob}/+ versus +/+ mice indicate that inheritance of a single copy of the mutated *Lep*^{ob} gene may cause subtle metabolic effects (Chung et al, 1998). Since one purpose of the present study was to determine when the enhanced insulin secretion response to acetylcholine first appears in *Lep*^{ob}/*Lep*^{ob} mice, it was important to determine if inclusion of pups with a single copy of the mutated *Lep*^{ob} gene would confound this determination. Pups with a single copy of the mutated *Lep*^{ob} gene were indistinguishable from +/+ pups (Table 1 and Fig. 8). This enabled us to pool results from +/+ or *Lep*^{ob}/+ mice in comparison with *Lep*^{ob}/*Lep*^{ob} mice.

Acetylcholine potentiates glucose-induced insulin secretion by activating muscarinic receptors to stimulate the phospholipase C-protein kinase C (PLC-PKC) pathway (Prentki and Matschinsky, 1987). This stimulatory pathway is already present in islets from neonatal mice to substantially elevate insulin secretion above rates observed in the presence of glucose alone (Figs. 8-10). In 4-day-old and 1-week-old pups, acetylcholine increased glucose-induced insulin secretion by 169% to 354% (Figs. 8-10). Acetylcholine continued to stimulate insulin secretion from islets of 2 and 4-week-old lean mice, but the percentage increases above glucose-induced insulin were less pronounced (i.e., 81% to 103%) (Figs. 8-10). In contrast to the age-associated decline in percentage increase in glucose-induced insulin secretion caused by exposure of islets from lean mice to acetylcholine, islets from *Lep*^{ob}/*Lep*^{ob} mice continued to respond to acetylcholine with increases in insulin secretion of 186% at 2 weeks of age (Figs. 9 and 10) and 125% at 4 weeks of age (Fig. 10). This failure of islets from *Lep*^{ob}/*Lep*^{ob} mice to decrease their stimulatory response to acetylcholine between 1 and 2 weeks of age as much as occurred in islets from lean mice explains why islets from 2-

week-old Lep^{ob}/Lep^{ob} mice secrete more insulin in response to acetylcholine than islets from lean mice (Figs. 9 and 10). This implies that during normal development some constraint of the PLC-PKC pathway in pancreatic islets emerges between 1 and 2 weeks of age to control insulin secretion. As discussed below, the presence of leptin in lean mice appears critically important at this stage of development.

Leptin acutely inhibited acetylcholine-induced insulin secretion from islets of 4-day-old lean and Lep^{ob}/Lep^{ob} pups (Fig. 10). This effect of leptin on islets occurred considerably earlier in development than effects of leptin on food intake or metabolic rate, which are not evident until after 2 weeks of age in these mice (Mistry et al, 1999). It is not clear whether the leptin signal transduction system *per se* matures earlier in islets than in the hypothalamus, or whether other components of these downstream physiological response pathways emerge at differential times during development.

Islets from 2-week-old lean mice continued to respond to acute exposure to leptin with lowered acetylcholine-induced insulin secretion, but this acute effect of leptin on insulin secretion was no longer evident in islets from 4-week-old lean mice (Fig. 10). Islets from 4-week-old, leptin-deficient Lep^{ob}/Lep^{ob} mice, however, continued to respond to leptin (Fig. 10). These results suggest that continued exposure of islets to leptin, as occurs *in vivo* in lean mice (Mistry et al, 1999), diminishes the acute effect of leptin on acetylcholine-induced insulin secretion. The chronic *in vivo* exposure of islets in lean mice to leptin may have also constrained the capacity of these islets to increase insulin secretion in response to acetylcholine. These effects of leptin on pancreatic islets, coupled with effects on food intake which emerge after 2 weeks of age (Mistry et al, 1999), help coordinate the regulation of insulin secretion in lean mice.

CHAPTER IV. LEPTIN CONSTRAINS PHOSPHOLIPASE C-PROTEIN KINASE C-INDUCED INSULIN SECRETION VIA A PHOSPHATIDYLINOSITOL 3-KINASE-DEPENDENT PATHWAY

A. ABSTRACT

Leptin-deficient *Lep^{ob}/Lep^{ob}* mice hypersecrete insulin in response to acetylcholine stimulation of the phospholipase C-protein kinase C (PLC-PKC) pathway, and leptin constrains this hypersecretion. Leptin has been reported to activate phosphatidylinositol 3-kinase (PI 3-K) and subsequently phosphodiesterase (PDE) to impair protein kinase A (PKA)-induced insulin secretion from cultured islets of neonatal rats. Present study determined if PKA-induced insulin secretion was altered in islets from *Lep^{ob}/Lep^{ob}* mice, and if leptin affected this pathway in islets from these mice. Additionally, the possible role for PI 3-K and PDE in leptin-induced control of acetylcholine-induced insulin secretion was examined. Stimulation of insulin secretion with GLP-1, forskolin (an activator of adenylyl cyclase), or IBMX (an inhibitor of PDE) did not cause hypersecretion of insulin from islets young *Lep^{ob}/Lep^{ob}* mice, and leptin did not inhibit insulin secretion from islets of mice. Inhibition of PDE with IBMX did not block leptin-induced inhibition of acetylcholine-mediated insulin secretion from islets of *Lep^{ob}/Lep^{ob}* mice. Preincubation of islets with wortmannin, an inhibitor of PI 3-K activity, blocked the ability of leptin to constrain acetylcholine- induced insulin secretion from islets of *Lep^{ob}/Lep^{ob}* mice. I conclude that the capacity of the PKA pathway is not altered in islets from *Lep^{ob}/Lep^{ob}* mice, and that leptin does

not regulate this pathway in islets from mice. Leptin may stimulate PI 3-K to constrain PLC-PKC-induced insulin secretion from islets of *Lep*^{ob}/*Lep*^{ob} mice.

B. INTRODUCTION

A mutation in the *Lep*^{ob} gene disrupts leptin synthesis and causes profound obesity in *Lep*^{ob}/*Lep*^{ob} mice (Zhang et al, 1994). These animals are hyperinsulinemic early in development, before they exhibit elevated food intake (Lin et al, 1977), decreased metabolic rates (Boissonneault et al, 1978; Trayhurn et al, 1977) or insulin resistance (Dubuc 1976). This suggests that leptin might directly regulate insulin synthesis or secretion. Several reports have shown that leptin decreases insulin mRNA abundance in islets and cell lines (Seufert et al, 1999; Kulkarni et al, 1997). Other studies have focused on insulin secretion. Leptin inhibits insulin secretion from islets and insulin-secreting cell lines (Emilsson et al, 1997; Fehmann et al, 1997a; 1997b; 1997c; Kieffer et al, 1997; Kulkarni et al, 1997; Ishida et al, 1997; Ookuma et al, 1998; Pallet et al, 1997; Poitout et al, 1998; Shimizu et al, 1997; Zhao et al, 1998), although there are conflicting reports (Leclercq-Meyer et al, 1996; Leclercq-Meyer and Malaisse, 1997).

Insulin secretion is stimulated by a variety of signals including nutrients, neurotransmitters and hormones that interact within the pancreatic islets (Zawalich and Rasmussen, 1990). Glucose-induced insulin secretion is potentiated by stimulation of the phospholipase C-protein kinase C (PLC-PKC) and protein kinase A (PKA) signal transduction pathways (Prentki and Matschinsky, 1987). Acetylcholine and cholecystokinin activate the PLC-PKC signaling pathway to stimulate insulin secretion

(Zawalich et al, 1989). Enhanced sensitivity of islets to this pathway has been suggested as a possible mechanism for initial development of hyperinsulinemia in *Lep*^{ob}/*Lep*^{ob} mice (Chen and Romsos, 1995). Islets from 2-week-old, as well as adult *Lep*^{ob}/*Lep*^{ob} mice, hypersecrete insulin in response to acetylcholine and cholecystokinin, and this hypersecretion of insulin is suppressible by leptin (Chen and Romsos, 1995; Chen et al, 1997; Lee and Romsos, 2001). The inhibitory effect of leptin was still present when insulin secretion was stimulated by a PKC agonist, phorbol-12-myristate-13-acetate (Chen et al, 1997; Ookuma et al, 1998), suggesting that this pathway is a possible target for leptin to regulate insulin secretion.

The possibility that the PKA pathway might also be activated to cause hypersecretion of insulin from islets of young *Lep*^{ob}/*Lep*^{ob} mice has not been as extensively investigated as the PLC-PKC pathway in these mice. Leptin was shown to activate phosphodiesterase (PDE) 3 B in islets from neonatal rats, suppress cAMP content of the islets, and inhibit glucagon-like peptide-1 (GLP-1) stimulated insulin secretion (Zhao et al, 1998). This raises the possibility that islets from leptin-deficient mice might exhibit an enhanced rate of insulin secretion when exposed to GLP-1 to activate adenylyl cyclase. The observation that leptin activates PDE in islets from neonatal rats (Zhao et al, 1998) also raises the possibility that the leptin-induced inhibition of PLC-PKC mediated insulin secretion observed in islets from *Lep*^{ob}/*Lep*^{ob} mice might occur via cross-talk between the PKA and PLC-PKC pathways (Chen and Romsos, 1995; Garrel et al, 1997).

Zhao et al, 1998 showed that the leptin-induced activation of PDE in islets from neonatal rats was mediated by activation of phosphatidylinositol 3-kinase (PI 3-K).

Inhibition of PI 3-K activity by wortmannin blocked the ability of leptin to inhibit GLP-1 induced insulin secretion. Other reports have linked the inhibitory effects of leptin on insulin secretion to activation of K^+_{ATP} channels via activation of PI 3-K activity (Harvey and Ashford, 1998; Harvey et al, 2000). Leptin also functions in other tissues to stimulate PI 3-K and affect glucose transport and glycogen synthesis (Berti et al, 1997; Kellerer et al, 1997). The possibility that the enhanced acetylcholine potentiation of insulin secretion from *Lep*^{ob}/*Lep*^{ob} mice is linked to PI 3-K activity to has our knowledge not been investigated.

The present study was undertaken first to determine if islets from neonatal, leptin-deficient *Lep*^{ob}/*Lep*^{ob} mice hypersecrete insulin in response to the PKA signaling pathway, as is observed when the PLC-PKC signaling pathway is stimulated in islets from these mice. Next, the effects of leptin on insulin secretion mediated by the PKA signaling pathway in islets from mice were examined. Finally, wortmannin, an inhibitor of PI 3-K, was used to investigate the possible role for PI 3-K in the enhanced acetylcholine-induced insulin secretion characteristic of islets from young *Lep*^{ob}/*Lep*^{ob} mice.

