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By

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ABSTRACT

Effects of Acetylcholine and Norepinephrine on Glucose-Induced Insulin Secretion from Ob/ob and Lean Mouse Pancreatic Islets

By

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Objectives of this study were to determine dynamics of 1) glucose-induced insulin secretion, and 2) acetylcholine potentiation and norepinephrine inhibition of glucose-induced insulin secretion, from pancreatic islets of 8-9 wk old ob/ob and lean mice. Ob/ob mouse islets were larger and were hypersensitive and hyperresponsive to glucose stimuli. In the presence of 15 mM glucose, ob/ob islets were 1) hyperresponsive but equally sensitive to acetylcholine stimulation, and 2) hypersensitive and hyperresponsive to norepinephrine inhibition compared to lean islets. In the presence of 5 mM glucose, acetylcholine potentiated insulin secretion from ob/ob but not lean islets. In ob/ob mice, both glucose and acetylcholine may be important contributing factors to hyperinsulinemia. In addition, there may be decreased sympathoadrenal inhibition of ob/ob islet B cells. Such decreased inhibition could contribute to hyperinsulinemia in ob/ob mice, at least prior to compensatory increases in islet sensitivity to catecholamines.

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I. LITERATURE REVIEW

A. Introduction

This literature review is intended to briefly introduce the problem of human obesity and the genetically obese (ob/ob) mouse as a model to study obesity. Since this research focuses on hyperinsulinemia in the ob/ob mouse as a major contributor to obesity, I have provided background information on general physiology of insulin secretion and clearance. I included alterations in physiology specific to the ob/ob mouse at the end of each section. I then focused more in detail on control of insulin secretion via circulating secretagogues such as glucose and via neural signals originating in the central nervous system (CNS). Specific evidence of altered glucose and neural regulation of insulin secretion is presented for only 3 well studied animal models of obesity, the ventral medial hypothalamic (VMH)-lesioned rat, the Zucker fa/fa rat, and the ob/ob mouse. This evidence forms the basis for my hypothesis and research objectives.

B. Obesity

Approximately 26% of U.S. adults are overweight and about 1/3 of these are considered extremely overweight

(VanItallie, 1985). Obesity in humans is a risk factor for hypertension, hypercholesterolemia, and type II diabetes, and is associated with increased morbidity and mortality (Kral, 1985; VanItallie, 1985). Although the etiology of obesity remains to be elucidated, there is increasing evidence that obesity may be a partially heritable trait (for review, see Dulloo and Miller, 1987). Most convincing evidence of a genetic component to obesity are studies with adopted children and/or adults. Stunkard et al, 1986, classified adopted subjects by body mass index into weight classes and correlated the weight class of the adopted subject with the body mass index of the biological or adoptive parents. He found a strong relationship between weight class of adopted subjects and body-mass index of biological parents; whereas, there was no relationship between weight class of adopted subjects and body-mass index of adoptive parents, indicating that a genetic component may determine later body weight.

Given the prevalence of obesity and the potential for a genetic contribution to the disease, studies on the mechanism of genetic obesity are important to the elucidation of treatments for obesity. However, since co-existance of genetic and dietary variability make human studies difficult to conduct and interpret, most obesity research has been conducted on obese animals as models for the human condition. The ob/ob mouse is one of the most common obese animals studied because of the extensive data base already established and because the obesity is genetic rather than

chemically or electrically induced. Thus, since these mice were available in our lab, I chose to study the etiology of obesity using the genetically obese ob/ob mouse.

C. Ob/ob Mice as Models for Human Obesity

In genetically obese C57BL/6J (ob/ob) mice, the obese phenotype is inherited as a Mendelian recessive trait on chromosome 6 (Coleman, 1978). Metabolic abnormalities displayed by ob/ob mice are similar to those of obese humans including hyperphagia, hyperinsulinemia, and peripheral insulin resistance (Karam et al, 1963; Bray and York, 1979; Olefsky and Kolterman, 1980; Dulloo and Miller, 1987). Additionally, ob/ob mice are characterized by mild hyperglycemia, decreased brown adipose tissue thermogenesis, increased plasma corticosterone, increased energy efficiency and many more metabolic abnormalities (Bray and York, 1979). Since hyperinsulinemia is 1) common to both obese humans and obese animals, 2) one of the first measurable metabolic abnormalities in ob/ob mice, and 3) implicated as an important causative factor in obesity, I chose to focus my efforts toward determining the cause of hyperinsulinemia in the ob/ob mouse. The remainder of this literature review will be focused on the role of hyperinsulinemia in contributing to obesity and then on the possible causes of hyperinsulinemia in ob/ob mice.

1. Hyperinsulinemia in Ob/ob Mice

Although the primary defect responsible for obesity in ob/ob mice has not been elucidated, hyperinsulinemia is apparent as early as 6 days of age (Dubuc, 1981), before increased adiposity (7 days; Boissonneault et al, 1978), increased plasma corticosterone (21 days; Dubuc, 1976a), hyperphagia (28 days; Lin et al, 1977), hyperglycemia (30 days; Westman, 1968), and peripheral insulin resistance (6 wks; Batchelor et al, 1975).

Early hyperinsulinemia has been implicated as the primary cause of excess adiposity in ob/ob mice since insulin is known to increase activity of lipogenic enzymes, decrease lipolysis, and promote adipocyte hypertrophy and adipose tissue cell proliferation (Bray and York, 1979; Geloen et al, 1989). Hyperinsulinemia could also contribute to obesity by causing hypoglycemia with resultant hyperphagia (MacKay et al, 1940).

Artificial lowering of plasma insulin in ob/ob mice using streptozotocin reduces adiposity through a decrease in lipogenesis (Loten et al, 1974). In fact, in every case where plasma insulin is lowered in ob/ob mice, adiposity is also decreased. Restricted feeding (Dubuc, 1976b), and treatment with β adrenergic agonist cimateral (Walker and Romsos, 1988) both lower plasma insulin and decrease severity of obesity. Adrenalectomy also lowers plasma insulin in ob/ob mice, but to various degrees depending on the diet fed. Decreases in adiposity correspond to decreases in plasma insulin (Warwick

and Romsos, 1988). Again, since hyperinsulinemia is one of the first metabolic defects that contributes to obesity and the primary cause of hyperinsulinemia in ob/ob mice is unknown, it is an important area to study in an attempt to elicit the etiology of obesity in ob/ob mice.

Plasma insulin concentrations are controlled by 2 factors, secretion, and clearance; therefore, I begin the next section with a brief description of the general physiology of insulin secretion, discussing alterations in physiology observed in the ob/ob mouse at the end of each section. I then focus on glucose and neurotransmitters as factors that influence insulin secretion, and finally, I discuss the role of insulin clearance in contributing to hyperinsulinemia.

D. Regulation of Plasma Insulin Concentration

1. Physiology of Insulin Synthesis and Secretion

The pancreas is composed of both exocrine, and endocrine tissue. Exocrine cells secrete digestive enzymes into a duct system which empties into the duodenum via the common bile duct. In contrast, endocrine cells are localized into clusters of 1000-2000 cells known as the islets of Langerhans and secrete hormones directly into the circulatory system. The islets of Langerhans, which make up only 1-2% of total pancreatic tissue, are composed of a core of B cells which secrete insulin, surrounded by A, D, and F cells which

secrete glucagon, somatostatin and pancreatic polypeptide, respectively. Approximately 60-80% of islet cells are B cells, 15-20% are A cells, 15-20% are F cells and 5-10% are D cells (Bonner-Weir, 1989). Islets are surrounded by a dense network of capillaries which supply nutrients and transport secretory products via portal blood directly to the liver, then into general circulation.

Insulin is a peptide hormone, synthesized within the islet B cell as single chain pro-insulin (MW 9000), which is packaged into secretory granules, then cleaved into C-peptide and insulin (MW 6000). Secretory granules serve as storage for insulin and upon stimulus, granules release their contents by exocytosis.

Pancreatic islets from adult ob/ob mice (> 8 wks of age) are distinctly different from islets of lean littermates. Not only are there more islets per pancreas in adult ob/ob mice (Coleman and Hummel, 1973), but ob/ob mouse islets tend to be larger (Hellman et al, 1961; Coleman and Hummel, 1973), contain a greater percentage B cells (>90%; Gepts et al, 1960; Baetens et al, 1978), and are supplied by a much more dense system of capillaries than islets of lean littermates (Rooth et al, 1985). Furthermore, whereas normal islets are highly granulated, ob/ob islets tend to be degranulated and display alterations in rough endoplasmic reticulum and golgi apparatus indicative of a constantly stimulated pattern of insulin synthesis and secretion (Gepts et al, 1960; Diani et al, 1984). Alterations in islet

morphology appear to occur developmentally since at 4 wks of age, islets of ob/ob mice are hypertrophied and degranulated, but at 8 wks of age these alterations in islet morphology are even more exaggerated (Coleman and Hummel, 1973). The exact mechanism of observed islet changes is unknown.

There are many factors that control insulin secretion including glucose, amino acids, hormones within the islet, islet blood flow, neuropeptides and neurotransmitters. These signals reaching the islet can be carried through the circulatory system or can be initiated in the brain and travel through neural pathways. Additionally, cells within the islet can directly influence B cell secretion through paracrine control systems.

If insulin secretion is increased in ob/ob mice, the factors that may contribute to insulin hypersecretion must be understood. I will discuss in detail only glucose and neurotransmitters since these are proposed to be important regulators of insulin secretion, and since my experiments involved these two regulators.

2. Glucose as a Regulator of Insulin Secretion

The primary stimulus for insulin secretion from the islet B cell is glucose. Upon glucose stimulus, insulin is released in a biphasic manner. Glucose metabolism is required for stimulus/secretion coupling; however, the exact mechanism whereby glucose stimulates insulin secretion is unclear. Glucose metabolism results in ATP production. ATP

sensitive K^+ channels appear to be involved in initial membrane depolarization (Arkhammer et al, 1987) which then activates voltage sensitive Ca^{++} channels and allows Ca^{++} entry (Atwater et al, 1980; Wollheim and Sharp, 1981; Henquin and Meissner, 1984). Increased cellular Ca^{++} is believed to be involved in translocation of secretory granules from the cell interior to the plasma membrane for exocytosis; however, mechanisms beyond Ca^{++} entry into the B cell remain to be elucidated (Draznin and Dahl, 1989).

