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DIRECT INFLUENCES OF GLUCOCORTICOIDS WITHIN THE CENTRAL NERVOUS SYSTEM ON REGULATION OF BROWN ADIPOSE TISSUE METABOLISM AND PLASMA INSULIN IN OB/OB MICE

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By

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ABSTRACT

DIRECT INFLUENCES OF GLUCOCORTICOIDS WITHIN THE CENTRAL NERVOUS SYSTEM ON REGULATION OF BROWN ADIPOSE TISSUE METABOLISM AND PLASMA INSULIN IN OB/OB MICE.

By

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Adrenalectomy stimulates the depressed brown adipose tissue metabolism and decreases hyperinsulinemia in ob/ob mice with minimal effects in lean mice. A single intracerebroventricular injection of 250 ng dexamethasone into adrenalectomized ob/ob mice completely reversed effects of adrenalectomy on BAT thermogenesis as assessed by mitochondrial GDP binding, approximately doubled plasma insulin, lowered whole body metabolic rates by 17%, and increased food intake by 19%. These responses were rapid in onset with changes in BAT metabolism and plasma insulin occurring within 30 minutes of dexamethasone injection. Adrenalectomized lean mice were much less responsive to dexamethasone than their ob/ob counterparts. The dexamethasone-induced decrease in BAT thermogenesis in adrenalectomized-ob/ob mice was associated with an organ-specific decrease in BAT sympathetic nerve activity as assessed by norepinephrine turnover, whereas the

dexamethasone-induced increase in plasma insulin was blocked by atropine, suggesting involvement of the parasympathetic nervous system. Intracerebroventricular injection of corticotropin-releasing factor did not affect BAT thermogenesis in dexamethasone-injected adrenalectomized ob/ob mice, but markedly lowered plasma insulin concentrations possibly by suppression of the parasympathetic nervous system. In conclusion, dexamethasone alters regulation of the autonomic nervous system in ob/ob mice.

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LIST OF ABBREVIATIONS

ACTH..... Adrenocorticotropic Hormone

ATP.....Adenosine 5'-triphosphate

BAT.....Brown Adipose Tissue

CNS.....Central Nervous System

CRF.....Corticotropin Releasing Factor

cAMP.....3',5'-cyclic Adenosine Monophosphate

DEX.....Dexamethasone

icv.....intracerebroventricular

GDP.....Guanosine 5'-diphosphate

ip.....intraperitoneal

MSG.....Monosodium Glutamate

NPY.....Neuropeptide Y

PVN.....Paraventricular Nucleus

mRNA.....messenger Ribonucleic Acid

VMH.....Ventromedial Hypothalamus

A. REVIEW OF LITERATURE

1. INTRODUCTION, DISSERTATION OBJECTIVES AND HYPOTHESIS.

Genetically obese (ob/ob) mice display a variety of abnormalities including hyperinsulinemia (2,29,101,102), low brown adipose tissue (BAT) thermogenesis (9) and high circulating concentrations of plasma corticosterone (156,181). These abnormalities remain even under conditions where food intake is restricted to the levels of leans (156,169). Adrenalectomy normalizes or reduces, depending on the age of animals, all these defects in ob/ob mice (94,138,156,169,185,203), and glucocorticoids replacement in adrenalectomized ob/ob mice reverses all the effects of adrenalectomy (66,180,181). Thus, the obesity in ob/ob mice depends on functional activity of the adrenal glands; the presence of glucocorticoids is necessary for full development of obesity. However, the sites and mechanisms of glucocorticoid action for development of obesity still remain unknown.

BAT and pancreas play important roles in development of obesity. BAT is an important thermogenic organ in rodents which can contribute up to 26% of total body heat

production under maximal stimulation (63), and its function is defective in obese rodents. Pancreatic hypersecretion of insulin has also been proposed as a primary factor in the etiology of obesity (101,102). Hyperinsulinemia causes increases in activation of most lipogenic pathways and these result in excess adiposity in ob/ob mice (11,29,69).

Several experimental reports suggest that there is no inherent defect in BAT thermogenesis of obese animals (86,89,188). Furthermore the direct effect of glucocorticoids on pancreatic insulin secretion is known to be inhibitory rather than stimulatory (23,104). These observations suggest that BAT and pancreas themselves do not contain the primary defect that causes obesity in ob/ob mice.

The central nervous system (CNS) receives many afferent signals from the periphery (neural-, substrate-, hormonally-mediated), some of which are related to the modulation of BAT thermogenesis or pancreatic insulin secretion. In addition, increases or decreases in sympathetic or parasympathetic activity cause changes in the functional activities of these two organs. Thus, restoration of high levels of BAT metabolism and low plasma insulin concentrations after adrenalectomy of ob/ob mice is possibly due to restoration of normal sympathetic and parasympathetic drive to the tissues.

Lesions placed in the CNS of normal animals produce abnormalities, including low BAT thermogenic activity and hyperinsulinemia, which closely resemble those of genetically obese rodents (151). Defective thermogenic capacity and increased substrate-induced insulin secretion in these animals is detectable almost immediately after ventromedial hypothalamus (VMH) lesions (20,163). Thus, a central defect or lesion could alter regulation of several organs such as BAT, pancreas and adrenal glands whose metabolism is governed by the CNS. This implies that the origin of defects in BAT and pancreas metabolism in ob/ob mice is located within the CNS and thus, the action site of glucocorticoid which causes development of obesity is also possibly located within the CNS where it controls BAT and pancreas of obese animals.

Glucocorticoid receptors are known to regulate gene expression in eukaryotes (153). Thus, it is possible that the action of glucocorticoids on BAT and pancreas metabolism may occur indirectly via influences on some other neuromodulator(s) which in turn exert actions within CNS. Corticotropin releasing factor (CRF) appears to be one of these neuromodulator candidates since glucocorticoids are known to feed back to control CRF release and production in the hypothalamus (39,45,47,56,168) and thus, the secretion and production of this neuropeptide are sensitive to removal of glucocorticoids.

Acute intracerebroventricular (icv) injection of CRF (1.2 ug) increased sympathetic nerve activity to interscapular BAT of rats in a dose-dependent manner, with a peak effect (approximately 56% increase from pre-injection basal level) 20-40 min after injection (55). Guanosine 5'-diphosphate (GDP) binding to BAT mitochondria of 21 h food deprived rats was also significantly increased (23%) 30 min after acute icv injection of CRF (5 ug) (7). Chronic infusion (icv, 4.8 ug/day for 7 days) of CRF also caused a significant increase in BAT mitochondrial GDP binding (7). Similarly, chronic treatment of genetically obese (fa/fa) rats with CRF (icv, 5 ug/day for 7 days) was also characterized by a decrease in basal hyperinsulinemia and an increase in BAT weight and activity (150). These results indicate that this peptide, which is controlled in part by glucocorticoids, is a possible neuromodulator of the sympathetic nervous system which control thermogenesis in BAT. However, there is no direct evidence which demonstrates that glucocorticoid action on BAT and pancreas metabolism occurs through the CNS, or (if it does) that its action on central nervous system is direct or via CRF.

Recently Debons et al showed that CNS administration of a single dose of glucocorticoids causes hyperphagia in gold thioglucose-treated adrenalectomized obese mice. The icv dose of cortisone required to induce hyperphagia was only 1/60th of that shown to be needed systemically to restore

hyperphagia (48). This suggests that the action of glucocorticoids on hyperphagia occurs through the CNS. It, took several days, however, to see the effect of glucocorticoids; dexamethasone, the most effective glucocorticoid, was not effective in increasing food intake until 4 days after the single injection and the maximal response occurred at 6 days.

Effects of glucocorticoids on food intake appear to occur slowly, but certain other actions of this hormone appear rapidly. BAT mitochondrial GDP binding was significantly increased and already reached its peak effect 60 minutes after injection of an antiglucocorticoid indicating that changes in BAT metabolism in response to glucocorticoids are rapid (82). These data suggest that the mechanism of action of glucocorticoids on metabolism of BAT and pancreas or other organs might be different than that on food intake.

The overall aim of my research was to examine action mechanisms of glucocorticoid on BAT thermogenesis and plasma insulin in ob/ob and lean mice. The objectives of my research were to 1) determine dose response and time course relationships for the effects of icv injection of dexamethasone on GDP binding to BAT mitochondria, and on plasma insulin and glucose concentrations. 2) examine the effects of central versus peripheral injection of dexamethasone on GDP binding to BAT mitochondria, and plasma

insulin and glucose concentrations. 3) examine effects of dexamethasone on whole animal oxygen consumption and food intake. 4) study the possible role of the sympathetic and parasympathetic nervous systems in mediating effects of dexamethasone on GDP binding to BAT mitochondria, and on plasma insulin and glucose concentrations. 5) determine the effects of CRF on GDP binding to BAT mitochondria, and on plasma insulin, glucose and free fatty acid concentrations. My hypothesis was that the action of glucocorticoids on plasma insulin and thermogenic activity of BAT take place through the CNS via alterations in the sympathetic nervous system and parasympathetic nervous system.

2. BAT THERMOGENESIS.

2.1 BAT THERMOGENESIS IN NORMAL ANIMALS.

BAT, which constitutes 1-2% of body weight of rats, is distributed in discrete depots in many locations in the body; the interscapular depot is a major depot in rodents (133). Brown adipocytes receive direct and exclusive innervation by sympathetic nerve fibers (15,61,133,170). These cells contain large numbers of mitochondria that have a unique pathway of energy dissipation to enable heat production (133).

The quantitative importance of BAT thermogenesis to whole body heat production was first shown by Foster and

Frydman; they demonstrated that the energy expended for thermogenesis in BAT can contribute substantially to total energy expenditure. According to their studies, BAT accounts for 60% or more of the rise in metabolic rate upon maximal stimulation by cold acclimation for 4 wks, and up to 26% of total body heat production under these conditions (63,64). Blood flow to BAT is also capable of a 24-fold increase and can account for up to 25% of total cardiac output under extreme cold stress (62,64). Thus, BAT could be an important thermogenic organ in energy balance and in the etiology of obesity.

In mitochondria of all cells, oxidation of substrates in the respiratory chain generates protons, which are ejected to the outside of the inner mitochondrial membrane. The electrochemical potential difference resulting from the asymmetric distribution of the protons is used for synthesis of ATP by driving a membrane-located ATP synthetase. Thus, electron transfer from substrates to oxygen leads to synthesis of stoichiometric amounts of ATP.

In brown fat cell mitochondria, there is also a specific proton-conductance channel across the inner mitochondrial membrane, which is regulated by an uncoupling protein of molecular weight 32000 which has been termed thermogenin. This protein is found exclusively in BAT mitochondria and binds purine nucleotides. The presence of this short-circuit for protons allows the proton gradient to be

dissipated without generation of ATP, and enables respiration to proceed at a high rate (38,136). This results in a form of heat production unique to brown fat cells.

BAT contains both beta-adrenergic receptors and alpha-adrenergic receptors. However, the nature of the specific beta-adrenergic receptors in BAT is still not clearly understood. On the basis of binding studies and of metabolic studies, most authors conclude that a mixture of beta 1 and beta 2-adrenergic receptors is present (120,161). Some studies, however, indicate that BAT may contain a third type (atypical kind) of beta-adrenergic receptor (8,80). In intact BAT, norepinephrine released from the nerves would interact with all types of adrenergic receptors. Synergism between beta- and alpha 1-adrenergic responses of BAT has been observed in vivo and in vitro. Acute thermogenic response of isolated BAT cells is in part, mediated by beta-adrenergic receptors (approximately 80%) and in part by alpha 1-adrenergic receptors (approximately 20%) (128).

Beta-adrenergic receptors are coupled to an adenylate cyclase system in the plasma membrane. Norepinephrine released from sympathetic nerve endings interacts with these receptors which activate adenyl cyclase and increase cAMP production. The principal effect of increased cAMP level is assumed to be activation of a protein kinase that

phosphorylates hormone sensitive lipase, resulting in accelerated lipolysis (35). Cold-induced increases in adenylate cyclase activity do not occur in denervated BAT (79), but do occur in BAT of animals chronically infused with norepinephrine (78). Thus the acute cold response of BAT is believed to be mediated by norepinephrine secreted by sympathetic nerves during acclimation to cold. Both beta- and alpha 1-adrenergic receptor responses are involved in this effect of chronic sympathetic stimulation (78).

Unlike the beta-adrenergic receptors, stimulation of alpha 1-adrenergic receptors present in BAT results in an increased operation of the phosphatidylinositol biphosphate (PIP_2) cycle. This increases production of two second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG) (165). Inositol triphosphate stimulates release of Ca^{2+} from intracellular stores, and this results in efflux of K^+ possibly via a calcium-activated cation channel (166). The other second messenger, diacylglycerol causes translocation of protein kinase C to the plasma membrane (14). A DAG-protein kinase C activation of Na^+/H^+ exchange results in Na^+ entry and further Ca^{2+} release (44). The altered levels of K^+ and Na^+ activate Na^+,K^+-ATPase, with an increase in hydrolysis of ATP. Approximately 20% of the thermogenic response of isolated BAT cells to norepinephrine is mediated by alpha 1-adrenergic receptors (98). This

portion of the response is associated with increased utilization of ATP for ion pumping needed to restore ion gradients dissipated by the opening of certain ion channels. Stimulation of alpha 1-adrenergic receptors by norepinephrine is also known to increase the synthesis of 5'-deiodinase (139). The intracellular mediator for this response is not known yet.