C. MATERIALS AND METHODS

Animals

Male and female *Lep*^{ob}/*Lep*^{ob} and lean mice were obtained from our breeding colony (C57BL/6J-*Lep*^{ob}/+). Care and treatment of the mice was according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1985) and local institutional guidelines. Mice were housed in solid-bottom cages with wood

shavings for bedding and were maintained at 25°C with a 12:12-hr light-dark cycle (lights on at 0700 hr). They were fed a nonpurified diet (Harlan Teklad Rodent Diet 8640; Madison, WI). Litters were adjusted to 6 pups per litter within a few days after birth. Mice were weaned at 3 weeks of age. Mice were used at 2, and 4 to 5 weeks of age. Littermate mice were used in selected trials as noted in the table footnotes and figure legends.

Experimental design

Experiment 1. Islets from 2-week-old leptin-deficient Lep^{ob}/Lep^{ob} pups respond normally to glucose, but increase insulin secretion more in response to activation of the PLC-PKC pathway than islets from lean littermates (Chen and Romsos, 1995; Lee and Romsos, 2001). This experiment utilized GLP-1, a peptide that activates adenylyl cyclase via a receptor-mediated process (Drucker et al, 1987; Göke and Conloon, 1988); forskolin, a direct activator of adenylyl cyclase (Seamon and Daly, 1981); and 3-isobutyl-1-metylxanthin (IBMX), an inhibitor of PDE (Beavo et al, 1970), to determine if insulin secretion mediated by the PKA signal transduction pathway was altered in islets from 2-week-old Lep^{ob}/Lep^{ob} mice.

Experiment 2. Leptin has been reported to suppress GLP-1-induced insulin secretion from islets of neonatal rats (Zhao et al, 1998). The ability of leptin (murine leptin, a generous gift from Pfizer Central Research, Groton, CT) to suppress GLP-1-induced insulin secretion from islets of 4-week-old lean mice was thus examined.

Experiment 3. Acetylcholine, via activation of the PLC-PKC signaling pathway, causes hypersecretion of insulin from islets of Lep^{ob}/Lep^{ob} mice, and leptin suppresses

this hypersecretion (Chen et al, 1997; Lee and Romsos, 2001). Leptin suppresses PKA activity in neonatal rat pancreatic islets by activating PDE (Zhao et al, 1998). To determine if PDE might, via cross-talk between the PKA and PLC-PKC signal transduction systems (Chen and Romsos, 1995; Garrel et al, 1997), influence acetylcholine-induced insulin secretion, acetylcholine-induced insulin secretion was measured in the presence and absence of leptin and IBMX, an antagonist of PDE.

Experiment 4. Wortmannin (20 nM), an inhibitor of PI 3-K activity, was used to examine the role of PI 3-K activity in acetylcholine-induced insulin secretion from islets of 4- to 5-week-old lean and Lep^{ob}/Lep^{ob} mice.

Islet isolation, insulin secretion, and insulin assay

Pancreatic islets were isolated with collagenase type V (Sigma Chemical, St. Louis, MO) as described previously (Lee and Romsos, 2001). Isolated islets were selected with the aid of a pipette under a stereoscopic microscope. Similar-sized islets from individual mice (10 islets/dish) were distributed into small (35 mm) black-bottom petri dishes. In experiment 4, islets from two mice were pooled, and then aliquoted (10 islets/dish) into 4 petri dishes. Islets were pre-incubated at 37°C for 30 min under a 95% O_2 - 5% CO_2 atmosphere in 1 ml Krebs-Ringer bicarbonate buffer (KRB, pH 7.4) containing 0.5 mM glucose and 0.1% bovine serum albumin (BSA, Amoresco, Solon, OH). Islets were then incubated in KRB containing 10 mM glucose (or 10 mM glucose + 20 nM wortmannin, as indicated in the legend to figure 6), during a second 30-min period, and then incubated in KRB containing various treatments during the last 30-min period, as indicated in the figure legends.

To measure insulin secretion, 0.5 ml of incubation media was collected. Insulin was quantified by an enzyme-linked-immunosorbent assay (Kekow et al, 1988).

Statistical analysis

Data are presented as means ± SE. Data from experiment 1 and experiment 2-4 were analyzed by two-way ANOVA and one-way ANOVA, respectively, in conjunction with LSD adjustment. Differences were considered statistically significant at $P < 0.05$.

D. RESULTS

Protein kinase A potentiated insulin secretion from islets of lean and Lep^{ob}/Lep^{ob} mice.

Islets from 2-week-old lean and Lep^{ob}/Lep^{ob} littermates secreted similar amounts of insulin in the presence of 10 mM glucose alone (Fig. 11), consistent with previous studies (Chen and Romsos, 1997; Lee and Romsos, 2001). Addition of 1 μM GLP-1, but not 0.1 μM GLP-1, significantly ($P < 0.05$) potentiated glucose-induced insulin secretion similarly from islets of 2-week-old lean and Lep^{ob}/Lep^{ob} littermates (Fig. 11). Stimulation of adenylyl cyclase with 2.5 μM forskolin markedly increased insulin secretion from islets of lean and Lep^{ob}/Lep^{ob} littermates (Fig. 11). Addition of IBMX, an inhibitor of PDE, also increased insulin secretion from islets of both groups of mice (Fig. 11). Milrinone (9 μM), a specific inhibitor of PDE 3 B (Harrison et al, 1986), increased insulin secretion as much as IBMX did from islets of 4-week-old lean mice (0.64 ± 0.09, 3.40 ± 1.12 and 3.77 ± 0.65 fmole · islet $^{-1}$ · min^{-1} in response to glucose,

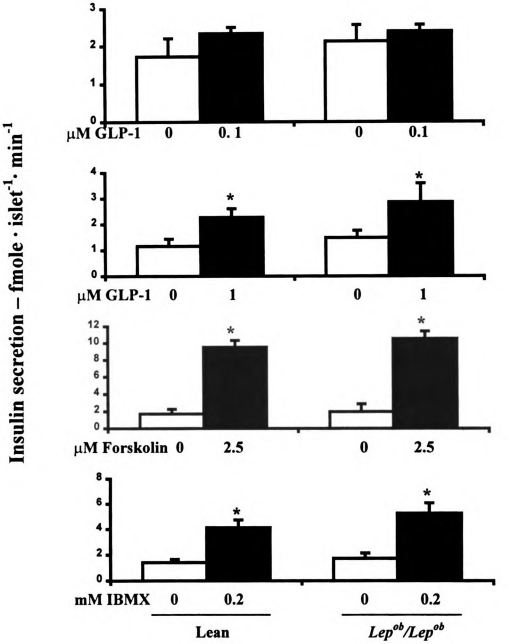

Figure 11. Stimulation of insulin secretion by the protein kinase A signaling pathway in islets from 2-week-old lean and *Lep*^{ob}/*Lep*^{ob} littermates (n=7-8). Islets were incubated in 10 mM glucose for 30 min, and then in 10 mM glucose plus 0.1 μM or 1 μM GLP-1, 2.5 μM forskolin (an activator of adenylyl cyclase), or 0.2 mM IBMX (an antagonist of phosphodiesterase) for 30 min. Data represent means ± SE. * Indicates significant (P <0.05) effects of 1.0 μM GLP-1, 2.5 μM forskolin, and 0.2 mM IBMX on insulin secretion, as determined by two-way ANOVA in conjunction with LSD test. Phenotype did not influence insulin secretion.

glucose plus milrinone, and glucose plus IBMX, respectively). The similar insulin secretion responses of islets from 2-week-old lean and Lep^{ob}/Lep^{ob} littermates to stimulators of the PKA signaling pathway (i.e., GLP-1, forskolin, and IBMX) (Fig. 11) suggest that the increased plasma insulin concentrations observed in these young Lep^{ob}/Lep^{ob} mice is not caused by an increased capacity for PKA-induced insulin secretion.

GLP-1- potentiated insulin secretion was not inhibited by leptin

Addition of 20 nM leptin to islets from 4-week-old lean mice did not inhibit GLP-1 (1 μM)-induced insulin secretion (data not shown). The concentration of GLP-1 (i.e., 1 μM) used in this trial may have been too high for leptin to exert an inhibitory action, as noted by Zhao et al, 1998. Because 0.1 μM GLP-1 failed to stimulated insulin secretion from islets of mice (Fig. 11), a subsequent trial utilized an intermediate GLP-1 concentration (i.e., 0.5 μM GLP-1). Insulin secretion was elevated by 0.5 μM GLP-1, but leptin failed to inhibit secretion (Fig. 12). These results suggest that the PKA signaling pathway may not be a target for leptin action in islets from mice.

Leptin inhibits acetylcholine-potentiated insulin secretion in the presence of IBMX.

The possibility that leptin may act via a PDE-dependent signaling pathway (Zhao et al, 1998) to regulate acetylcholine-induced insulin secretion was investigated. As shown previously (Chen et al, 1997; Lee and Romsos, 2001), leptin suppressed 10 μM acetylcholine-potentiated, glucose-induced insulin secretion from islets of 4- to 5-

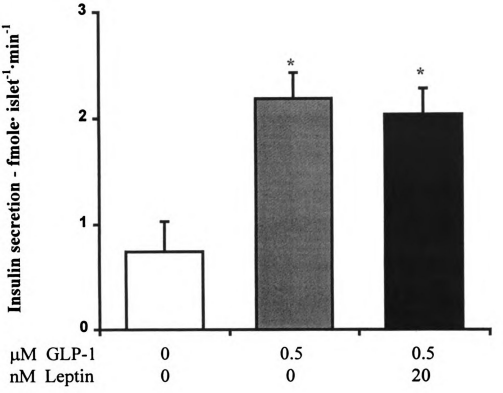

Figure 12. Leptin did not affect GLP-1-induced insulin secretion. Islets from 4-week-old lean mice were incubated in 10 mM glucose for 30 min, followed by 10 mM glucose + 0.5 μM GLP-1 ± 20 nM leptin for 30 min. Data represent means ± SE (n= 7). * Indicates that 0.5 μM GLP-1 significantly (P<0.05) increased insulin secretion, as determined by one-way ANOVA in conjunction with LSD. Leptin did not influence GLP-1 induced insulin secretion.

week-old Lep^{ob}/Lep^{ob} mice, but not from islets of lean mice (Fig. 13). In the presence of 0.2 mM IBMX, a PDE inhibitor, acetylcholine-induced insulin secretion was further increased in islets from both lean and Lep^{ob}/Lep^{ob} mice. Addition of 0.2 mM IBMX did not affect the ability of 20 nM leptin to suppress acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice (Fig. 13). This suggests that the leptin-induced reduction of acetylcholine-potentiated insulin secretion from islets Lep^{ob}/Lep^{ob} mice is independent of PDE activity.