Islets from adult ob/ob mice secrete insulin in a typical biphasic manner but are both hyperresponsive and hypersensitive to glucose stimulus (Lavine et al, 1977). Mechanisms involved in this altered secretory response are unknown. This hypersecretion in response to glucose could partially explain the observed hyperinsulinemia in ob/ob mice; however, glucose may not be the sole cause of insulin hypersecretion since at the earliest time point of hyperinsulinemia (6 days of age) and until weaning, ob/ob mice are hypoglycemic yet are still hyperinsulinemic (Dubuc, 1976c; 1981).

3. Neurotransmitters as Regulators of Insulin Secretion

Neural pathways have been proposed as an important system to modulate glucose-induced insulin secretion. In fact, meal-induced insulin release in rats has been estimated to be approximately 26% neurally mediated. (Berthoud, 1984). Pancreatic islets are innervated by both parasympathetic and

sympathetic fibers which have been observed in close vicinity of B cell membranes (Esterhuizen et al, 1968).

Sympathetic fibers innervating the pancreas originate in the spinal cord, travel through the splanchnic nerve and release norepinephrine at the B cell membrane (Bereiter et al, 1981; Miller, 1981). Epinephrine, released by the adrenal medulla into the bloodstream, provides additional sympathetic control of insulin secretion. In normal rats, there appears to be a tonic inhibition of insulin secretion through the sympathetic nervous system. Some researchers have estimated that the sympathetic nervous system may tonically inhibit in vivo insulin secretion in fed rats by as much as 47% (Curry, 1983). Thus, the sympathetic nervous system may be an important regulatory system for overall insulin response.

Although both norepinephrine and epinephrine can potentially act at β (stimulatory) or α (inhibitory) receptors, sympathetic innervation in rats or mice results in overall inhibition of insulin secretion (for review, Miller, 1981). Inhibitory effects appear to be mediated by the α_2 subclass of α receptors since effects of epinephrine or norepinephrine on insulin secretion from mouse islets can be blocked by a nonspecific α antagonist, phentolamine (Coll-Garcia and Gill, 1968), and more specifically blocked by an α_2 antagonist yohimbine (Nakadate et al, 1980).

The mechanism of norepinephrine inhibition of insulin secretion is not completely understood. Binding at α_2

receptors was originally believed to act through an inhibitory G protein which decreased cellular cAMP; however, many studies have dissociated the effects of norepinephrine on cAMP from its effects on insulin release (Ullrich and Wollheim, 1984). More recent evidence suggests that binding to α_2 receptors may act through a more distal site in stimulus-secretion coupling, perhaps involving desensitizing the secretory apparatus to Ca^{++} (Morgan and Montague, 1985; Metz, 1988; Nilsson et al, 1988).

Parasympathetic fibers innervating the pancreas appear to originate in the nucleus ambiguus and dorsal motor nucleus of the main brainstem, travel through the vagus nerve, and release acetylcholine at B cell membranes (Bereiter et al, 1981). Parasympathetic nerves innervating islets release acetylcholine which acts at muscarinic receptors and results in potentiation of glucose-induced insulin secretion (Bergman and Miller, 1973; Loubatieres-Mariani et al, 1973; Miller, 1981). Parasympathetic nervous system activity at the pancreas is believed to be responsible for cephalic phase insulin secretion (insulin secretion that occurs just prior to a meal and before an increase in plasma glucose) since cephalic insulin secretion can be blocked by vagotomy (Storlien, 1985). In fact, parasympathetic input to the pancreas may be responsible for over 26% of the insulin response to a meal (Berthoud, 1984).

Potentiation of glucose-induced insulin secretion by acetylcholine appears to require the presence of a glucose

concentration above threshold for insulin release (Hermans et al, 1987). Furthermore, effects of acetylcholine are proposed to be greater at higher concentrations of glucose (Campfield and Smith, 1980).

The mechanism of acetylcholine potentiation of glucose-induced insulin secretion is also not completely understood. Binding of acetylcholine to muscarinic receptors on the B cell membrane is believed to result in activation of the phosphoinositide second messenger system with resultant release of intracellular Ca^{++} (Best and Malaisse, 1984). Increased cellular Ca^{++} then initiates insulin release. Recent evidence suggests that acetylcholine mediates an early transient Ca^{++} entry required for its potentiating effect (Henquin, et al, 1988; Sanchez-Andres et al, 1988). Yet, exact mechanisms of acetylcholine action remain unclear.

Alterations in insulin secretion, controlled by glucose, neurotransmitters, and other stimuli could contribute to hyperinsulinemia; however, insulin clearance must also be considered as a possible contributing factor.

4. Insulin Clearance

Approximately 50% of insulin leaving the pancreas is immediately cleared during the first pass through the liver (Karakash et al, 1976). Since plasma insulin is so excessive in ob/ob mice, decreased clearance of insulin has been suggested as a potential contributing factor. Although insulin clearance is decreased in ob/ob compared to lean

livers, decreased clearance appears to be secondary to hyperinsulinemia rather than a primary contributing factor to the hyperinsulinemia. This was concluded because reduction of plasma insulin using streptozotocin in ob/ob mice resulted in increased insulin clearance, indicating that ob/ob livers were normal in their ability to clear insulin when plasma insulin was normalized (Karakash et al, 1976). As a result, increased insulin secretion must be the primary initiator of hyperinsulinemia in ob/ob mice.

Since neural input appears to play a major role in control of insulin secretion, alterations in parasympathetic or sympathetic activity could potentially contribute to hyperinsulinemia in ob/ob mice. Additionally, because neural input to the pancreas is originally signalled from the brain, evidence of alterations in neural regulation of insulin secretion from ob/ob islets may indicate a defect in the brain. This would support a current hypothesis that a central neural defect is the primary etiology of obesity in ob/ob mice. This central neural defect hypothesis is discussed in the next section. Evidence in support of this hypothesis has been collected on 3 models of obesity; thus, specific evidence will be provided on the VMH-lesioned rat, the Zucker fa/fa rat and the ob/ob mouse.

E. Relationship Between Central Nervous System, Hyperinsulinemia, and Obesity

Several animal models of obesity have been studied in an attempt to locate a primary defect responsible for widespread

metabolic abnormalities seen in obese animals. Genetically obese animals, ob/ob mice, and Zucker fa/fa rats, share many common metabolic abnomalities with rats made obese by electrical lesioning of the ventromedial hypothalamus (VMH-lesioned rats). Ob/ob mice, fa/fa rats and VMH-lesioned rats are all characterized by hyperinsulinemia, hyperphagia, obesity, decreased thermoregulatory thermogenesis (through decreased sympathetic neural activity at brown adipose tissue), and hypertrophy and hypersecretion of pancreatic islets (Bray and York, 1979; Jeanrenaud, 1985). A single gene defect in genetically obese rodents results in such diverse abnormalitities, which so closely resemble those of rodents made obese through a lesion in the CNS, that researchers have suggested all 3 obese states may result from a primary central neural abnormality. A central neural defect or lesion could alter neural regulation of many organs including the pancreas which could explain existing hyperinsulinemia and resultant obesity (Jeanrenaud, 1985).

The VMH and lateral hypothalamic regions of the hypothalamus are proposed to reciprocally control metabolism through the sympathetic and parasympathetic nervous systems, respectively (Bray, 1986). Alterations in these brain areas could lead to excess insulin secretion by B cells through increased stimulation by acetylcholine or decreased inhibition by norepinephrine and epinephrine, or both. Techniques used to measure whether obese animals display altered neural regulation of insulin secretion include; 1)

effects of vagotomy or neurotransmitters and neurotransmitter antagonists on *in vivo* insulin secretion, 2) effects of neurotransmitters on insulin secretion from isolated pancreatic islets *in vitro*, and 3) norepinephrine turnover as a measure of sympathetic activity in whole pancreas.

1. *VMH-Lesioned Rats*

Islets isolated from VMH-lesioned rats 8-10 wks after lesioning are hypertrophied and hypersecrete insulin in response to both low (5 mM glucose) and high (20 mM) concentrations of glucose (Inoue et al, 1977). By 8-10 wks after lesioning islet hypertrophy could be the result of hyperphagia; however, there is some evidence that this is not the case. When VMH-lesioned, hypophysectomized rats were pair-fed to control rats, islets of VMH-lesioned, hypophysectomized rats were still larger than islets of control rats (Han et al, 1970). Additionally, islets from VMH-lesioned rats are hyperresponsive to glucose stimulus within 1 day after lesioning, before increased food intake or increased islet size (Campfield et al, 1986). Thus, the lesion itself must somehow be signaling the islets such that they become hyperresponsive to glucose stimulus and increase in size. Altered neural signalling to the islets may be one cause of these observed islet changes.

There is considerable evidence for alterations in the parasympathetic and sympathetic neural activity to the pancreas in the VMH-lesioned rat. Within minutes after

lesioning there is an increase in plasma insulin which can be reversed by vagotomy (Berthoud and Jeanrenaud, 1979). These results suggest that increases in parasympathetic nerve activity after VMH-lesioning mediate the hyperinsulinemia. Evidence for alterations in both sympathetic and parasympathetic innervation of islets from VMH-lesioned rats is presented by Campfield and Smith, 1983. By incubating isolated pancreatic islets from VMH and control rats in varying concentrations of neurotransmitters and 10 mM glucose, sensitivity to neurotransmitters was measured. VMH-lesioned rat islets were less responsive and less sensitive to acetylcholine, and more responsive and more sensitive to norepinephrine than islets of non-lesioned rats. These data suggest possible up-regulation of norepinephrine receptors and down-regulation of acetylcholine receptors, perhaps in response to decreased sympathetic and increased parasympathetic tone at the islets in vivo. Additional time course studies demonstrated simultaneous increases in glucose and norepinephrine responsiveness at day 1 after lesion; whereas, acetylcholine responsiveness decreased at day 2. Authors suggest a possible role for norepinephrine in altering glucose responsiveness (Campfield et al, 1986). An additional study by Campfield et al, 1984, suggests that the parasympathetic nervous system may play a role in regulating islet glucose sensitivity since vagotomy in normal rats increased islet sensitivity to glucose.

