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Intracerebroventricular Injection of Dexamethasone Rapidly Increases NPY Secretion Via Type II-Like Glucocorticoid Receptors to Suppress Brown Adipose Tissue Thermogenesis and Increase Plasma Insulin in Adrenalectomized ob/ob Mice

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INTRACEREBROVENTRICULAR INJECTION OF DEXAMETHASONE RAPIDLY INCREASES NPY SECRETION VIA TYPE II-LIKE GLUCOCORTICOID RECEPTORS TO SUPPRESS BROWN ADIPOSE TISSUE THERMOGENESIS AND INCREASE PLASMA INSULIN IN ADRENALECTOMIZED OB/OB MICE

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ABSTRACT

INTRACEREBROVENTRICULAR INJECTION OF DEXAMETHASONE RAPIDLY INCREASES NPY SECRETION VIA TYPE II-LIKE GLUCOCORTICOID RECEPTORS TO SUPPRESS BROWN ADIPOSE TISSUE THERMOGENESIS AND INCREASES PLASMA INSULIN IN ADRENALECTOMIZED OB/OB MICE

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A single intracerebroventricular injection of dexamethasone decreases brown adipose tissue (BAT) thermogenesis (as assessed by GDP-binding) and increases plasma insulin concentration rapidly (within 30 min) in adrenalectomized ob/ob mice, with minimal effects in lean mice. The present study was to determine 1) the type of glucocorticoid receptor(s) which mediate these effects, 2) if protein synthesis is a prerequisit for these alterations, and 3) how dexamethasone acts on NPY release to exert these rapid metabolic effects in adrenalectomized ob/ob mice. Intracerebroventricular injection of aldosterone (type I glucocorticoid receptor agonist) was ineffective in altering peripheral metabolism in adrenalectomized ob/ob mice whereas RU-486 (type II glucocorticoid receptor antagonist) abolished effects of dexamethasone. Thus, type II-like receptors, not type I receptors, mediated rapid effects of dexamethasone in adrenalectomized ob/ob mice. Anisomycin (a

translation blocker) almost completely suppressed (-93%) cerebral protein synthesis, but did not influence dexamethasone-induced BAT metabolism and plasma insulin. Since dexamethasone-induced actions are independent of protein synthesis, I postulated that dexamethasone exerts these rapid effects by modulating neurotransmitter release.

Since intracerebroventricular injection of NPY suppresses BAT thermogenesis and increases plasma insulin concentration in adrenalectomized ob/ob mice, NPY is a logical candidate to mediate downstream rapid dexamethasone effects. In support of this hyothesis, PYX-2, a NPY receptor antagonist, blocked effects of dexamethasone on BAT activity and on plasma insulin in adrenalectomized ob/ob mice. Intracerebroventricular injection of dexamethasone selectively reduced NPY concentrations in the hypothalamus by 35% within 30 min, but not in the brain stem or hippocampus of adrenalectomized ob/ob mice or in any of these 3 regions of the lean counterparts. NPY concentrations decreased in the arcuate nucleus region (-70%), dorsomedial (-60%) and ventromedial (-40%) hypothalami. This finding suggests that dexamethasone induced rapid transport of NPY from cell bodies to the terminal regions for secretion in adrenalectomized ob/ob mice to increase vagal drive to the pancreas and suppress sympathetic input to the brown adipose tissue to cause development of obesity in ob/ob mice.

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LIST OF ABBREVIATIONS

BAT..... Brown Adipose Tissue

CNS.....Central Nervous System

CRH..... Corticotropin Releasing Hormone

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

DEX.....Dexamethasone

GDP.....Guanosine 5'-Diphosphate

icv.....Intracerebroventricular

LHRH.....Lutenizing Hormone-Releasing hormone

mRNA.....Messenger Ribonucleic Acid

NPY.....Neuropeptide Y

PVN.....Paraventricular Nucleus of Hypothalamus

VMH.....Ventromedial Hypothalamus

CHAPTER I. INTRODUCTION

Obesity is a prevalent nutrition-related health risk in the United States. National Health and Nutrition Examination Survey (NHANES III) showed that one third (33.4%) of Americans aged 20 or older are overweight (BMI > 27.8 for men and 27.3 for women) (Berg 1994). Several health problems, such as cardiovascular disease, diabetes mellitus, gallbladder disease, and psychosocial disability have long been linked to obesity (Van Itallie 1979). Additional research focused on understanding the metabolic basis of obesity is necessary for successful prevention and treatment of human obesity.

Genetically obese rodents, including the ob/ob mice used in this study, have been extensively used for obesity research. These rodents exert metabolic defects that are also manifested in human obesity, such as hyperphagia, hyperinsulinemia, insulin resistance, hyperadrenocorticolism, and suppressed whole body energy expenditure (Bray and York 1979). The mutated obese (ob) gene in ob/ob mice has recently been cloned (Zhang et al. 1994). Functions of the peptide product of this defective gene remain to be established. Therefore, further research in obesity development in ob/ob mice is necessary to

understand the specific biochemical consequences of this mutated gene.

Suppressed energy expenditure and hyperphagia contribute to obesity. Whole body energy expenditure is partially (26% during maximal stimulation) contributed by brown adipose tissue (BAT) thermogenic activity (Foster and Frydman 1978). Although BAT thermogenesis in ob/ob mouse is defective as early as 6 d of age, studies showed that there is no inherent defect in BAT of obese animals (Boissonneault et al. 1987, Bray and York 1979, Himms-Hagen et al. 1986). These findings suggest that the external drive, such as the sympathetic nerve activity, for BAT thermogenesis is suppressed in ob/ob mice. When food intake is restricted to the same level as lean littermates, ob/ob mice still deposit excess fat (Alonzo and Maren 1955). Fat deposition is activated by insulin. Increased vagal nerve activity to the pancreas, which stimulates insulin secretion, may be another cause of obesity in ob/ob mice (Assimacopoulos-Jeannet et al. 1974, Geloen et al. 1989). Therefore, the origin of the metabolic defects in ob/ob mice may lie in regulation of BAT and pancreatic islets by the central nervous system (CNS) .

Adrenalectomy (ADX) generally normalizes or reduces metabolic defects in obese rodent models including ob/ob mice (Bruce et al. 1982, Kim and Romsos 1987, Romsos et al. 1987, Saito and Bray 1984, Rothwell et al. 1984). It is now known that glucocorticoids, but not aldosterone, secreted by

the adrenal glands are involved in the expression of obesity in these animal models (Saito and Bray 1984, Tokuyama and Himms-Hagen 1987). Therefore, glucocorticoid may act on the CNS to influence BAT thermogenesis and insulin secretion in ob/ob mice.

Both long-term and short-term effects of glucocorticoids in ob/ob mice have been studied (Saito and Bray 1984, Tokuyama and Himms-Hagen 1987, Walker and Romsos 1992). Chronic replacement of ADX ob/ob mice with glucocorticoids increased their adipose tissue mass, body weight and food intake to nearly the levels of intact ob/ob mice (Saito and Bray 1984, Tokuyama and Himms-Hagen 1987). Acute studies further reveal the time of onset of glucocorticoid-induced metabolic effects. A single intracerebroventricular (icv) injection of dexamethasone (DEX) into ADX ob/ob mice rapidly (within 30 min) reversed effects of ADX on BAT thermogenic activity and on plasma insulin concentration (Walker and Romsos 1992). A single subcutaneous injection of corticosterone also dramatically decreased BAT thermogenesis, increased plasma insulin and food intake within 15 h in ADX ob/ob mice (Tokuyama and Himms-Hagen 1989). These results suggest that glucocorticoid can modulate metabolic parameters as early as 30 min if administered directly to the CNS.

Although ob/ob mice have higher plasma glucocorticoid concentration than lean mice, the reduced BAT thermogenic activity and hyperinsulinemia in ob/ob mice are not caused

only by elevated serum glucocorticoids, but also by hypersensitivities of ob/ob mice to glucocorticoids. Long term, acute peripheral and acute central replacements of various doses of glucocorticoids each reveals greater potency for glucocorticoid in decreasing BAT thermogenesis and increasing plasma insulin concentration in ADX ob/ob mice than in lean mice (Tokuyama and Himms-Hagen 1987, 1989, Walker and Romsos 1992). Therefore, the influence of glucocorticoids in CNS activity may be greater in ob/ob than in lean mice.

The role of the CNS in rapid effects of glucocorticoids on BAT and pancreas has been illustrated (Walker and Romsos 1992). The turnover rate of norepinephrine as an indicator of sympathetic nerve activity indicated a 45% lower activity in icv DEX-injected ADX ob/ob mice than in saline injected counterparts (Walker and Romsos 1992). Atropine, a parasympathetic nerve blocker, lowered plasma insulin concentrations of DEX-injected ADX ob/ob mice to the level of saline-injected counterparts (Walker and Romsos 1992). These studies support the involvement of CNS in rapid regulation of BAT and pancreas by glucocorticoids.

There are two major subtypes of glucocorticoid receptors in the brain, type I and type II, that may mediate the glucocorticoid-induced metabolic effects (Funder and Sheppard 1987). The type I receptor has high affinity but low binding capacity for the endogenous glucocorticoid such as corticosterone (Funder and Sheppard 1987). The type II

receptor has low binding affinity and large binding capacity for corticosterone. DEX, a synthetic glucocorticoid, has high affinity for the type II receptor but also has affinity for the type I receptor (Funder and Sheppard 1987). Therefore, it is unclear which type of glucocorticoid receptor is involved in the rapid metabolic actions of icv-administered DEX in ob/ob mice.

RU-486, a type II glucocorticoid receptor antagonist, has helped determine the type of receptor involved in glucocorticoid-induced metabolic changes. Acute icv injection of RU-486 raised overall metabolic rate, as assessed by oxygen consumption, 2-3 h after the injection in Sprague Dawley rats (Hardwick et al. 1989). This rapid effect of RU-486 also appears to be mediated by the CNS since it is inhibited by a beta-adrenergic antagonist-propranolol and by surgical denervation of the sympathetic nerves innervating the BAT (Hardwick et al. 1989). Thus, I propose that type II-like glucocorticoid receptors are likely to mediate the metabolic changes induced by a single icv injection of DEX in ADX ob/ob mice.

Glucocorticoid receptors are known to regulate gene expression mostly with long lag (hours to days) in eukaroyates (McEwen 1986, 1991). However, emerging evidence suggest that steroids, including glucocorticoids, also elicit membrane effects that occur within sec (McEwen 1991). Thus, rapid effects of DEX on BAT thermogenesis and on plasma insulin concentration in ADX ob/ob mice may not be

compatible with alterations in gene expression of DEX-regulated proteins, but may be mediated by acute actions at the neuronal plasma membranes. Therefore, I proposed to determine if the rapid effects of icv DEX required protein synthesis. I also propose that DEX may influence release of neuromodulators which in turn regulates the neuronal drives to the BAT and pancreas.

Neuropeptide Y (NPY) appears to be one of these neuromodulator candidates. NPY has been suggested to be an important central regulator of energy homeostasis (White 1993). Administration of porcine NPY into the brain induced rapid (within 15 min of injection) and dose-dependent increases in food intake and insulin secretion (Clark et al. 1984, 1985, Moltz and McDonald 1985). Further, a single icv injection of porcine NPY induced food intake by 400%, reduces BAT activity by 25% and increased plasma insulin by almost 100% within 30 min of injection, in both ADX ob/ob and lean mice (Walker and Romsos 1993). Although icv injection of DEX does not rapidly increase food intake as NPY does, the common effects of NPY and DEX on BAT thermogenesis and plasma insulin concentration in ADX ob/ob mice suggest that DEX may modulate NPY release via glucocorticoid receptors in the CNS. It is possible that icv-administered DEX stimulated more NPY release in the CNS of ADX ob/ob mice than in ADX lean mice, which leads to rapid alterations of BAT thermogenesis and plasma insulin concentrations in ADX ob/ob mice. Thus, I also propose to

determine if DEX rapidly modulates NPY content and release in the CNS of ADX ob/ob and lean mice.