Inhibition of PI 3-K and insulin secretion

To determine if PI 3-K activity affects insulin secretion from islets of mice, insulin secretion was examined in the presence of wortmannin, an inhibitor of PI 3-K. Wortmannin tended to increase 10 mM glucose-induced insulin secretion (1.12 ± 0.22 and 1.96 ± 0.32 fmole insulin released $\cdot$ islet $^{-1} \cdot$ min^{-1} in the absence and presence of wortmannin, respectively, P=0.059) from islets of 4-week-old lean mice, as determined by Student's *t*-test. Wortmannin significantly increased acetylcholine-potentiation of insulin secretion from islets of 4-week-old lean mice (Fig. 14).

In another trial, 20 nM wortmannin was again shown to stimulate acetylcholine-induced insulin secretion from islets of lean mice (Fig. 15). In contrast, the presence of wortmannin did not stimulated acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice (Fig. 15).

Consistent with the finding in Fig. 13, leptin suppressed acetylcholine-potentiated insulin secretion from islets of Lep^{ob}/Lep^{ob} mice, but not from islets of lean mice (Fig. 15). Islets incubated with wortmannin alone secreted more insulin in

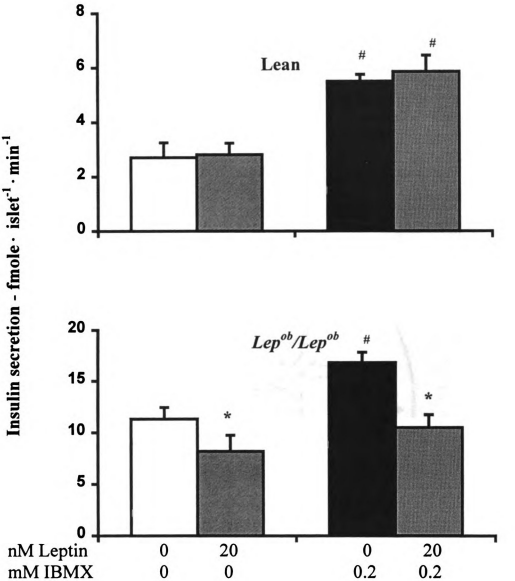

Figure 13. Leptin inhibited acetylcholine-induced insulin secretion from islets of *Lep*^{ob}/*Lep*^{ob} mice, but not in lean mice, in the absence or presence of IBMX. Islets from 4- to 5-week-old lean and *Lep*^{ob}/*Lep*^{ob} mice were incubated in 10 mM glucose for 30 min. Insulin secretion averaged 0.74 ± 0.17 and 4.93 ± 0.42 fmole · islet $^{-1}$· min^{-1} in lean and *Lep*^{ob}/*Lep*^{ob} mice, respectively. All islets were then exposed to 10 mM glucose plus 10 μM acetylcholine ± 20 nM leptin, and ± 0.2 mM IBMX as indicated in the figure. Data represent means ± SE (n=5-7). * Indicates a significant effect of leptin on acetylcholine-induced insulin secretion in the absence or in the presence of IBMX, and # indicates a significant stimulatory effect of IBMX on insulin secretion, as determined by one-way ANOVA in conjunction with LSD test (P<0.05).

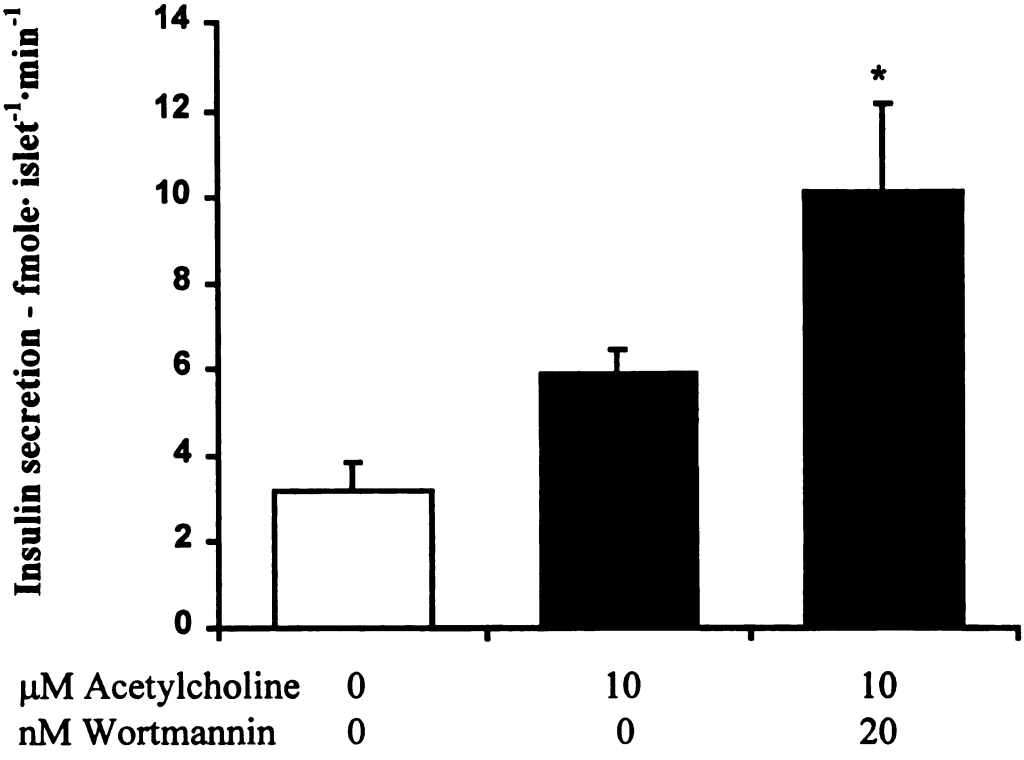

Figure 14. Inhibition of PI 3-K with wortmannin increased acetylcholine-induced insulin secretion. Islets from 4- to 5-week-old lean mice were incubated in10 mM glucose for 30 min, and then in 10 mM glucose plus 10 μM acetylcholine ± 20 nM wortmannin for 30 min. Data represent means ± SE (n=6). Significant (P<0.05) effect of wortmannin on acetylcholine-induced insulin secretion, indicated with an asterisk, as determined by one-way ANOVA in conjunction with post-hoc LSD test.

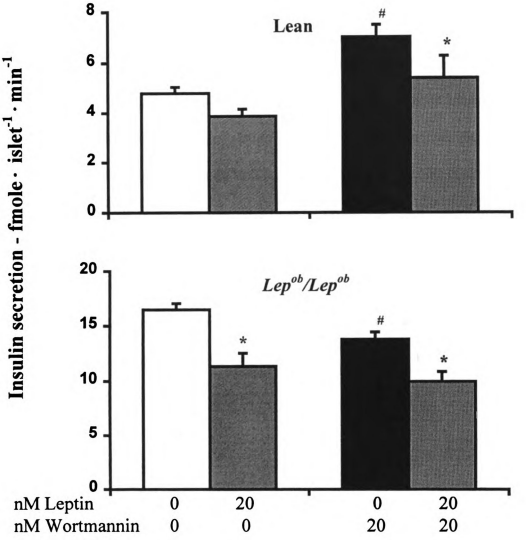

Figure 15. Wortmannin stimulated acetylcholine-induced insulin secretion from islets of lean, but not *Lep*^{ob}/*Lep*^{ob}, mice, and acetylcholine-induced insulin secretion was lower in islets simultaneously co-exposed to leptin and wortmannin than in islets exposed to wortmannin alone. Islets from 4- to 5-week-old lean and *Lep*^{ob}/*Lep*^{ob} mice were incubated in 10 mM glucose for 30 min. Glucose-induced insulin secretion averaged 2.02 ± 0.40 and 6.40 ± 0.83 fmole · islet $^{-1}$· min^{-1} for lean and *Lep*^{ob}/*Lep*^{ob} mice, respectively. All islets were then simultaneously exposed to10 mM glucose plus 10 μM acetylcholine ± 20 nM leptin, and ± 20 nM wortmannin, as indicated in the figure. Data represent means ± SE (n=5-6). # Indicates significant (P<0.05) effect of wortmannin on acetylcholine-induced insulin secretion, and * indicates significant (P<0.05) inhibitory effect of leptin on insulin secretion, as determined by one-way ANOVA in conjunction with post-hoc LSD test.

response to acetylcholine than islets simultaneously coincubated with wortmannin and leptin (Fig. 15).

Wortmannin inhibits PI 3-K in pancreatic islets with a time lag of as long as 20 min (Eto et al, 2002; Zawalich and Zawalich, 2000), whereas leptin inhibits acetylcholine-induced insulin secretion within 3 minutes (Chen et al, 1997). Thus, the more rapid-onset actions of leptin to block insulin secretion may be overroad the stimulatory effects of wortmannin on insulin secretion (Fig. 15).

To determine if leptin would inhibit acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice when PI 3-K was pre-inhibited, islets were preincubated with wortmannin for 30 min prior to incubation with acetylcholine ± leptin. Under these conditions, wortmannin blocked the ability of leptin to inhibit acetylcholine-induced insulin secretion (Fig. 16).