These studies provided indirect estimates of neural

activity to the islet; however, more direct studies performed *in vivo* support the *in vitro* results in suggesting that alterations in islet sensitivity and responsiveness to neurotransmitters are compensatory responses to decreased sympathetic and increased parasympathetic tone. Infusion of an acetylcholine analog into VMH-lesioned rats 1 wk after lesioning resulted in less of an increase in plasma insulin in lesioned rats compared to sham-operated rats. Again this indicated decreased responsiveness to acetylcholine after VMH-lesioning. After infusion of norepinephrine and 0.5 g glucose/kg body weight, plasma insulin decreased much more in VMH-lesioned rats compared to control rats. This confirms increased responsiveness to norepinephrine in VMH-lesioned rats (Smith and Campfield, 1986). Finally, direct recordings from pancreatic nerves have shown that after VMH lesion, firing rate of sympathetic nerves is decreased and firing rate of parasympathetic nerves is increased (Yoshimatsu et al, 1984).

2. *Zucker (fa/fa) Rats*

Zucker (fa/fa) rats are similar to ob/ob mice in that their obesity is inherited as an autosomal recessive trait. Plasma insulin of fa/fa rats has been reported at 3-30X greater than that of lean littermates (Curry and Stern, 1985). As with ob/ob mice, pancreatic islets of fa/fa rats are both hypertrophied (Shino et al, 1973) and hyperplastic (Larsson et al, 1977) and are hypersensitive and

hyperresponsive to glucose stimulus (Schade and Eaton, 1975; Pansini and Tolman, 1981; Curry and Stern, 1985).

CNS alterations have been suggested as contributing to insulin hypersecretion in fa/fa rats. Glucose infusion into pre-obese, 17 day old fa/fa rats results in hypersecretion of insulin compared to lean littermates. This increased responsiveness to glucose in pre-obese rats could be blocked by pre-treatment with atropine suggesting increased vagal activity in fa/fa rats (Rohner-Jeanrenaud et al, 1983). Additionally, electrical stimulation of the vagus nerve in 6-9 wk old fa/fa and lean rats resulted in much greater stimulation of insulin secretion from fa/fa rats than lean littermates (Rohner-Jeanrenaud et al, 1983). This evidence is supported by recent research using a fully innervated pancreatic perfusion method on Zucker fa/fa rats. Insulin secretion in response to 200 mg glucose/dl was greatest from perfused pancreas of CNS-intact fa/fa rats and was decreased by 50% in CNS-ablated fa/fa rats. Vagotomy of CNS-intact rats decreased insulin secretion to levels of CNS-ablated rats. No effect of ablating CNS or vagotomy were observed from lean rats (Lee et al, 1989). This supports the view that increased activity of the vagus nerve in fa/fa rats is responsible for much of the hyperinsulinemia.

3. Ob/ob Mice

There are several reasons to postulate that neurotransmitter mediated insulin secretion may be altered in

ob/ob mice. Ahren and Lundquist, 1982, administered 26 μmol methylatropine (a muscarinic cholinergic antagonist) per kg body weight intraperitoneally (IP) to ob/ob and control NMRI mice. Measurements of plasma insulin at 15, 30 and 60 mins post-injection showed significantly greater absolute decreases in plasma insulin in ob/ob mice compared to controls at all time points. In addition, when expressed as a percentage of baseline insulin concentration, which is significantly higher in ob/ob than controls, by 15 mins plasma insulin had decreased 60% in ob/ob mice compared to 30% in controls. Furthermore, phentolamine and L-propranolol, α and β adrenergic antagonists respectively, were administered to determine possible alterations in insulin response to sympathetic activity. Phentolamine administration IP at 53 μmol/kg resulted in greater absolute increases in plasma insulin concentration in ob/ob mice compared to controls. Percentage increases in plasma insulin were 200% over baseline in both ob/ob and controls. L-propranolol (68 μmol/kg) IP resulted in a 40 % decrease in plasma insulin concentration in both ob/ob and controls; however, absolute decreases in plasma insulin were greater in ob/ob than controls.

Although these results support the hypothesis of altered insulin response to neurotransmitters in ob/ob mice, several limitations must be considered. Control mice used by Ahren and Lunquist, 1982, were of different genetic background (NMRI mice) than ob/ob mice used; thus, differences in

response to adrenergic and cholinergic blockade may have resulted from genetic background differences rather than differences in expression of the ob/ob gene itself. Furthermore, drugs were administered based on body weight. Since ob/ob mice were up to 40 g heavier than controls, absolute amounts of drug administered were greater in ob/ob than controls. Furthermore, phentolamine has recently been shown to have a direct stimulatory effect on insulin secretion (Schulz and Hasselblatt, 1988).

A recent report from Kuhn et al, 1987, supports the data of Ahren and Lunquist, 1982, as evidence for possible abnormalities in neural control of insulin secretion in ob/ob mice. Epinephrine, an adrenergic agonist, injected subcutaneously (3 μg/10 g body weight), resulted in a rapid 50% decrease in plasma insulin in ob/ob mice; whereas, no change in plasma insulin was observed in lean mice. To evaluate islet insulin response to endogenous neural activity, a nonspecific α adrenergic antagonist, phentolamine, was administered. Absolute increases in plasma insulin were greater in ob/ob than controls supporting results seen with exogenous epinephrine administration. Furthermore, ob/ob mice exhibited greater decreases in plasma insulin than controls in response to immobilization stress, implying exaggerated responsiveness to catecholamines or sympathetic hypertonicity with stress (Kuhn et al, 1987). Again, a problem with this work is that phentolamine has a direct stimulatory effect on islet insulin secretion (Schulz

and Hasselblatt, 1988).

Norepinephrine turnover, a measure of sympathetic nervous system activity, in ob/ob mouse pancreas is comparable to control mouse pancreas (Knehans and Romsos, 1983). Although a normal norepinephrine turnover in whole pancreas would appear to argue for normal sympathetic activity to the islets, islets make up only 1-2% of pancreatic tissue. Thus, sympathetic activity to islets could be altered and remain undetectable when measuring whole pancreas. Additionally sympathetic epinephrine from the adrenal medulla could be decreased as has been shown for rats with hypothalamic knife cuts (Vander Tuig et al, 1987).

Additional evidence for altered neural regulation of insulin secretion in ob/ob mice is provided by studies on islet blood flow. Norepinephrine injection resulted in immediate inhibition of blood flow to ob/ob islets with little effect on lean islets (Rooth et al, 1985). Furthermore, in response to epinephrine, ob/ob mouse islets secreted glucagon at 4X basal levels; whereas, the same dose of epinephrine had no effect on lean islets (Beloff-Chain et al, 1977).

F. Hypothesis and Objectives

I hypothesized that islets of ob/ob mice would hypersecrete insulin in response to glucose stimulus, and would be less sensitive to acetylcholine and more sensitive to norepinephrine stimulus compared to islets of lean

littermates. Throughout this report, I use the term hypersensitive to refer to islets that secrete insulin in response to lower concentrations of secretagogue when compared to control islets. On the other hand, I use hyperresponsive to refer to islets that secrete larger amounts of insulin in response to a given concentration of secretagogue as compared to control islets. My objectives were to determine 1) the dynamics of glucose-induced insulin secretion, and 2) the dynamics of norepinephrine inhibition and acetylcholine potentiation of glucose-induced insulin secretion, from islets of 8-9 wk old ob/ob and lean mice.

II. MATERIALS AND METHODS

A. Animals and Materials

Female obese (ob/ob) mice and lean littermates (ob/+ or +/+) from our breeding colony (C57BL/6J - ob/+) were group-housed in a temperature controlled room (25°C) with a 12 hr light-dark cycle (lights on at 0700 hr). Wood shavings were provided for bedding. Stock diet (Wayne Lab-Blocks, Continental Grain, Chicago, IL) and water were allowed *ad libitum* from weaning at 3 wks of age until mice were used at 8-9 wks of age.

Collagenase, type V, lot # 18F-6840, bovine serum albumin (fraction V, radioimmunoassay grade), yohimbine hydrochloride, prazosin hydrochloride, acetylcholine chloride, norepinephrine (arterenol hydrochloride), eserine, atropine methyl nitrate, and HEPES, were from Sigma Chemical Company, St. Louis, MO; ^{125}I insulin was from ICN Biomedicals, Irvine, CA; ascorbic acid was from Fisher Scientific Company, Fair Lawn, NJ; anti-porcine insulin guinea pig serum and rat insulin standard were from Novo BioLabs, Danbury, CT. Krebs Ringer bicarbonate buffer for isolation of islets and islet incubations was freshly oxygenated, pH 7.4.

B. Islet Preparation

We isolated pancreatic islets using a modification of the method by Lacy and Kostianovsky, 1967. Mice, (8-9 wks of age) were killed by cervical dislocation in the fed state between 1000 and 1200 hr. Upon opening the abdominal cavity, we ligated the common bile duct at the duodenal entrance, and by inserting a 30 guage needle into the hepatic portion of the bile duct, we inflated the pancreas with 3 mls of 37°C Krebs Ringer bicarbonate buffer containing 2.5 mg collagenase/ml and 2.5 mM glucose. We quickly dissected the pancreas and without chopping, placed it in a small glass tube containing an additional 0.5 ml of a 10 mg collagenase/ml solution. Each tube containing 1 pancreas was gently shaken by hand in a 37°C water bath for 3-4 mins, then briskly shaken about 10 times to loosen islets from surrounding connective tissue. To stop the digestion, we added ice-cold buffer containing 2.5 mM glucose, then washed the islets several times to remove digested acinar tissue and collagenase. Islet yield averaged 60 islets per lean mouse and 100 islets per ob/ob mouse.