2.2 BAT THERMOGENESIS IN OBESE ANIMALS.

Many studies of various obese rodents have demonstrated that these animals usually have a decreased energy expenditure. Low rates of BAT thermogenesis appear to be present in most of these animals. Consequently, many studies have focused on regulation of BAT metabolism in obese animals.

Genetically obese (ob/ob) mice have a lower BAT sympathetic nervous system activity than lean mice (203). Norepinephrine turnover in BAT of these ob/ob mice is approximately 50% lower when they are housed at 20-26°C and fed stock diets than in lean mice (109-112,204). These lowered rates of norepinephrine turnover in BAT of ob/ob mice are observed before visual signs of gross obesity are evident, indicating that low sympathetic nervous system activity in BAT of ob/ob mice is not simply a secondary consequence of gross obesity (112).

Decreased sympathetic activity results in atrophy of BAT, low levels of GDP binding to BAT mitochondria (an indicator of thermogenic activity of the tissue), and a reduced capacity to respond to norepinephrine or to acute cold exposure by an increase in thermogenesis (89). Most of the atrophic changes in BAT of ob /ob mice are merely secondary to low sympathetic nervous system activity because they are reversed by procedures which increase sympathetic nerve activity such as acclimation to mild cold (89), feeding a diet that is palatable or high in unsaturated fatty acids (86), or by treatment with beta-adrenergic agonists (188).

Because there is no inherent defect in either cold-induced or diet-induced thermogenesis in BAT of the ob/ob mouse, these obese rodents are presumed to have CNS-mediated abnormalities in sympathetic outflow. The abnormally low sympathetic nerve activity in ob/ob mice is, however, not a consequence of a generalized depression of sympathetic nervous system activity because norepinephrine turnover in tissues other than BAT, e.g. pancreas, liver, and white adipose tissue, is not diminished (111,112,198). These data suggest that in ob/ob mice CNS-mediated and organ specific decreases in sympathetic outflow are present.

Sympathetic nervous system activity in BAT of genetically obese (fa/fa) rats is also depressed (201,202), as it is in ob/ob mice. Thermogenic activity of BAT,

assessed by mitochondrial GDP binding, is reduced in the Zucker obese (fa/fa) rat as early as 2 days of age as well (17), and this is thought to contribute to the high energy efficiency and development of obesity in these animals. Diet-induced thermogenesis also fails to occur in adult obese Zucker fa/fa rats (96,183). When fa/fa rats are exposed to cold, however, their BAT can be thermogenically activated (96,183). This supports the hypothesis that the primary defect is not in BAT of these animals, but more likely resides in CNS control of BAT metabolism.

Obese (fa/fa) rats, like ob/ob mice, are rather cold intolerant, possibly in association with a reduced response of their BAT thyroxine 5'-deiodinase to cold (193). 3,5,3'-triiodothyronine is required for the acute thermogenic response of BAT to beta-adrenergic stimulation (69,164,173). Initiation of BAT thermogenesis during cold exposure is defective in the absence of thyroid hormone (88). 3,5,3'-triiodothyronine influence on the acute thermogenic response is thought to be permissive since 3,5,3'-triiodothyronine requires the simultaneous action of norepinephrine to exert this effects (167). Norepinephrine can eventually bring about the thermogenic changes in the absence of an increase in thyroxine 5'deiodinase activity, as in cafeteria-fed rats (10,193). The density of alpha 1-adrenergic receptors, which serve to mediate this response of the deiodinase is low in the fa/fa rat (144).

Decreased thermogenic activity has also been observed in VMH-lesioned obesities. VMH-lesioned (electrolytic) rats are unable to activate diet-induced thermogenesis in BAT when they eat a palatable cafeteria diet (90). A similar lack of diet induced thermogenesis in BAT occurs in rats with chemically-induced lesions of the VMH, although they can activate BAT thermogenesis when they are exposed to cold (154). Similarly, several stress-induced sympathetic nerve mediated metabolic responses are blunted in VMH-lesioned animals (163). The defective thermogenic capacity in these animals is detectable very early after VMH-lesions, and spontaneous electrical activity of the efferent sympathetic nerve of BAT is reduced by 80% within 30 min after VMH lesions (137). Therefore, the underlying abnormalities of these particular CNS regulated sympathetic out flow responses occur early in the evolution of syndrome and are part of an overall impairment in the regulation of the autonomic nervous system.

2.3 EFFECTS OF GLUCOCORTICOID ON BAT THERMOGENESIS.

Adrenalectomy is known to prevent the further development of obesity in several animal models regardless of whether the origin of their obesity is genetic (156,169), dietary (152), or induced by lesions in the brain (33,151). The role of adrenal status in the control of energy balance is quite pronounced in these obese rodents. BAT which plays

an important role in the control of energy balance in these species is impaired in intact obese animals (see A.2.2), and adrenalectomy restores normal energetic efficiency (156,169) as well as normal BAT function (185). The relationships between BAT thermogenesis and the action of glucocorticoid in various obese animals have been under active investigation by several laboratories.

Adrenalectomy increases both thermogenesis and sympathetic nervous system activity in BAT of ob/ob mouse (101,102). Chronic or acute corticosterone replacement abolishes or reduces these effects as well as many other effects of adrenalectomy. Recent studies have established a dose response relationship for the effect of blood corticosterone on development of obesity in adrenalectomized ob/ob mice (181). Adrenalectomized ob/ob mice were treated with different doses of corticosterone implanted for 2 wk to allow the restoration of blood corticosterone, and the relationship between serum corticosterone concentrations and other parameters involved in development of obesity were determined. In adrenalectomized ob/ob mice, but not in lean mice, low physiological levels of serum corticosterone (up to 10 ug/dl) markedly decreased BAT mitochondrial GDP binding. Higher levels of corticosterone (12-22 ug/dl) suppressed BAT mitochondrial GDP binding in lean mice also, but to a lesser extent than in ob/ob mice. Therefore the suppression of BAT function in ob/ob mice is possibly

not only due to the high circulating level of glucocorticoids, but also due to an excessive sensitivity of the ob/ob mice to a suppressive action of glucocorticoids. Effects of adrenalectomy, however, may be primarily limited to animals fed high-carbohydrate diets because adrenalectomy is only partially effective when very high fat diets are provided (169).

BAT mitochondrial GDP binding, an index of thermogenic activity, of fa/fa rats is reduced from an early age in the preobese fa/fa rat (17). After weaning, the BAT progressively develops the appearance of an hypoactive tissue as mitochondrial content, GDP binding and uncoupling protein concentration are reduced, while triacylglycerol deposits increase (4,17,96). Adrenalectomy of fa/fa rats rapidly restores both the morphological appearance and the function of the tissue to normal. Chronic and acute diet-induced thermogenesis in BAT of adult fa/fa rat is also restored by adrenalectomy (201).

Since adrenalectomy is a somewhat stressful and nonselective procedure, a recent study utilized a potent selective glucocorticoid receptor antagonist, RU-486 (82). Acute injection (sc) of RU-486 (5-10 mg/kg) stimulated oxygen consumption and BAT activity, assessed by mitochondrial GDP binding, in the rat with a peak effect at 60 min after injection. Chronic administration (5 mg/kg, sc, twice daily for 8 days) of antiglucocorticoid also

caused an increase in BAT activity, and this effect was maintained for at least 18 hr after the last treatment. The action of RU-486 on oxygen consumption and BAT metabolism appears to be exerted centrally since a significant thermogenic response to RU-486 was elicited with a very much smaller (1/50th) dose when this compound was injected into the third ventricle. Furthermore the thermogenic response produced by RU-486 was markedly inhibited by beta-adrenergic antagonist propranolol. The effect of RU-486 on BAT was also abolished by surgical denervation of the sympathetic supply to the tissue. These results together suggest that the effect of glucocorticoid is mediated by the sympathetic nervous system (82).

Effects of adrenalectomy in fa/fa rats appear to be due to the deprivation of glucocorticoid, not due to the deprivation of mineralocorticoid which is also released from the adrenal gland. Replacement of glucocorticoid, but not aldosterone, in adrenalectomized fa/fa rats reverses all these effects of adrenalectomy and restores the development of obesity (65,66,96).

Adrenalectomy in VMH-obese animals eliminates all excess weight gain and decreases food intake to below the level of control (both adrenalectomized-sham VMH and sham adrenalectomized-sham VMH) groups (33). In a similar way, VMH lesions in adrenalectomized animals also did not produce the characteristic weight gain associated with VMH damage

(33). Replacement of corticosterone abolished all these effects of adrenalectomy. Adrenalectomy increases sympathetic nervous system activity in rats with electrolytic lesions in the ventromedial hypothalamus (177), as well as in rats with hypothalamic knife cuts (151). GDP binding to BAT mitochondria, an indicator of the thermogenic capacity of the tissue, is also known to increase after adrenalectomy in hypothalamic-lesioned rats (151). Although effects of glucocorticoid replacement on BAT function in adrenalectomized hypothalamic-lesioned rodents have not been reported, it is very likely that glucocorticoid suppress BAT metabolism in these animals as has been observed in adrenalectomized fa/fa rats and ob/ob mice.

Recently, effects of adrenalectomy on obesity were also studied in glutamate-induced obese animals. Mice treated with monosodium glutamate (MSG) in the neonatal period develop into obese adults without over-eating (131). The principal energy conserving feature of the MSG-mouse is thermoregulation at a lower than normal body temperature during the nocturnal and early diurnal period (178). Adrenalectomy prevents the obesity of this animal model without altering either food intake or thermogenic activity of BAT (179).

Overall, BAT is an important contributor to obesity, and metabolism of BAT depends on adrenal status. Adrenalectomy increases BAT thermogenesis, and glucocorticoid replacement

reverses these effects of adrenalectomy. Obese animals appear to be more sensitive to glucocorticoid. The mechanism of glucocorticoid action is still unclear, but it probably involves the CNS.

3. PANCREATIC INSULIN SECRETION.

3.1 PANCREATIC INSULIN SECRETION IN NORMAL ANIMALS.

The endocrine pancreas is composed of the islets of Langerhans which contain four major different cell types, A,B,D, and F cells. B cells, which make up the large central mass of the islet, are the most predominant cell type in the islets, and are the cells that synthesize and secrete insulin. The pancreas receives direct parasympathetic and sympathetic innervation (19). Parasympathetic stimulation via the vagus nerve increases, whereas sympathetic stimulation via the splanchnic nerve inhibits, insulin release (19).

Electrical stimulation of the dorsal motor nucleus of the vagus nerve and of the nerve itself stimulates insulin secretion from the beta-cells in the pancreatic islets in several species of animals (18,67,92). Parasympathetic agents, muscarinic agonists, also stimulate insulin secretion in mice (123), in dogs (105), and in humans (103). Similarly stimulation of the lateral hypothalamus, which accelerates parasympathetic drive to the vagus nerve,

and inhibits sympathetic drive to the splanchnic nerve, enhances insulin secretion (28). Electrical stimulation of the splanchnic nerve or VMH, on the other hand, causes suppression of insulin secretion (24,60,147).

Parasympathetic nerves innervating pancreatic islets release acetylcholine (18,127). Both in vivo and in vitro studies show that acetylcholine stimulates insulin and glucagon secretion, the same effect observed after electrical stimulation of the vagus (36,84,106,175). Insulin and glucagon release elicited by cholinergic or vagal stimulation occurs via activation of muscarinic receptors since these effects are blocked by the muscarinic antagonist, atropine (25,106).

Norepinephrine, the sympathetic neurotransmitter, and epinephrine released concomitantly with norepinephrine by the adrenal medulla influence insulin release via direct stimulation of the alpha 2-, and beta-receptors (145). Although both norepinephrine and epinephrine can potentially act at alpha 2- or beta-receptors, the overall inhibitory effect of catecholamines on insulin secretion appears to be exercised via the interaction with alpha 2-receptors. Effects of epinephrine or norepinephrine on insulin secretion from mouse islets can be blocked specifically by an alpha 2-antagonist, yohimbine (132). Agonists that affect primarily the beta-adrenergic receptors actually

stimulate release of insulin (190). Alpha 2 effects of catecholamines dominate under ordinary circumstances (190).

3.2 HYPERINSULINEMIA IN OBESE ANIMALS.

One of the main defects in genetically obese rodents with a recessive single gene mutation is hyperinsulinemia. Hyperinsulinemia in these genetically obese rodents has been proposed as a key factor in the etiology of obesity (101, 122). This defect in ob/ob mice is apparent as early as 6 days of age (53), which is before other abnormalities such as increased adiposity (26), increased plasma corticosterone (52), hyperphagia (121) and peripheral insulin resistance (16) are visualized. This early hyperinsulinemia contributes to the excess adiposity in ob/ob mice through its action to increase lipogenic enzyme activity ,decrease lipolysis, and promote adipocyte hypertrophy and adipose tissue cell proliferation (29,69). The accelerated lipogenesis in liver and adipose tissue of ob/ob mice is normalized after treatment of these mice with streptozotocin to destroy pancreatic beta-cells, or with anti-insulin serum to decrease plasma insulin concentration (122). The hypothesis that insulin is a primary cause of obesity is also supported by results of several manipulations including restricted feeding (51), administration of beta-adrenergic

agonist (188) and adrenalectomy which lower plasma insulin in ob/ob mice and also decrease adiposity.