E. DISCUSSION

The present study was undertaken to further examine the basis for hypersecretion of insulin from islets of leptin-deficient Lep^{ob}/Lep^{ob} mice. We focused that the capacity for PKA-induced insulin secretion was not elevated in islets of young Lep^{ob}/Lep^{ob} mice. But insulin secretion associated with modulation of the PI 3-K signal transduction pathway was altered in these mice. Wortmannin, an inhibitor of PI 3-K, stimulated acetylcholine-induced insulin secretion from islets of lean mice, but not islets of Lep^{ob}/Lep^{ob} mice. This suggested that PI 3-K might be inactive in islets from Lep^{ob}/Lep^{ob} mice, and that this low PI 3-K activity might contribute to their hypersecretion of insulin. Leptin, an activator of PI 3-K, inhibited the acetylcholine-

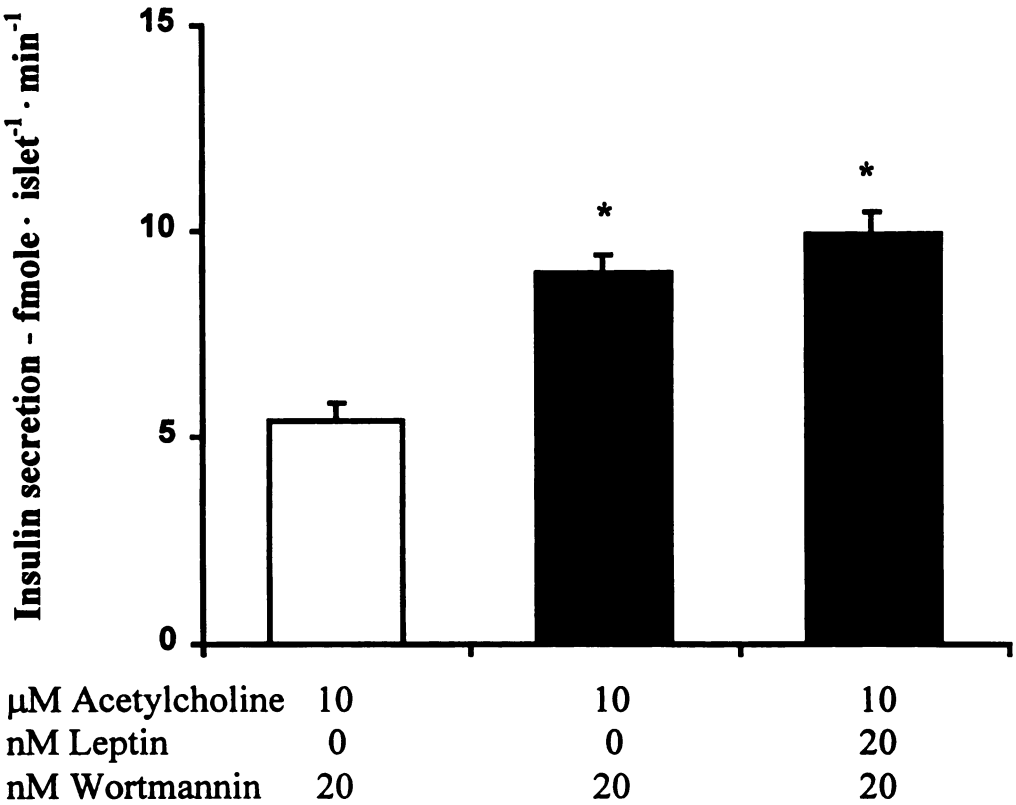

Figure 16. Preincubation of islets from *Lep^{ob}/Lep^{ob}* mice with wortmannin blocked the ability of leptin to inhibit acetylcholine-induced insulin secretion. Islets from 4- to 5-week-old *Lep^{ob}/Lep^{ob}* mice were incubated in 10 mM glucose plus 20 nM wortmannin for 30 min, and then incubated in 10 mM glucose + 10 μM acetylcholine + 20 nM wortmannin ± 20 nM leptin for an additional 30 min, as indicated in the figure. * Indicates a significant (P<0.05) stimulatory effect of acetylcholine on insulin secretion in the presence of wortmannin, as determined by one-way ANOVA in conjunction with post-hoc LSD test. Leptin failed to influence insulin secretion.

induced hypersecretion of insulin from islets of *Lep*^{ob}/*Lep*^{ob} mice, and this effect of leptin was blocked in islets preincubated with wortmannin.

Several approaches were used to determine if PKA-induced insulin secretion was abnormally elevated in islets from leptin-deficient *Lep*^{ob}/*Lep*^{ob} mice. Islets from leptin-deficient *Lep*^{ob}/*Lep*^{ob} mice responded similarly to GLP-1 (two doses), forskolin, and IBMX (Fig. 11). These data suggested that PKA-induced insulin secretion is not altered in islets from young *Lep*^{ob}/*Lep*^{ob} mice, even though islets from older *Lep*^{ob}/*Lep*^{ob} mice do hypersecrete insulin in response to forskolin (Black et al, 1986). Likewise, islets from young *Lep*^{ob}/*Lep*^{ob} mice do not hypersecrete insulin in response to glucose (Fig. 11), whereas islets from older *Lep*^{ob}/*Lep*^{ob} mice are very hyperresponsive to glucose (Chen and Romsos, 1997). We conclude that leptin deficiency does not directly target glucose and PKA-induced insulin secretion pathways in mouse islets, but rather target a pathway associated with the PLC-PKC signaling pathway, which is altered very early in development of *Lep*^{ob}/*Lep*^{ob} mice (Chen and Romsos, 1995; Lee and Romsos, 2001). The increases in glucose-induced and PKA-induced insulin secretion appear to the secondary compensatory responses to prolonged hyperphagia and other consequences of leptin deficiency in these *Lep*^{ob}/*Lep*^{ob} mice.

In contrast to the observation in islets from neonatal rats where leptin inhibited GLP-1-induced insulin secretion (Zhao et al, 1998), leptin did not influence GLP-1-induced insulin secretion from islets of mice (Fig. 12). This failure of leptin to inhibit GLP-1-induced insulin secretion from islets of mice is consistent with our observation that GLP-1-induced insulin secretion is not elevated in islets from young leptin-deficient *Lep*^{ob}/*Lep*^{ob} mice. If this pathway was a primary target for leptin action, leptin

deficiency would be expected to enhance GLP-1-induced insulin secretion. GLP-1 appears to be a more potent stimulator of insulin secretion from islets of rats than mice (Zawalich, 1996). Zhao et al, 1998 demonstrated a significant inhibitory effect of leptin on GLP-1-induced insulin secretion from islets of neonatal mice in the presence of 0.1 nM GLP-1, but higher concentration of GLP-1 (i.e., 1 μM) blocked the inhibitory effects of leptin. In the present study 0.1 nM GLP-1 failed to increase insulin secretion (data not presented) and even 100 nM GLP-1 was ineffective (Fig. 11). I concluded that the initial hyperinsulinemia characteristic of young Lep^{ob}/Lep^{ob} mice is unlikely to be caused by enhanced PKA-induced insulin secretion.

Leptin activates PI 3-K, which leads to activation of PDE 3B and inhibition of GLP-1-induced insulin secretion from islets of neonatal rats (Zhao et al, 1998). While I was unable to demonstrate a similar linkage in islets from mice, it is possible that leptin-induced activation of PDE 3B might lead to inhibition of acetylcholine-induced insulin secretion via cross-talk between PKA and PLC-PKC signal transduction pathways. I thus used IBMX to inhibit PDE 3B (Fig. 13), and then determined whether leptin would inhibit acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice (Fig. 13). Leptin was as effective in inhibiting acetylcholine-induced insulin secretion in the presence of IBMX as in the absence. It appears that leptin function to inhibit PLC-PKC-induced insulin secretion independent of PDE regulation. Others have also reported inhibitory effects of leptin on glucose-induced insulin secretion in the presence of IBMX or a specific inhibitor of milrinone, a specific PDE 3B inhibitor (Poitout et al, 1998 and Cases et al, 2000). These observations further support my conclusion that leptin does not act via the PKA pathway to inhibit insulin secretion from islets of mice.

Leptin has been shown to activate PI 3-K in number of tissues including pancreatic islets. I used wortmannin, an inhibitor of PI 3-K, to determine if inhibitory effects of leptin on acetylcholine-induced insulin secretion were mediated by a PI 3-K linked pathway. First, I determined wortmannin-induced inhibibtion of PI 3-K would stimulate acetylcholine-induced insulin secretion from mouse islets (Fig. 14 and Fig. 15). Zawalich and Zawalich, 2000 had earlier shown that wortmannin increased carbochol-induced insulin secretion from islets of rats, although conflicting reports have been published (Gao et al, 1996). Wortmannin stimulated acetylcholine-induced insulin secretion from islets of lean mice, but not from islets of Lep^{ob}/Lep^{ob} mice (Fig. 15). These findings parallel the report of Zawalich and Zawalich where wortmannin stimulated carbochol-induced insulin secretion from islets of Sprague-Dawley or lean Zucker rats, but not from islets of leptin and insulin resistant Zucker fatty rats. The failure of wortmannin treatment to increase insulin secretion from islets of Lep^{ob}/Lep^{ob} mice suggested that PI 3-K activity might be inherently low in these islets. This low PI 3-K activity might possibly explain why islets from Lep^{ob}/Lep^{ob} mice hypersecrete insulin in response to acetylcholine.

The observation that leptin suppresses acetylcholine-induced insulin secretion from islets of Lep^{ob}/Lep^{ob} mice, but not from islets of lean mice (Chen et al, 1997; Lee and Romsos, 2001 and Fig. 15) is consistent with the noted effects of wortmannin on insulin secretion from these islets (Fig. 15). That is, leptin would be predicted to lower insulin secretion from islets of Lep^{ob}/Lep^{ob} mice where PI 3-K activity is predicted to be low, and would be expected to be loss effective in islets from lean mice where PI 3-K activity is predicted to be high based on the insulin secretion responsiveness to

wortmannin (Fig. 15). When I simultaneously co-administered wortmannin and leptin to islets from *Lep*^{ob}/*Lep*^{ob} and lean mice, insulin secretion was lower than when wortmannin alone was administered (Fig. 15). Leptin, which inhibits acetylcholine-induced insulin secretion within minutes (Chen et al, 1997), presumably activated PI 3-K in islets from *Lep*^{ob}/*Lep*^{ob} mice, and prevented the wortmannin-induced inhibition of PI 3-K activity in islets from lean mice. As a result, insulin secretion from islets of *Lep*^{ob}/*Lep*^{ob} and lean mice exposed to leptin and wortmannin was lower than insulin secretion from islets exposed to leptin alone (Fig. 15). This conclusion is based in part on the assumption that insulin secretory responses to wortmannin occur with a delay of 10-20 minutes (Eto et al, 2002; Zawalich and Zawalich, 2000) whereas leptin acts within a few minutes (Chen et al, 1997; Attoub et al, 2000).

Preincubation of islets from *Lep*^{ob}/*Lep*^{ob} mice with wortmannin totally blocked the activity of leptin to suppress acetylcholine-induced insulin secretion (Fig. 16). This finding supports my conclusion that leptin deficiency might elevate insulin secretion secondary to lowered stimulation of PI 3-K activity. Studies are now needed to directly evaluate PI 3-K activity in islets from *Lep*^{ob}/*Lep*^{ob} mice, and to determine the mechanism whereby activation of PI 3-K might target the PLC-PKC signal transduction pathway that participate in the regulation of insulin secretion.