C. Experimental Design

1. General Design

Each experiment consisted of mean results from 5 or 6 experimental days, (n=5 or 6). On one experimental day, 3 lean and 2 ob/ob mice were alternately killed and their

islets isolated. Islets from the 3 lean mice were pooled and distributed 10 islets per dish with 1 or 2 dishes per treatment. Ob/ob mouse islets were also pooled and distributed across identical treatments. For treatments with 2 dishes of islets, the 2 values for insulin secretion for that experimental day were averaged and considered a single replication (n). Otherwise, insulin secretion from the one dish (10 islets) for each treatment was considered a single replication (n). A stereoscopic microscope and a 200 μl micropipetter were used to transfer islets into small, black bottom petri dishes. Care was taken to distribute islets across dishes such that all dishes within one phenotype received 10 representative islets of approximately equal size. Islets for all experiments underwent 3, consecutive, 30 min incubations at 37°C under a continuous 95% O_2, 5% CO_2 atmosphere. For the first 30 min, islets were pre-incubated in 1 ml of 2.5 mM glucose Krebs Ringer bicarbonate buffer with 1 mg/ml bovine serum albumin. Islets were then transferred, using a 200 μl micropipetter and visualizing under a stereoscopic microscope, to 1 ml of fresh but identicle incubation buffer. After a second 30 min incubation, 0.5 ml of buffer was sampled for determination of basal, non-stimulated immunoreactive insulin (referred to as insulin throughout this paper) release. This 30 min incubation in 2.5 mM glucose was included in each experiment as a method of determining islet damage incurred during collagenase digestion. The 0.5 mls of sample buffer

collected for determination of islet damage was then replaced with 0.5 ml of Krebs Ringer bicarbonate buffer containing the desired concentrations of glucose, neurotransmitters, or antagonists, depending on the experiment. Islets were then incubated for another 30 min. Following this last, 30 min incubation, medium was collected for determination of insulin released.

At the end of each experiment, representative islets were extracted for determination of islet insulin content (Curry, 1986). Since ob/ob islets secrete more insulin under some conditions than lean islets and this would influence final islet insulin content, we only extracted islets that were incubated in 2.5 mM glucose, or 15 mM glucose plus 3 or 30 μM norepinephrine. Under these conditions, insulin secretion from ob/ob and lean islets was similar.

As stated earlier, insulin secreted during the second, 2.5 mM glucose incubation was used as an index of islet damage. Hahn et al, 1976, demonstrated that over-digested islets secreted up to 20 fold more insulin under basal conditions than intact islets and then up to 2 fold more insulin than intact islets in response to 20 mM glucose stimulus. In our preliminary tests, both ob/ob and lean islets that released greater than 0.3 ng insulin/islet (10 fold greater than normal) during basal conditions also secreted excessive quantities of insulin when challenged with glucose; therefore, non-stimulated secretion over 0.3 ng insulin/islet was considered an indication of islet damage

and these data were discarded. As an additional control, islet viability was evaluated by islet ability to secrete insulin in response to glucose (Krause et al, 1973; Lacy and Kostianovsky, 1967). If islets from an experimental day showed no secretory response to glucose, data was discarded.

On each of 5 randomly chosen experimental days, we measured diameters of approximately 20 representative islets. These data were cumulated to determine the distribution of various islet sizes used from ob/ob and lean mice. A total of 98 ob/ob islets and 92 lean islets were measured. Non-spherical islets were measured at their longest and shortest axes and the average of the two was recorded.

Additional mice were killed between 1000-1200 hr for determination of pancreatic insulin content, plasma insulin and plasma glucose. For pancreatic extraction of insulin, each carefully dissected pancreas was chopped, sonicated for 5 min, and incubated overnight at 4°C in 10 ml acid ethanol (composition 7.5 ml 12 N HCl to 492.5 ml 75% ethanol). Trasylol, 1000 K/pancreas, was added to inhibit proteolytic enzymes. Samples were centrifuged at approximately 3000 g: Supernatants were removed and pellets re-extracted (Curry, 1986). Following three overnight extractions, all samples were diluted in Krebs Ringer bicarbonate buffer and stored at -20°C for subsequent insulin determination.

2. Experiment 1 - Effects of Glucose on Insulin Secretion

For experiment 1, islets were incubated in 2.5, 5, 7, 10, 12, 15, or 20 mM glucose during the last 30 min period to characterize the glucose/insulin dose response curves for both ob/ob and lean mouse islets. Based on these results, we selected the concentrations of glucose to use for studies with acetylcholine and norepinephrine.

Acetylcholine may be more effective at higher concentrations of glucose. Therefore, although alterations in sensitivity to glucose of ob/ob islets is well documented (Lavine et al, 1977), we felt it was important to characterize the effects of glucose on insulin secretion from our ob/ob and lean mouse islets. Due to alterations in sensitivity, what may be a high concentration of glucose for ob/ob islets (eliciting near 100% of maximum insulin secretion), may be a low concentration of glucose for lean islets (eliciting less than 50% of maximum secretion). Thus, in an attempt to eliminate this potential source of bias, we selected a concentration of glucose that had approximately the same effect on both ob/ob and lean islets (as a % of maximum secretion).

3. Experiment 2 - Effects of Neurotransmitters on Glucose-Induced Insulin Secretion

Experiment 2 was conducted in 3 parts. It was designed to determine islet responsiveness and sensitivity to 1) varying concentrations of acetylcholine in the presence of

high (15 mM) glucose, 2) varying concentrations of acetylcholine in the presence of low (5 mM) glucose, and 3) varying concentrations of norepinephrine in the presence of high (15 mM) glucose. Thus, for the 2 trials with acetylcholine, during the last 30 min incubation, each dish contained either 15 or 5 mM glucose and 0, 0.005, 0.01, 0.05, 0.1, 1, or 10 μM acetycholine. All treatment dishes including the control (0 μM acetylcholine) dishes contained 10 μM eserine to antagonize acetylcholine esterase activity. For the trial with norepinephrine, 0, 0.0003, 0.003, 0.03, 0.3, 3, or 30 μM norepinephrine in addition to 15 mM glucose was used for the last, 30 min incubation. All treatment dishes including the control (0 μM norepinephrine) dishes contained 1 mM ascorbic acid to prevent oxidation of norepinephrine.

4. Experiment 3 - Effects of Neurotransmitter Antagonists on Insulin Secretion

Experiment 3 was conducted in 2 parts and employed acetylcholine and norepinephrine antagonists to determine if, in previous experiments, neurotransmitters were interacting with specific receptors to produce the observed stimulatory and inhibitory effects. Treatment groups included; 15 mM glucose alone; 15 mM glucose plus neurotransmitter; 15 mM glucose plus neurotransmitter plus antagonist; and 15 mM glucose plus antagonist. First, 10 μM atropine (a muscarinic acetylcholine antagonist) was used to block the stimulatory

effects of 1 μM acetylcholine. And second, 30 μM yohimbine (an α_2 adrenergic antagonist) or 30 μM prazosin (an α_1 adrenergic antagonist) was used to block the inhibitory effects of 3 μM norepinephrine.

D. Sample Analysis

Insulin in incubation buffer and plasma was determined using radioimmunoassay (Novo Research Laboratories, Bagsvaerd, Denmark). Intra-assay and inter-assay coefficients of variation were 5 and 12%, respectively. Plasma glucose was determined using the glucose oxidase method (Boehringer Mannheim, Indianapolis, Indiana).

E. Statistical Analysis

Data were analyzed by two factor factorial analysis of variance (phenotype x concentration of glucose or neurotransmitter) or by one way analysis of variance. Dunnett's t-test was used to detect the lowest effective dose of drug on insulin secretion, and Students t-test to detect differences in insulin secretion between ob/ob and lean mouse islets at a given drug dose (Gill, 1981). Means were considered statistically significant at $P< .05$.

III. RESULTS

At 8-9 wks of age, ob/ob and lean mice weighed approximately 35 and 22 g, respectively. Plasma insulin and glucose concentrations of 8-9 wk old ob/ob and lean mice are presented in Figure 1. Plasma insulin was 95 fold higher and plasma glucose 30% higher in ob/ob compared to lean mice. Additionally, pancreatic islets of ob/ob mice were on average larger in diameter than islets of lean littermates, and were distributed across a wider size range (Figure 2). Mean islet volumes, calculated using the formula $4/3\pi r^3$, were 29.6×10^{-3} for ob/ob islets and 7.2×10^{-3} mm^3 for lean islets. Although ob/ob islets were significantly larger, they contained the same quantity of insulin as lean islets (Figure 3). Total pancreatic insulin content was 25% greater in ob/ob than lean mice (Figure 3).

A. Experiment 1 - Effects of Glucose on Insulin Secretion

Ob/ob and lean mouse islets responded to glucose in a dose-dependent manner (Figure 4). Basal insulin secretion in response to 2.5 mM glucose was not significantly different between ob/ob and lean islets. However, 5 and 7 mM glucose stimulated insulin secretion from ob/ob islets, while having no stimulatory effect on insulin secretion from lean islets (Figure 4). The glucose dose response curve for ob/ob islets

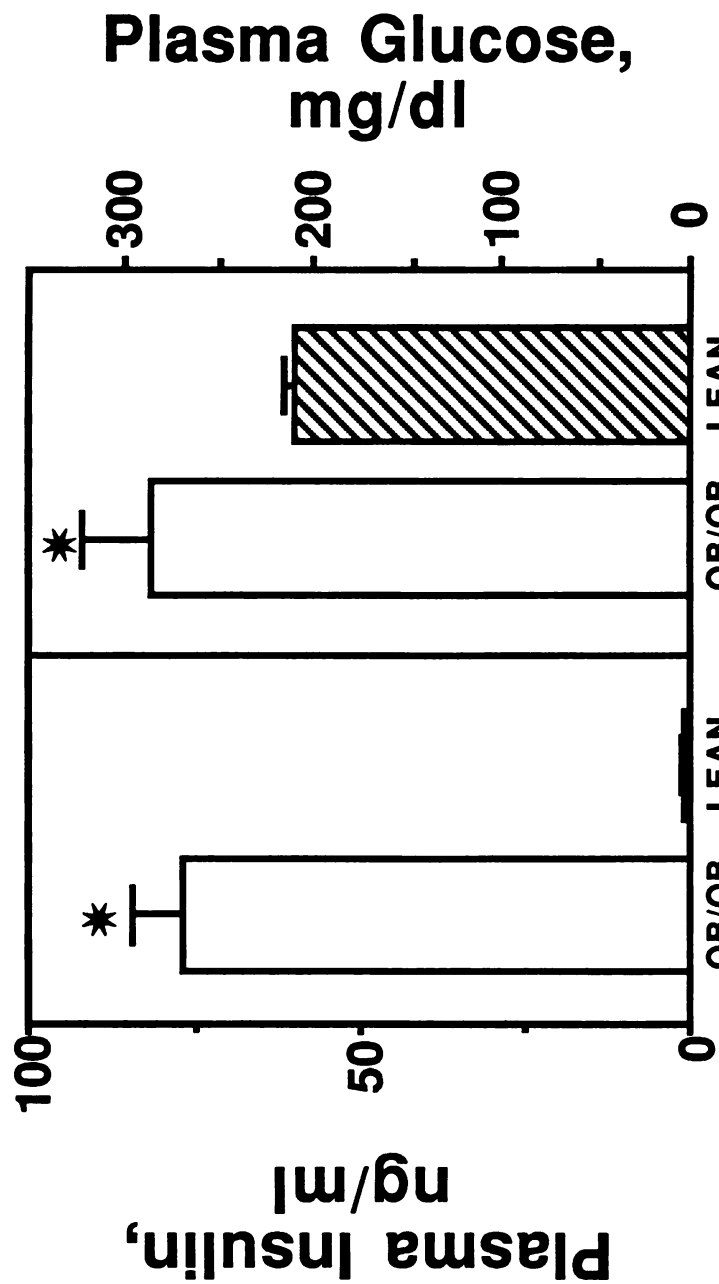

Figure 1. Plasma insulin and glucose of 8-9 wk old ob/ob and lean mice. Data are means ± SE (n = 7). Asterick indicates significant effect of phenotype.