Although both the sympathetic and parasympathetic nervous system play important roles in regulation of pancreatic insulin secretion, the occurrence of hyperinsulinemia in obese animals has been thought to be due to abnormal CNS regulation of the parasympathetic nervous system. Ahren et al studied the autonomic nervous system regulation of basal insulin secretion by the means of receptor blockade (2). The modulation of basal insulin secretion was investigated by recording plasma insulin levels for 2 h after administration of adrenergic or cholinergic blockade in ob/ob mice. Beta-adrenoceptor blockade by L-propranolol transiently inhibited basal insulin secretion in both ob/ob and lean mice by a maximum about 40%. Further, in both ob/ob and lean mice, alpha 2-adrenoceptor blockade by phentolamine induced a sustained elevation of plasma insulin levels to about 200% over saline-injected control values. Thus no impressive abnormality in the adrenergic regulation of insulin secretion in this obese mouse was detected.

Cholinergic blockade by methylatropine, however, induced a pronounced and sustained reduction (about 60%) of plasma insulin concentration in ob/ob mice without influencing insulin secretion in lean mice (2). These results suggest that the basal hypersecretion of insulin in ob/ob mice is

mainly governed by enhanced responsiveness to normal and/or increased vagal activity but not adrenergic activity. A similar study was conducted in ob/ob mice using a muscarinic agonist as well as antagonist. Bethanechol chloride, a muscarinic agonist (0.5 ug/g), produced a profound hyperglycemia accompanied by an equally remarkable hyperinsulinemia within 10 min of injection (68). Atropine significantly attenuated the baseline hyperinsulinemia seen in these ob/ob mice. Lean mice also showed an increase in plasma insulin in response to bethanechol chloride, but this was associated with a fall in blood glucose, and they displayed no response to atropine (68). These findings suggest that ob/ob mice have an exaggerated muscarinic receptor sensitivity and/or a tonic elevation in vagal activity that contributes to the persistent hyperinsulinemia seen in these animals. Obese mice are thus much more sensitive to cholinergic blockade, and have an enhanced cholinergic tone and/or an increased cholinergic responsiveness in promoting insulin secretion. Similarly, increased plasma insulin in genetically pre-obese rat pups was also completely abolished by acute cholinergic blockade (148,149), again indicating participation of vagus nerve in regulation of insulin secretion in obese animals.

Hyperinsulinemia is also found in VMH-lesioned obese rodents. The increased fat accretion that follows VMH-lesions has been ascribed to the presence of

hyperinsulinemia (12,27). Acute lesion of the VMH area markedly increase substrate-induced insulin secretion in normal anesthetized rats and leads to stimulation of most lipogenic pathways (20,146). These changes take place within minutes after the lesions, and are rapidly abolished by superimposed vagotomy. Again, this effect of vagotomy to decrease plasma insulin concentration was not observed in normal animals, suggesting an abnormally increased parasympathetic nerve activity in VMH-induced obese animals. Thus, hyperinsulinemia in obese rodents (regardless of whether they are genetic or CNS-induced) may result from the same origin, primary CNS abnormalities.

3.3 EFFECTS OF GLUCOCORTICOID ON PANCREATIC INSULIN SECRETION.

Several studies demonstrate that the vagus nerve mediates neural regulation of insulin secretion. Hyperinsulinemia in genetically obese animals is thus possibly due to an overactive parasympathetic nerve activity or/and due to hyperresponsiveness to a normal parasympathetic nerve activity in these animals (see A.3.2). One of the possible candidates responsible for the expression of this defect (overactive parasympathetic nerve or hyperresponsiveness to parasympathetic nerve activity) has been thought to be glucocorticoids. It is well known that adrenalectomy alleviates the hyperinsulinemia as well

as other defects of genetic and surgically- or chemically-induced obesity in rats and mice. These effects of adrenalectomy can be reversed by chronic or acute replacement of corticosterone.

Adrenalectomized ob/ob mice receiving corticosterone implants for 2 wk returned certain of their abnormalities (including hyperinsulinemia) in virtually full force when the level of corticosterone in their blood was in a physiological range (<10 ug/dl) (181). Lean mice required much higher concentrations of plasma corticosterone to produce a similar level of hyperinsulinemia. Acute responses to corticosterone in genetically ob/ob mice were also studied to determine whether chronic presence of corticosterone was necessary, or whether a single injection would also have the same effects (180). A single injection of corticosterone (1 mg/10 g body wt sc) markedly accelerated hyperinsulinemia in ob/ob mice 15 hr after treatment, with less effect in lean mice. These results suggest that plasma insulin concentrations in ob/ob mice have an excessive sensitivity and responsiveness to corticosterone.

Genetically obese fa/fa rats are also more sensitive /responsive to corticosterone than lean rats. Corticosterone 21-acetate (2.0 mg) given by gastric intubation induced a relative hyperinsulinemia in adrenalectomized obese Zucker rat 24 hr after treatment

(58). The same dose of corticosterone 21-acetate did not increase plasma insulin concentrations of adrenalectomized lean animals. Hyperinsulinemia of the intact or glucocorticoid-treated adrenalectomized obese rat was not accompanied by differences in plasma glucose concentration compared with lean animals. These results indicate the differential effects of glucocorticoid replacement on insulin secretion by adrenalectomized lean and obese rats.

Recently, studies have been done to demonstrate the possible role for the parasympathetic nervous system in mediating effects of glucocorticoids on insulin secretion. A single injection of atropine (0.3 mg), a cholinergic blocker, completely abolished the effects of corticosterone replacement on plasma insulin of adrenalectomized obese rats with its effect lasting 45 min after injection. There were no significant changes in plasma glucose concentrations during this treatment (59). Atropine also prevented the greater increment in plasma insulin concentration of corticosterone-treated adrenalectomized obese rats compared with similarly-treated lean animals after a glucose load (59). Since atropine antagonizes the muscarinic actions of the parasympathetic nervous system on insulin secretion (192), these results suggest that the differential effects of glucocorticoid replacement on insulin secretion by adrenalectomized lean and obese rats are due, at least in part, to increased stimulation of the pancreas of obese

animals by the parasympathetic nervous system. Similarly, atropine administration to intact obese rats also reduced their plasma insulin concentrations although they did not restore their insulinemia to the levels found in intact lean animals (59). These findings provide some evidence that the parasympathetic nervous system contributes to the hyperinsulinemia of intact obese rats.

Overall, it is likely that deprivation of glucocorticoids alleviates the hyperinsulinemia of genetic obesity by preventing expression of the defect, which results in an overactive parasympathetic nervous system. It is also likely that genetically obese rodents are more sensitive and/or hyperresponsive to glucocorticoid mediated parasympathetic nerve activity.

4. POSSIBLE ACTION MECHANISM OF GLUCOCORTICOIDS IN DEVELOPMENT OF OBESITY.

Adrenalectomy increases sympathetic nerve activity, and thermogenic activity, assessed by mitochondrial GDP binding, in BAT of genetically obese rodents (101,102,185). Corticosterone replacement after adrenalectomy in these animals causes restoration of low sympathetic activity as well as low thermogenic activity in this tissue (see A.2.3). Adrenalectomy also decrease high plasma insulin concentrations in obese animals, and glucocorticoid

replacement abolishes these effects of adrenalectomy (see A.3.3). Neither the site nor the mechanism of these actions of glucocorticoid on BAT and pancreas metabolism are known.

4.1 POSSIBLE ACTION SITE OF GLUCOCORTICOID

Although BAT does possess glucocorticoid receptors it is not likely BAT per se is the primary action site of glucocorticoid. There is no inherent defect in either cold-induced or diet-induced thermogenesis in BAT of obese animals (see A.2.2). It is also known that glucocorticoids do not influence beta-adrenergic receptors in BAT, as they do in other tissues (162). Inhibition of BAT thermogenic activity by glucocorticoids thus likely is due to CNS-mediated abnormalities in sympathetic outflow to BAT.

Effects of glucocorticoid on pancreatic insulin secretion also can not be explained by direct effects of the hormone on the beta-cell, since such effects are inhibitory (23,104). Hyperinsulinemia induced by glucocorticoids in obese rodents is completely abolished by treatment with the cholinergic blocker, atropine. Lesions placed in the CNS of normal animals produce hyperinsulinemia which closely resembles that of genetically obese rodents. Thus, a central defect or lesion could alter regulation of many organs including the pancreas.

4.2 POSSIBLE ACTION MECHANISMS OF GLUCOCORTICOIDS AS A NEUROMODULATOR.

Although the precise molecular mechanisms by which glucocorticoid modulate autonomic nervous systems remain unclear at this point, one can consider two different possible action mechanisms involved in this process. First, direct actions of glucocorticoid to modulate sympathetic and parasympathetic activity, and second indirect actions of glucocorticoid via influences on other neuromodulators which in turn exert actions within CNS to regulate autonomic nervous system activity. This is possible because glucocorticoid receptors control gene expression in eukaryotes (153).

4.3 POSSIBLE DIRECT ACTIONS OF GLUCOCORTICOID ON CNS.

It is well known that the stress-induced activation of the pituitary-adrenocortical axis, which in rat results in enhanced circulating levels of corticosterone, occurs rapidly after stressful stimuli are encountered (73,83). Centrally within the limbic system, the septo-hippocampal cholinergic pathway with its cell bodies in the medial septum and axon terminals in the hippocampus, is also rapidly activated in response to stress (57,72). Thus, the possible link between this rapid rise in glucocorticoid and neural activation after stress has been examined.

Parenteral administration of exogenous corticosterone in high doses which lead to plasma levels similar to those induced by stress, activates the hippocampal cholinergic terminals as rapidly as stress does (70). Thus, glucocorticoid could be directly involved in the activation of hippocampal presynaptic cholinergic terminals. This result is supported by the observations that in the periphery dexamethasone enhances presynaptic choline uptake in stimulated nerve-muscle preparations in vitro (186) and centrally, direct application of glucocorticoids can rapidly alter the spontaneous electrophysiological activity of hippocampal neurones (46,141). Further evidence comes from a study by Gilad et al with synaptosomal preparations from rat hippocampus (71). They demonstrated that high glucocorticoid (methylprednisolone as well as dexamethasone) concentrations, but not adrenocorticotropic hormone (ACTH), can directly enhance acetylcholine release and can reduce dopamine uptake by dopaminergic synaptosomes. These results imply that increased glucocorticoid levels during stress or disease can directly modulate neuronal activity of specific cholinergic and dopaminergic systems in brain.

The molecular basis of direct action of glucocorticoid on the presynaptic neurotransmitter regulatory mechanisms is not yet known. However, it is unlikely that these actions of glucocorticoid are exerted via modulation of gene expression because glucocorticoid are able to exert action

as a neuromodulator in synaptosomal preparations which lack nuclei. Some studies suggest that glucocorticoid interact with specific binding sites on plasma membranes of brain synaptosomes (3,182). The membrane response of such binding is not clearly understood. It has been demonstrated however that cortisol binding is directly related to increased Na^+-K^+ stimulated ATPase activity, and that this effect is related to the increase in membrane fluidity caused by cortisol (49). A direct action of glucocorticoid on presynaptic terminals is also supported by the observations that glucocorticoids can stimulate uptake of tryptophan, the amino acid precursor for serotonin biosynthesis, by brain synaptosomes (134). Moreover, cortisol was found to inhibit acetylcholine-induced release of CRF from hypothalamic synaptosomes (54). Therefore, it is possible that glucocorticoid exerts some actions directly through interactions with specific binding sites on the plasma membrane of brain synaptosomes. This will possibly cause changes in membrane fluidity which may lead to modulation of neuronal activity of specific cholinergic and dopaminergic systems in the brain.

4.4 POSSIBLE INDIRECT ACTIONS OF GLUCOCORTICOID ON CNS.

It is known that altered hypothalamic pituitary adrenal axis is one of the CNS-mediated abnormalities in obesity syndromes (29,157). Such defects could originate in sites

involved in corticosteroid production and eventually result in the observed adrenal hypertrophy and increased plasma glucocorticoids levels in the obese rodents (29). It has long been appreciated that corticosteroids exhibit negative feedback effects on pituitary ACTH (76,197) and hypothalamic CRF production (39,45,47,56,168). Hypothalamic implantation of corticosteroids has been shown to inhibit secretion of ACTH (45,47,56,168) and decrease hypothalamic content of bioassayable CRF (39). Therefore, it is possible to think that glucocorticoid may exert central effects through one of these peptides. However any potential role for ACTH can be eliminated for a couple of reasons. First, hypophysectomy has a similar effect to adrenalectomy in preventing obesity (143), and second, corticosterone replacement to hypophysectomized obese rats restores the defects in BAT function (93). Thus, the possibility that CRF contributes to CNS regulation of obesity has received considerable attention.