CHAPTER V. LEPTIN ADMINISTRATION NORMALIZES INSULIN SECRETION FROM ISLETS OF *LEP^{OB}/LEP^{OB}* MICE BY FOOD INTAKE DEPENDENT AND INDEPENDENT MECHANISMS

A. ABSTRACT

Leptin-deficient *Lep^{ob}/Lep^{ob}* mice specifically hypersecrete insulin in response to acetylcholine within the first 2 weeks of age. The present study tested the hypothesis that absence of leptin during neonatal development permanently program islets from these mice to hypersecrete insulin. Administration of leptin to young adult *Lep^{ob}/Lep^{ob}* mice for 8 days normalized their food intake, plasma insulin concentration and insulin secretion in response to glucose, acetylcholine, and leptin. Restriction of food intake of *Lep^{ob}/Lep^{ob}* mice lowered, but did not normalized plasma insulin concentration. Food restricted *Lep^{ob}/Lep^{ob}* mice continued to hypersecrete insulin in response to glucose, but islets from these mice did not hyperrespond to acetylcholine or leptin as occurs in *ad libitum* fed *Lep^{ob}/Lep^{ob}* mice. I conclud that neonatal leptin deficiency does not permanently program islets from mice to hypersecrete insulin. The hyperphagia associated with leptin-deficiency contributes substantially the hypersecretion of insulin, but leptin also appears to have more direct effects on regulation of insulin secretion.

B. INTRODUCTION

Leptin-deficient Lep^{ob}/Lep^{ob} mice exhibit elevations in plasma insulin early in developemt (Dupuc, 1976), before alterations in food intake are evident (Lin et al, 1977). Pancreatic islets from 1- to 2-week-old Lep^{ob}/Lep^{ob} mice do not hypersecrete insulin in response to glucose (Chapter III) or activators of the protein kinase A (PKA) signal transduction pathway, such as GLP-1 (Chapter IV), but specifically hypersecrete insulin in response to activators of the phospholipase C-protein kinase C (PLC-PKC) signal transduction pathway such as acetylcholine and cholecystokinin (Chen and Romsos, 1995). Addition of leptin to the media suppresses this hypersecretion of insulin in response to acetylcholine (Chen et al, 1997; Chapter III), possibly via activation of phosphatidylinositol 3-kinase (PI 3-K) (Chapter IV). These results indicate that leptin functions early in development (i.e., within the first several weeks of age) to regulate a pathway important in the control of plasma insulin concentrations.

Young adult Lep^{ob}/Lep^{ob} mice have severe hyperinsulinemia and insulin resistance. It is possible to marginally lower the extent of hyperinsulinemia in these mice with nutritional manipulation (Dubuc, 1981), but most treatments have been unable to normalize plasma insulin concentration in Lep^{ob}/Lep^{ob} mice. Two successful approaches have been reported. Adrenalectomy corrects many of metabolic abnormalities in Lep^{ob}/Lep^{ob} mice, including a normalization of plasma insulin concentration (Okuda and Romsos, 1994; Kang et al, 1992; Walker and Romsos, 1992; Walker and Romsos, 1993), and chronic leptin administration to these mice also normalizes their plasma insulin concentrations (Harris, 1998; Harris et al, 1998; Levin et al, 1996; Pelleymounter et al, 1995).

The late fetal and early postnatal periods are critical in development of the pancreas (Kaung, 1994). The presence or absence of specific stimuli during this period may have permanent effects on the ability of islets to regulate insulin secretion. For example, a brief exposure to a high carbohydrate diet during the suckling periods causes rat pups to hypersecrete insulin as adults (Aalinkeel et al, 1999; Aalinkeel et al, 2001; Srinivasan et al, 2000; Laychock et al, 1995; Vadlamudi et al, 1993). Adrenalectomy of young adult Lep^{ob}/Lep^{ob} mice normalizes their plasma insulin, but when these mice are fed a high glucose diet their hyperinsulinemia reappears (Kang et al, 1992 ; Walker and Romsos, 1992; Walker and Romsos, 1993). And, when islets from young adult adrenalecotmized Lep^{ob}/Lep^{ob} mice are exposed to acetylcholine, the islets still hypersecrete insulin (Okuda and Romsos, 1994).

These results raise the possibility that the absence of leptin durning neonatal development of Lep^{ob}/Lep^{ob} mice permanently programs their islets to hypersecrete insulin when challenged. Pancreatic islets from young adult Lep^{ob}/Lep^{ob} mice that have been chronically administered leptin have not previously been challenged with acetylcholine to determine if the absence of leptin during neonatal development permanently alters their ability to respond normally to acetylcholine.

The present study was undertaken to determine the effects of chronic administration of leptin to Lep^{ob}/Lep^{ob} mice on the regulation of insulin secretion from their isolated islets. Islets from these mice were exposed to acetylcholine and leptin. Because leptin lowers food intake, which would be expected to lower insulin secretion, a group of Lep^{ob}/Lep^{ob} mice were pair-fed to the leptin-treated Lep^{ob}/Lep^{ob} mice to

assess the effects of lowered food intake *per se* versus food intake independent effects of leptin on insulin secretion.

C. MATERIALS AND METHODS

Animals

Lep^{ob}/*Lep*^{ob} and lean mice were obtained from our C57BL/6J-*Lep*^{ob}/+ breeding colony. Mice were housed in plastic cages with wood shavings for bedding and were maintained in a room at 25°C with a 12:12 h light-dark cycle (lights on at 0700 h). They were fed a nonpurified, commercial diet (Harlan Teklad Rodent Diet 8640; Madison, WI). Litters were adjusted to 6 pups per litter within a few days after birth. Mice were weaned at 3 weeks of age. Mice were used at 4 to 5 weeks of age. They were individually housed for 3 days prior to study and throughout the 8 day experiments. The care and treatment were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1985) and local institutional guidelines.

Leptin Administration

Leptin (PeproTech Inc., NJ) was administered to *Lep*^{ob}/*Lep*^{ob} mice to lower their food intake to approximate the intake of lean mice. Intraperitoneal injections of leptin (100 μg/day for 4 days, and then 50 μg/day for the last 4 days of the 8 day study) and vehicle (150 μl of PBS) were made with a 30-gauge needle twice daily (at 0900-0930 h, and at 1730-1800 h).

Experimental Design

Experiment 1. *Lep^{ob}/Lep^{ob}* mice were administered with leptin twice daily for 8 days and fed *ad libitum*. Control *Lep^{ob}/Lep^{ob}* mice were injected with vehicle and fed ad libitum, or pair-fed to the leptin-treated *Lep^{ob}/Lep^{ob}* mice. Lean mice (*Lep^{ob}*/ + or +/+ mice) were also injected with vehicle and fed *ad libitum*. Food intake and body weight were measured daily at 0830-0900 h. Pair-fed *Lep^{ob}/Lep^{ob}* mice were fed half their daily food allotment at 0900 h and half at 1800 h. All mice were decapitated at ~ 0930 h on day 9, approximately 16 h after the last leptin or vehicle injection. Blood was collected for insulin assay (Kekow et al, 1988), liver and abdominal fat pad weights were recorded, and pancreatic islets were obtained for measurement of insulin secretion.

Experiment 2. Islets from pair-fed *Lep^{ob}/Lep^{ob}* mice in experiment 1 unexpectedly exhibited a minimal insulin secretion response to acetylcholine, and failed to suppress insulin secretion in response to leptin addition to the media. This observation was in sharp contrast to the robust response of islets from *ad libitum* fed *Lep^{ob}/Lep^{ob}* mice to acetylcholine stimulation of insulin secretion, and to the suppressive effects of leptin. Additional *Lep^{ob}/Lep^{ob}* mice were thus pair-fed for an 8 day period, and killed at ~ 0930 h on day 9 to obtain pancreatic islets for examination of insulin secretion.

Islet isolation, and incubation

Pancreatic islets were isolated with collagenase type V (Sigma Chemical, St. Louis, MO) digestion as described previously (Lacy and Kostianovsky, 1967 and Lee and Romsos, 2001). Isolated islets were selected with the aid of a pipette under a stereoscopic microscope.

Similar-sized islets from single mice (10 islets/dish) were distributed into 3.5 mm black-bottom petri dishes. Islets were statically incubated at 37°C for 30 min under a 95% O_2 - 5% CO_2 atmosphere in 1 ml Krebs-Ringer bicarbonate buffer (KRB, pH, 7.4) containing 0.5 mM glucose and 0.1% bovine serum albumin (BSA, Amoresco, Solon, OH). Consecutively, islets were incubated in KRB containing 10 mM glucose for 30 min, and then 10 mM glucose + 10 μM acetylcholine ± 20 nM leptin, or 10 mM glucose + 10 μM acetylcholine ± 20 nM leptin and ± 20 nM wortmannin for 30 min as indicated in the figures.

To measure insulin secretion, 0.5 ml of incubation media was collected. Insulin analyzed by ELISA (Kekow et al, 1988). Diameters of islets were measured with the aid of eyepiece micrometer of stereoscopic microscope.

Statistical analysis

Data are presented as means ± SE. Data were analyzed by one-way ANOVA in conjunction with LSD adjustment. Differences were considered statistically significant at $P < 0.05$.

D. RESULTS

As expected, *Lep^{ob}/Lep^{ob}* mice were hyperphagic, and leptin administration lowered their food intake (Table 2). We attempted to administered a dose of leptin that would lower food intake of the *Lep^{ob}/Lep^{ob}* mice to approximate food intake of lean

Table 2. Food intake, body and tissue weights, and plasma insulin in leptin-treated *Lep*^{ob}/*Lep*^{ob} mice

	Lep^{ob}/*Lep*^{ob}			Lean
	vehicle (2/3)	leptin (3/3)	pairfed (2/3)	vehicle (2/4)
Cumulative food intake - g	41 ± 2^{a}	20 ± 1^{b}	20 ± 2^{b}	26 ± 1^{c}
Body weight change - g/8 d	6.7 ± 0.5^{a}	-1.0 ± 0.7^{b}	-1.2 ± 0.8^{b}	1.1 ± 0.5^{c}
Fat pad - g	2.94 ± 0.23^{a}	1.15 ± 0.11^{b}	1.60 ± 0.11^{c}	0.27 ± 0.04^{d}
Liver weight - g	2.38 ± 0.15^{a}	1.14 ± 0.04^{b}	1.04 ± 0.04^{b}	1.07 ± 0.06^{b}
Islet diameter - μm	144 ± 7^{a}	113 ± 3^{b}	129 ± 6^{c}	115 ± 2^{b}
Plasma insulin - ng/ml	79 ± 26^{a}	1.1 ± 0.4^{b}	11 ± 5^{b}	0.5 ± 0.2^{b}

Values are means ± SE. Numbers of male/female animals are indicated in parentheses. All mice were injected intraperitoneally twice-daily with leptin (100 μg /day for the first 4 days, and then 50 μg/day for the second 4 days) or vehicle for 8 days. Food was provided *ad libitum*, except for *Lep*^{ob}/*Lep*^{ob} mice pair-fed to the leptin-treated mice. Initial body weights were 21.8 ± 0.58 and 17.4 ± 0.64 for *Lep*^{ob}/*Lep*^{ob} and lean mice, respectively. Islet diameter was obtained by measuring 12-20 islets from each mouse. Data were analyzed by one-way ANOVA in conjunction with LSD. Superscripts indicate significant differences among groups ($P<0.05$).

mice. Because administration of 100 μg leptin per day for 4 days lowered food intake of *Lep*^{ob}/*Lep*^{ob} mice below intakes of lean mice (2.07 ± 0.30 g versus 3.37 ± 0.28 g on day 4), the dose of leptin was lowered to 50 μg/day for the last 4 days of the experiment. Food intakes of leptin injected *Lep*^{ob}/*Lep*^{ob} mice and lean mice averaged 2.36 ± 0.16 g and 3.23 ± 0.18 g on day 8 of the experiment. Body weight changes paralleled food intake, as did fat pad and liver weights. Treatment of *Lep*^{ob}/*Lep*^{ob} mice with leptin lowered their islet diameter and plasma insulin to approximate values in lean mice, whereas, as expected (Koyoma et al, 1997), restriction of food intake alone of *Lep*^{ob}/*Lep*^{ob} mice was less effective (Table 2).