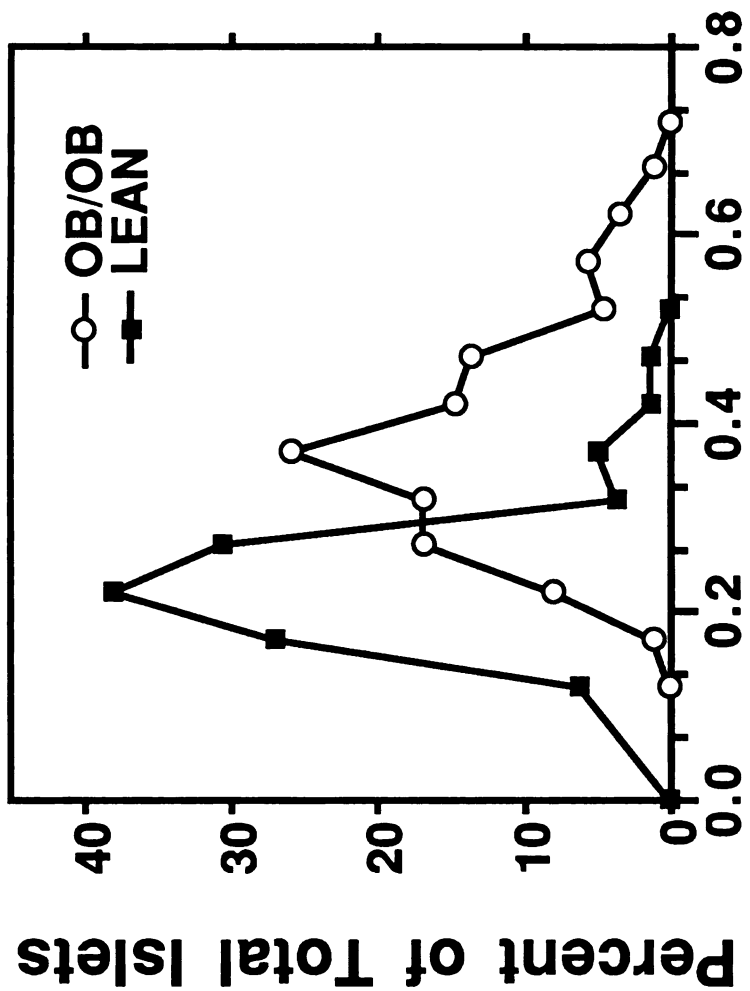

Figure 2. Distribution of islet size from ob/ob and lean mice. Data are expressed as percentage of 98 ob/ob or 92 lean islets.

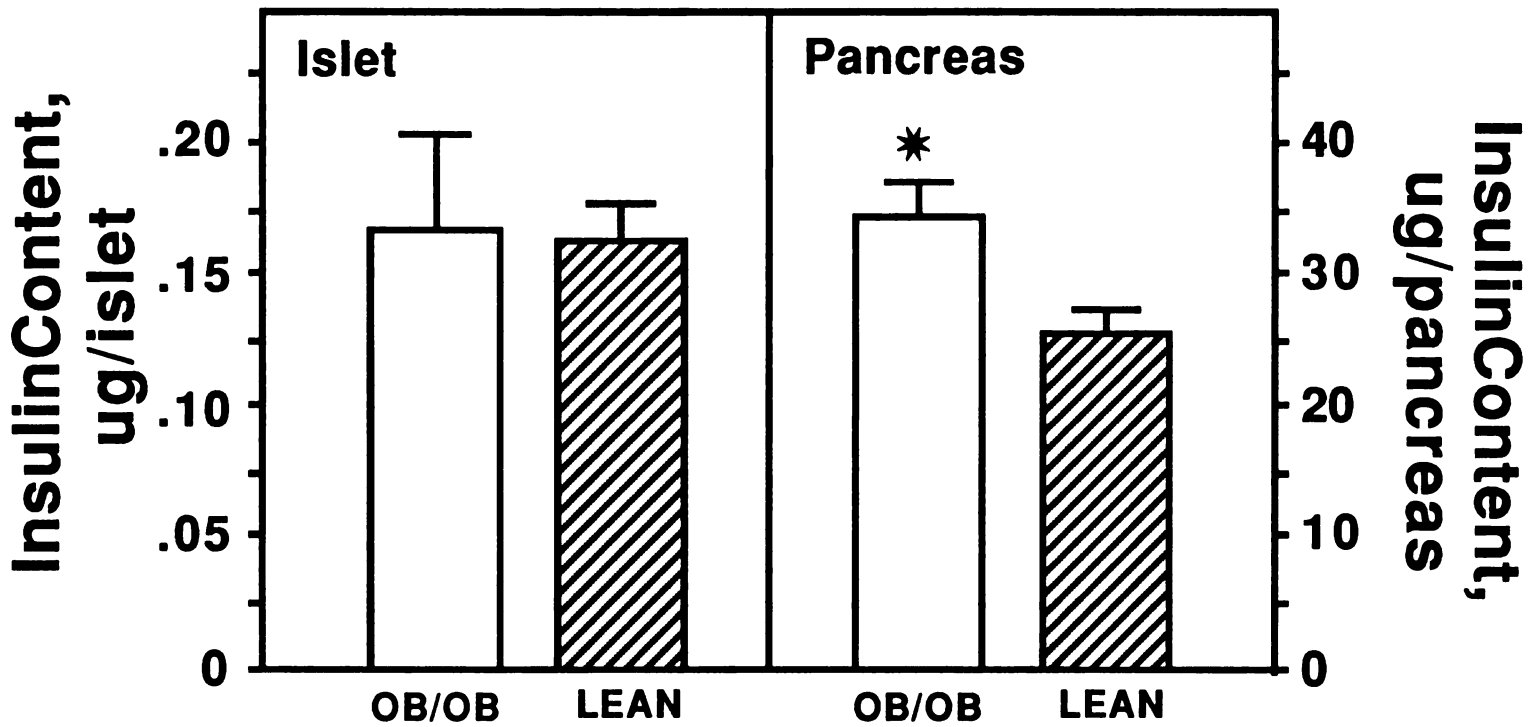

Figure 3. Islet and pancreatic insulin content from ob/ob and lean mice. Data are means ± SE (n = 22 for islets, 8 for pancreata). Each observation (n) for islet insulin content is 10 islets extracted as a group with results expressed as insulin content per islet. Asterick indicates significant effect of phenotype.

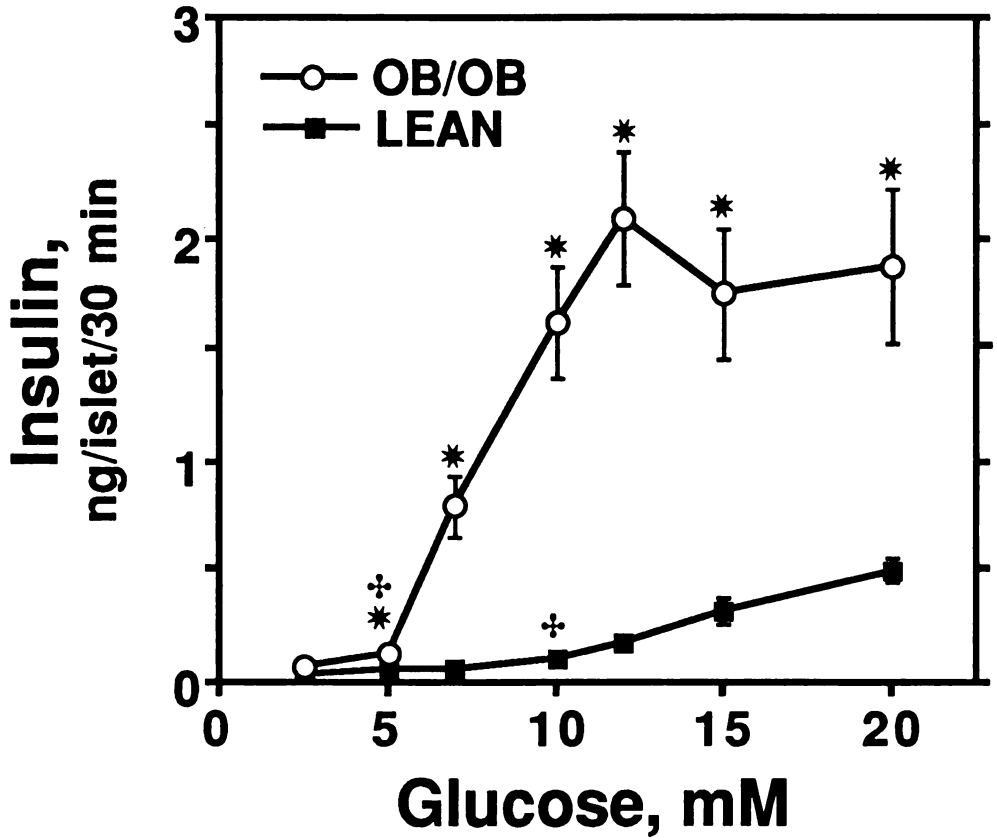

Figure 4. Glucose-induced insulin secretion during 30 min incubation of 8 wk old ob/ob and lean mouse islets. Data are means ± SE (n = 4-7). Cross symbols indicate the lowest glucose concentration to significantly increase insulin secretion above baseline (2.5 mM glucose) secretion within phenotype and asterick symbols indicate significant effect of phenotype at a given concentration of glucose.

was shifted up and to the left. Half-maximal insulin secretion occurred in response to 8.5 mM glucose for ob/ob islets and 12 mM glucose for lean islets. When incubated in either 15 mM or 7 mM glucose, ob/ob islets secreted approximately 5.5 fold and 15 fold more insulin than lean islets, respectively. Since 15 mM glucose appeared to elicit near maximal insulin secretion from both ob/ob and lean islets (Figure 4), we selected 15 mM glucose for subsequent experiments with acetylcholine and norepinephrine.