Corticotropin releasing hormone (CRH), may also be involved in the relationship of glucocorticoids and NPY. Glucocorticoids were known to inhibit CRH neuron activity through a negative feedback loop (Antoni 1986, Taylor and Fishman 1988). CRH administered ventricularly has been shown to depress food intake and increase metabolic rate in rats (Arase et al. 1988). Therefore, effects of icv DEX in ADX ob/ob mice may be mediated by rapid inhibition of CRH release. Studies also show that CRH seems to inhibit NPY secretion (Morley et al. 1985). Thus, the DEX-induced rapid decrease in CRH secretion may increase NPY secretion in the CNS, which in turn decreases metabolic rate and increases plasma insulin concentrations in ADX ob/ob mice. The overall scope of my research is to examine acute action mechanisms of glucocorticoid on BAT thermogenesis and plasma insulin concentration in ADX ob/ob and ADX lean mice.

The objectives of this dissertation were to determine if:

1) effects of DEX in ADX ob/ob mice were mediated via type II-like glucocorticoid receptors in the CNS.

2) the rapid-onset responses to icv injection of DEX in ADX ob/ob mice require protein synthesis.

3) DEX elicits rapid actions in ADX ob/ob mice via regulations of NPY release in the brain.

CHAPTER II. REVIEW OF LITERATURE

A. ACTIONS OF GLUCOCORTICOIDS

1. EFFECTS OF GLUCOCORTICOIDS ON OBESITY.

Adrenalectomy (ADX) reverses or reduces the symptoms of obesity in genetic, food-induced and brain lesion-induced models of obesity (Bruce 1982, Saito and Bray 1984, Romsos 1987). Chronic treatment of ADX ob/ob mice with cortisone acetate increased muscle weight, spleen weight and brain weight while both cortisone and deoxycorticosterone acetate increased food intake, adipose tissue mass and growth rate (Saito and Bray 1984). A similar study in which circulating corticosterone was restored to physiological concentrations in ADX ob/ob mice confirmed that corticosterone caused hyperphagia, hyperinsulinemia and increased weight gain in ADX ob/ob mice, but corticosterone was less effective in ADX lean mice (Tokuyama and Himms-Hagen 1987). The physiological concentrations of blood corticosterone (up to 10 μg/dl plasma) markedly decreased BAT GDP binding only in ADX ob/ob mice, but not in lean mice (Tokuyama and Himms-Hagen 1987). This oversensitivity of ob/ob mice to glucocorticoid is also observed after acute replacement of

glucocorticoids (Tokuyama and Himms-Hagen 1989, Walker and Romsos 1992). A single subcutaneous injection of corticosterone dramatically decreased BAT thermogenesis and increased plasma insulin concentration within 15 h to a greater extent in ob/ob mice than in lean mice (Tokuyama and Himms-Hagen 1989). Again, a single icv injection of DEX into ADX ob/ob mice rapidly (within 30 min) reverses the effect of ADX on BAT thermogenic activity and on plasma insulin, with only minimal effects in lean counterparts (Walker and Romsos 1992). These reports where various doses of glucocorticoids were employed suggest that ADX ob/ob mice are more sensitive to glucocorticoids. Therefore, the suppressed BAT thermogenic activity and hyperinsulinemia in ob/ob mice are not caused only by elevated serum glucocorticoids, but also by excessive sensitivity of ob/ob mice to glucocorticoids.

2. ROLE OF GLUCOCORTICOID RECEPTORS IN OBESITY DEVELOPMENT.

Glucocorticoids are thought to exert physiological responses by interacting with specific receptors. There are two major subtypes of glucocorticoid receptors in the CNS, type I and type II (Funder and Sheppard 1987). The type I receptor in the brain is structurally homogenous to the classical mineralocorticoid receptor found in the kidney. This type of receptor has high affinity but low binding capacity for endogenous glucocorticoids such as corticosterone (Funder and Sheppard 1987, Krozowski and

Funder 1983). The type II receptor in the CNS is identical to the classical glucocorticoid receptor found in liver, and it has low binding affinity and large binding capacity for glucocorticoids (Funder and Sheppard 1987, Krozowski and Funder 1983). Dexamethasone (DEX), has high binding affinity with type II receptor but also binds to type I receptor. The type I receptor is abundant in the septum and hippocampus whereas the type II receptor is widespread in the paraventricular nuclei (PVN), supraoptic nucleus, arcuate nucleus, thalamic nuclei, and all nuclei of the ascending aminergic pathways (Funder and Sheppard 1987).

Effects of adrenalectomy appear to be due to activation of type II glucocorticoid receptors because only glucocorticoid, not aldosterone, reverses all the effects of adrenalectomy and restores the development of obesity (Freedman et al. 1986). Peripheral administration of RU-28362, a specific type II receptor agonist, to ADX VMH-lesioned obese rat again suggests the role of type II receptor in restoring hyperphagia, weight gain and hyperinsulinemia (Thomas et al. 1994). The role of type II glucocorticoid receptor was further investigated by administering a type II receptor antagonist, RU-486 (Langley and York 1990, Okada et al. 1992, Harwick et al. 1989). Peripheral administration of RU-486 prevents the hyperphagia and obesity in Zucker (fa/fa) rats and diet-induced obese Osborne-Mendel rats (Langley and York 1990, Okada et al. 1992). Acute central application of RU-486 in Sprague-

Dawley rat increased overall metabolic rate, as assessed by oxygen consumption, by 2-3 h after the injection (Harwick et al. 1989). Nevertheless, it was suggested that type I receptor may be involved in energy metabolism. Type I receptor has been shown to stimulate fat intake in ADX rats whereas type II receptor activation restores carbohydrate intake (Tempel et al. 1991, Tempel and Leibowitz 1989). The type of receptor that mediates rapid effects of DEX on BAT thermogenesis and plasma insulin concentration in ADX ob/ob mice was unknown. Therefore, I tested the type of glucocorticoid receptors involved in rapid responses in ADX ob/ob mice following a single icv injection of DEX (see Chapter III).

3. LONG TERM ACTIONS OF GLUCOCORTICOIDS

Glucocorticoids belong to the steroid receptor superfamily that is best recognized for their genomic roles as transcription factors (McEwen 1986, 1991). Steroids bind to their receptors and then these steroid-receptor complexes regulate gene transcription and subsequently alter the amounts of glucocorticoid-regulated proteins (McEwen 1991). The time required for the onset of these physiological changes is usually hours to days (McEwen 1991). For example, treatment of adipose cells with DEX for 2 h decreased glucose transport activity and the amount of cytochalasin B-labeled glucose transporter in plasma membrane of adipocytes (Carter-Su and Okamoto 1987). This effect was not evident

when a transcription blocker (actinomycin D) or translation blocker (cycloheximide) was present (Carter-Su and Okamoto 1987). Glutamine synthetase activity is enhanced by glucocorticoid within 1 d of treatment (Holbrook et al. 1981). This increase in enzyme activity requires both transcription and translation to occur (Holbrook et al. 1981). These data suggest that protein synthesis is required for these glucocorticoid-induced effects. On the basis of these reports, it was unclear if rapid actions of DEX (within 30 min of icv injection) in ADX ob/ob mice on BAT thermogenesis and plasma insulin concentration were dependent on alterations in DEX-induced protein synthesis. Therefore, I propose to test whether protein synthesis was a prerequisite for DEX-induced metabolic responses in ADX ob/ob mice.

4. RAPID ACTIONS OF GLUCOCORTICOIDS

Besides long-term effects, emerging evidence suggest that steroids, including glucocorticoids, also elicit rapid effects on turnover of neurotransmitters, electrophysiologic characteristics and signal transductions by binding to specific membrane binding sites or receptors (Grollman and Huang 1973, Hardwick et al. 1989, Hollenberg et al. 1985, Hua and Chen 1989, Tempel and Leibowitz 1989, Orchinik et al. 1991). Some of these responses are proven independent of genomic actions of steroids.

4.1 STEROID-MODULATED NEUROTRANSMITTER TURNOVER

The well-known acute stress responses of glucocorticoids may be mediated through a non-genomic pathway. Parenteral administration of corticosterone in a dose that leads to a stress level of circulating corticosterone activates hippocampal cholinergic neurons within 10 min after the treatment (Gilad et al. 1985). *In vitro* incubation of stimulated nerve-muscle preparations with DEX for only 2 min enhances the presynaptic choline uptake (Veldsema-Currie et al. 1985). However, it remains unclear if these responses require genomic effects of glucocorticoid. In order to illustrate non-genomic effects of glucocorticoids in the CNS, synaptosomal preparations from the nerve endings lacking nuclei were prepared. Synaptosomal preparations from the rat hippocampus displayed increased acetylcholine release and reduced dopamine uptake within 10 min of methyl-prednisolone or DEX treatment (Gilad et al. 1987). These studies support nongenomic, rapid actions of glucocorticoids in regulating other neurotransmitter metabolism in the CNS. One of my objectives of study will be to determine if rapid effects of icv-administered DEX are dependent of protein synthesis (see Chapter III).

4.2 STEROID-INDUCED MEMBRANE EFFECTS (ION-CHANNELS)

Glucocorticoids were shown to modulate membrane

characteristics such as membrane potential, membrane resistance and ion-channel conductance (Hua and Chen 1989, Chen et al. 1991). Cortisol inhibits the norepinephrine-induced depolarization in the PVN of rats (Kasai and Yamashita 1988). Corticosterone, cortisol, DEX and triamcinolone, but not gonadal steroids, were found to stimulate $^{45}Ca^{2+}$ uptake in stimulated synaptosomes isolated from the cerebral cortex (Sze and Iqbal 1994a). These studies suggest that rapid effects of steroids are specific and possibly relate to membrane ion channels that in turn trigger membrane-coupled signal transduction pathways.

Progesterone also rapidly modulates neural activity by a mechanism that possibly involves ion channels (McEwen 1991). Progesterone and its metabolites have been reported to rapidly activate calcium channels in plasma membrane of human sperm (within sec), increase dopamine release from striatal tissue and increase LHRH release from the hypothalamus (Schumacher 1990). Infusion of progesterone into the ventromedial nuclei of hypothalamus increases oxytocin receptor binding (fmol/mg protein) within 30 min (Schumacher et al. 1991). This effect is not blocked by anisomycin, a protein synthesis inhibitor, suggesting a nongenomic mode of action (Schumacher et al. 1991).

Emerging evidence also show that 1,25-dihydroxyvitamin D3 rapidly (seconds to minutes) increases cytosolic Ca^{2+} levels in hepatocytes, osteoblasts and a variety of other cell types (Baran and Milne 1986, Walters 1992). An

osteoblast-like cell line incubated with 2 pM of 1,25-dihydroxyvitamin D_3 for 15 min increased $^{45}Ca^{2+}$ accumulation in the cells by 10-20% (Walters 1992). Actinomycin did not alter these rapid actions, suggesting that de novo protein synthesis was not required.