Adrenalectomy enhances the staining for CRF-41 in parvicellular subdivision of the paraventricular nucleus (PVN) (126,159). Signs of increased neuronal activity caused by adrenalectomy are also detected in the terminal region of the CRF cells in the median eminence (34,205). Recently Kovacs et al reported that increased immunostaining of CRF 41 after adrenalectomy was blocked by unilateral implantation of dexamethasone in the vicinity of the PVN

(115). Further evidence that glucocorticoids are capable of modifying brain CRF dynamics is provided by the observation that systemic administration of dexamethasone, a potent synthetic steroid, resulted in a decrease in hypothalamic CRF by bioassay (129,187). All these observations suggest that glucocorticoids can modify CRF release and concentrations in the brain.

The action site of glucocorticoid to modulate CRF release and production is not clearly known yet. However, since paraventricular nucleus of the hypothalamus contains the highest content of immunoreactive CRF in the brain (5,184) and is known to have a high concentration of nuclear glucocorticoid receptor (1) it is likely to be the site of control of CRF release. Local implants of dexamethasone prevented adrenalectomy-induced enhancement of CRF immunoreactivity in parvicellular neurosecretory neurons of the paraventricular nucleus of the hypothalamus (158). Furthermore, the increase in release of CRF-41 in the median eminence due to adrenalectomy was abolished when adrenalectomy was combined with the surgical lesions of (PVN) suggesting that adrenalectomy-induced CRF-41 release occurs from parvicellular axons of PVN neurons (91).

CRF appears to be involved in regulation of the autonomic nervous system. CRF administered into the lateral ventricles in rats produced immediate and sustained elevations in plasma concentrations of epinephrine and

norepinephrine (30,100,116,140). It is also known that the CRF receptor antagonist alpha-helical CRF 9-41 suppresses adrenal epinephrine secretion following ether-stress and insulin-induced hypoglycemia (30-32). CRF given intracerebroventricularly suppresses parasympathetic activity (174). These findings resulted in the hypothesis that endogenous brain CRF may play a physiologic role in regulation of the autonomic nervous system. This hypothesis is confirmed by the observation that ganglionic blockade by chlorisondamine completely abolished CRF-induced elevations in plasma NE concentrations (100). This effect of CRF on sympathetic nerve activity seems to be dissociated from the activation of the pituitary-adrenal axis by CRF. CRF-induced suppression of natural killer cell activity which is correlated with an increase in circulating norepinephrine, was not affected by ACTH (100). Furthermore, ganglionic blockade did not suppress CRF-stimulated secretion of ACTH, although it did suppress CRF-induced plasma norepinephrine concentrations (100). Further evidence to support this is the effect of hypophysectomy on CRF-induced increase in sympathetic nerve activity. ICV injection of CRF (6.4 nMol) resulted in a dose dependent increase in adrenal sympathetic nerve activity within several min after injection, and this response of nerve activity to CRF was not influenced by hypophysectomy (116). Furthermore this increase in ongoing activity of the adrenal nerve was not produced by

intravenous administration of the same dose (6.4 nMol) of CRF indicating that the action site of CRF in regulation of autonomic nervous system activity is within the brain, and is dissociated from its action on the pituitary (116).

CRF receptors are found not only in pituitary, but also in several brain regions (50,196). Since CRF action on the autonomic nervous system within the brain must be expressed through specific binding sites in the central nervous system, studies have recently been done to characterize receptors for CRF in the brain. It is found that specific and high affinity binding sites for CRF are present in discrete areas of the rat brain (cerebral cortex and limbic system), with affinity constant in the nanomolar range, similar to that reported in pituitary CRF receptor (194,195). Such binding sites are coupled to adenylate cyclase, and are located in areas involved in the control of hypothalamic and autonomic nervous system function consistent with CRF-mediated central responses to stress.

Interestingly, CRF receptors in the brain are regulated differently after adrenalectomy from those in anterior pituitary gland. CRF receptors in the anterior pituitary showed a marked reduction in concentration after adrenalectomy, whereas, CRF receptor concentration in the brain and intermediate pituitary lobe was unaffected by adrenalectomy (196). This suggests that the regulatory mechanisms in the intermediate lobe and brain differ from

those in the anterior pituitary. Although immunoreactive CRF is present in the paraventricular areas and paraventricular nuclei of the hypothalamus in high density (5,184), no CRF receptors have been found in these areas (196). Thus, CRF actions within the brain, if there is any, likely do not occur within PVN. It is also possible that adrenal feed back on CRF production is adaptable to only hypothalamic CRF production (that act on pituitary), but not to extrahypothalamic CRF production. That may be the reason why adrenalectomy (which would be expected to increase CRF) did not affect the number of CRF receptors in the brain, although it did in the pituitary. This possibility is supported by a recent study on the regulation of CRF mRNA in different brain sites, where only the PVN mRNA for CRF responds to alterations of peripheral glucocorticoid status. Northern gel analysis showed that the mRNA for CRF is present in the bed nucleus of the stria terminals, the central nucleus of the amygdala and the supraoptic nucleus as well as the PVN of hypothalamus, and that all are the same size (21). In response to adrenalectomy, the level of hybridizable mRNA increased 2.75 fold over 7 days in the PVN, but no changes in concentrations of mRNA for CRF were found in other brain regions (21). Glucocorticoid replacement by dexamethasone injection (360 ug/100 g, ip) or by corticosterone implant (sc) also decreased the CRF mRNA in the PVN, but not in other regions. These results imply

that only CRF from the PVN is involved in feed back regulation of glucocorticoid. However, this conclusion is tentative since only limited areas of the brain were examined.

Recently Kovacs et al carried out similar studies to investigate the involvement of different brain sites in the mediation of glucocorticoid feed back action. Dexamethasone implants placed into arcuate nucleus or lateral septum as well as PVN caused a significant reduction of adrenalectomy-induced hypersecretion of ACTH. Corticosterone implants in the dorsal hippocampus also reduced adrenalectomy-induced hypersecretion of ACTH (114). This study indicates that CRF from areas other than PVN are also involved in feed back regulation by glucocorticoid to control ACTH secretion. Thus, the assumption that only CRF from PVN is involved in feed back regulation of glucocorticoid is not likely true. The other possibility is that the interaction of CRF with its brain receptor may not cause receptor down regulation, as it does in the anterior pituitary.

The PVN has received considerable attention as a possible site for CRF-mediated actions on the autonomic nervous system. Liebowitz et al have demonstrated the presence of a glucocorticoid-dependent alpha 1-adrenergic system for noradrenergic feeding in the paraventricular nucleus (PVN) of rats (118). The PVN is also the site of CRF-containing neurons which control ACTH secretion from the

pituitary (160). However, it is unlikely that the PVN is the major site for CRF, or CRF-mediated glucocorticoid action on central nervous system, for several reasons. First, as mentioned above CRF receptors were not found in PVN (196). Second, it has not been possible to demonstrate any functional connection between the PVN and sympathetic nerves innervating BAT (97). Third, the obesity observed after lesions of the PVN is also prevented by adrenalectomy (177). Likewise, the ventromedial hypothalamus (VMH) is also unlikely to be the site of action of glucocorticoid or CRF in controlling autonomic nervous system modulation of metabolism in BAT and pancreas since adrenalectomy prevents the obesity which occurs from damage to this region (33). Recently, York suggested that the lateral hypothalamus may be the site of glucocorticoid action in regulating diet-related sympathetic activity (200). However, further studies are needed to test this hypothesis.

Although many studies suggest that impaired CRF activity by high circulating glucocorticoid is a key factor in development of obesity, CRF alone appears to be unable to totally mimic effects of adrenalectomy. Chronic icv infusion of CRF into obese rats prevented further weight gain and reduced plasma insulin levels (150). However, although CRF given icv increased the firing rate of sympathetic nerves innervating the interscapular BAT pad in a dose-dependent manner in both lean and obese (fa/fa) rats,

the maximal response to CRF still remained substantially reduced in the obese rat (95). This suggests that CRF action alone can not totally explain effects of adrenalectomy, and that other mechanisms are probably involved in the action of glucocorticoid in mediating autonomic nerve system activity. It is possible that some other neuropeptide whose production and secretion is sensitive to glucocorticoid removal is involved.

4.5 SUMMARY.

From the summary of all these data one can conclude two possible mechanisms for the action of glucocorticoid. First, glucocorticoid may exert actions directly on the CNS. In this case, it is unlikely that a hormone-receptor complex exerts these effects through an interaction with nuclear DNA. In this case, it is more likely that the hormone interacts with specific binding sites on plasma membrane of brain synaptosomes, and thus possibly causes changes in membrane fluidity which may lead the modulation of neuronal activity of specific cholinergic and dopaminergic systems in the brain. The second general mechanism of glucocorticoid action is via hormone-receptor complex interactions with nuclear DNA. Glucocorticoid may exert its action on CNS, for example, via suppression of CRF production. If this is true, the action is unlikely to occur in PVN or VMH. In this case, glucocorticoid action is

likely to occur through interaction with its intracellular receptors to regulate the expression of CRF gene.

B. MATERIALS AND METHODS

1. ANIMALS AND DIET.

Male obese (ob/ob) and lean mice were obtained from our breeding colony of C57 BL/6J-ob/+ mice. They were weaned at 3 wks of age, housed at 23 to 25°C in solid-bottom plastic cages with wood shavings for bedding, and fed a nonpurified diet ad libitum (Wayne Lab-Blox, Continental Grain Company, Chicago, IL). Room lights were on from 0700 to 1900 h daily.

At 4.5 wk of age, bilateral adrenalectomies were performed through dorsal incisions while mice were under ether anesthesia. Sham-operated mice were exposed to the same surgical procedure, except the adrenal glands were left intact. Adrenalectomized mice received 0.9% NaCl solution to drink following surgery.

A high-starch purified diet was available ad libitum postsurgery. The high-starch diet contained (in g/100 g): starch 62.9; casein, 21.2; methionine, 0.3; corn oil, 5.3; mineral mixture, 3.7 (23); vitamin mixture, 1.1 (23); choline chloride, 0.2; and cellulose, 5.3. This diet provided 3.9 kcal metabolizable energy/g with 66% of

metabolizable energy as carbohydrate, 22% as protein and 12% as fat.

Fourteen days after surgery mice were used for each experiment. All mice were killed at the end of the experiment and plasma corticosterone concentrations were determined.

2. CHEMICALS.

Dexamethasone phosphate solution (3 mg dexamethasone/ml) was obtained from the Schering Corp. (Kenilworth, NJ), and atropine methylnitrate obtained from Sigma Chemical Co. were diluted in isotonic saline before use. CRF was obtained from the Bachem Inc. (Torrance, CA) and diluted in isotonic saline containing 0.1% bovine serum albumin.

3. EXPERIMENTAL DESIGN.

3.1 EXPERIMENT 1.

Experiment 1 was designed to determine dose response and time course relationships for effects of icv injection of dexamethasone on GDP binding to BAT mitochondria, and on plasma insulin and glucose concentrations. First, sham-operated or adrenalectomized mice were injected with either dexamethasone (3, 6, 12, 25, 50, 100, 200, 250 or 1000 ng/mouse) or saline between 1000 and 1100 h. Three h

after injection, mice were decapitated, blood was collected for insulin and glucose assays, and BAT interscapular depots were rapidly removed for measurement of BAT mitochondrial protein content and GDP binding to mitochondria. Next, mice were injected icv with 25 ng dexamethasone and killed 1/2, 1, 3 or 24 h later for measurement of food intake, GDP binding to BAT mitochondria, and plasma insulin and glucose concentrations.

3.2 EXPERIMENT 2.

Experiment 2 was designed to examine effects of central versus peripheral injection of dexamethasone on GDP binding to BAT mitochondria, and on plasma insulin and glucose concentrations. The same general procedures used for experiment 1 were followed here, except that mice were injected icv or intraperitoneally (ip) with 25 ng dex and killed 30 min later.

3.3 EXPERIMENT 3.

Experiment 3 examined effects of dexamethasone on whole animal oxygen consumption. Fourteen days after surgery adrenalectomized ob/ob and lean mice were injected icv with 0 or 250 ng of dexamethasone, and oxygen consumption was measured 3 h later at 25°C.

3.4 EXPERIMENT 4.

Experiment 4 was performed to study effects of dexamethasone on norepinephrine turnover in BAT, pancreas and heart. L-[ring-2,5,6-^{3}H] norepinephrine (52.9 Ci/mmol, New England Nuclear) in 0.2 ml saline was injected (ip) into unanesthetized mice 30 min after icv saline or dexamethasone injection (250 ng) to measure norepinephrine turnover. Each mouse received 250 uCi ^{3}H-norepinephrine/kg body weight. Mice were decapitated 1, 3 or 5 h after ^{3}H-norepinephrine injection. BAT (interscapular pads), pancreas and heart were immediately removed, frozen on dry ice and stored at -70°C until NE assay was performed (within 1 wk).