B. Experiment 2 - Effects of Neurotransmitters on Glucose-Induced Insulin Secretion

Acetylcholine enhanced glucose-induced insulin secretion from both lean and ob/ob islets in a dose-dependent manner; however, both baseline insulin secretion in response to 15 mM glucose, 0 acetylcholine, and insulin secretion potentiated by acetylcholine were significantly greater from ob/ob islets compared to lean islets (Figure 5,A). In fact, insulin secreted from ob/ob islets in response to 1 μM acetylcholine was 9 fold greater than insulin secreted from lean islets. The lowest effective dose of acetylcholine was 0.01 μM for lean and 0.05 μM for ob/ob islets.

Because of the large difference in baseline insulin secretion between lean and ob/ob islets, direct comparisons between the two are difficult. Therefore, baseline insulin secretion, in response to 15 mM glucose, 0 acetylcholine (Figure 5,A) was subtracted from secretion in response to each dose of acetylcholine to give absolute increases in

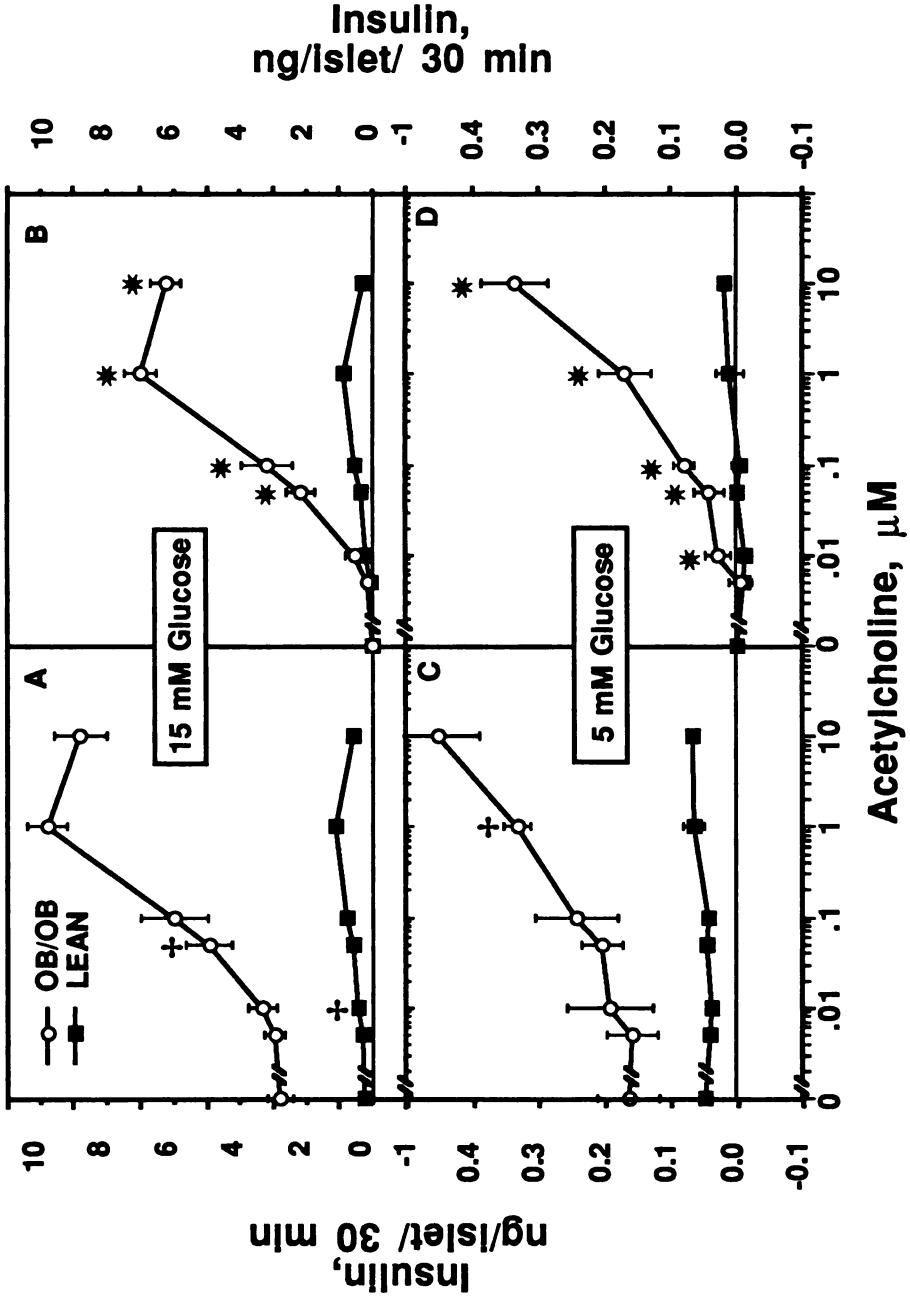

Figure 5. Insulin secretion from ob/ob and lean mouse islets in ressponse to 15 mM glucose (A,B) or 5 mM glucose (C,D) and acetylcholine. Results are expressed as absolute insulin secretion (A,C) and as absolute increase in insulin secretion above baseline (B,D). Baseline is defined as secretion in 0 acetylcholine (A,C). Data are mean ± SE (n = 4-6 except for 10 µM acetylcholine in A and C; ob/ob, n = 3, lean, n = 1). Cross symbols indicate lowest acetylcholine concentration to significantly increase insulin secretion above baseline secretion within phenotype, and asterick symbols indicate significant effect of phenotype within treatment.

insulin secretion due to acetylcholine (Figure 5,B). Acetylcholine (0.05 μM and higher) resulted in much greater absolute increases in insulin secretion from ob/ob islets than from lean islets. Acetylcholine, (1 μM), potentiated insulin secretion by approximately 7 ng/islet from ob/ob islets, compared to approximately 0.9 ng/islet from lean islets (Figure 5,B).

Since acetylcholine acts in vivo to stimulate insulin secretion just prior to a meal (termed cephalic phase insulin secretion; Storlien, 1985), when plasma glucose would be low, 5 mM glucose was selected to further study the effects of acetylcholine on glucose-induced insulin secretion. Only ob/ob islets responded in a dose-dependent manner to acetylcholine and 5 mM glucose (Figure 5,C). The lowest effective dose of acetylcholine on insulin secretion from ob/ob islets was 1 μM.

Baseline insulin secretion, in response to 5 mM glucose, 0 acetylcholine (Figure 5,C) was again significantly different between ob/ob and lean islets; therefore, baseline secretion was subtracted from all secretion values. Thus, when expressed as absolute increase in insulin secretion over baseline secretion, acetylcholine at concentrations of 0.01 μM and above resulted in significantly greater potentiation of insulin secretion from ob/ob than lean islets (Figure 5,D).

Data on effects of norepinephrine on glucose-induced insulin secretion are presented in Figure 6. Norepinephrine

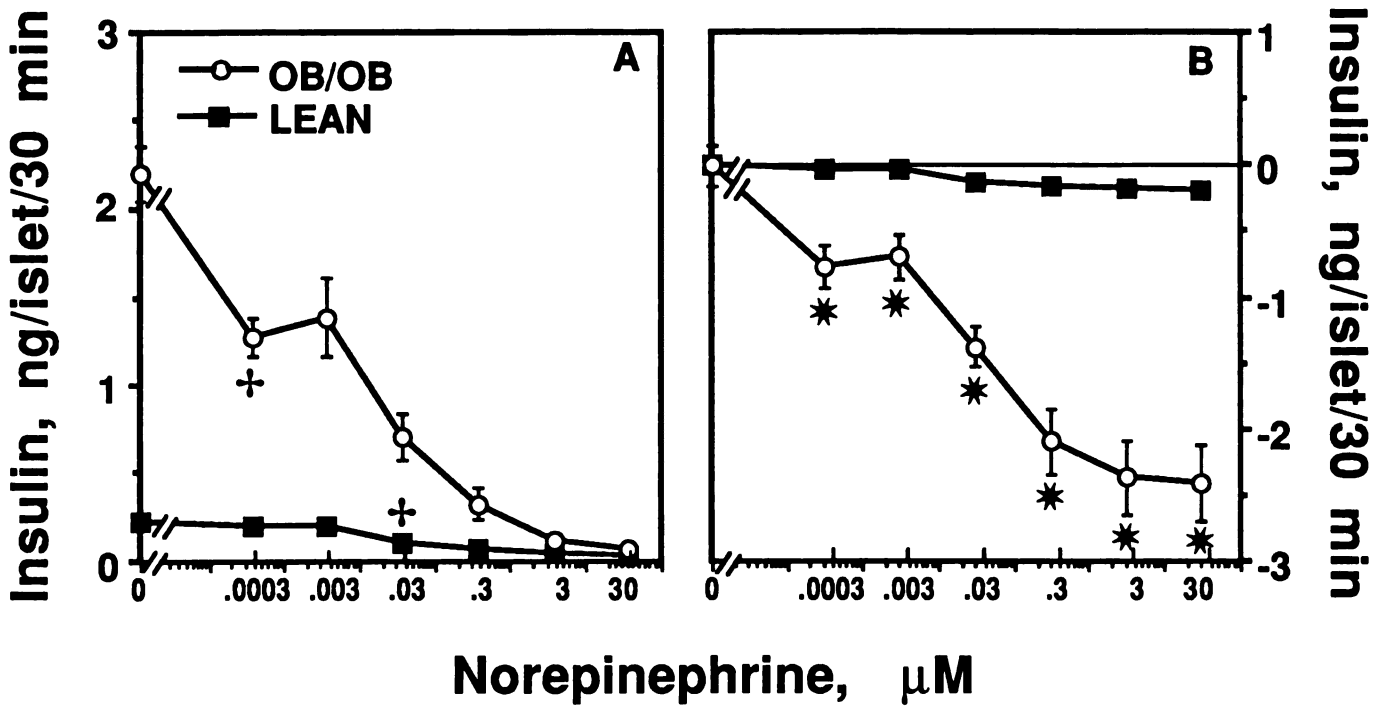

Figure 6. Insulin secretion from ob/ob and lean mouse islets in response to 15 mM glucose and norepinephrine expressed as absolute insulin secretion (A) or absolute decrease below baseline secretion (B). Baseline is defined as insulin secretion in 15 mM glucose, 0 mM norepinephrine (graph A). Data are mean ± SE (n = 5-6). Cross symbols indicate the lowest concentration of norepinephrine to significantly decrease insulin secretion below baseline secretion, and asterick symbols indicate significant effect of phenotype within treatment.