4.3 STEROIDS-MODULATED SIGNAL TRANSDUCTIONS

Studies have suggested that glucocorticoids may modulate signal transduction pathways, such as adenylate cyclase, protein kinase C, calcium channel and calmodulin systems (Harrelson et al. 1987, McEwen et al. 1986, Kato et al. 1992, Sze and Iqbal 1994a and b). ADX enhanced vasoactive intestinal peptide (VIP)- and isoproterenol-stimulated cAMP accumulations and decreased the response to histamine. Treatment with DEX for 48 h induced the opening of dihydropyrine-sensitive Ca^{2+} channels in A7r5 rat aortic smooth muscle cell line (Kato et al. 1992). This effect was abolished by protein kinase C inhibitor, staurosporine and UCN-01, suggesting protein kinase C as a second messenger (Kato et al. 1992). These studies, however, suggest that induction of protein synthesis is required (Harrelson et al. 1987, Kato et al. 1992).

Recent evidence suggests that corticosterone acts on outer plasma membrane of synaptosomes to enhance calmodulin binding by increasing affinity and decreasing dissociation (Sze and Iqbal 1994a). Further study also suggests that corticosterone, DEX, cortisol, and triamcinolone increase

depolarization-dependent Ca^{2+} uptake (Sze and Iqbal 1994b). Nitrendipine and nifedipine (20 μM) inhibit glucocorticoid-induced Ca^{2+} intake, suggesting that the dihydropyridine site of calcium channel is associated with this effect (Sze and Iqbal 1994b). Disrupted synaptic membranes also respond to the glucocorticoid preincubation if Ca^{2+}-calmodulin and Mg^{2+}-ATP are added to the preparation (Sze and Iqbal 1994b). Therefore, it is possible that glucocorticoids acts on membrane receptor-like proteins to enhance depolarization-dependent Ca^{2+} influx by promoting calmodulin-dependent modulation of Ca^{2+} channels.

The rapid effect of 1,25-dihydroxyvitamin D3 on intracellular Ca^{2+} concentration in osteoblast-like cells may also relate to signal transduction systems that would enhance the opening of Ca^{2+} channels since EGTA and calcium channel blockers abolished this event (Lieberherr 1987). Further studies suggest that 1,25-dihydroxyvitamin D_3 rapidly enhanced membrane phospholipase C activity that caused an increase in inositol triphosphate, a mediator involved in hormone-induced cellular calcium increment (Civitelli et al. 1990).

4.4 MEMBRANE BINDING SITES/RECEPTORS OF STEROIDS.

Since several lines of evidence point to nongenomic mechanisms that involve the plasma membrane, these steroid hormones may have binding sites or receptors on the plasma membrane. A binding study suggests the presence of a

specific binding site for glucocorticoids on synaptic plasma membranes of rat brain (Towle and Sze 1983). Some rapid glucocorticoid actions may be mediated through a type II-like membrane receptor since RU-486 blocked these effects (Hua et al. 1989). Binding of corticosterone to plasma membrane has been suggested in neurons of amphibian (Orchinik et al. 1991) and coeliac ganglion preparations of adult male guinea pigs (Hua et al. 1989).

Besides the discoveries of membrane sites for glucocorticoids, a plasma membrane progesterone binding protein also has been reported recently (Tischkau and Ramirez 1993). Vitamin D may also elicit nongenomic actions through a receptor different from the previously characterized vitamin D receptor. 1,25-dihydroxyvitamin D_3 increases intracellular calcium concentration in an osteoblast-like cell line lacking the classical vitamin D receptor, suggesting distinctive receptors for the rapid, nongenomic effect (Baran et al. 1991). One-beta epimer of 1,25-dihydroxyvitamin D3 does not interact with the classic cytosolic/nuclear vitamin D receptor, but it inhibited the rapid effect of 1,25-dihydroxyvitamin (Baran et al. 1991). This finding again suggests that rapid actions of 1,25-dihydroxyvitamin D3 may be mediated through a membrane binding site (Holick et al. 1980, Baran et al. 1991).

The DEX-induced metabolic alterations in ADX ob/ob mice occur relatively rapidly, within 30 min of icv injection (Walker and Romsos 1992). This rapid onset may not be

compatible with alterations in DEX-regulated proteins, but may be mediated by acute actions at the neuronal plasma membranes.

4.5 POSSIBLE RELATIONSHIP BETWEEN GENOMIC AND NONGENOMIC EFFECTS OF STEROIDS

The connection between nongenomic and genomic actions of glucocorticoid is yet to be established. However, the 1α,25-dihydroxyvitamin D_3-induced transcription at 1 hour was attenuated when its rapid effects were inhibited by another vitamin D metabolite (Baran et al. 1992). This result indicates that the rapid nongenomic actions of 1α,25-dihydroxyvitamin D_3 have a functional role in its genomic actions.

B. ACTIONS OF NEUROPEPTIDE Y (NPY)

1. INTRODUCTION OF THE NPY SYSTEM IN THE CNS.

NPY belongs to a peptide family that consists of peptide tyrosine-tyrosine and pancreatic polypeptide (Tatemoto et al. 1982). NPY is the most prevalent neuropeptide in the brain and it also exists in the peripheral nervous system (Tatemoto et al. 1982). The precursor of biologically active NPY, proNPY, is produced in the endoplasmic reticulum and is further cleaved to produce free acid NPY and C-flanking peptide of the proNPY (Mutt

1989). The free acid NPY is further amidized at the C-terminal (Mutt 1989). NPY distributes widely in the CNS including the cortex, brain stem, hippocampus, hypothalamus, amygdala and thalamus. There are 3 major NPY projection systems (Chronwall 1989). One system projects from ventromedial geniculate nucleus that innervates the suprachiasmatic nucleus (Chronwall 1989). Another system coexists with catecholaminergic cells in the pons-medulla and projects to the hypothalamus (Chronwall 1989). The other system projects within the hypothalamus from the arcuate nucleus to the PVN (mainly parvocellular subdivision), dorsomedial hypothalamic nuclei and the medial preoptic nucleus and mesencephalic gray (Chronwall 1989).

2. SUBTYPES OF NPY RECEPTORS IN THE CENTRAL NERVOUS SYSTEM

There are 3 major subtypes of NPY receptors, Y1, Y2 and Y3, based on functional and binding studies (Wahlestedt et al. 1990, Larhammar et al. 1992). The Y1 receptor binds to NPY and PYY but not C-terminal fragment of NPY while Y2 receptor binds to NPY, PYY and C-terminal fragment of NPY (Wahlestedt et al. 1990). Y3 receptor can be activated by NPY but not by PYY (Larhammar et al. 1992). The agonist for Y1 receptor, [Ile^{31}, Pro^{34}]NPY and [Pro^{34}]NPY and the agonist for Y2 receptor, NPY13-36, are used as tools to localize the subtypes of NPY receptors and the functions the receptors mediate (Wahlestedt et al. 1990).

Y1 receptor subtype is found most abundant in cortex, the dentate gyrus of the hippocampal formation, the claustrum, and the reuniens nucleus of the thalamus (Dumont et al. 1993). The Y2 receptor subtype is present in most other brain regions including hypothalamus (Dumont et al. 1993). Y3 receptors have not been mapped. However, Y3 receptors may mediate catecholamine release from chromaffin cells, atrial natriuretic factor secretion and cardiovascular responses (Larhammar et al. 1992). Thus, its binding sites possibly exist in chromaffin cells and myocardium (Larhammar et al. 1992).

Studies have suggested that Y1- and Y2-receptors belong to the G-protein coupled superfamily of receptors (Wahlestedt et al. 1990). Y1 receptors are coupled to increases in Ca^{2+} influx, phosphoinositidase activity and decreases in cAMP (Wahlestedt et al. 1990). In contrast, Y2 receptor relates to increase in cAMP and decrease in Ca^{2+} influx (Wahlestedt et al. 1990). The signalling pathways of Y3 receptor remain to be investigated.

3. NPY AND FOOD INTAKE

NPY is an important regulator of energy balance. It exerts rapid actions in feeding behavior and other metabolic events. Administration of porcine NPY into the third ventricle induced immediate (within 15 min of injection) and dose-dependent increases in food intake in both satiated female and male Sprague-Dawley rats (Clark et al. 1984,

Clark et al. 1985). Injections of NPY to various specific sites indicate that the perifornical hypothalamus at the level of caudal PVN is the most sensitive site to enhance feeding (Stanley et al. 1993). An single icv injection of porcine NPY was shown to increase food intake rapidly (within 30 min after injection) in both ADX ob/ob and ADX lean mice in contrast with ineffectiveness of icv-administered DEX in these mice (Walker and Romsos 1993).

NPY also exerts long-term effects on feeding. Chronic (2 wk) icv infusion of NPY (0.44 μg/h) in male adult Long-Evans rats increased food intake starting on the first d and lasting for the entire 9 d of continuous NPY infusion (Beck et al. 1992). The overall feeding behavior of chronically NPY-infused normal rats mimic feeding behavior in obese rodents; hyperphagia, overweight, and altered diurnal pattern of food intake were evident.

These studies suggest that NPY exerts both immediate and long-lasting effects that would cause obesity. Therefore, altered NPY synthesis and release could play a role in the etiology of obesity.

NPY-induced feeding is regulated by glucocorticoids and other hypothalamic neurotransmitter systems (Kalra et al. 1988, Stanley et al. 1989, Heinrichs et al. 1993, Menzaghi et al. 1993, Morley et al. 1985). Adrenalectomy reduces NPY-induced feeding while glucocorticoid replacement restores the full effects of NPY on feeding (Kalra et al. 1988, Stanley et al. 1989). It is not clear if the effect

of adrenalectomy is due to glucocorticoid per se or partially through corticotropin-releasing hormone (CRH) since adrenalectomy is known to enhance hypothalamo-pituitary-adrenal axis activity. Administration of α-helical CRH (CRH antagonist) to the PVN causes inactivation of PVN CRH neurons and enhances NPY-induced feeding (Heinrichs et al. 1993, Menzaghi et al. 1993). Conversely, dynorphin, which is known to stimulate feeding through κ opioid receptors, may potentiate the effect of NPY on feeding (Morley et al. 1985). Since glucocorticoid and CRH regulate NPY actions, I hypothesize that these two factors may both rapidly regulate NPY release.

NPY also seems to play a role in macronutrient selection. Short-term studies support the role of NPY in the preference for carbohydrate diets (Stanley et al. 1985, Morley et al. 1987). However, long-term administration of NPY (1 μg, every 8 h for 6 d) into the PVN increased both daily carbohydrate (+26.4 kcal/d) and fat (+48.5 kcal/d) intake with no significant potentiation of protein intake in female rats (Stanley et al. 1989). The preference for fat intake seems to be greater than for carbohydrate intake with long-term treatment of NPY, which is in contrast to the acute effect of NPY. Furthermore, NPY may actually induce whatever nutrient the animal normally prefers (Welch et al. 1993). Therefore, the diet selection effect of NPY is not conclusive.

4. NPY, INSULIN SECRETION AND BAT THERMOGENESIS

NPY not only promotes food intake, it also enhances insulin secretion and suppresses energy expenditure, which possibly causes obesity. The role of NPY in insulin secretion depends on the site of action. In rats, ADX ob/ob and lean mice, icv injections of porcine NPY significantly elevated circulating insulin within 5-30 min (Moltz and McDonald 1985, Dunbar et al. 1992, Walker and Romsos 1993). This increase may be mediated by the nucleus tractus solitarius (Dunbar et al. 1992). However, the secretion of insulin from isolated pancreatic islets incubated with NPY was inhibited (Moltz and McDonald 1985, Opara et al. 1991). These observations imply that the role of NPY in insulin secretion is site-specific. Centrally-administered NPY enhances insulin secretion or decreases insulin turnover through the CNS while NPY receptors on pancreatic islets mediate suppressive effects on insulin secretion.

NPY administered icv rapidly suppressed BAT thermogenesis as assessed by GDP-binding to uncoupling proteins in both ADX lean and ob/ob mice (Walker and Romsos 1993). Long-term infusion of NPY to the PVN down-regulates the mRNA of the uncoupling protein in rats (Billington et al. 1991). These findings suggest that central effects of NPY down regulate BAT activity.