3.5 EXPERIMENT 5.

Experiment 5 was designed to study the possible role of the parasympathetic nervous system in mediating effects of dexamethasone on GDP binding to BAT mitochondria, and on plasma insulin and glucose concentrations. The same general procedures used for experiment 1 were followed, but mice were injected with either dexamethasone (250 ng) or saline (icv) and atropine nitrate (0, 5, 20 and 200 ug atropine in 0.2 ml saline, ip). Mice were killed 30 min later.

3.6 EXPERIMENT 6.

Experiment 6 was designed to determine the effects of CRF on GDP binding to BAT mitochondria, and on plasma

insulin, glucose and free fatty acid. Sham-operated and dexamethasone (250 ng, icv)-injected adrenalectomized mice were injected icv with saline or CRF (5 ug). Mice were killed 30 min after injection.

4. ICV INJECTION.

Icv injections were carried out by the method described by Laursen et al (117) with slight modifications. A Hamilton 701RN syringe fitted with a 2-in.,26- gauge needle was used. Stiff Tygon tubing 15 mm in length was slipped over the needle and was secured with epoxy to control the depth of the injection and also to stiffen the needle. The needle was then cut 4.5 mm from the end of the tubing and sharpened until the needle was 3.7 mm long at the end of the bevel and 3.2 mm long at the center of the bore.

Each animal was slightly anesthetized with ether. The syringe was held at an angle approximately 45° to the skull, with the needle bevel facing up and pointing toward the tail of the animal. The bregma was located by lightly rubbing the point of the needle over the skull until the suture was felt through the skin. The needle was inserted about 1.5 mm lateral to the midline (Figure 1). Because the skull is relatively thin at this point, only mild pressure was required to insert the needle.

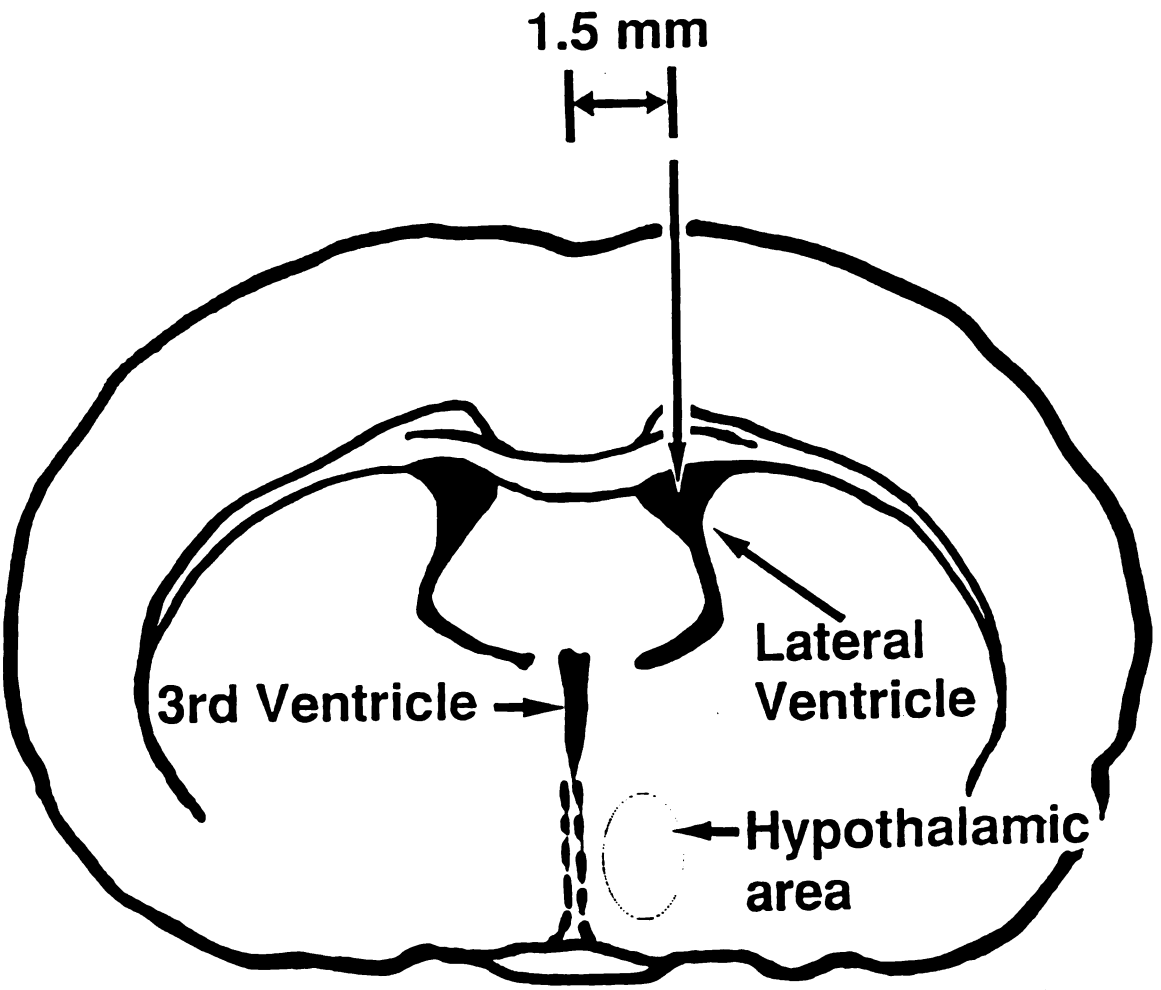

Figure 1. Injection target spot of lateral ventricle.

Solutions containing dexamethasone, CRF or saline were injected in a volume of 2 ul between 1000 and 1100 h. After animals were killed the brain was removed and sliced coronally along the needle tract to ascertain if the lateral ventricle had been entered. The success rate of icv injection by this method was approximately 90%.

5. GDP BINDING.

Mice were killed at approximately 1300 h, and the interscapular BAT depot was rapidly removed and placed in ice-cold sucrose buffer. Mitochondria were isolated in buffer containing 250 mM sucrose and 5 mM K-TES (PH 7.2) by the procedure of Cannon et al (37). Protein content of mitochondrial preparations was measured by a modified Lowry method (124).

Binding of ^{3}H-GDP to BAT mitochondria was measured by the method described by Nicholls (135) with slight modifications. In brief, mitochondria (0.5 to 1 mg mitochondrial protein/ml) were incubated at room temperature for 7 min in a media containing 100 mM sucrose, 20 mM K-TES, 1 mM EDTA, 2 uM rotenone, 100 uM potassium atractyloside, 2.5 x 10^{6} dpm/ml ^{3}H-GDP (New England Nuclear, Boston, MA. 10.2 mCi/mmol) and unlabeled GDP (10 uM). ^{14}C-sucrose, 5.55 x 10^{5} dpm/ml (New England Nuclear, Boston, MA. 673 mCi/mM) were included in the incubation media to calculate the

volume of media trapped in the final mitochondrial pellet. Nonspecific binding was assessed by ^{3}H-GDP binding in the presence of excess unlabelled GDP (200 uM). Tubes were quickly centrifuged at the end of the 7 min incubation. The supernatant was removed, and the mitochondrial pellet was subsequently dissolved (Beckman tissue solubillizer-450) and counted in a liquid scintillation counter.

6. MEASUREMENT OF PLASMA CORTICOSTERONE, INSULIN, GLUCOSE AND FREE FATTY ACID CONCENTRATIONS.

Plasma corticosterone concentrations were determined by radioimmunoassay (RIA; Endocrine Sciences, Tarzana, CA) with modifications. Plasma was diluted 1:9 with borate buffer (0.5 M, pH 8.0) and incubated for 30 min at 60°C to denature corticosterone binding proteins. Ethanol was then added to precipitate proteins and extract corticosterone. After drying an aliquot of the ethanol extract, the radioimmunoassay was conducted. The lower limit of detection with this assay was 0.15 ug corticosterone/dl plasma. Adrenalectomized mice with nondetectable plasma corticosterone in this assay were assigned a value of 0.15 ug corticosterone/dl. Adrenalectomized mice with plasma corticosterone concentrations > 1 ug/dl were excluded from the study; 12% of the adrenalectomized mice were excluded

on this basis. Plasma corticosterone concentrations averaged 0.52±0.02 ug/dl in adrenalectomized ob/ob mice and 0.47 ±0.03 ug/dl in adrenalectomized lean mice. Sham operated ob/ob and lean mice had 13.9±1.7 and 5.2±0.7 ug corticosterone/dl plasma, respectively. Plasma insulin concentrations were determined by radioimmunoassay (Novo Laboratory, Denmark). Plasma glucose was assayed by the glucose oxidase-peroxidase method (Boehringer Mannheim, Indianapolis, IN). Plasma free fatty acid was measured by modified Dole method (113).

7. MEASUREMENT OF OXYGEN CONSUMPTION.

Oxygen consumption was measured between 1400 and 1500 h. Soda lime was used to absorb expired CO_2 from the chamber atmosphere (189). Food was available to the mice until oxygen consumption measurements commenced. Three h after icv dexamethasone (250 ng) injection, mice were placed in flasks immersed in a water bath maintained at 25 ± 1° C. After 5 min adaptation, oxygen consumption was recorded five times within 2 min. The average of the five readings were calculated as milliliters of oxygen consumed per gram of body weight (189).

8. NOREPINEPHRINE TURNOVER.

Norepinephrine content, specific radioactivity, fractional rates (k) of norepinephrine turnover and calculated rates of norepinephrine turnover were determined as previously described (111). Briefly, tissues were homogenized in perchloric acid, and norepinephrine was eluted from the alumina and determined by high-performance liquid chromatography (HPLC) with electrochemical detection (Bioanalytical Systems, West Lafayette, IN). Norepinephrine was collected from the column and counted in a liquid scintillation counter to measure [^{3}H]-norepinephrine specific radioactivity. Slopes of linear regressions describing the disappearance of ^{3}H-labeled norepinephrine from tissue were used to calculate fractional rates of norepinephrine turnover. Rates of norepinephrine turnover were calculated as the product of fractional turnover rate for each experimental group and norepinephrine content of organs of individual animals.

9. STATISTICS.

Data were presented as means ± SE. Data were analyzed by one way analysis of variance, and statistical comparisons with control means were made with Dunnett's test.

Statistical comparisons between two specific treatment groups were also made with Student t-test (74).

C. RESULTS

1. EXPERIMENT 1 - DOSE RESPONSE AND TIME COURSE RELATIONSHIPS FOR EFFECTS OF ICV INJECTION OF DEXAMETHASONE ON GDP BINDING TO BAT MITOCHONDRIA, AND ON PLASMA INSULIN AND GLUCOSE CONCENTRATIONS.

Sham-operated ob/ob mice had 0.5 times lower GDP binding to BAT mitochondria and 30 times higher plasma insulin concentrations than sham-operated lean mice (Figure 2). In agreement with earlier reports (107), adrenalectomy increased GDP binding to BAT mitochondria over 100%, and decreased plasma insulin concentrations by 80% in ob/ob mice, with no effects in lean mice. Plasma glucose concentrations were unaffected by adrenalectomy. A single icv injection of dexamethasone decreased GDP binding to BAT mitochondria in adrenalectomized ob/ob mice in a dose-dependent manner so that GDP binding to BAT mitochondria in adrenalectomized ob/ob mice receiving 250 ng dexamethasone icv was almost as low as in sham-operated ob/ob mice (Figure 2). Dexamethasone also increased plasma insulin concentrations in a dose-dependent manner in adrenalectomized ob/ob mice. Injection of 250 ng of

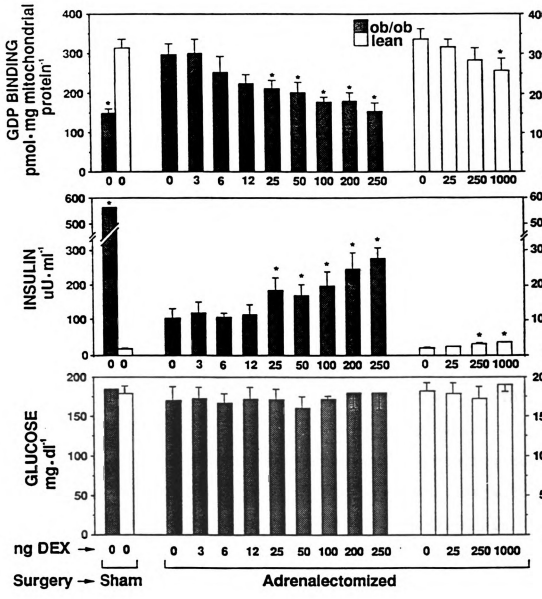

Figure 2. Dexamethasone dose-response relationships for GDP binding to BAT mitochondria, and for plasma insulin and glucose concentrations. Adrenalectomized ob/ob and lean mice were injected icv with 0 to 1000 ng dexamethasone and killed 3 h later. The left 2 bars represent sham-operated mice injected with saline. Each bar represents means ± SE of 9-28 mice, except for adrenalectomized ob/ob mice injected with 3 ng dexamethasone (n=5). Asterisks indicate significant differences ($p < 0.05$; Dunnett's test) within phenotype from corresponding adrenalectomized mice injected with 0 ng dexamethasone.

dexamethasone increased plasma insulin concentrations by 160% in adrenalectomized ob/ob mice; these dexamethasone-induced increases in insulin occurred without any change in plasma glucose concentrations. Dexamethasone injections had much less influence in adrenalectomized lean mice; doses of 250 or 1000 ng dexamethasone were required to demonstrate significant changes in GDP binding to BAT mitochondria, and in plasma insulin; and even at these high doses of dexamethasone changes were modest relative to responses observed in adrenalectomized ob/ob mice (Figure 2).