inhibited glucose-induced insulin secretion from both ob/ob and lean islets in a dose-dependent manner. Norepinephrine, (0.0003 μM), significantly inhibited glucose-induced insulin secretion from ob/ob islets while having no effect on lean islets. Insulin secretion from lean islets was not significantly inhibited until 0.03 μM norepinephrine was used (Figure 6,A). Norepinephrine, 0.0003 μM, resulted in a 0.8 ng/islet decrease in insulin secretion from ob/ob islets which is a 4 fold greater decrease in insulin secretion than observed when lean islets were exposed to the maximally effective dose of norepinephrine (30 μM). Additionally, despite large differences in baseline insulin secretion, both 3 and 30 μM norepinephrine completely inhibited insulin secretion from ob/ob islets to the same absolute level of secretion as lean islets (Figure 6,A).

Data from Figure 6,B are absolute decreases in insulin secretion below baseline secretion (15 mM glucose, 0 norepinephrine). When exposed to maximally effective doses (30 μM) of norepinephrine, insulin secretion from ob/ob islets was decreased by approximately 2.4 ng/islet; whereas, the same concentration of norepinephrine decreased secretion by only approximately 0.2 ng/islet for lean islets (Figure 6,B). All concentrations of norepinephrine resulted in greater absolute decreases in insulin secretion from ob/ob islets than from lean islets.

C. Experiment 3 - Effects of Neurotransmitter Antagonists on Insulin Secretion

Finally, in experiment 3, atropine (10μM) completely blocked the potentiating effect of 1 μM acetylcholine on 15 mM glucose-induced insulin secretion (Figure 7,A,C). Atropine itself had no effect on glucose-induced insulin secretion (Figure 7,A,C). The inhibitory effect of norepinephrine (3 μM) on 15 mM glucose-induced insulin secretion was completely blocked by 30 μM yohimbine (α_2 antagonist), while yohimbine itself had no significant effect on glucose-induced insulin secretion (Figure 7,B,D). Prazosin (30 μM; α_1 antagonist) had no effect on norepinephrine inhibition of glucose-induced insulin secretion from lean or ob/ob islets (Figure 7,B,D).

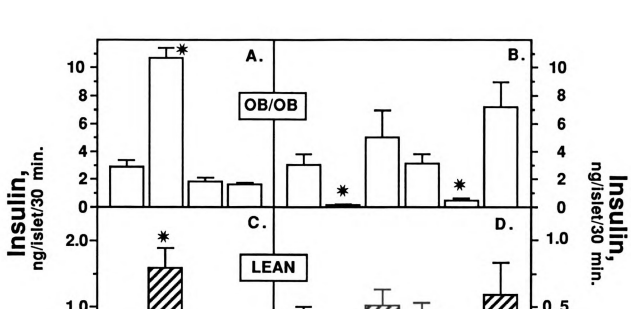

Figure 7. Insulin secretion from ob/ob (A,B) and lean (C,D) mouse islets in response to neurotransmitter antagonists. For A,C, islets were incubated for 30 mins in 15 mM glucose, 1 μM acetylcholine (ACH) and 30 μM atropine (ATR; cholinergic antagonist), and for B,D in 15 mM glucose, 3 μM norepinephrine (NE) and 30 μM yohimbine (YO; α_2 adrenergic antagonist) or 30 μM prazosin (PRA; α_1 adrenergic antagonist). Asterick symbols indicate significant difference from 15 mM glucose control group within phenotype.

IV. DISCUSSION

I conclude from these results that when compared to lean islets, ob/ob mouse islets are 1) both hyperresponsive and hypersensitive to glucose stimulus (Figure 4), 2) hyperresponsive to acetylcholine stimulus in the presence of 15 or 5 mM glucose (Figure 5), and 3) hyperresponsive and hypersensitive to norepinephrine inhibition in the presence of 15 mM glucose (Figure 6). These alterations in insulin secretion from ob/ob islets in response to neurotransmitters may contribute to the extremely high plasma insulin concentrations observed in 8-9 wk old ob/ob mice (Figure 1). Hyperinsulinemia as well as moderate hyperglycemia (Figure 1) observed in ob/ob mice in the present experiment, are consistent with previous reports (Coleman and Hummel, 1973; Dubuc, 1976c).

Even though islets from ob/ob mice were on average larger (Figure 2), insulin content of ob/ob mouse islets was similar to that of lean mouse islets (Figure 3). Total pancreatic insulin was higher in ob/ob compared to lean mice (Figure 3). In previous studies, both ob/ob islet insulin content and pancreatic insulin content have been reported as either higher or lower than that of lean littermates (Findley et al, 1973; Loten et al, 1974; Dunbar and Walsh, 1979; Kakita et al, 1982; Black et al, 1988). These discrepencies

in insulin content of islets or pancreas may be due to differences in sex, age, condition of mice (fed vs. fasted), technique used to isolate islets, technique used to determine total insulin content, or part of the pancreas studied (dorsal vs. ventral). For example, Black et al, 1988, using islets from only the splenic portion of the pancreas of 8-12 wk old male mice reported islet insulin contents of 20 ng per ob/ob islet and 32 ng per lean islet; contrary to reports by Dunbar, on islets from whole pancreas of 24-32 week old fasted mice of approximately 280 ng per ob/ob islet and 60 ng per lean islet.

Results in Figure 3 can be used to calculate approximate islet number per pancreas by dividing pancreatic insulin content by islet insulin content. Thus, on average, one ob/ob mouse pancreas contains approximately 200 islets compared to approximately 150 islets per lean mouse pancreas. This corresponds to 25% more islets per ob/ob mouse pancreas, which is consistent with histological studies by Gepts et al, 1960, who found 15% more islets in the pancreata of 16 wk old ob/ob mice compared to lean littermates.

Islets from ob/ob mice were larger than islets from lean littermates (Figure 2), consistent with previous work (Lavine et al, 1977; Dunbar and Walsh, 1980; Black et al, 1988). The etiology of islet hypertrophy in ob/ob mice is unknown and has not been well studied. However, in general, pancreatic islet hypertrophy is consistently observed when excessive demands are placed on insulin secretion, such as

the case of partial pancreatectomy in rats (Chen et al, 1989), or constant stimulation of islet insulin secretion with glucose, (King et al, 1978). Islet hypertrophy also occurs in rats as a result of VMH-lesion. In fact, islet hypertrophy in VMH-lesioned rats occurs after increased insulin responsiveness to glucose stimulus, and after increases in parasympathetic activity and decreases in sympathetic activity to VMH-lesioned rat islets (Campfield et al, 1986). This suggests excess stimulus for insulin secretion as a contributing factor to islet hypertrophy. Islet hypertrophy in ob/ob mice is also likely to be the result of excessive demands on insulin secretion.

Ob/ob mouse islets may be constantly stimulated to secrete insulin under both fasted (5 mM glucose) and fed (15 mM glucose) conditions, unlike lean islets, which are stimulated to secrete insulin only at 10 mM glucose or above (Figure 4). The 15 fold higher or 5.5 fold higher insulin secretion from ob/ob islets compared to lean islets at 10 and 15 mM glucose, respectively, cannot be fully explained by an average 4 fold greater ob/ob islet volume. I conclude from these results that increased insulin secretion from ob/ob islets is not due exclusively to increased islet size, nor is it due to increased islet insulin content, as discussed previously. Therefore, there must be some as yet unidentified factor that is responsible for altered glucose-induced insulin secretion from ob/ob mouse islets.

Increased sensitivity and responsiveness to glucose of

ob/ob islets may be an important contributing factor to hyperinsulinemia in adult ob/ob mice. These alterations could also play a role in the development of hyperinsulinemia in ob/ob mice, especially if hypersensitivity to glucose is present prior to weaning, when ob/ob mice are hypoglycemic, but plasma insulin is increased (Dubuc, 1981). Unfortunately studies on islets of younger ob/ob mice have not been conducted. However, results from studies with VMH-lesioned rats clearly demonstrate a role for islet hyperresponsiveness in initiating hyperinsulinemia. Islets of VMH-lesioned rats hypersecrete insulin in response to glucose as early as one day after lesioning, before any islet hypertrophy can occur (Campfield et al, 1986).

The hypersensitivity to glucose observed in ob/ob mice may contribute to hyperinsulinemia in other ways, perhaps by making the B cells of ob/ob mice more susceptable to parasympathetic stimulation than are B cells of lean mice. Since stimulatory concentrations of glucose appear to be needed before acetylcholine can potentiate insulin secretion (Hermans et al, 1987), lean islets may not respond to acetylcholine at 5 or 7 mM glucose; whereas, ob/ob islets may be responsive to acetylcholine stimulus at both 5 and 7 mM glucose. This idea will be discussed further with regard to experiments performed with 5 mM glucose and acetylcholine.

Even if ob/ob mouse pancreata contain 25% more islets, and those islets are up to 15 fold more responsive to glucose stimulus, this cannot completely explain the 95 fold higher

plasma insulin concentration in ob/ob compared to lean mice. Alterations in neural control of insulin secretion may be a second contributing factor.