In conclusion, NPY administered centrally exerts similar rapid effects on BAT thermogenesis and on plasma insulin concentration in ADX ob/ob and ADX lean mice. These

effects of NPY are similar to the effects exerted by a icv injection of DEX in ob/ob mice while ADX lean mice are irresponsive to DEX. Thus, I proposed that NPY may mediate effects of DEX on BAT thermogenesis and on plasma insulin concentration in ADX ob/ob mice. DEX may regulate endogenous NPY secretion to a greater extent in ADX ob/ob mice that in ADX lean mice.

5. NPY AND NEUROENDOCRINE SYSTEM

NPY modulates actions of other neurotransmitters or neurohormones. Central injection of NPY decreases the concentration of serotonin (appetite suppressor) and epinephrine (appetite enhancer) in the CNS (Shimizu and Bray 1989). These observations suggest that NPY rapidly modulate endocrine systems that are related to feeding.

NPY may contribute to the infertility of ob/ob mice. NPY rapidly inhibits sexual behavior while elevating feeding behavior (Clark et al. 1985). Icv injection of NPY (0.85-20 μg) to adult male and ovariectomized female Sprague-Dawley rats drastically suppressed sexual behavior 10 min following the injection (Clark et al. 1985). NPY induces luteinizing hormone (LH) secretion from the pituitary in gonad-intact rats and rabbits, and in ovariectomized rats pretreated with ovarian steroids (Kalra et al. 1990). Further studies indicate that NPY acts on both the hypothalamus and pituitary to regulate LH secretion. NPY stimulates LH-releasing hormone (LHRH) release at the hypothalamus and

potentiates the release of LH induced by LHRH at the pituitary (Kalra et al. 1990). Pre-existing gonadal hormone seems to be necessary for communication between hypothalamic NPY and LHRH neurons. In general, NPY fails to change the basal LHRH release from the medial basal hypothalamus of ovariectomized rats, but priming with either estradiol 17ß alone or in combination with progesterone resulted in stimulation of LHRH release by NPY. The enhancing effect of NPY at the pituitary level is very weak as compared to that of the physiological releaser LHRH (Kalra et al. 1990). However, NPY augments release of LH when LHRH is present in a dose-dependent fashion, which is likely due to enhanced LHRH binding to its receptors on pituitary membrane (Kalra et al. 1990).

6. ABNORMAL NPY AND mRNA LEVELS IN OBESE ANIMALS.

Based on the stimulatory effect of NPY on energy intake, hyperphagic characteristic of obese animals may result from altered NPY synthesis or secretion. Several studies support this theory (Sanacora et al. 1992, McKibbin et al. 1991, Williams et al. 1989, Wilding et al. 1992, Wilding et al. 1993). The levels of preproNPY mRNA and NPY content are elevated in obese Zucker rats in the whole hypothalamus as well as specific hypothalamic regions such as PVN, dorsomedial nucleus, and arcuate nucleus (Sanacora et al. 1992, McKibbin et al. 1991). Streptozocin-induced diabetic rats also have increased NPY concentrations in the

PVN, dorsomedial nucleus and lateral hypothalamic area (Williams et al. 1989). In diet-induced obese rats, NPY contents were elevated in the PVN, arcuate nucleus, medial preoptic area, and the anterior hypothalamus, with no change in NPY mRNAs (Wilding et al. 1992). Ob/ob mice also have higher levels of hypothalamic NPY mRNA than in lean mice (Wilding et al. 1993). However, NPY content in the whole brain has been shown similar in ob/ob and lean mice, possibly because changes only occur in certain specific hypothalamic areas or the release of NPY is greater in ob/ob mice than in lean mice (Wilding et al. 1993). Therefore, continuous greater release of NPY could possibly contribute to obesity development.

7. REGULATIONS OF NPY mRNA LEVEL, CONTENT AND SECRETION BY FOOD INTAKE

NPY content in the suprachiasmatic nucleus displays a circadian rhythm with a peak at 12:00 midnight and an abrupt increase at dark-light transition point (Shinohara et al. 1993). NPY content in the parvocellular subdivision of PVN of normal rats elevates before the dark and decreases rapidly after the onset of the dark period (Jhanwar-Uniyal et al. 1990). These reports suggest that NPY synthesis or secretion corresponds to NPY-stimulated feeding.

As a powerful modulator of food intake, synthesis and release of NPY are greatly influenced by metabolic states. Several lines of studies have shown reciprocal changes in

mRNA level or NPY content upon food deprivation and refeeding. As short as a 24 h of food deprivation increases hypothalamic NPY mRNA by 200% over the levels in the ad libitum fed C57BL/6J mice (the genetic background of ob/ob mice) (Chua et al. 1991). Refeeding for 24 or 48 h after 24-h food deprivation tends to decrease NPY mRNA level, but not to the level in the satiated counterparts (Chua et al. 1991). Two-day food deprivation significantly increases NPY mRNA level by 130% in the arcuate nucleus of hypothalamus where NPY cell bodies aggregate (Schwartz et al. 1993). Longer term (2 wk) food restriction significantly raised NPY mRNA in both lean and obese Zucker rats to a similar level (Pesonen et al. 1992). Therefore, I conclude that food deprivation (at least 1 d) generally increases NPY mRNA abundance that is gradually reversed by refeeding.

Hypothalamic NPY content also elicits reciprocal changes with food deprivation and refeeding regimen. Two-day food deprivation drastically increased NPY content in the arcuate nucleus-medium eminence region by 11 fold and NPY content in the PVN region by 4 fold as compared to the levels in the satiated animals (Beck et al. 1990a). A period of 6-h refeeding was unable to lower the NPY content in the PVN, but was able to restore the NPY content in the arcuate nucleus-medium eminence region to the level of satiated controls (Beck et al. 1990a). However, NPY contents in the dorsomedial, ventromedial, suprachiasmatic and supraoptic nuclei did not respond to this treatment

(Beck et al. 1990a). Longer-term food deprivation (3-4 d) followed by one day refeeding in male rats exerts site-specific changes in NPY content (Sahu et al. 1988). NPY content in the arcuate nucleus of the hypothalamus increased at end of food deprivation and remained high even after 1 d of refeeding (Sahu et al. 1988). NPY level in the PVN increased gradually with food deprivation, but fell dramatically to the control level after 1 day of refeeding (Sahu et al. 1988). Medial preoptic area of hypothalamus does not respond to food deprivation, but its NPY level increases with refeeding (Sahu et al. 1988). Consistant with the observation of Beck et al. (1990a), NPY contents in the dorsomedial and ventromedial nuclei did not change with food deprivation (Sahu et al. 1988). Therefore, NPY mRNA and NPY content consistently increase in the arcuate nuclei with food restriction and either decrease or remained high even after 1 day of refeeding. NPY content in the PVN increased gradually with food deprivation and was restored to the satiated level after refeeding. The increases in both mRNA and content of NPY suggest an increase in secretion with food deprivation.

It has been suggested that *in vivo* NPY release in the PVN, as assessed by a push-pull cannula, tightly relates to increased appetite for food (Kalra et al. 1991). When rats were trained to eat only in 4 h every day, NPY release in the PVN was 30% greater during the non-feeding period than the feeding period. NPY secretion decreased with feeding to

the level of ad libitum-fed rats at the end of the feeding period (Kalra et al. 1991). When rats were deprived of food for 3 d and then refed for only 2 h, NPY release in the PVN was consistently greater (by four fold increase) than that secreted by ad libitum-fed rats over the 2-h refeeding period (Kalra et al. 1991). An in vitro study confirmed the role of food deprivation in NPY secretion in the hypothalamus using the micropunched PVN and the dorsomedial hypothalamus (Dube et al. 1992). The basal as well as KCl-stimulated in vitro NPY secretion from the PVN in 4-day food deprived rats were greater than that in satiated rats (Dube et al. 1992). Consistent with other studies on NPY content in the DMH, neither the basal nor KCl-stimulated in vitro secretion of NPY from the DMH was influenced by food deprivation (Dube et al. 1992). Therefore, I conclude that long-term food restriction and increased appetite consistently enhance NPY synthesis and release.

The types of macronutrients that modulate NPY gene expression and content upon macronutrient restriction were therefore examined (Beck et al. 1990b, Jhanwar-Uniyal et al. 1993, White et al. 1994). Long-Evans rats were fed a balanced, high carbohydrate (69.9% energy from carbohydrate), or high fat (67.9% energy from fat) diet for 2 wk (without energy restriction) and then NPY contents in several hypothalamic and extra-hypothalamic regions were determined (Beck et al. 1990b). NPY contents in all the extra-hypothalamic regions and most hypothalamic regions

including the dorsomedial, ventromedial, suprachiasmatic nuclei, magnocellular part of PVN, arcuate nucleus, supraoptic nucleus and medial eminence were not different from each other (Beck et al. 1990b). However, NPY content in the parvocellular part of PVN was the lowest in the high carbohydrate group whereas NPY content in the lateral hypothalamus was the lowest in the high fat group. When free selections of pure macronutrient diets were provided for 3 wks, NPY levels in the parvocellular part of the PVN, arcuate nucleus and dorsomedial hypothalamus were higher in the rats consuming more carbohydrate then the rats consuming less carbohydrate (Jhanwar-Uniyal et al. 1993). The effects carbohydrate on NPY content in the PVN in these two studies seem contradictory. A recent study that manipulates the macronutrient content and energy intake, suggests that low protein intake, instead of carbohydrate or fat, enhances NPY gene expression in the basomedial hypothalamus of rat (White et al. 1994).

The response of hypothalamic NPY content to metabolic conditions lacking carbohydrate or fat metabolism were also examined. 2-deoxy-D-glucose and mercaptoacetate which blocks carbohydrate and fat metabolism, respectively, were injected intraperitoneally (Akabayashi et al. 1994). This study found that only 2-deoxy-D-glucose, not mercaptoacetate, significantly potentiated NPY levels in the arcuate nucleus and suprachiasmatic nuclei of the hypothalamus (Akabayashi et al. 1994), suggesting that lack

of carbohydrate metabolism signals NPY synthesis. Therefore, the roles of macronutrients in NPY content or synthesis need more investigation.

8. REGULATION OF NPY CONTENT, mRNA AND SECRETION BY GLUCOCORTICOIDS.

Glucocorticoids have been shown to modulate NPY level and gene expression. Daily intraperitoneal administration of DEX to intact female rats for 5 d increased NPY content in the mediobasal hypothalamus by 117% (Corder et al. 1988). In another chronic (7 d) study, administration of DEX significantly raised NPY concentrations in the PVN (by 45%), but not in the basomedial hypothalamus (including arcuate, dorsomedial and ventromedial hypothalamus nuclei), lateral hypothalamus and medial preoptic area (McKibbin et al. 1992). In an acute study where plasma insulin concentration and body weight remain similar to the control levels, peripheral injection of DEX to intact Wistar rats increased NPY level in the lateral hypothalamus, after 4 h of injection (McKibbin et al. 1992). Therefore, long-term effect of DEX seems to increase NPY content in the PVN and short-term effect of DEX seems to increase NPY in the LH. In these *in vivo* studies, the changes of NPY level in the PVN and arcuate nucleus are not consistent with DEX treatment. NPY content relates to the balance between synthesis, transport and release. Thus, the effects of DEX on NPY synthesis and release are not conclusive from these studies. However, NPY-like immunoreactivity of cultured

hypothalamic neurons increased with DEX in a dose-dependent fashion (Corder et al. 1988) suggesting stimulatory mode of action.