The lowest dose of dexamethasone to decrease GDP binding to BAT mitochondria, and to increase plasma insulin in adrenalectomized ob/ob mice was 25 ng (Figure 2). This dose was used to examine the time course of response to dexamethasone. Dexamethasone depressed GDP binding to BAT mitochondria in adrenalectomized ob/ob mice as effectively within 30 min after injection as after 60 or 180 min. Plasma insulin concentrations tended to be increased 30 min after dexamethasone injection, and were significantly elevated by 60 min (Figure 3). Again, these changes in plasma insulin concentrations occurred without changes in plasma glucose.

The single icv injection of dexamethasone caused relatively rapid changes in GDP binding to BAT mitochondria and plasma insulin concentrations in adrenalectomized ob/ob

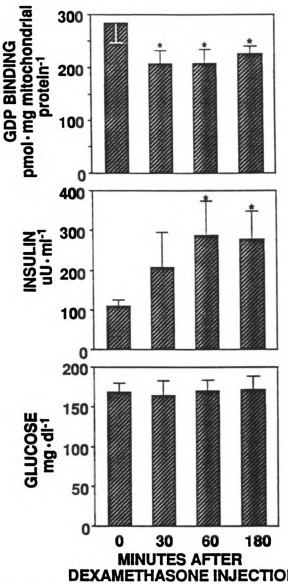

Figure 3. Time course for dexamethasone-induced changes in GDP binding to BAT mitochondria, and in plasma insulin and glucose concentrations. Adrenalectomized ob/ob mice were injected icv with 25 ng dexamethasone and killed 30, 60, or 180 min later. Adrenalectomized ob/ob mice injected icv with saline and killed 180 min later served as controls. Sham-operated ob/ob mice injected icv with saline and killed 180 min later had BAT GDP binding values of 162 ± 12 pm/mg mitochondrial protein, plasma insulin values of 981 ± 196 uU/ml and plasma glucose values 180 ± 20 mg/dl. Each bar represents means ± SE of 7 mice. Asterisks indicate significant differences ($p < 0.05$; Dunnett's test) from corresponding adrenalectomized ob/ob mice killed at 0 time.

mice (Figure 3). To determine if these dexamethasone-induced changes were sustained for a longer time mice received a single icv injection of dexamethasone and were killed 24 h later. Adrenalectomy depressed food intake in ob/ob mice as expected (Figure 4). Food intake increased in adrenalectomized ob/ob mice during the 24 h period after the single icv injection of dexamethasone (250 ng). Consistent with the responses observed within 1/2 to 3 h after dexamethasone administration, GDP binding remained depressed and insulin remained elevated in adrenalectomized ob/ob mice 24 h after dexamethasone injection, although only the higher dose was effective. Adrenalectomized lean mice were unaffected by dexamethasone injection.

2. EXPERIMENT 2 - EFFECTS OF CNS VERSUS PERIPHERAL INJECTION OF DEXAMETHASONE.

Sham-operated ob/ob mice had approximately 39% lower GDP binding to BAT mitochondria and 614% higher plasma insulin concentrations than ADX ob/ob mice. Icv injection of dexamethasone (25 ng) into adrenalectomized ob/ob mice decreased GDP binding to BAT mitochondria by 35%, and increased plasma insulin concentrations by 62% within 30 min with no change in plasma glucose concentrations. This dose of dexamethasone administered intraperitoneally did not influence either GDP binding or insulin (Figure 5).

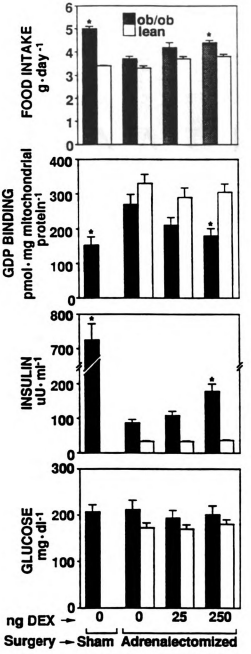

Figure 4. Food intake, GDP binding to BAT mitochondria, and plasma insulin and glucose concentrations in adrenalectomized ob/ob and lean mice injected icv with 0, 25 or 250 ng dexamethasone and killed 24 h later. The left two bars represent sham-operated mice injected with saline and killed 24 h later. Each bar represents means ± SE of 9-15 mice. Asterisks indicate significant differences ($p < 0.05$; Dunnett's test) from corresponding adrenalectomized mice injected with 0 ng dexamethasone.

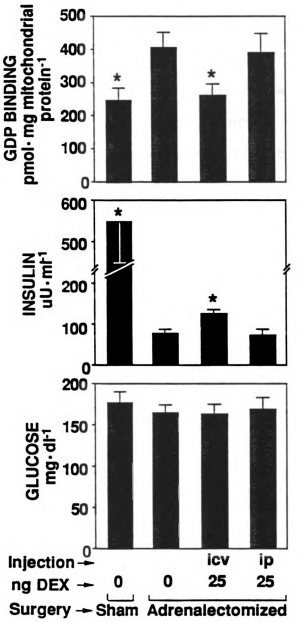

Figure 5. Effects of icv versus intraperitonial injection of dexamethasone on GDP binding to BAT mitochondria, plasma insulin and glucose concentrations. Adrenalectomized ob/ob mice were injected icv or ip with 0 or 25 ng dexamethasone, and killed 30 min later. The left bar represents sham-operated ob/ob mice injected with 0 ng DEX (saline) and killed 30 minutes later. Values for 0 ng dexamethasone-injected sham or adrenalectomized mice are combined averages of icv and ip injections. Each bar represents means ± SE of 7-9 mice. Asterisks indicate significant differences ($p<0.05$; Dunnett's test) from adrenalectomized mice injected with 0 ng dexamethasone.

3. EXPERIMENT 3 - EFFECTS OF DEXAMETHASONE ON WHOLE ANIMAL OXYGEN CONSUMPTION.

Dexamethasone (250 ng) decreased whole body oxygen consumption by 17% in adrenalectomized ob/ob mice 3 h after icv injection (Figure 6). Oxygen consumption in lean mice was unaffected by dexamethasone.

4. EXPERIMENT 4 - EFFECTS OF DEXAMETHASONE ON NOREPINEPHRINE TURNOVER IN BAT, PANCREAS AND HEART.

To determine if the observed changes in BAT metabolism and plasma insulin were associated with dexamethasone-induced changes in sympathetic nervous system activity to BAT and pancreas, norepinephrine turnover (an indicator of sympathetic nervous system activity) was measured. Fractional rates (k) of norepinephrine turnover, assessed by injection of [^{3}H]-norepinephrine were approximately 37 and 27% lower in BAT and heart, respectively, of adrenalectomized ob/ob mice injected with dexamethasone than in those injected with saline (Figure 7). Fractional rates of norepinephrine turnover in pancreas were unaffected by dexamethasone injection. Calculated rates of norepinephrine turnover (norepinephrine content x k) were approximately 45% lower in BAT of dexamethasone-injected ob/ob mice than in saline injected mice (Figure

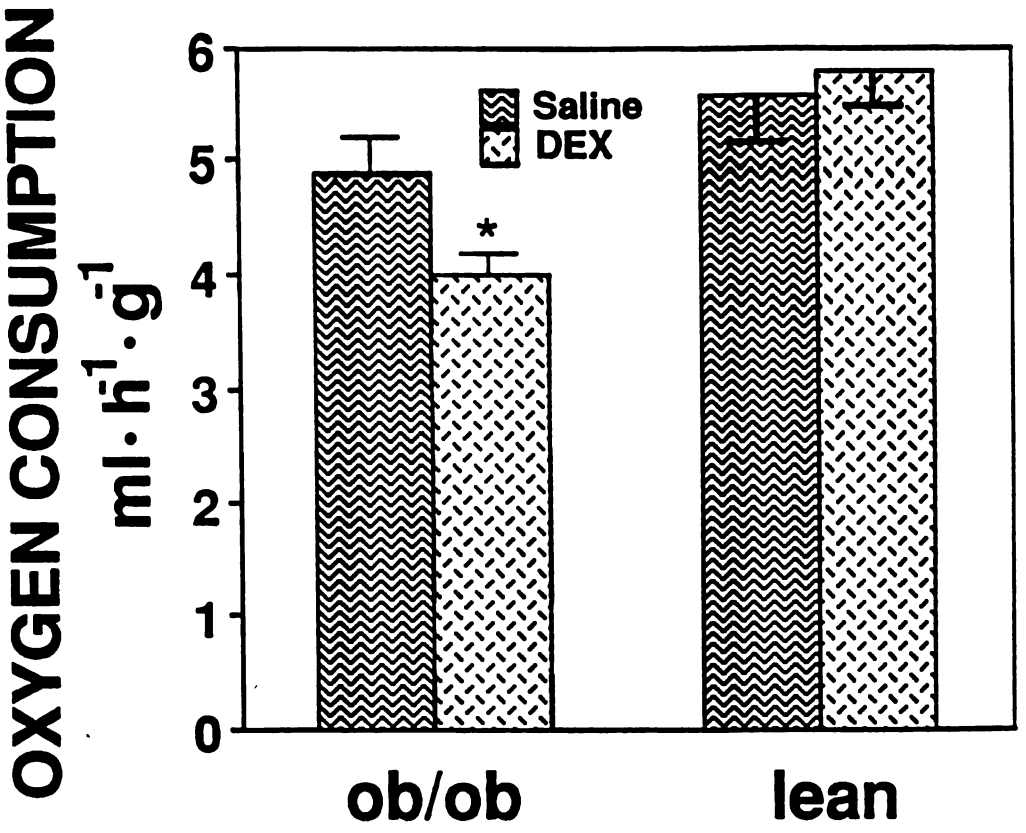

Figure 6. Oxygen consumption in adrenalectomized ob/ob and lean mice injected icv with saline or 250 ng of dexamethasone; oxygen consumption was measured 3 h after icv injection at 25° C. Body weights averaged 25.2 ± 1.0 and 25.4± 1.1 g for adrenalectomized ob/ob mice and 20.8 ± 0.9 and 20.3 ± 1.1 for lean mice injected with saline or dexamethasone, respectively. Each bar represents means ± SE of 13-14 mice. Asterisks indicate significant differences ($p<0.05$; Student's t-test) between treatments within phenotype.

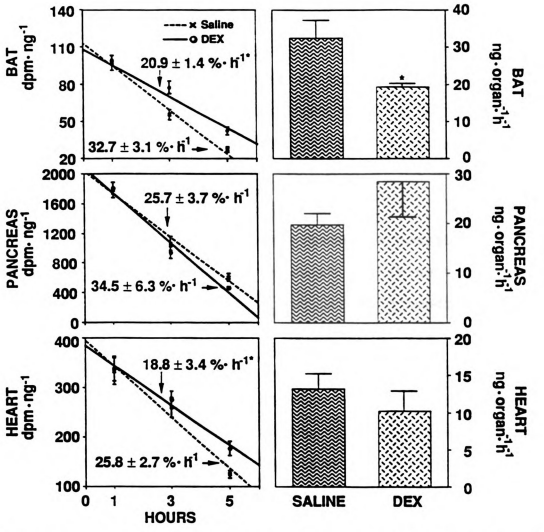

Figure 7. Norepinephrine specific activity and calculated rates of norepinephrine turnover in adrenalectomized ob/ob mice injected icv with 0 or 250 ng dexamethasone; 30 min later they were injected ip with ^{3}H-norepinephrine. Points represent means ± SE for 7-10 mice killed 1, 3 or 5 h after ^{3}H-norepinephrine injection. Values in the left panels represent fractional rates of norepinephrine turnover (k)±SE calculated from the slopes (b) of each regression line (k=b/0.434). The norepinephrine turnover rates (right panels) were calculated as the product of k and the norepinephrine content of each tissue. Each bar represents means ± SE of 25-30 mice. Norepinephrine content of BAT, pancreas and heart averaged 98±6, 77±4 and 50±5, respectively, for saline-injected mice; and 93±7, 80±8 and 53±5, respectively, for dexamethasone-injected mice. Asterisks indicate significant differences ($p < 0.05$; Student's t-test) from mice injected with 0 ng dexamethsone.

7). Calculated rates of norepinephrine turnover in pancreas and heart were unaffected by dexamethasone.

5. EXPERIMENT 5 - POSSIBLE ROLE OF THE PARASYMPATHETIC NERVOUS SYSTEM IN MEDIATING EFFECTS OF DEXAMETHASONE ON GDP BINDING TO BAT MITOCHONDRIA, AND ON PLASMA INSULIN AND GLUCOSE.