Islets from adult *Lep*^{ob}/*Lep*^{ob} mice, as expected, were much more responsive to glucose- and acetylcholine-induced insulin secretion than islets from lean mice (Fig. 17). Again, as expected, leptin effectively suppressed acetylcholine-induced insulin secretion from islets of adult *Lep*^{ob}/*Lep*^{ob} mice, but not from islets of lean mice (Fig. 17).

Consistent with the lowering of plasma insulin concentration in *Lep*^{ob}/*Lep*^{ob} mice treated with leptin for 8 days, insulin secretion responses of islets from these mice to glucose, acetylcholine, and leptin were similar to islets from lean mice (Fig. 17). The lowered food intake of leptin-treated *Lep*^{ob}/*Lep*^{ob} mice *per se* would be expected to lower their insulin secretion. Consequently, a group of *Lep*^{ob}/*Lep*^{ob} mice were pair-fed. Restriction of food intake *per se* did not lower glucose-induced insulin secretion from islets of *Lep*^{ob}/*Lep*^{ob} mice, but markedly blunted the stimulating effect of acetylcholine on glucose-induced insulin secretion (Fig. 17). Additionally, leptin failed to suppress

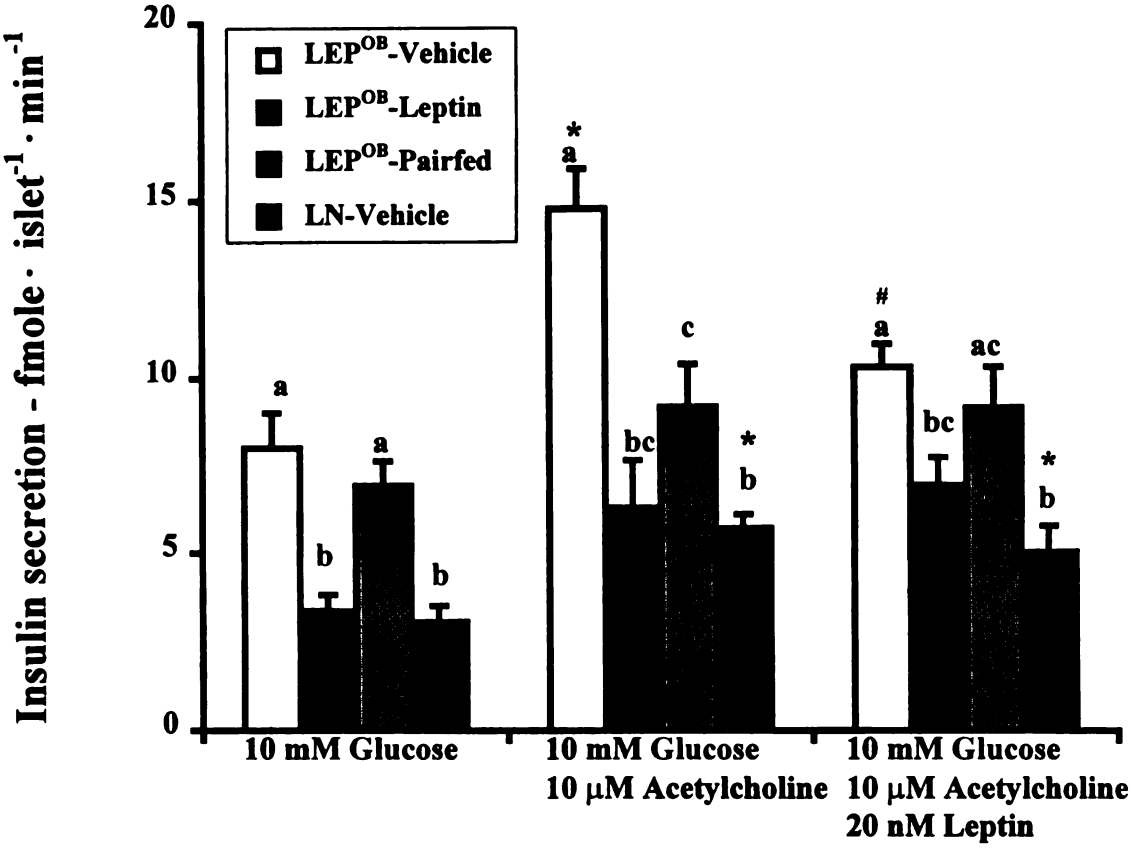

Figure 17. Effect of chronic administration of leptin to *Lep^{ob}/Lep^{ob}* mice on insulin secretion. *Lep^{ob}/Lep^{ob}* and lean mice at 4 to 5 weeks of age were treated for 8 days as indicated in figure. Islets were then isolated and incubated with 10 mM glucose for 30 min, followed by 10 mM glucose plus 10 μM acetylcholine ± 20 nM leptin for 30 min. Data represent means ± SE (n=5-6). Bars with different letters are significantly ($P<0.05$) different, as determined by one-way ANOVA in conjunction with the LSD post-hoc test. Comparisons were made within the same incubation media (i.e. glucose, glucose +acetylcholine, or glucose + acetylcholine + leptin). * Indicates significantly increased acetylcholine-induced insulin secretion, and # indicates a significant inhibitory effect of leptin on acetylcholine-induced insulin secretion, as determined by one-way ANOVA in conjunction with the LSD post-hoc test.

acetylcholine-induced insulin secretion from islets of pair-fed *Lep^{ob}/Lep^{ob}* mice (Fig. 17).

The earliest detectable alteration in islets that occurs in *Lep^{ob}/Lep^{ob}* mice is an enhanced response to acetylcholine-induced insulin secretion, which is suppressible by addition of leptin to the islets (Lee and Romsos, 2001). This alteration in insulin secretion occurs by 2 weeks of age (Lee and Romsos, 2001). We did not expect that food restriction *per se* would correct this alteration, as occurred in experiment 1 (Fig. 17). A second experiment was thus conducted (Fig. 18). Again, restricted food intake *per se* of *Lep^{ob}/Lep^{ob}* mice blunted the effectiveness of acetylcholine to stimulate insulin secretion ($P>0.05$), as well as the ability of leptin to suppress acetylcholine-induced insulin secretion ($P>0.05$). Wortmannin, an inhibitor of PI-3 K, stimulated acetylcholine-induced insulin secretion from islets of food-restricted *Lep^{ob}/Lep^{ob}* mice (Fig. 18), in contrast to our earlier observation in *ad libitum* fed *Lep^{ob}/Lep^{ob}* mice where wortmannin failed to increase insulin secretion (Chapter IV). Co-stimulation of islets from food-restricted *Lep^{ob}/Lep^{ob}* mice with leptin and wortmannin blocked the wortmannin-induced increase in insulin secretion (Fig. 18).

E. DISCUSSION

The main finding of this study was that leptin administration to *Lep^{ob}/Lep^{ob}* mice for 8 days normalized every aspect of insulin secretion measured (i.e., plasma insulin, islet diameter, and insulin secretion responses to glucose, acetylcholine, and leptin). The food intake suppressive effect of leptin accounted for a portion of these change, but

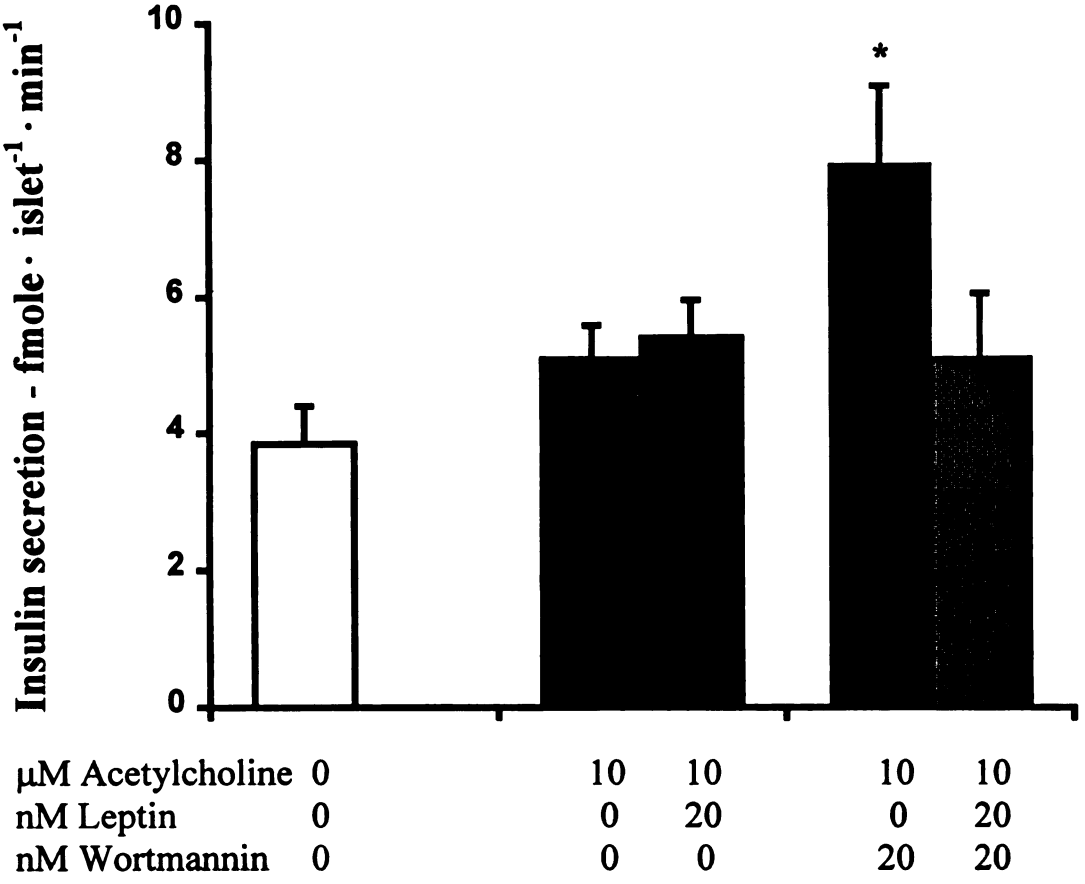

Figure 18. Wortmannin increased acetylcholine-induced insulin secretion from islets of pair-fed *Lep*^{ob}/*Lep*^{ob} mice. Islets from pair-fed *Lep*^{ob}/*Lep*^{ob} mice were isolated and incubated with 10 mM glucose for 30 min. All islets were then exposed to 10 mM glucose plus 10 μM acetylcholine ± 20 nM leptin and, ± 20 nM wortmaninn for 30 min, as indicated in the figure. Data represent means ± SE (n=6). * Indicates significant (P<0.05) effect of wortmannin on acetylcholine-induced insulin secretion, as determined by one-way ANOVA in conjunction with post-hoc LSD test.