Studies regarding effects of glucocorticoid on NPY mRNA may give more direct support to NPY synthesis. Adrenalectomy in Sprague-Dawley rats decreased NPY mRNA level in the arcuate nucleus, but not in the rest of hypothalamus (excluding the arcuate nucleus) or the brain stem (White et al. 1990). This decrease in NPY gene expression was reversed by corticosterone replacement (White et al. 1990). Administrations of type I and type II glucocorticoid receptor agonists have suggested that type II receptor is responsible for this increased NPY gene expression in the basomedial hypothalamus (White et al. 1994). The regulatory roles of glucocorticoid in NPY content and mRNA level are modes of glucocorticoid actions in development of obesity.

As discussed previously, obese rodent models possessing high plasma corticosterone level often exhibit greater NPY gene expression. ADX seems to decrease the NPY mRNA and restores metabolic balance in obese rodents. A single icv injection of DEX rapidly (within 30 min) suppresses BAT thermogenesis and increases plasma insulin concentration in ADX ob/ob mice. This rapidity of effects may not allow alterations in NPY gene expression caused by the genomic role of DEX. Thus, metabolic effects of DEX on BAT thermogenesis and on plasma insulin concentration are

probably due to an increase in NPY secretion without an increase in NPY synthesis. Unchanged synthesis and increased secretion that result from DEX administration should cause a decrease in NPY content. I therefore proposed a decrease in NPY content and increase in release induced by icv DEX treatment in ADX ob/ob.

9. REGULATION OF NPY CONTENT, mRNA AND SECRETION BY CRH.

Altered hypothalamo-pituitary-adrenal axis is one of the CNS-mediated abnormalities in obesity (Saito and Bray 1984). It has long been appreciated that corticosterone and DEX exhibit negative feedback on hypothalamic CRH production (Moldow and Fischman 1982). Adrenalectomy therefore enhances the CRH content, as assessed by immunostaining technique, in the parvocellular subdivision of PVN (Merchenthaler et al. 1983). A single injection of CRH (5 μg) to the third ventricle rapidly (within 30 min) decreases food intake and increases GDP binding to mitochondria from interscapular brown adipose tissue in normal rats (Arase et al. 1988). In the VMH-lesioned obese rat, 5 μg of CRH rapidly (within 1 h) increased GDP binding and suppresses food intake even after deprivation (Arase et al. 1988). However, the same dose of CRH administered icv did not increase GDP binding to BAT in intact ob/ob mice but decreased plasma insulin concentrations to 1/7 of that in the saline-injected ob/ob mice (Walker and Romsos 1992, Drescher et al. 1994). When CRH release is enhanced by ADX

in ob/ob mice, a CRH receptor antagonist, α-helical CRH_{9-41}, was able to increase plasma insulin concentrations by 50% but still fail to affect BAT thermogenesis (Walker and Romsos 1992). On the basis of these reports, CRH may partially modulate rapid effects of DEX in the CNS of ADX ob/ob mice.

CRH may modulate NPY neuron activities since NPY terminals at the PVN are in proximity to CRH-containing cell bodies (Sawchenko et al. 1984). CRH may act to diminish NPY-induced effect. Co-administration of CRH and NPY blunts NPY-induced feeding (Morley et al. 1985). Co-administration of α-helical CRH and NPY to the PVN significantly potentiates the NPY-induced feeding (Heinrichs et al. 1993). Similarly, inactivation of CRH neurons in the PVN augments NPY-induced feeding (Menzaghi et al. 1993). It has also been suggested that CRH may inhibit NPY secretion (Morley et al. 1985). Further study showed that NPY-induced feeding was potentiated in Wistar rats with 6 hr of dexamethasone pretreatment at a dose known to downregulate CRH neuron activity (Heinrichs et al. 1993). Since DEX could act rapidly to downregulate CRH that presumably inhibits NPY secretion (Moldow and Fischman 1982), I plan to examine if CRH antagonist, α-helical CRH, rapidly enhances NPY secretion and decreases NPY content in the CNS as does DEX.

CHAPTER III. TYPE II GLUCOCORTICOID RECEPTORS IN THE CNS REGULATE METABOLISM IN OB/OB MICE INDEPENDENT OF PROTEIN SYNTHESIS

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A. Abstract

A single intracerebroventricular injection of dexamethasone rapidly (within 30 min) decreases brown adipose tissue thermogenesis by 25% as assessed by GDP binding and increases plasma insulin twofold in adrenalectomized ob/ob mice. The present study investigated the type of corticoid receptor(s) that mediate these effects and determined whether protein synthesis was necessary for expression of these glucocorticoid actions in ob/ob mice. Intracerebroventricular injection of aldosterone (a type I-corticoid receptor agonist) was ineffective in altering peripheral metabolism in adrenalectomized ob/ob mice, whereas RU-486 (a type II-corticoid receptor antagonist) abolished the effects of dexamethasone. Thus, type II-like corticoid receptors, not type I receptors, mediated the rapid effects of dexamethasone in adrenalectomized ob/ob mice. Anisomycin (0.5 mg) administered subcutaneously almost completely suppressed (-92%) cerebral protein synthesis but anisomycin did not abolish the rapid effects of dexamethasone in adrenalectomized ob/ob mice. Thus,

protein synthesis is not a prerequisite for rapid effects of dexamethasone in adrenalectomized ob/ob mice.

B. INTRODUCTION

Genetically obese (ob/ob) mice display many abnormalities including suppressed brown adipose tissue (BAT) thermogenesis, hyperinsulinemia and high plasma corticosterone (Bray 1984, Bray and York 1979, Kim and Romsos 1988). Removal of the adrenal glands normalizes these alterations (Bray 1984, Kim and Romsos 1988), and peripheral replacement of glucocorticoids reverses these effects of adrenalectomy within 6 to 15 h (Tokuyama and Himms-Hagen 1989). In follow-up studies, we demonstrated that a single intracerebroventricular injection of dexamethasone rapidly (within 30 min) decreased brown adipose tissue (BAT) thermogenesis by 25% and increased plasma insulin twofold in adrenalectomized ob/ob mice, with minimal effects in the lean counterpart (Walker and Romsos 1992). Injection of a similar amount of dexamethasone intraperitoneally failed to influence metabolism within 30 min in ob/ob mice (Walker and Romsos 1992). These results suggest that glucocorticoids are likely to act within the central nervous system to stimulate the initiation of obesity development in ob/ob mice.

There are two major types of corticoid receptors in the central nervous system, type I and type II (Ahima and Harlan 1990, Ahima et al. 1991, Arriza et al. 1981, Hollenberg et

al. 1985, Krozowski and Funder 1983). The type I receptor in the brain is identical to the classical mineralocorticoid receptor found in the kidney, and it has high affinity and low binding capacity for endogenous glucocorticoids (Ahima et al. 1991, Krozowski and Funder 1983). The type II receptor in the central nervous system is identical to the classical glucocorticoid receptor in liver, and it has lower affinity and greater binding capacity for endogenous glucocorticoids (Ahima and Harlan 1990, Krozowski and Funder 1983). The Type II receptor has greater affinity for dexamethasone, the synthetic glucocorticoid we injected intracerebroventricularly in ob/ob mice (Walker and Romsos 1992, 1993), than does type I receptor (Krozowski and Funder 1983). Therefore, the rapid changes in BAT thermogenesis and in plasma insulin in ob/ob mice observed after intracerebroventricular injection of dexamethasone may be mediated by the type II receptor, although these findings do not rule out a role for the type I receptor. RU-486, a specific type II-like corticoid receptor antagonist with little interaction with type I receptor (Philibert 1984), has been shown to affect metabolic rates within 40 min of intracerebroventricular injection in rats (Harwick et al. 1989). This rapid effect of RU-486 also suggests a role for type II-like corticoid receptors in these actions.

Glucocorticoid-receptor complexes are translocated from the cytosol to the nucleus to modulate gene expression. The time-frame for glucocorticoid-induced changes in the amount

of glucocorticoid-regulated peptides normally exceeds 30 min (McEwen 1991). Therefore, the rapid changes observed in BAT activity and in plasma insulin after intracerebroventricular injection of dexamethasone in adrenalectomized ob/ob mice seem inconsistent with a genomically-mediated glucocorticoid action (Walker and Romsos 1992, 1993).

The present study was undertaken to 1) evaluate the relative contributions of type I vs. type II receptors in the rapid-onset, dexamethasone-induced metabolic effects in ob/ob mice and 2) determine if protein synthesis is necessary for these dexamethasone-induced effects. The role of type I corticoid receptors was examined by comparing intracerebroventricular administration of the type I receptor agonist aldosterone vs. administration of equal molar dexamethasone. RU-486, a type II receptor antagonist (Philibert 1984), was administered intracerebroventricularly simultaneously with dexamethasone to evaluate the role of type II receptors in ob/ob mice. RU-486 was also administered intracerebroventricularly to intact ob/ob mice to determine if blockage of type II receptors would rapidly reverse their low metabolic rates and high plasma insulin concentrations. Finally, anisomycin, a protein synthesis inhibitor (Flood et al. 1973, Grollman and Huang 1973), was administered to determine if cerebral protein synthesis is required for the rapid metabolic changes induced by dexamethasone in ob/ob mice.

C. MATERIALS AND METHODS

1. Animals

Male obese (ob/ob) mice were obtained from our breeding colony of C57 BL/6J- ob/+ mice. These mice were weaned at 3 wk of age and housed at 23-25°C in solid-bottom plastic cages with wood shavings for bedding. They were fed a non-purified diet (Rodent Laboratory Chow 5001, Purina Mills, St. Louis, MO) ad libitum. Room lights were on from 0700 h to 1900 h daily. National Research Council (NRC) guidelines (NRC 1985) for the care and treatment of mice were followed, and institutional approval was received.

Mice were adrenalectomized under ether anesthesia through dorsal incisions at 4.5-5 wk of age. Plasma corticosterone concentrations were determined to verify adrenalectomy (Walker and Romsos 1993). Mice with plasma corticosterone concentrations > 1 μg/dl (29 nmol/l) were excluded for data analyses. Plasma corticosterone concentrations averaged 0.44 ± 0.03 μg/dl in adrenalectomized ob/ob mice in this study. The adrenalectomized ob/ob mice received 0.9% NaCl to drink after surgery and were housed in groups of four mice per cage until experiments were conducted 2 wk later. Mice were individually housed with food and physiological saline (or water for intact mice), which were available on the day of the experiment.

2. Chemicals

Dexamethasone 21-phosphate disodium salt, aldosterone and anisomycin were obtained from Sigma Chemical Co. (St. Louis, MO). RU-486 (RU-38486; 17-hydroxy-1ß-(4-dimethylamino-phenyl)17ß-(1-propynyl)estra-4,9- dien-3-one) was donated by Roussel-UCLAF (Paris, France). Dexamethasone in experiment 1 and 2 and aldosterone and RU-486 were dissolved in an ethanol/saline mixture (5/95,v/v). Dexamethasone and anisomycin in experiment 3 were dissolved in saline.

3. Experimental Design

Experiment 1 was conducted to examine the roles of type I and type II corticoid receptors in mediating effects of intracerebroventricularly injected dexamethasone on GDP binding to BAT mitochondria and on plasma insulin. To determine involvement of the type I receptor, adrenalectomized ob/ob mice were injected with either vehicle, 250 ng (637 pmoles) dexamethasone or 230 ng (637 pmoles) aldosterone intracerebroventricularly between 1000 and 1100 h. To examine involvement of the type II receptor, the type II-like corticoid-receptor antagonist RU-486 (Philibert 1984) was employed. Adrenalectomized ob/ob mice were injected intracerebroventricularly with either vehicle, 250 ng dexamethasone, 28 µg RU-486 (63.7 nmoles, 100 fold more than the dexamethasone dose administered), or a combination of dexamethasone and RU-486. They were

decapitated 45 min later. Blood was collected for insulin, glucose and corticosterone assays. Interscapular and subscapular BAT depots were removed for subsequent measurement of BAT thermogenic activity as assessed by GDP binding to mitochondria.