Atropine was injected to determine the possible role of the parasympathetic nervous system in mediating the effects of dexamethasone on GDP binding to BAT mitochondria, and on plasma insulin in adrenalectomized ob/ob mice. As observed earlier (Figure 3,5) dexamethasone-injected adrenalectomized ob/ob mice had 37% lower GDP binding to BAT mitochondria and 123% higher plasma insulin concentrations than saline-injected adrenalectomized mice, with no difference in plasma glucose level (Figure 8). Atropine did not affect GDP binding to BAT mitochondria in adrenalectomized ob/ob mice, except for the highest dose (200 ug) of atropine which increased GDP binding to BAT mitochondria of dexamethasone-injected adrenalectomized ob/ob mice by 46%. Atropine lowered plasma insulin concentrations in both adrenalectomized and dexamethasone-injected adrenalectomized ob/ob mice in a dose-dependent manner. However, effects of atropine were much greater in dexamethasone-injected

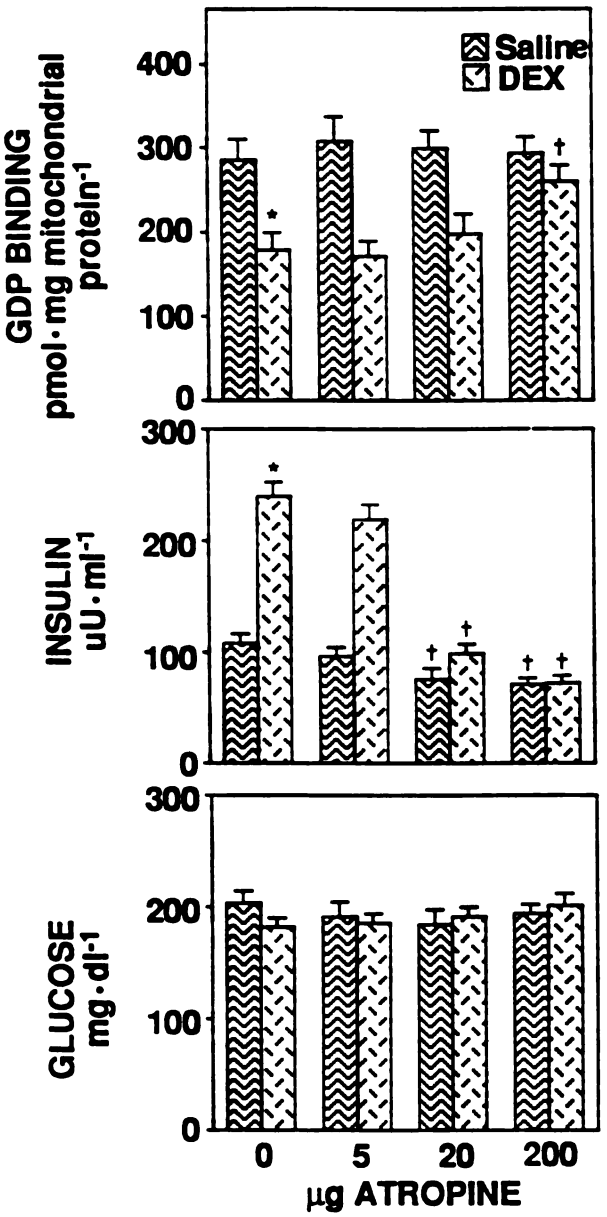

Figure 8. Effects of atropine on GDP binding to BAT mitochondria, and on plasma insulin and glucose concentrations. Adrenalectomized ob/ob mice were injected ip with 0, 5, 20 and 200 ug of atropine. At the same time, mice were injected icv with saline or 250 ng dexamethasone. Mice were killed 30 min later. Each bar represents means ± SE of 6-13 mice. Asterisks indicate significant differences ($p < 0.05$; Student's t-test) between saline and dexamethasone groups receiving o ng atropine. Crosses indicate significant differences ($p<0.05$; Dunnett's test) within treatment from corresponding 0 ng atropine value.

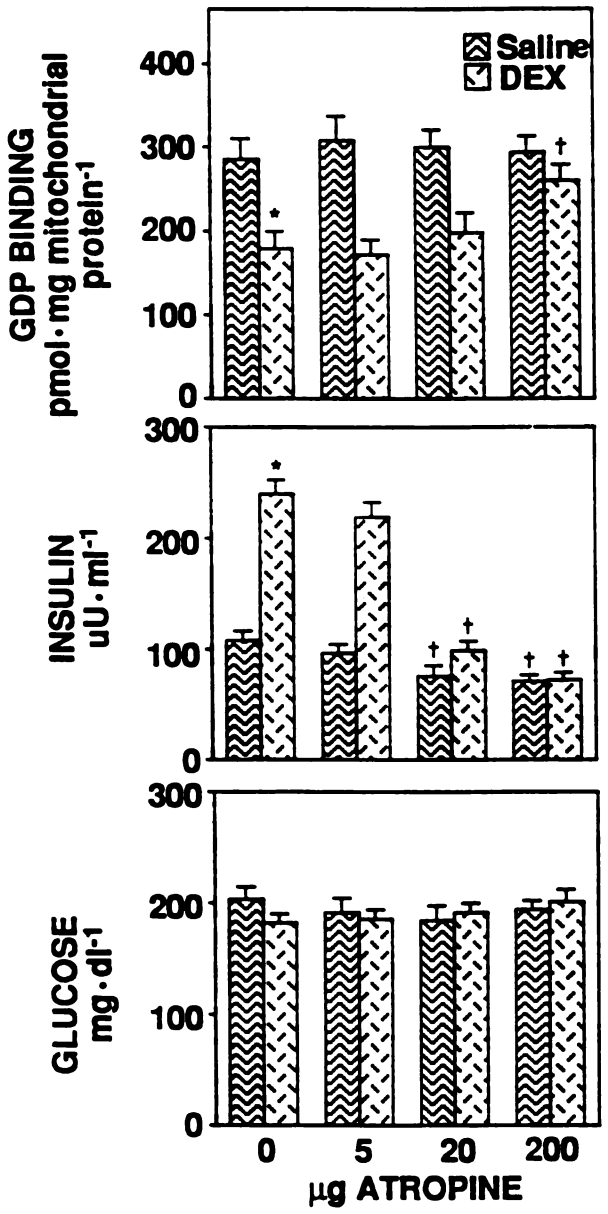

Figure 8. Effects of atropine on GDP binding to BAT mitochondria, and on plasma insulin and glucose concentrations. Adrenalectomized ob/ob mice were injected ip with 0, 5, 20 and 200 ug of atropine. At the same time, mice were injected icv with saline or 250 ng dexamethasone. Mice were killed 30 min later. Each bar represents means ± SE of 6-13 mice. Asterisks indicate significant differences ($p < 0.05$; Student's t-test) between saline and dexamethasone groups receiving o ng atropine. Crosses indicate significant differences ($p<0.05$; Dunnett's test) within treatment from corresponding 0 ng atropine value.

adrenalectomized ob/ob mice, so that plasma insulin concentration in dexamethasone-injected adrenalectomized ob/ob mice injected with 200 ug atropine was the same as that of adrenalectomized ob/ob mice. Atropine did not influence plasma glucose concentrations.

6. EXPERIMENT 6 - EFFECTS OF CRF ON GDP BINDING TO BAT MITOCHONDRIA, AND ON PLASMA INSULIN, GLUCOSE, AND FREE FATTY ACID.

CRF (icv, 5 ug) did not affect GDP binding to BAT mitochondria in either sham-operated or dexamethasone-injected adrenalectomized ob/ob mice (Figure 9). CRF markedly lowered plasma insulin concentrations in both sham-operated and dexamethasone-injected adrenalectomized ob/ob mice, so that plasma insulin concentrations in sham-operated and dexamethasone-injected adrenalectomized ob/ob mice were only 14 and 17% of those of saline-injected mice. These changes in plasma insulin occurred without changes in plasma glucose. CRF increased plasma free fatty acid concentrations by 56 and 22% in sham-operated and dexamethasone-injected adrenalectomized ob/ob mice, respectively, as compared to their saline injected controls.

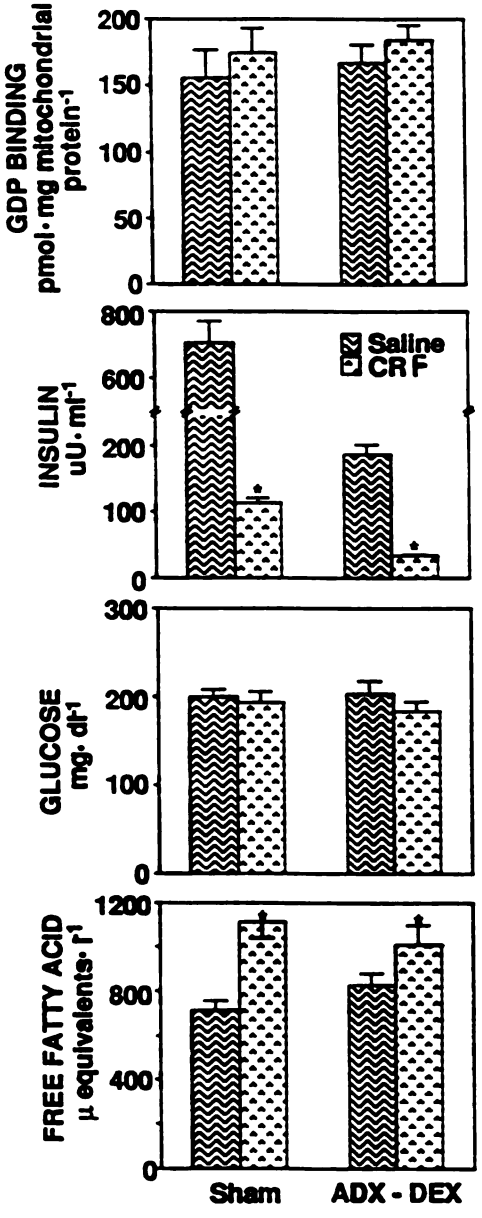

Figure 9. Effects of CRH on GDP binding to BAT mitochondria, and on plasma insulin, glucose and free fatty acid concentrations. Sham-operated and dexamethasone (250 ng,icv)-injected adrenalectomized ob/ob mice were injected icv with saline or CRF (5 ug). Mice were killed 30 min after injection. Each bar represents means ± SE of 10-12 mice. Asterisks indicate significant differences ($p<0.05$; Student's t-test) from corresponding mice injected with saline.

D. DISCUSSION

The main findings of this study were that a single icv injection of dexamethasone in adrenalectomized ob/ob mice completely reversed effects of adrenalectomy on BAT thermogenesis as assessed by mitochondrial GDP binding (Figure 2), approximately doubled plasma insulin concentrations (Figure 2-4), lowered whole body metabolic rates by 17% (Figure 6), and increased food intake by 19% (Figure 4). These responses were rapid in onset with changes in BAT metabolism and plasma insulin occurring within 30 minutes of dexamethasone injection. Adrenalectomized lean mice were much less sensitive and responsive to icv dexamethasone injection than were ob/ob counterparts.

Evidence was obtained to support the hypothesis that glucocorticoids influence metabolism in ob/ob mice via direct interactions within the CNS. An intraperitonial injection of 25 ng dexamethasone failed to change BAT metabolism or plasma insulin whereas icv injection of 25 ng dexamethasone was effective (Figure 5). Debons et al also reported that glucocorticoids act via the CNS to cause hyperphagia in goldthioglucose-treated adrenalectomized

obese mice (48). The icv dose of cortisone required to induce hyperphagia was 1/60th of the intraperitoneal dose to restore hyperphagia in these mice. Dexamethasone exhibited the most pronounced response among the several glucocorticoids examined (48). The minimal effective dose of dexamethasone (25 ng) to decrease BAT thermogenesis and to increase plasma insulin concentration in the present study was similar to the minimal effective dose of dexamethasone needed to restore hyperphagia in their study; they used 50 ng dexamethasone which was reported to be approximately double the minimal effective dose.

A single icv injection of dexamethasone decreased GDP binding to BAT mitochondria in adrenalectomized ob/ob mice to values as low as in sham-operated ob/ob mice within 30 to 180 min (Figure 2,3). Tokuyama et al also found decreased GDP binding to BAT mitochondria of adrenalectomized ob/ob mice 15 h, but not 6 h, after subcutaneous injection of corticosterone (180). The longer lag time in their study may relate to the delay in corticosterone reaching the CNS after subcutaneous administration.

The dexamethasone-induced decrease in BAT thermogenesis in adrenalectomized ob/ob mice was associated with an organ-specific decrease in BAT sympathetic nerve activity; icv-administered dexamethasone decreased norepinephrine turnover in BAT, but not in pancreas or heart (Figure 7). The same

organ-specific decrease in sympathetic nerve activity occurs in 8-wk old intact ob/ob mice, and adrenalectomy of these mice leads to an organ-specific increase in BAT norepinephrine turnover (107). Changes in BAT metabolism are often associated with alterations in whole body metabolic rates (188). Consistent with this association, the icv dexamethasone-induced lowering of BAT norepinephrine turnover in adrenalectomized ob/ob mice was also associated with a lowering of whole body metabolic rates in these animals (Figure 6).