The parasympathetic nervous system has tremendous potential to cause excess insulin secretion from pancreata of ob/ob mice (Figure 5). In fact, high concentrations of acetylcholine (1 μM) in the presence of 15 mM glucose potentiated insulin secretion by 7 ng per ob/ob islet compared to 0.9 ng per lean islet, corresponding to an 8 fold greater potentiation of insulin secretion from ob/ob islets (Figure 5,B). Furthermore, even under glucose conditions representative of the fasting state (5 mM glucose), acetylcholine is able to potentiate glucose-induced insulin secretion only from ob/ob islets (Figure 5,D); whereas, lean islets do not respond to acetylcholine at 5 mM glucose. The reason for this difference in response to acetylcholine may be that 5 mM glucose alone is able to stimulate insulin secretion from ob/ob islets but not from lean islets. The potentiating effect of 1 μM acetylcholine on ob/ob islets in the presence of 5 mM glucose (+0.34 ng/islet; Figure 5,D) is as great as the stimulatory effect of 15 mM glucose alone on lean mouse islets (+0.32 ng/islet; Figure 4). Again establishing that acetylcholine could be playing a major role in hyperinsulinemia of ob/ob mice.

Data from Figure 5 are also consistent with <u>in vivo</u> studies using acetylcholine antagonists in ob/ob mice. Ahren and Lundquist, 1982, found that cholinergic blockade with

atropine resulted in much greater decreases in plasma insulin in ob/ob mice compared to control NMRI mice. This suggests the possibility of increased parasympathetic nerve activity in ob/ob mice, or increased responsiveness to existing parasympathetic nerve activity.

In addition to alterations in responsiveness to acetylcholine, ob/ob islets may be slightly less sensitive to acetylcholine stimulus. Higher concentrations of acetylcholine were needed to achieve significant potentiation of glucose-induced insulin secretion from ob/ob islets compared to lean islets (Figure 5,A). However, contrary to these statistical results, the dose response curve of ob/ob islets does not appear to be shifted to the right compared to leans which suggests sensitivity is not altered. Additionally, in Figure 5,B, absolute increase in insulin secretion from ob/ob islets at 0.01 μM acetylcholine is actually 2 fold higher than that of lean islets. Therefore, I conclude that ob/ob and lean islets are probably equally sensitive to acetylcholine stimulus. As a result, I suggest that parasympathetic tone at the islets of ob/ob mice is probably not altered compared to islets of lean mice.

This is in contrast with what has been observed with other obese rodents. In both Zucker fa/fa rats and VMH-lesioned rats, research suggests that increased parasympathetic nerve activity to the pancreas contributes to hyperinsulinemia (Rohner-Jeanrenaud et al, 1983; Yoshimatsu et al, 1984).

Ob/ob mouse islets are also hyperresponsive to norepinephrine inhibition of insulin secretion (Figure 6). Norepinephrine (30 μM) had a much greater effect on ob/ob islets than on lean islets. Even though baseline secretion (15 mM glucose, 0 norepinephrine) was much different between ob/ob and lean islets, when incubated in 30 μM norepinephrine, secretion from ob/ob and lean islets was the same. This was a decrease of 2.4 ng per ob/ob islet, but only 0.2 ng per lean islet, corresponding to a 12 fold greater effect on ob/ob islets (Figure 6,B).

Ob/ob mouse islets are also more sensitive to norepinephrine inhibition, as seen by the leftward shift of the ob/ob islet norepinephrine dose response curve (Figure 6,B). Furthermore, ob/ob islet insulin secretion was inhibited by 0.0003 μM norepinephrine, a 100 fold lower concentration of norepinephrine than was required to produce inhibition in lean mouse islets, 0.03 μM. In support of these data, Kuhn et al, 1987, also found that ob/ob mice were hyperresponsive to catecholamines. Kuhn and coworkers injected epinephrine into ob/ob and lean mice and found a large decrease in plasma insulin of ob/ob mice with no effect on plasma insulin of lean mice.

Increased ob/ob islet sensitivity to norepinephrine is indicative of up-regulation of adrenergic receptors or hypersensitization of the second messenger system for adrenergic receptors. Such increased sensitivity could be in response to decreased <u>in vivo</u> sympathetic stimulus at the

ob/ob islet B cell. In ob/ob mice, if there is a decreased sympathetic inhibition of insulin secretion, hyperinsulinemia could result, at least prior to compensatory increases in islet sensitivity to catecholamines.

Alterations in islet response to sympathetic stimuli has been demonstrated with other obese rodents. Campfield and Smith, 1983, demonstrated that islets from VMH-lesioned rats were hypersensitive to norepinephrine. In this case, increased sensitivity to norepinephrine could be confirmed by studies in vivo as a compensatory mechanism for decreased in vivo sympathetic input to the islets. (Yoshimatsu, 1984; Smith and Campfield, 1986).

In contrast to my data, which suggests decreased sympathetic tone at islets of ob/ob mice, evaluation of data on norepinephrine turnover in pancreas of ob/ob mice suggests that whole pancreas sympathetic activity is the same in ob/ob and lean mice (Knehans and Romsos, 1983). However islet cells comprise only 1-2% of total pancreatic tissue. Therefore, sympathetic activity specifically at the ob/ob islet cells, could still be decreased. An alternative explanation could be that there is less epinephrine from the adrenal medulla reaching the islets of ob/ob mice compared to lean littermates, resulting in the observed hypersensitivity to norepinephrine stimulus. Evidence of decreased epinephrine in obese animals is provided by experiments on rats made obese by hypothalamic knife-cuts. Urinary epinephrine was measured and found to be lower in obese

knife-cut rats compared to lean rats (Vander Tuig et al, 1987). One could also argue that B cells of ob/ob mice have an intrinsic defect which results in alterations in sensitivity to norepinephrine. However, this is probably not the case since the ob/ob B cell is not the only site where increased sensitivity to catecholamines is observed. Epinephrine inhibits islet blood flow to a much greater extent in ob/ob islets than in lean islets (Rooth et al, 1985). Additionally, epinephrine results in a 4 fold increase in glucagon secretion from islets of ob/ob mice with no effect on glucagon secretion from islets of lean littermates (Beloff-Chain et al, 1977). Thus, alterations in sensitivity to norepinephrine are not isolated to an intrinsic defect in the ob/ob islet B cell. Another explanation could be that in ob/ob mice both α_1 and α_2 adrenergic receptors rather than just α_2 receptors may be functioning to enhance the signals produced by norepinephrine. Results in Figure 7 dispel this theory since only α_2 receptor antagonism blocked effects of norepinephrine in both lean and ob/ob islets.

Intrinsic islet defects have been suggested by Black et al, 1988, who demonstrated that ob/ob islets may have impaired function of voltage dependent Ca^{++} channels. Also, the electrical pattern produced in B cells of ob/ob mice upon glucose stimulus is quite different from that observed with lean mouse islets (Rosario et al, 1985). However, these authors did not establish whether these islet alterations are

primary or whether they may be secondary to the multiple metabolic abnormalities in ob/ob mice.

In summary, alterations in sensitivity to neurotransmitters in the present experiment suggest normal parasympathetic tone and decreased sympathetic tone or sympathoadrenal activity at the islets of ob/ob mice compared to islets of lean littermates. Additionally, these results suggest a role for both parasympathetic and sympathetic nervous systems in the hyperinsulinemia of ob/ob mice. The parasympathetic nervous system, via release of acetylcholine, can result in much greater increases in insulin secretion and at lower glucose concentrations from ob/ob than lean islets. The sympathetic nervous system, via decreased sympathetic or sympathoadrenal inhibition of insulin secretion, could contribute to hyperinsulinemia in ob/ob mice, at least prior to compensatory increases in islet sensitivity to catecholamines.

V. RECOMMENDATIONS FOR FURTHER STUDIES

To better understand the present results, and to continue searching for the cause of hyperinsulinemia and obesity in the ob/ob mouse, I propose the following studies.

A. Epinephrine in Ob/ob Mice

Plasma epinephrine turnover or urinary epinephrine should be measured in 8-9 wk old ob/ob and lean mice to determine if decreased plasma epinephrine in ob/ob mice may

be responsible for increased ob/ob islet sensitivity to catecholamines.

B. Interactive Effects of Neurotransmitters

The interaction profile of acetylcholine and norepinephrine on islet insulin secretion should be measured in ob/ob and lean islets to establish the relative importance of inhibitory and stimulatory input. Sympathetic dominance could indicate that increases in sensitivity and responsiveness to norepinephrine of the ob/ob islet B cell might be compensating for increased acetylcholine potentiated insulin secretion.

C. Pancreatic Insulin of Pre-Obese Ob/ob Mice

As a preliminary study to experiment D, pancreata of 2 wk old ob/ob mice should be extracted for total pancreatic insulin and compared with total pancreatic insulin of lean littermates. Differences in pancreatic insulin content would be indicative of altered islet insulin content or islet number early in development. This would suggest that insulin secretion from islets may also be altered by 2 wks of age in ob/ob mice.

D. Insulin Secretion from Pre-Obese Ob/ob Mouse Islets

Pancreatic islets should be isolated from pancreata of 2 wk old ob/ob and lean mice. Dose response of insulin to glucose should be performed for these islets to determine if

during a period of *in vivo* hypoglycemia, islets are hypersensitive or hyperresponsive to glucose stimulus. If islets are hypersensitive to glucose at this early age, we would suspect that altered islet insulin secretion in response to glucose is a primary factor initiating hyperinsulinemia and obesity in ob/ob mice. Selected concentrations of norepinephrine should also be used to determine islet sensitivity to norepinephrine. If alterations in sensitivity to norepinephrine is apparent at 2 wks of age without alterations in glucose sensitivity, we would suspect that alterations in glucose sensitivity could be a consequence of alterations in sympathetic input to the islets.

E. Islets of Adrenalectomized Ob/ob Mice

Since adrenalectomy normalizes plasma insulin in ob/ob mice, the effects of adrenalectomy on islet size and insulin secretion should be studied. Ob/ob mice should be adrenalectomized at 4 wks of age. At 8 wks of age, islets should be isolated and sensitivity to glucose and norepinephrine measured. If adrenalectomy normalizes islet size, sensitivity to glucose and sensitivity to norepinephrine, we would suspect that the increased glucocorticoid levels in ob/ob mice may be the cause of alterations in islet response to glucose and altered neural activity to the islets of ob/ob mice.

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