Experiment 2 was carried out to examine effects of type II-like corticoid receptor blockage on the whole body metabolic rate, as assessed by whole body oxygen consumption. BAT thermogenesis, and plasma insulin in 6.5- to 7-wk-old intact ob/ob mice. Mice were injected intracerebroventricularly with vehicle or 28 μg RU-486. Oxygen consumption was measured at 25 ± 1°C before injection, and 45 min and 120 min after injection. BAT thermogenesis and plasma insulin were measured 45 and 120 min after injection. To examine the possibility that RU-486 leaked from the brain into the general circulation to exert peripheral actions, other mice received vehicle or 28 μg of RU-486 subcutaneously. Oxygen consumption was determined 45 min after subcutaneous injection.

Experiment 3 was conducted to determine if protein synthesis is a prerequisite for acute effects of dexamethasone on BAT activity and on plasma insulin. First, an appropriate dose of the protein synthesis inhibitor anisomycin was established to block cerebral protein synthesis in adrenalectomized ob/ob mice. Preliminary studies showed that 0.5 mg anisomycin was adequate. To ensure that protein synthesis was maximally inhibited before

examination of dexamethasone effects, we waited 30 min after injection of anisomycin before intracerebroventricular injection of dexamethasone. To measure effects of anisomycin on protein synthesis during the period for which we would examine effects of dexamethasone, 0.6 μCi [^{3}H]-leucine (179.6 μCi/mmole, New England Nuclear, Boston, MA) was injected intracerebroventricularly. To examine the time course of anisomycin action, animals were killed 40 or 75 min after anisomycin was injected subcutaneously (10 or 45 min after intracerebroventricular injection of [^{3}H]-leucine, respectively). Cerebral proteins were extracted according to Flood et al. (Flood et al. 1973). Protein synthesis inhibition = 1-(disintegration per min (dpm) brain in anisomycin-treated mouse/dpm in brain of control mouse). Next, we determined if the effects of dexamethasone were sustained after inhibition of protein synthesis. Adrenalectomized ob/ob mice received saline or 0.5 mg anisomycin subcutaneously 30 min before they were injected intracerebroventricularly with saline or 250 ng dexamethasone. Mice were lightly anesthetized with ether for both subcutaneous and intracerebroventricular injections. Animals were subsequently killed 45 min after intracerebroventricular injection. GDP binding to mitochondria, plasma insulin, plasma glucose and plasma corticosterone were determined.

4. Methods

Intracerebroventricular injection. Injections (2 μl) to one of the lateral ventricles were performed as previously described (Laursen and Belknap 1986, Walker and Romsos 1992). In brief, a Hamilton 701RN syringe was used. A 2-in. 26-gauge needle with 15 mm stiff Tygon tubing glued on the base of the needle was prepared. The needle was then cut 4.5 mm from the end of the Tygon 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. The depth of intracerebroventricular injection was therefore controlled. Each animal was lightly anesthetized with ether before intracerebroventricular injection. The needle tip was slightly rubbed on the midline of the skull until reaching the bregma, then the needle was moved 1.5 mm lateral to the midline. The skull is relatively thin at that location; therefore, only mild pressure was required to insert the needle into the brain. After mice were decapitated a drop of Evans Blue dye (Fisher Scientific) was applied into the needle tract of the skinned skull. The brain was then removed, preserved in 10% formalin for at least 1 day, and then sectioned along the needle tract. The position of the needle tract in the brain was traced by the Evans Blue dye. Only animals where the needle tract entered the ventricle were included in data analyses.

GDP binding. Thermogenic activity of BAT was assessed by measuring specific GDP binding to isolated BAT mitochondria

by methods described previously (Cannon and Lindberg 1979, Walker and Romsos 1992). Briefly, specific GDP binding was determined by subtracting total [^{3}H]-GDP binding measured in the presence of 10 μM unlabelled GDP from nonspecific binding measured in the presence of excess unlabelled GDP (200 μM). [^{14}C]sucrose is included in the incubation media (5.55 x 10^{5} dpm/ml, 673 mCi/mmol, New England Nuclear) to calculate the amount of trapped incubation media in the final mitochondrial pellet. Mitochondrial protein concentration was determined by a modified Lowry method (Markwell et al. 1981).

Measurement of plasma insulin, glucose and corticosterone. Plasma insulin concentrations were determined by a competitive enzyme-linked immunoabsorbent assay (ELISA) described by Kekow et al. (Kekow et al. 1988). Plasma glucose was measured with a glucose oxidase-peroxidase kit (Sigma Chemical). Plasma corticosterone concentrations were determined by radioimmunoassay (Endocrine Sciences, Tarzana, CA, see Walker and Romsos 1992). The minimum concentration for detection with this assay was 0.15 μg corticosterone/dl plasma. When the plasma corticosterone concentration was below detection, the concentration was assumed to be 0.15 μg.

Measurement of oxygen consumption. Oxygen consumption were determined before and 45 and 120 min after intracerebroventricular injection between 1300-1600 h. A glass flasks (7.8 cm diameter and 7.0 cm in height)

contained soda lime to absorb expired CO_2 was immersed in a water bath maintained at 25 ± 1°C. Mice were placed in the flask for 5 min, and oxygen consumption was recorded five times during the next 5- to 10-min period (Watt and Gourley 1953).

5. Statistics. Data are presented as means ± SE and were analyzed by one-way analysis of variance or two-way analysis of variance (see legends for Figures 1-4) with a Systat program (Systat, Evanston, IL). Multiple comparisons to the control groups were made with Dunnett's test. Pairwise comparisons were analyzed by Student's t test. All differences were considered significant at $P < 0.05$.

D. RESULTS

A single intracerebroventricular injection of the type II corticoid receptor agonist dexamethasone exerted relatively rapid effects on GDP binding to BAT mitochondria and on plasma insulin concentrations in adrenalectomized ob/ob mice (Figure 1A), in agreement with previous studies (Walker and Romsos 1992, 1993). GDP binding to BAT mitochondria decreased by 30% and plasma insulin concentrations increased by 159% within 45 min of intracerebroventricular dexamethasone injection (Figure 1A).

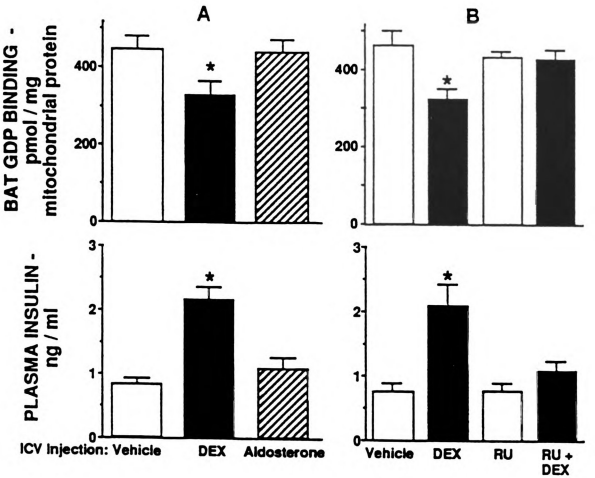

Figure 1. A: Effect of the type II corticoid receptor agonist dexamethasone (DEX) and the type I receptor agonist aldosterone on brown adipose tissue (BAT) thermogenesis and plasma insulin concentrations. Adrenalectomized ob/ob mice weighing 16.9 ± 0.5 g were injected icv with vehicle, 250 ng DEX (637 nmoles) or 230 ng aldosterone (637 nmoles) and were killed 45 min later. Each bar represents mean ± SE of 9-10 mice. B: RU-486 (type II corticoid receptor antagonist) antagonized effects of DEX on BAT thermogenesis and on plasma insulin concentrations. Adrenalectomized ob/ob mice were injected icv with vehicle, 250 ng DEX, 28 μg (63.7 nmoles, 100 fold of DEX amount) RU-486, or both dexamethasone and RU-486. Mice were killed 45 min later. Each bar represents mean ± SE of 8-12 mice. Two-way ANOVA showed significant effects of dexamethasone and of dexamethasone/RU-486 interaction. * Significant differences ($p < 0.05$; Dunnett's test) from vehicle-injected adrenalectomized mice for each panel. * Significant differences ($p<0.05$; Dunnett's test) from vehicle-injected adrenalectomized mice.

Intracerebroventricular injection of equal molar amounts of aldosterone, a type I receptor agonist, did not influence GDP binding to BAT mitochondria and only slightly ($P > 0.05$) increased plasma insulin concentrations (by 33%) within 45 min of intracerebroventricular injection (Figure 1A). This ineffectiveness of aldosterone excluded the involvement of type I corticoid receptors in mediating the rapid changes seen in dexamethasone-injected, adrenalectomized ob/ob mice.

The role of type II-like corticoid receptors was then further examined in adrenalectomized ob/ob mice by employing a type II receptor antagonist-RU-486. As expected, intracerebroventricularly administered dexamethasone decreased BAT GDP binding by 30%, and increased plasma insulin concentrations by 164% (Figure 1B). Administration of RU-486 (63.7 nmoles, 100 fold more than the dose of dexamethasone) did not change GDP binding to BAT mitochondria or plasma insulin concentrations (Figure 1B). RU-486 totally blocked effects of dexamethasone when both were co-administered (Figure 1B). These results support the hypothesis that effects of dexamethasone on BAT GDP binding and on circulating insulin concentrations in ob/ob mice are likely mediated by type II-like receptors.

To determine if antagonism of the type II corticoid receptor would rapidly reverse low metabolic rates and the

hyperinsulinemia characteristic of intact ob/ob mice, RU-486 was injected intracerebroventricularly and intact mice were examined 45 and 120 min later. Oxygen consumption of control and dexamethasone-injected intact ob/ob mice averaged 92 ± 3 ml/h before intracerebroventricular injection. Oxygen consumption increased by 16% compared to vehicle-injected mice within 45 min after administration of RU-486, and this elevated oxygen consumption was sustained for at least 120 min (21% increase compared to vehicle-injected mice) (Figure 2). BAT activity increased 48% and plasma insulin decreased 45% within 120 min after intracerebroventricular injection of RU-486 into intact ob/ob mice, although changes were not evident within 45 min (Figure 2). Subcutaneous administration of 28 μg RU-486 was ineffective in increasing oxygen consumption 45 min postinjection; values averaged 85 ± 6 and 78 ± 4 ml oxygen consumed per h per mouse in control and RU-486-treated intact ob/ob mice, respectively. These results imply that intracerebroventricularly administered RU-486 did not elicit its effects via peripheral corticoid receptors and that a site of RU-486 action was in central nervous system.