The mechanism whereby icv dexamethasone depresses sympathetic nervous system activity in BAT of ob/ob mice remains to be established. Centrally, insulin is known to inhibit sympathetic nervous system activity in BAT (155). Thus, one could speculate that the dexamethasone-induced increase in plasma insulin caused the suppression of BAT metabolism. This mechanism appears unlikely because administration of 20 ug atropine to dexamethasone-treated, adrenalectomized ob/ob mice blocked the increase in plasma insulin, but not the depression in BAT metabolism (Figure 8). A higher dose of atropine (200 ug) prevented the dexamethasone-induced decrease in BAT metabolism, possibly because atropine methylnitrate in high doses may pass through the brain blood barrier and cause CNS stimulation leading to an increase in BAT thermogenesis (191).

Increased plasma insulin concentrations in adrenalectomized ob/ob mice following a single icv injection of dexamethasone were not secondary to an increase in plasma glucose since plasma glucose concentrations were unaffected by dexamethasone (Figure 2-5). Changes in pancreatic sympathetic nervous system activity can not explain the observed increases in plasma insulin either since norepinephrine turnover in the pancreas was unaffected by dexamethasone. It appears more likely that icv administration of dexamethasone increased parasympathetic nervous system activity to the pancreas, resulting in enhanced insulin secretion. Atropine completely blocked the dexamethasone-induced increase in plasma insulin concentrations (Figure 8) supporting the conclusion that dexamethasone-induced rises in plasma insulin were mediated by increased parasympathetic nerve activity. This result is consistent with a recent study by Fletcher et al where atropine also reduced plasma insulin concentrations in corticosterone-replaced (for 24 h) adrenalectomized fa/fa rats (59).

Clearly, adrenalectomized ob/ob mice were more sensitive and responsive to icv dexamethasone than lean counterparts. These findings provide direct evidence for a primary defect in the CNS of ob/ob mice that leads to altered regulation of food intake, whole body metabolic rates, BAT metabolism, and plasma insulin. An understanding of the mechanism(s)

whereby glucocorticoid exert these effects within the CNS should help identify the primary defect in ob/ob mice.

Glucocorticoids are known to exert feedback control on CRF production in the hypothalamus (39,45,47,56,168). CRF administered icv activate the sympathetic nervous system (55,100) as well as decreases food intake (7) in rat. This has lead to the hypothesis that glucocorticoid influences on development of obesity are mediated via suppressed synthesis and release of CRF. However, CRF injected into lateral ventricle did not increase GDP binding to BAT mitochondria in either sham-operated ob/ob mice or in dexamethasone-treated adrenalectomized ob/ob mice (Figure 9). This result contrasts with studies in 21 h food deprived rats (7) and VMH-lesioned rats (6) where icv administration of CRF increased GDP binding to BAT mitochondria within 30 min and 6 h, respectively. Although the type (human/rat CRF) and dose (5 ug) of CRF used these studies were the same as those used in the present study, it is possible that the dose or effective time of CRF to increase BAT metabolism in mice are different from those in rats.

CRF administered into lateral ventricle dramatically lowered plasma insulin in sham-operated ob/ob mice to the level found in adrenalectomized ob/ob mice (Figure 9). CRF also completely abolished the dexamethasone-induced increase in plasma insulin. Plasma glucose concentrations were unaffected by CRF; thus, changes in plasma insulin

concentrations were not secondary to changes in plasma glucose. A similar effect on plasma insulin has been reported in genetically obese (fa/fa) rats treated chronically (for 7 days) with CRF, however in that study it was not possible to determine whether CRF exerted direct effects on plasma insulin or indirect effects via chronic alterations in metabolism and body composition. The present report demonstrates that CRF has rapid effects (within 30 min) on plasma insulin concentrations in ob/ob mice. These effects parallel those observed when atropine is administered (i.e. decreases in plasma insulin without increases in BAT metabolism) and suggest that CRF might act by suppressing parasympathetic nervous system activity in pancreas.

CRF administration into the lateral ventricle also raised plasma free fatty acid concentration in both sham-operated and dexamethasone-injected adrenalectomized ob/ob mice (Figure 9). Gunion (82) earlier noted that plasma free fatty acids increased after central injection of CRF into rats. Based on the failure of CRF to stimulate BAT metabolism in the mouse, I speculate that CRF did not increase plasma free fatty acid concentration via enhanced lipolysis subsequent to activation of the sympathetic nervous system. Rather, the increase in plasma free fatty acid may have been secondary to CRF-induced depressions in plasma insulin concentrations.

Overall, the results of this study suggest that the action of glucocorticoid on BAT thermogenesis and pancreatic insulin secretion in ob/ob mice occurs through the CNS. Centrally, glucocorticoid appears to increase parasympathetic nervous system via suppression of CRF which causes decreases in plasma insulin and leads to lipolysis from adipose tissue. Glucocorticoid action to decrease BAT metabolism in adrenalectomized ob/ob mice likely involves mechanisms other than via CRF. Direct action of glucocorticoid on plasma membrane or other neuropeptides may be involved in the action mechanism of this hormone to increase sympathetic nervous system activity in BAT.

E. RECOMMENDATIONS FOR FUTURE STUDIES.

To better understand the present results, and to continue searching for the effects and action mechanisms of glucocorticoid in development of obesity in ob/ob mice, I propose the following studies.

1. DETERMINE WHETHER THE ACTION MECHANISM OF GLUCOCORTICOID IS VIA NUCLEAR DNA (GENE EXPRESSION) OR THROUGH OTHER MECHANISMS.

In the present study, central injection of glucocorticoid significantly decreased BAT thermogenesis and increased plasma insulin concentrations within 30 min in adrenalectomized ob/ob mice. Two possible action mechanisms (direct and indirect) of glucocorticoid are discussed in the literature review section.

The first study which should follow the present study is to determine whether the action of glucocorticoid on BAT metabolism and plasma insulin is via induction of certain proteins through its interaction with DNA, or through some other mechanism. For this purpose, effects of dexamethasone should be determined in adrenalectomized ob/ob mice treated

with transcriptional or translational inhibitors such as actinomycin or cyclohexamide.

If dexamethasone exerts its action on BAT metabolism and plasma insulin in the presence of transcriptional and translational inhibitors, then, one can conclude that the action of dexamethasone on BAT metabolism and plasma insulin are not related to induction of protein synthesis but is related to other mechanism within the CNS (see E.2 for potential studies).

If the action of dexamethasone on BAT metabolism and plasma insulin requires production of certain peptides (or proteins), activity will be blocked in the presence of transcriptional or translational inhibitors because these inhibitors will block the synthesis of mRNA or any peptide. In this case, one needs to determine the peptides (or proteins) which are responsible for mediation of glucocorticoid action (see E.3 for potential studies).

2. POTENTIAL STUDIES TO CONDUCT IF GLUCOCORTICOID ACTION DOES NOT REQUIRE mRNA OR PROTEIN SYNTHESIS.

If glucocorticoid action does not require mRNA or protein synthesis, glucocorticoid action likely occurs through non-genomic mechanisms. Glucocorticoid may exert rapid acting effects after binding to plasma membranes. The rapidity with which glucocorticoid exert its effects on BAT

metabolism and plasma insulin (within 30 min) support this possibility.

To attempt to identify rapid membrane effects of glucocorticoid, one should determine the following parameters after icv administration of glucocorticoid in ob/ob mice. 1) changes in the activity of the membrane-bound adenylate cyclase, a possible messenger system for the rapid effects of glucocorticoid. 2) changes in potassium (K^+) permeability of membrane or activation of calcium (Ca^{2+}) channels which are effector systems that may transduce the signal of steroid-membrane interactions in the brain. 3) changes in firing frequency of specific nerves to BAT and pancreas, or the modulation of neurotransmitter release from nerve terminals which result from a direct action of steroids on the plasma membrane. To confirm that if the action is direct, all effects should occur in the presence of potent protein synthesis inhibitors.

3. POTENTIAL STUDIES TO CONDUCT IF GLUCOCORTICOID ACTION IS SHOWN TO REQUIRE mRNA OR PROTEIN SYNTHESIS.

The protein which mediates the effects of glucocorticoid needs to have the following characteristics.

1) this neuropeptide should be synthesized and released within the CNS.

2) the production and release of this peptide should be sensitive to glucocorticoids removal and replacement.

3) the action of peptide includes modulation of CNS.

Candidate peptides which qualify for all these conditions include neuropeptide Y and CRF.

Neuropeptide Y (NPY), a 36 amino acid peptide (177), is an abundant and important regulator in mammalian nervous system. In the brain, NPY-containing neurons are widely distributed, particularly in the cortex, hypothalamus, and striatum, and are probably involved in autonomic regulation and higher cognitive functions (125).

Recently Hisano et al reported that glucocorticoid receptor positive neurons in the arcuate nucleus show NPY-like immunoreactivity (87). They also reported the presence of synaptic contacts of NPY axons upon PVN neurons containing CRF which are target neurons of glucocorticoid (42,159). Light microscopic immuno-histochemistry has also shown that immunoreactive NPY fibers make dense networks in the PVN, where glucocorticoid receptors are abundant (13,40). This localization of glucocorticoid receptors in NPY-containing neurons suggests some functional connection between glucocorticoid and NPY.

Central injection of NPY is known to selectively elicit a marked eating response in satiated animals (43). One of the most responsive sites for this in brain is the PVN (171), a nucleus with abundant concentrations of endogenous

NPY (41) and an important role in control of feeding behavior (119). Recently, a study was conducted to examine whether the NPY-elicited feeding response is dependent on corticosterone. Adrenalectomy or hypophysectomy reduced the NPY-elicited feeding response by 60-71%. Corticosterone replacement, via subcutaneous implant normalized the NPY-induced feeding response in both the adrenalectomized and hypophysectomized rats (172). This finding suggests that the hypothalamic effect of NPY on the feeding system is largely dependent upon circulating corticosterone and that no other adrenal or pituitary hormone is essential.

Regulation of the prepro-NPY mRNA abundance was also studied by Higuchi et al. in several rodent neural cell lines (85). Treatment of PC12 rat pheochromocytoma cells, which possess low basal levels of prepro-NPY mRNA, with dexamethasone increased the prepro-NPY mRNA level 2-3 fold. Treatment of these cells with the protein kinase C activator, phorbol ester plus forskolin which elevated cAMP, increased prepro-NPY level by 20-70 fold. This increase was further enhanced to over 200-fold by dexamethasone and calcium ionophore A23187 which can elevate intracellular Ca^{2+} ion and thereby potentiate actions of phorbol esters and other Ca^{2+}-dependent protein. Phorbol ester or A23187 alone had no effect on the NPY mRNA abundance. These results indicate that NPY gene expression is very sensitive to glucocorticoid and can be positively

regulated by synergistic action of glucocorticoid, cAMP elevation, and protein kinase C activation (85).

Recently Egawa et al showed that icv injection of NPY to rats suppressed sympathetic nerve activity to interscapular brown adipose tissue in a dose-dependent manner indicating that this brain peptide is a neuromodulator of the sympathetic nervous system which may control energy expenditure in interscapular brown adipose tissue (55). Central injection of NPY also increased plasma insulin concentrations in rats. Moltz et al observed a significant elevation of circulating insulin 30 minutes after icv administration of NPY in rats (130). All these data suggest that NPY is a possible neuromodulator mediating the action of glucocorticoid on BAT thermogenesis and plasma insulin. Thus, I would examine effects of icv injections of NPY on BAT thermogenesis and plasma insulin concentration in adrenalectomized ob/ob mice. If glucocorticoid exert its action on BAT thermogenesis (and pancreatic insulin secretion) through the induction of NPY in the brain, icv injection of this peptide will exert the same effect as glucocorticoid. I would also recommend a NPY antagonist in dexamethasone injected-adrenalectomized ob/ob mice so that one could determine if NPY is necessary for mediation of glucocorticoid action on BAT thermogenesis and plasma insulin. If NPY is necessary for mediation of the

glucocorticoid action, glucocorticoid will not exert its action under the presence of NPY antagonist.

CRF, the production of which is sensitively regulated by glucocorticoid at the hypothalamic level, is another candidate for a possible neuromodulator mediating the action of glucocorticoid (see A.4.4). However, the present study showed that CRF is not likely involved in the action of glucocorticoid on BAT thermogenesis. I had expected that a high dose of CRF administered icv to either intact or glucocorticoid-injected adrenalectomized ob/ob mice would exert a similar effect as adrenalectomy since adrenalectomy causes a dramatic increase in brain CRF. It is possible that other neuromodulators are involved in mediating the action of glucocorticoid, or some other mechanism which is not related to gene expression of peptide through the binding of hormone-receptor complex to nuclear DNA.

In the present study, CRF dramatically lowered plasma insulin concentrations in both saline and dexamethasone-injected adrenalectomized ob/ob mice. Thus, one may also need to confirm that the effect of glucocorticoid on plasma insulin concentration is directly related to the changes in CRF level in the brain, or independent from the action of CRF on plasma insulin concentrations. For this reason, a CRF antagonist should be used to determine whether CRF is necessary for the action of glucocorticoid to increase plasma insulin concentrations or not.

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