Lep^{ob}/*Lep*^{ob} mice pair-fed to the leptin-treated *Lep*^{ob}/*Lep*^{ob} mice still exhibited hyperinsulinemia and elevated insulin secretion in response to 10 mM glucose.

I had postulated that the absence of leptin during neonatal development might program islets from *Lep*^{ob}/*Lep*^{ob} mice to permanently hyperrespond to acetylcholine. Contrary to this expectation, islets from 4- to 5- week-old *Lep*^{ob}/*Lep*^{ob} mice treated with leptin for only 8 days exhibited a response to acetylcholine similar to that of islets from lean control mice. A deficiency of leptin during neonatal development does not appear to causes a permanent change in the ability of the islets to respond normally to acetylcholine. Although removal of corticosterone from *Lep*^{ob}/*Lep*^{ob} mice by adrenalectomy lowered plasma insulin concentration as effectively as did leptin treatment, adrenalectormy failed to correct the hyperresponsiveness of the islets to acetylcholine. Glucocorticoids are known to antagonize some actions of leptin (Jang et al, 2000). Leptin would appear to have distinct effects in regulation of insulin secretion independent of glucocorticoids interactions because the absence of glucocorticoids did not totally mimic effects of leptin on insulin secretion.

The impact of the leptin-induced reduction in food intake *per se* in leptin-treated *Lep*^{ob}/*Lep*^{ob} mice was examined by inclusion of *Lep*^{ob}/*Lep*^{ob} mice pair-fed to the leptin-treated mice. Food restriction *per se* attenuated the acetylcholine-induced insulin secretion from islets of these mice, and blocked the ability of leptin to suppress acetylcholine-induced insulin secretion. These results suggest that the failure of leptin to inhibit acetylcholine-induced insulin secretion from islets of leptin-treated *Lep*^{ob}/*Lep*^{ob} mice, or from lean mice, is not secondary to "leptin resistance" *per se* because islets from leptin-deficient, food restricted *Lep*^{ob}/*Lep*^{ob} mice also failed to

decrease acetylcholine-induced insulin secretion in response to leptin. Leptin, secondary to constraint of food intake, appears to assist in the regulation of acetylcholine-induced insulin secretion.

I showed in Chaper IV that wortmannin, an inhibitor of PI 3-K, increased acetylcholine-induced insulin secretion from islets of lean mcie, but not from islets of *ad libitum* fed Lep^{ob}/Lep^{ob} mice. These findings are consistent with results in Zucker lean and fatty rats (Zawalich and Zawalich, 2000), suggesting that islets from Lep^{ob}/Lep^{ob} mice and fatty rats have low PI 3-K activity. Zawalich and Zawalich, 2000 suggest that PI 3-K inhibits acetylcholine-induced insulin secrtion. They attributed low PI 3-K activity in the Zucker fatty rats to insulin resistance. These animals also are leptin resistant, secondary to a mutation in the leptin receptor. When islets from pair-fed Lep^{ob}/Lep^{ob} mice were exposed to wortmannin, acetylcholine-induced insulin secretion increased. This suggests that PI 3-K is active in islets from food restricted Lep^{ob}/Lep^{ob} mice to help constrain acetylcholine-induced insulin secretion.

Leptin is known to activate PI 3-K in islets. I hypothesized that leptin via activation of PI 3-K constrains acetylcholine-induced insulin secretion in islets from mice. I showed in Chapter IV that preincubation of islets with wortmannin blocks the ability of leptin to inhibit acetylcholine-induced insulin secretion. Islets from Lep^{ob}/Lep^{ob} mice presumably have low PI 3-K activity because wortmannin does not increase acetylcholine-induced insulin secretion from islets of these mice (Chapter IV). Addition of leptin to the islets would be expected to activate PI 3-K and this may explain the lowered acetylcholine-induced insulin secretion in the presence of leptin. In contrast islets from food restricted Lep^{ob}/Lep^{ob} mice appear to have high PI 3-K activity

because wortmannin increases insulin secretion (Fig 18). If PI 3-K activity is already high, leptin would be expected to be ineffective *in vitro*, as was observed (Fig 18). Food restriction of Lep^{ob}/Lep^{ob} mice lowered plasma insulin and may have reduced insulin resistance. This may have activated PI 3-K in the islets to constrain acetylcholine-induced insulin secretion. Others have reported that food deprivation for 48 h activated PI 3-K in peripheral tissues of obese neonates (Heydrick et al, 1993). It remanined to be established how food restriction *per se* affect PI 3-K activity in islets of mice.

Food restricition per se did not totally normalize insulin secretion of Lep^{ob}/Lep^{ob} mice (Fig. 17). Leptin lowers insulin mRNA abundance and in some studies is reported to affect ATP-sensitive K^+ and Ca^{2+} channels, which leads to a reduction in glucose-induced insulin secretion. It is not clear whether leptin treatment, but not pair-feeding, acted via these mechanisms to normalize insulin secretion.

CHAPTER VI. SUMMARY AND CONCLUSIONS

Obesity causes multiple physiological alterations. *Lep^{ob}/Lep^{ob}* mice are extensively used to investigate these obesity-related multiple alterations. These mice as adults weigh 3 times more than normal mice, have over 50% body fat, and have multiple metabolic alterations, including hyperphagia, decreased thermogenesis, hyper-corticosteronemia and hyperinsulinemia (Leibel et al, 1997). It is now clear that obese syndromes in *Lep^{ob}/Lep^{ob}* mice are caused by a deficiency in leptin synthesis, which is due to a nonsense mutation (substitution of T for C) at the coding region of the *Lep^{ob}* gene (Zhang et al, 1994).

Hyperinsulinemia is an early-onset metabolic alteration (Dubuc, 1981), and precedes an increase in body fat, onset of overeating or overweight and insulin resistance in *Lep^{ob}/Lep^{ob}* mice (Lin et al, 1977; Rath and Thenen, 1979; Boissonneault et al, 1978; Dubuc 1976). This is likely due to lack of a direct inhibitory control of insulin secretion in these mice.

Islets from 2-week-old *Lep^{ob}/Lep^{ob}* mice do not yet hypersecrete insulin in response to glucose, but hyperrespond to acetylcholine (Chen and Romsos, 1995). This suggests that the PLC-PKC post-receptor signaling system may function as a primary mechanism in the development of hyperinsulinemia in *Lep^{ob}/Lep^{ob}* mice. However, the metabolic and/or hormonal signals that contribute to the development of obesity-associated early-onset hyperinsulinemia are still not clear.

Leptin has been noted to have multiple physiological functions, including direct effects to modulate insulin secretion (Chen et al, 1997; Kieffer et al, 1997; Poitout et al, 1998; Zhao et al, 1998), in addition to a key role as a satiety factor (Halaas et al, 1995; Pelleymounter et al, 1995). A characterization of the temporal relationship between the initial development of alterations in insulin secretion from islets of neonatal Lep^{ob}/Lep^{ob} mice and of the effects of leptin on this pathway may help to understand how hyperinsulinemia develops in these mice.

I first determined when the enhanced insulin secretion response to acetylcholine initially appears in Lep^{ob}/Lep^{ob} mice by examining mice younger than 2 weeks of age. I also determined the role of leptin to modulate acetylcholine-induced insulin secretion from islets of these young Lep^{ob}/Lep^{ob} and lean mice.

Addition of acetylcholine similarly increased insulin secretion from islets of 1-week-old Lep^{ob}/Lep^{ob} mice and lean littermates. Consistent with an eariler report (Chen and Romsos, 1995), islets from 2-week-old Lep^{ob}/Lep^{ob} mice secreted more insulin in the presence of acetylcholine than did islets from lean littermates (Fig. 9). Thus, the enhanced acetylcholine-induced insulin secretion in Lep^{ob}/Lep^{ob} pups first occurs between 1 and 2 weeks of age. Leptin rapidly suppressed acetylcholine-induced insulin secretion from islets of 4-day-old, 2-week-old, and 4-week-old leptin-deficient mice at each stage of development (Fig. 10). Whereas this effect of leptin became progressively less effective in 4-day-old, 2-week-old, and 4-week-old lean mice with development (Fig. 10). These findings suggested that leptin targets pancreatic islets early in development to specifically constrain the overall capacity for acetylcholine-induced insulin secretion, and to acutely modulate this secretion.

Leptin has also been reported to activate phosphatidylinositol 3-kinase (PI 3-K) and subsequently phosphodiesterase (PDE) to inhibit protein kinase A (PKA)-induced insulin secretion from neonatal rat islets (Zhao et al, 1998). I thus determined if the PKA pathway was also altered to cause hypersecretion from islets of young Lep^{ob}/Lep^{ob} mice. I also determined the effects of leptin on PKA-induced insulin secretion. Additionally, I examined that leptin-induced inhibition the PLC-PKC-induced hypersecretion of insulin from islets of these mice was mediated by cross-talk between the PKA and PLC-PKC pathways, or by more direct effects of PI 3-K on the PLC-PKC pathway.