Appropriate conditions to inhibit protein synthesis were then established to further examine the mechanism of acute dexamethasone action in ob/ob mice. Incorporation of [^{3}H]leucine into cerebral proteins in ob/ob mice averaged 40,657 dpm/whole brain in the control mice within 10 min of

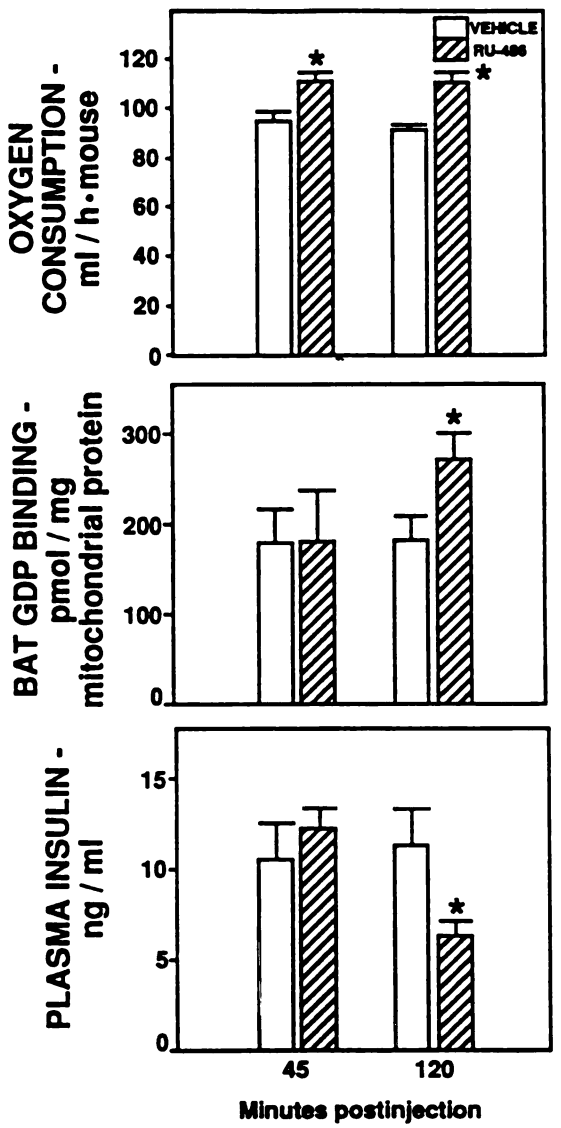

Figure 2. Effects of intracerebroventricular injection of RU-486 on whole body oxygen consumption, BAT thermogenesis and plasma insulin. Intact 6.5-7 wk old ob/ob mice weighing 27 ± 1 g were injected intracerebroventricularly with vehicle or 28 μg RU-486. Whole body oxygen consumption was measured repetitively at 25 ± 1°C before injection, and 45 min and 120 min after injection. Oxygen consumption of the intact vehicle-injected and RU-486-injected groups of ob/ob mice averaged 92 ± 3 ml/h prior to injection. Other groups of mice were killed at 45 min and 120 min postinjection for measurement of BAT metabolism and plasma insulin. Each bar represents mean ± SE for 7 mice. * Significant differences ($p<0.05$; Student's t test) within the same time interval.

intracerebroventricular injection of [^{3}H]leucine and 73,868 dpm/whole brain during the first 10 min and throughout the 45-min period during which effects of dexamethasone would subsequently be examined (Figure 3).

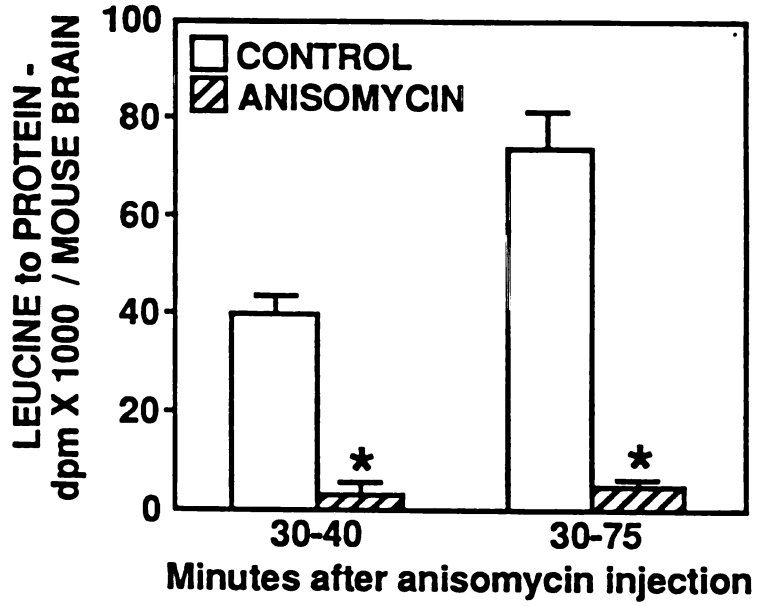

Figure 3. Blockage of cerebral protein synthesis with anisomycin. Adrenalectomized ob/ob mice were first injected subcutaneously with 0 (control) or 0.5 mg anisomycin, and then all mice were injected intracerebroventricularly with [^{3}H]-leucine 30 min later. Mice were killed 40 min or 75 min after anisomycin was injected. Each bar represents mean ± SE of 3-5 mice. * Significant difference ($p < 0.05$, Student's t test) between control and anisomycin-treated mice.

Effects of intracerebroventricular dexamethasone on peripheral metabolism in adrenalectomized ob/ob mice with suppressed protein synthesis were then tested. Again, dexamethasone alone decreased GDP binding to BAT mitochondria by 29% and increased plasma insulin concentrations by 143% in adrenalectomized ob/ob mice (Figure 4). Administration of anisomycin alone slightly increased BAT thermogenesis (Figure 4) which was possibly in response to the 1.6°C decrease in the core temperature of the anisomycin-treated mice by as assessed by a rectal probe (data not shown). Dexamethasone was as effective in the presence of anisomycin as when protein synthesis was not suppressed; GDP binding to BAT mitochondria was 25% lower and plasma insulin concentration was 123% higher in anisomycin- and dexamethasone-treated mice than in anisomycin- and saline-treated mice. The increases in plasma insulin concentrations were not secondary to increases in plasma glucose concentrations because dexamethasone did not increase plasma glucose concentrations and anisomycin actually depressed the concentrations (Figure 4).

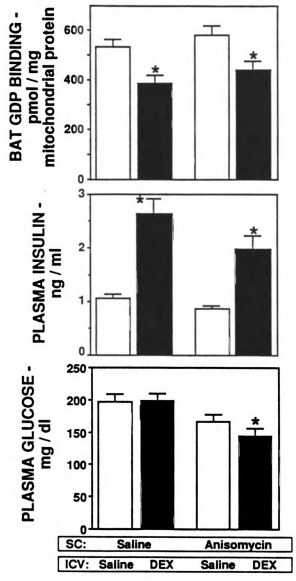

Figure 4. Effects of DEX on BAT thermogenesis, plasma insulin concentrations and plasma glucose concentrations with unaltered or suppressed protein synthesis. Adrenalectomized ob/ob mice were injected subcutaneously with saline or 0.5 mg anisomycin 30 min before icv injection of saline or DEX. Mice were killed 45 min after icv injection. Each bar represents mean ± SE of 11-17 mice. Two-way ANOVA indicated significant effects of DEX on BAT metabolism and on plasma insulin concentrations. * Significant differences ($p < 0.05$; Dunnett's test) from adrenalectomized mice injected with saline only.

D. DISCUSSION

Three major findings arose from the present study. First, the rapid effects of dexamethasone in the central nervous system on BAT thermogenesis and on plasma insulin in adrenalectomized ob/ob mice are mediated specifically by type II-like corticoid receptors, not type I receptors (Figure 1). Second, blockage of the type II corticoid receptors in the central nervous system of intact ob/ob mice rapidly increased whole body oxygen consumption (Figure 2). And third, the dexamethasone-induced rapid decreases in BAT thermogenic activity and increases in plasma insulin concentrations in adrenalectomized ob/ob mice are independent of protein synthesis since suppression of protein synthesis did not attenuate these effects (Figure 4).

The relative role of type I and type II corticoid receptors in dexamethasone-induced effects were examined because dexamethasone has some affinity to type I corticoid receptors (Krozowski and Funder 1983), and aldosterone seems to be involved in diet-selection and food intake regulation (Tempel and Leibowitz 1989, Tempel et al. 1991). To do so, equal moles of type I receptor (aldosterone) and type II receptor (dexamethasone) agonists were compared. Aldosterone has greater affinity for type I corticoid receptors than does dexamethasone (Krozowski and Funder 1983). However, intracerebroventricular administered aldosterone failed to change BAT thermogenesis or plasma

insulin in ob/ob mice. This suggests that the effects of dexamethasone were not through type I receptors and that there is some steroid specificity in the response. RU-486 is an antagonist to type II corticoid, pregesterone, and androgen receptors (Phillert 1984). Administration of RU-486 alone did not affect any of the parameters that we measured, suggesting that progesterone and androgen receptors were not involved. But, intracerebroventricular coadministration of RU-486 with dexamethasone abolished the rapid effects of dexamethasone in adrenalectomized ob/ob mice. Based on these results, we conclude that dexamethasone exerts its rapid physiological effects on BAT and on plasma insulin in ob/ob mice through type II-like receptors.

The adrenalectomized ob/ob mice used to demonstrate the rapid onset of glucocorticoid action were essentially devoid of endogenous glucocorticoid for several weeks before study. Responses of these adrenalectomized mice to glucocorticoids might differ from responses of intact mice which have had continuous exposure to endogenous glucocorticoids. The possibility that intact ob/ob mice possess a rapid-onset glucocorticoid action was thus tested by intracerebroventricular administration of the type II receptor antagonist RU-486. Whole body oxygen consumption and GDP binding to BAT mitochondria increased, whereas plasma insulin concentrations decreased after RU-486 administration (Figure 1). The time course for these

actions in intact mice appear slightly longer than in adrenalectomized mice. Intact ob/ob mice have elevated plasma corticosterone and corticoid receptors that are well occupied even in the morning (Tsai and Romsos 1991). Therefore, some lag in response to intracerebroventricularly injected RU-486 in intact ob/ob mice would be expected because many of the glucocorticoid receptors would have been occupied by corticosterone at the time of RU-486 administration (1300-1600 h). Rapid increases in metabolic rates have also been demonstrated following intracerebroventricular administration of RU-486 to normal rats (Hardwick et al 1989). Thus, it appears that at least one component of glucocorticoid action in intact ob/ob mice is via a rapid responding system and dependent on type II-like corticoid receptors.

Steroid hormones are commonly thought to exert their regulatory roles via regulation of genes (McEwen 1991). The time frame required for activation of corticoid receptor changes in gene expression and subsequent changes in the effector protein is usually hours or even days (McEwen 1991). Therefore, we hypothesized that protein synthesis is not a prerequisite for rapid-onset alterations in ob/ob mice observed after intracerebroventricular injection of dexamethasone. Consistent with this hypothesis, dexamethasone was as effective in mice where protein synthesis was blocked as in control mice (Figure 4).

The mechanism whereby intracerebroventricular dexamethasone elicits rapid peripheral metabolic changes without protein synthesis remains to be established. Rapid effects of glucocorticoids (within min) in hypothalamic brain slice preparations, and in isolated coeliac ganglion have been demonstrated (Chen et al. 1991, Hua and Chen 1989, Kasai and Yamashita 1988). Glucocorticoids may modulate membrane potential (Chen et al. 1991, Hua and Chen 1989), and ion-channel conductance (Hua and Chen 1989). Some of these rapid effects of glucocorticoids may be mediated via corticoid receptors in plasma membranes (Hua and Chen 1989, Orchinik et al. 1991). These reports raise the possibility that glucocorticoids exert phenotype-specific (only in ob/ob mice, not lean mice) metabolic effects (Tokuyama an Himms-Hagen 1989, Walker and Romsos 1992) on plasma membranes in the central nervous system. The cellular nature of these actions, the specific brain sites involved, and the phenotype specificity of response all remain to be investigated.