I observed similar rates of insulin secretion after PKA activation by stimulators (i.e., GLP-1, forskolin, an activator of AC or IBMX, an inhibitor of PDE) were present in islets of 2-week-old Lep^{ob}/Lep^{ob} mice and lean littermates (Fig. 11), and that secretion was unaffected by addition of leptin (Fig. 12). Leptin constrained the PLC-PKC-induced hypersecretion of insulin from islets of Lep^{ob}/Lep^{ob} mice, even when PDE activities were inhibited by IBMX (Fig. 13). Wortmannin, an inhibitor of PI 3-K, addition to islets increased acetylcholine-induced insulin secretion from islets of lean, but not Lep^{ob}/Lep^{ob} mice. With simultaneous co-additon of wortmannin and leptin to islets from Lep^{ob}/Lep^{ob} and lean mice, insulin secretion was lower than when wortmannin alone was applied (Fig. 15). Leptin, which inhibits acetylcholine-induced insulin secretion within minutes (Chen et al, 1997; Attoub et al, 2000), presumably activated PI 3-K in islets from Lep^{ob}/Lep^{ob} mice prior to wortmannin-induced inhibition of PI 3-K activity in islets from lean mice. Inhibition of PLC-PKC-induced insulin secretion via leptin-induced PI 3-K activation was abolished when PI 3-K activity was

block prior to leptin action (Fig. 16). These results suggest that leptin may not target the PKA signaling pathway, but that leptin specifically targets a PI 3-K dependent pathway to constrain the PLC-PKC-induced insulin secretion. I conclude that leptin deficiency inherently causes hypersecretion of insulin secondary to low PI 3-K activity which failed to constrain the PLC-PKC-induced insulin secretion in early development of leptin-deficient *Lep*^{ob}/*Lep*^{ob} mice.

The various stimuli during the pancreatic ontogenic period may change permanently the ability of islets to regulate insulin secretion (Aalinkeel et al, 1999; Aalinkeel et al, 2001; Srinivasan et al, 2000; Laychock et al, 1995; Vadlamudi et al, 1993). I hypothesized that the absence of leptin during neonatal development of *Lep*^{ob}/*Lep*^{ob} mice permanently programs hypersecretion of insulin specifically in response to acetylcholine. I thus determined if chronic admistration of leptin to *Lep*^{ob}/*Lep*^{ob} mice would correct their hypersecretion of insulin. Since leptin reduces food intake, which would be expected to lower insulin secretion, I also determined insulin secretion response from islets of pair-fed *Lep*^{ob}/*Lep*^{ob} mice to leptin-treated *Lep*^{ob}/*Lep*^{ob} mice.

Chronic administration of leptin to young adult *Lep*^{ob}/*Lep*^{ob} mice normalized insulin secretion from isolated islets in response to glucose, acetylcholine and leptin similar to lean mice (Fig. 17). This result was consistent with the lowering plasma insulin concentration in these mice (Table 2). Food restriction *per se* lowered insulin secretion in response to acetylcholine and leptin, but not glucose (Fig. 17). Wortmannin induced an increases in insulin secretion response to acetylcholine in food-restricted *Lep*^{ob}/*Lep*^{ob} mice, and co-addition of leptin and wortmannin blocked the potentiating

effect of wortmannin on acetylcholine-induced insulin secretion (Fig 18). Considered with my earlier report (Chapter IV) that wortmannin failed to increase insulin secretion in *ad libitum* fed Lep^{ob}/Lep^{ob} mice, food restriction *per se* thus may help islets to activate PI 3-K to suppress the hyperresponsiveness to acetylcholine in Lep^{ob}/Lep^{ob} mice. These results suggest that leptin controls secretion of insulin by preventing hyperphagia, and by food intake independent mechanisms.

I conclude from my dissertation research that leptin activates a PI 3-K-dependent pathway to specifically regulate the PLC-PKC-induced insulin secretion. This action of leptin is required to prevent development of hyperinsulinemia early in life of leptin-deficient Lep^{ob}/Lep^{ob} mice.

CHAPTER VII. RECOMMENDATIONS FOR FUTURE STUDIES

I recommend that the following studies be considered to better understand, and to continually investigate, mechanisms whereby leptin acts to prevent hyperinsulinemia in Lep^{ob}/Lep^{ob} mice early in development.

A. Study to directly determine PI 3-K activity and PI 3-K expression in islets from lean and Lep^{ob}/Lep^{ob} mice

There is evidence for altered PI 3-K expression and activity in peripheral tissues of insulin-resistant animals (Anai et al, 1998; Kerouz et al, 2000; Heydrick et al, 1995). In the present study, the failure of wortmannin, an inhibitor of PI 3-K, to induce stimulation of insulin secretion from islets of Lep^{ob}/Lep^{ob} mice suggested that PI 3-K activity might be inherently low in these islets. This low PI 3-K activity might explain the hypersecretion of insulin in response to acetylcholine. This would parallel another report where reduced PI 3-K activity in islets of obese Zucker *fatty* rats was associated with hypersecretion of insulin (Zawalich and Zawalich, 2000). However, PI 3-K expression and activity has not been directly measured in islets of Lep^{ob}/Lep^{ob} mice. I therefore suggest that PI 3-K expression and activity in isolated islets from Lep^{ob}/Lep^{ob} mice be measured. To determine this, lean and Lep^{ob}/Lep^{ob} mice (4- to 5-week-old) will be used, and they will be fed standard chow diet and water *ad libitum*. Islets will be isolated from mice fed *ad libitum*, where high PI 3-K activity would be expected or mice fasted for 12 h, where low PI 3-K would be expected. After treatment of the islets

with or without leptin for 5 to10 min, I would use Western blotting and immunopreciptation to determine PI 3-K expression and PI 3-K activity, respectively. PI 3-K activity associated with anti-phosphotyrosine immunoprecipitates will be measured by Western blotting. (Kerouz et al, 1997)

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B. Study to determine if change of PI 3-K activity develops over the same time course with change of leptin effect on the PLC-PKC-induced insulin secretion.

Leptin-deficient *Lep*^{ob}/*Lep*^{ob} mice develop enhanced PLC-PKC-induced insulin secretion between 1 and 2 weeks of age. I propose that low PI 3-K activity in islets from *Lep*^{ob}/*Lep*^{ob} mice may lead to this hypersecretion of insulin in response to acetylcholine. This would indicate that the alteration in PI 3-K activity in *Lep*^{ob}/*Lep*^{ob} mice should occur at the same time (i.e., between 1 and 2 weeks of age), or before leptin-deficient mice develop enhanced insulin secretion.

The inhibitory effect of leptin became progressively less effective in leptin-sufficient lean mice between one and two weeks of age, suggesting an increase in PI 3-K activity with development. Leptin effect was no longer evident in islets of 4-week-old lean mice when PI 3-K activity is predicted to be high. In contrast, leptin suppressed acetylcholine-induced insulin secretion from islets of 4-day, and 2- and 4-week-old *Lep*^{ob}/*Lep*^{ob} mice, but this effect of leptin is even more evident in 4 week-old *Lep*^{ob}/*Lep*^{ob} mice. This suggests that PI 3-K activity is low in islets from *Lep*^{ob}/*Lep*^{ob} mice, and remains low. I therefore would determine PI 3-K expression and activity in the islets from neonatal lean and *Lep*^{ob}/*Lep*^{ob} mice, and in 2- and 4-week-old mice under same condition described above. This would help us understand if the initial

development of hypersecretion of insulin and leptin function are associated with change in PI 3-K activity.

C. Study to determine if administration of leptin change PI 3-K expression and activity in islets from *Lep^{ob}/Lep^{ob}* mice

Chronic administration (8 days) of leptin to *Lep^{ob}/Lep^{ob}* mice normalized insulin secretion response to glucose, acetylcholine and leptin from their isolated islets. Presumably part of this effect is ascribed to a leptin-induced change in PI 3-K activity as suggested in *in vitro* evidence that addition leptin to islets from *Lep^{ob}/Lep^{ob}* mice, with presumably low PI 3-K activity, may activate PI 3-K to inhibit hypersecretion of insulin secretion. I therefore would examine PI 3-K expression and PI 3-K activity in islets from leptin-administered *Lep^{ob}/Lep^{ob}* mice. *Lep^{ob}/Lep^{ob}* mice (4- to 5-week-old) will be fed *ad libitum*, and injected with leptin or vehicle for 8 days. Food will be withdrawn 12 h before isolating islets. After treatment of the islets with or without leptin for 5 to 10 min, PI 3-K expression and PI 3-K activity will be measured by using techniques described above.

D. Study to determine the mechanism whereby PI 3-K might target the PLC-PKC signal transduction pathway to regulate insulin secretion.

Present study proposed that leptin inhibits specifically PLC-PKC-induced insulin secretion by activation of a PI 3-K dependent pathway. However it is not clear how PI 3-K activation targets the PLC-PKC signaling system to regulate insulin secretion. Direct activation of PKC with phorbor-12-myristate-13-acetate (PMA), an

agonist of PKC, mimics acetylcholine-induced insulin secretion (Chen and Romsos, 1997). I therefore would examine whether leptin-induced PI 3-K activation directly affects PKC-induced signaling system to regulate insulin secretion. To determine this, islets from *Lep*^{ob}/*Lep*^{ob} mice will be preincubated with or without wortmannin, an inhibitor of PI 3-K, for 30 min, and then incubated with PMA ± leptin ± wortmannin for 30 min. Insulin secretion will be measured by an enzyme-linked-immunosorbent assay (Kekow et al, 1988). I will also measure whether leptin-induced PI 3-K affects distribution of PKC activity between soluble and membrane fraction. Islets will be preincubated with or without wortmannin for 30 min, and then incubated with PMA ± leptin ± wortmannin for 5 to 10 min. Cytosol and pellet fractions from islet homogenates will be prepared. PKC activity from cytosolic or pellet fraction will be measured by phosphorylation of histon in the presence of [γ-^{32}P] ATP. Also, PKC protein level will be determined by western blotting (Deeney et al, 1996).

E. Study to determine if leptin-induced PI 3-K activation regulates insulin synthesis

Single injection of leptin reduced preproinsulin mRNA levels in islets by reducing transcriptional activity of the rat insulin I gene promoter (Seufert et al, 1999). Insulin triggered IRS-PI 3-K signal transduction regulates their own insulin gene transcription in β-cells (Leibiger et al, 1998). This raises the possibility that leptin-induced PI 3-K activity might mediate regulation of preproinsulin mRNA expression in islets from leptin-adminstered *Lep*^{ob}/*Lep*^{ob} mice. It would be interesting to examine if leptin-induced PI 3-K activation may be involve in regulating insulin synthesis. It may

in part explain one of mechanisms whereby leptin normalized insulin secretion response in leptin-administered Lep^{ob}/Lep^{ob} mice. To test this, Lep^{ob}/Lep^{ob} mice (4- to 5-week-old) will be fed *ad libitum*. Food will be withdrawn 12 h before isolating islets. Isolated islets will be pretreated with or without wortmannin, an inhibitor PI 3-K, for 30 min, and then islets will be cultured in RPMI medium supplemented with leptin ± wortmannin for 12 h. Preproinsulin mRNA level *in vitro* in islets will be detected by semiquantative reverse transcription-PCR (Seufert et al, 1999).

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