CHAPTER IV. INTRACEREBROVENTRICULAR INJECTION OF DEXAMETHASONE CAUSES RAPID INCREASES IN HYPOTHALAMIC NEUROPEPTIDE Y SECRETION IN OB/OB MICE

A. Abstract

A single icv injection of DEX (250 ng) in ADX ob/ob mice, but not lean mice, rapidly (within 30 min) suppresses BAT thermogenesis by 25% and increases plasma insulin concentrations by nearly 100%. A single icv administration of NPY (2.0 μg) exerts similar rapid (within 30 min) metabolic effects on BAT activity and insulin secretion in both ADX ob/ob and lean mice. We therefore hypothesize that NPY mediates rapid actions of DEX in the ADX ob/ob mice. A single icv injection of PYX-2, a NPY receptor antagonist, blocked effects of DEX on BAT activity and on plasma insulin in ADX ob/ob mice, supporting the role of NPY in mediating the rapid actions of DEX in ob/ob mice. These rapid actions of DEX do not require DEX-induced protein synthesis and may be mediated by nongenomic actions that induce NPY release. Thus, we investigated if NPY secretion, especially in the hypothalamus, is enhanced by icv-administered DEX. Icv injection of DEX selectively reduced NPY concentrations in the hypothalamus by 35 % within 30 min, but not in the brain stem or hippocampus in ADX ob/ob mice. None of these three

regions responded to the DEX treatment in the ADX lean mice. A single icv injection of aldosterone (230 ng), a type I glucocorticoid receptor agonist, was unable to influence NPY concentrations in any of the three brain regions examined, suggesting that the type II glucocorticoid mediates these regulatory roles of DEX on NPY secretion. Specific hypothalamic regions of DEX-induced NPY secretion were then determined. Among micropunched hypothalamic regions, NPY concentrations decreased most dramatically (-70%) in the arcuate nucleus region. This suggests that DEX acts via the type II glucocorticoid receptor to evoke transport of NPY from cell bodies (the arcuate nucleus) to the terminal regions including dorsomedial and ventromedial hypothalamus for release in ADX ob/ob mice, which possibly leads to rapid alterations of BAT activity and plasma insulin concentration.

2. Introduction

Glucocorticoids secreted by the adrenal glands are involved in the expression of obesity in genetically obese (ob/ob) mice (Saito and Bray 1984, Tokuyama and Himms-Hagen 1987, Walker and Romsos, 1992). Adrenalectomy (ADX) normalizes many of the metabolic defects in ob/ob mice, including suppressed brown adipose tissue (BAT) thermogenic activity and hyperinsulinemia (Bray and York 1979, Kim and Romsos 1987, 1988, Smith and Romsos 1985). Intracerebroventricular (icv) injections of dexamethasone

(DEX) into ADX ob/ob mice rapidly (within 30 min) reverses these metabolic effects of adrenalectomy by decreasing BAT thermogenic activity by 25% and increasing plasma insulin concentration twofold in a dose-response manner. Minimal effects of icv-administered DEX were noted in ADX lean mice (Walker and Romsos 1992). Since injection of a similar dose of DEX intraperitoneally rather than icv failed to influence metabolism within 30 min in ADX ob/ob mice (Walker and Romsos 1992), it is likely that DEX acts within the CNS to rapidly stimulate the initiation of obesity development.

These rapid-onset glucocorticoid actions in ADX ob/ob mice seem incompatible with the time frame of nuclear actions of glucocorticoids (hours to days) (McEwen 1991). In support of a nongenomic mechanism of glucocorticoid action in ob/ob mice, we observed that these rapid effects of DEX do not require protein synthesis (Chen and Romsos 1994). The type of glucocorticoid receptor mediating the rapid actions of DEX in ob/ob mice has also been explored (Chen and Romsos 1994). Administrations of a type I glucocorticoid receptor agonist, aldosterone, did not alter BAT thermogenesis and plasma insulin concentration whereas a type II glucocorticoid antagonist, RU-486, blocked effects of DEX in ADX ob/ob mice. These studies suggest a role for the type II-like receptor in mediating the rapid effects of DEX in ADX ob/ob mice (Chen and Romsos 1994).

DEX may exert rapid-onset actions by regulating secretion of mediator neurotransmitters that in turn

influence BAT thermogenesis and plasma insulin concentration. NPY is a candidate to mediate DEX actions in ob/ob mice because DEX and NPY have similar acute effects on BAT activity and on plasma insulin in these mice (Walker and Romsos 1993). Icv injection of NPY into ADX ob/ob mice rapidly (within 30 min) reduces BAT activity by 25% and increases plasma insulin by almost 100%. This raises the possibility that icv-administered DEX rapidly stimulates NPY release from specific sites within the CNS in ADX ob/ob mice, and that this DEX-induced NPY release could presumably lead to decreased BAT thermogenesis and increased plasma insulin concentrations. The present study was designed to test this working hypothesis. ADX lean mice respond to icv injection of NPY, but not to DEX (Walker and Romsos 1992, 1993), possibly because DEX is ineffective in enhancing NPY secretion in these mice.

Corticotropin releasing hormone (CRH) is another candidate that may mediate the rapid-onset actions of glucocorticoids in ob/ob mice. Glucocorticoids inhibit CRH neuron activity through a negative feedback loop (Antoni 1986, Taylor and Fishman 1988) and CRH seems to inhibit at least certain NPY actions (Morley et al. 1985). Thus, DEX-induced rapid decreases in CRH secretion might lead to increases in NPY secretion in the CNS, and subsequent to decreases in BAT activity and increases in plasma insulin concentrations in ADX ob/ob mice.

The present study was undertaken to determine:

1) if NPY in the CNS mediates rapid effects of icv-administered DEX on BAT thermogenic activity and on plasma insulin concentration in ADX ob/ob mice. Assumimg that NPY is an intermediate in the DEX-induced metabolic changes reported in ADX ob/ob mice, it should be possible to block these DEX actions by blocking NPY action. This was tested by utilizing an antagonist of NPY receptors (Tatemoto et al 1992). 2) how icv-administered DEX regulates NPY concentrations in different brain regions in ADX ob/ob and lean mice. The glucocorticoid receptor type (i.e. type I or II) responsible for enhanced NPY secretion was examined, as was the possibility that corticotropin releasing hormone (CRH) participates in the glucocorticoid-stimulated NPY release. Finally, discrete hypothalamic regions were examined to localize the areas exhibiting the most pronounced glucocorticoid-induced decreases in NPY concentrations.

3. Materials and Methods

3.1 Animals and diet.

Male ob/ob mice and lean (+/+ or ob/+) littermates were obtained from our breeding colony of C57BL/6J mice or were purchased at 4 wk of age from Jackson Laboratories (Bar Habor, ME). The Guide for the Care and Use of Laboratory Animals (NRC 1985) and local institutional guidelines were followed for care and treatment of mice. Mice were weaned at 3-4 wk of age. Weaned mice were given free access to a

non-purified diet (Teklad Rodent Diet 8640), and group housed at 23-25 °C in solid-bottom plastic cages with wood shavings for bedding. Room lights were on from 0700 h to 1900 h daily.

3.2 Chemicals

Dexamethasone 21-phosphate disodium salt, aprotinin, α-helical CRH, aldosterone, avidin peroxidase conjugate, peroxidase-labelled insulin, and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma Chemical Inc.(St. Louis, MO). Rat NPY was purchased from Bachem (Torrance, CA). Rabbit anti-(rat, human)-NPY IgG, biotinylated (rat, human)-NPY and PYX-2 were purchased from Pennisula Lab, Inc. (Belmont, CA). Goat anti-rabbit antiserum for NPY ELISA and rabbit anti-guinea pig antiserum for insulin ELISA were obtained from EY Lab. Inc. (San Mateo, CA). Guinea anti-insulin antiserum was purchased from the Linco Research Inc. (St. Louis, MO).

3.3 Experimental Design

Experiment 1 was conducted to examine if NPY indeed mediates the rapid actions of icv-administered DEX on BAT activity and on plasma insulin concentration in ADX ob/ob mice. This was tested by administering an antagonist of NPY receptors at the same time DEX was administered. The NPY receptor antagonist selected, PYX-2 (Tatemoto et al 1992), blocks NPY-induced feeding (Leibowitz et al. 1992). ADX ob/ob mice were injected icv with saline, DEX (250 ng, 637

pmol), PYX-2, or a combination of DEX and PYX-2. Mice were killed 30 min after the injection. Food was not available after icv injections. Brown adipose tissue thermogenesis was assessed by GDP-binding to mitochondrial protein. Plasma insulin concentrations were determined with an ELISA.

Experiment 2 was undertaken to examine the effects of DEX on NPY concentrations in selected brain regions of ADX ob/ob and lean mice, to determine whether type I or type II glucocorticoid receptors are involved in this effect, and to examine the possibility that DEX influences NPY concentration secondary to inhibition of CRH action. Feeding status influences hypothalamic NPY content (Beck et al. 1990, Sahu et al 1988). Thus, daily food intakes over 3-d period prior to this experiment were also measured. ADX ob/ob and lean mice were injected icv with 250 ng (637 pmol) of DEX, 230 ng (637 pmol) of aldosterone, 10 μg α-helical CRH (2.61 nmol) or saline vehicle and were then decapitated 30 min after icv injection. Food was not available during the post-injection period. Trunk blood was collected for determinations of insulin and corticosterone.

NPY release in the hypothalamus where is closely associated with energy homeostasis may possibly mediate DEX-induced metabolic actions on BAT thermogenesis and on plasma insulin concentration. Two NPY systems terminate in the hypothalamus. One system originates in the arcuate region of hypothalamus and terminates within the hypothalamus (Chronwall 1989). The other NPY system is from the brain

stem (Chronwall 1989). Thus, the NPY concentrations in the hypothalamus and brain stem were determined. The hippocampus is enriched with glucocorticoid receptors but lacks a NPY projection system to the hypothalamus (Funder and Sheppard 1987, Chronwall 1989). Thus, hippocampal NPY concentrations were also determined to serve as a negative control for this experiment.

Experiment 3 was undertaken to determine the specific hypothalamic regions involved in the DEX-induced decreases in NPY concentration. ADX ob/ob and lean mice received icv injection of 250 ng (637 pmol) DEX or saline and were killed 30 min later. Food was not available after icv injections. Trunk blood was collected for determinations of insulin, glucose and corticosterone. Discrete hypothalamic regions in the rostracaudal order including the medial preoptic area (MPO), suprachiasmatic nucleus (SC), anterior hypothalamus (AH), lateral hypothalamus (LH), paraventricular nucleus (PVN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), and arcuate nucleus (ARC) were micropunched.

3.4 Methods

Adrenalectomy. Mice were ADX through dorsal incisions under ether anesthesia at 4.5-5 wk of age. Incisions were closed with wound clips. ADX mice were given free access to food and physiological saline to drink. Experiments were conducted 2-3 wk after the surgery. Adrenalectomy was verified by measurement of plasma corticosterone

concentrations with a radioimmunoassay (Endocrine Sciences, Tarzana, CA; Walker and Romsos, 1992). The cutoff concentration for successful adrenalectomy was set at 1 μg/dL. Values below the limit of detection (0.15 μg/dL) were assumed to be 0.15 μg/dL. Only mice with plasma concentrations < 1 μg/dL were included for data analysis. Plasma corticosterone concentration averaged 0.63 ± 0.03 μg/dL.

Intracerebroventricular injection. Injections (2 μL) to the right lateral ventricles were performed as previously described between 0930 h and 1100 h (Chen and Romsos 1994). Mice were killed 30 min after icv injection. The success of injections was verified after mice were decapitated.

GDP binding. Thermogenic activity of brown adipose tissue was assessed by measuring specific GDP binding to the isolated mitochondria with the method described previously (Kim and Romsos 1987).

Measurement of insulin and NPY with competitive enzyme-linked immunosorbent assays (ELISA). Ninety-six well, Immulon 4 microtiter plates were used for the ELISA. The preparation of coating, washing, citrate buffers for both insulin and NPY assays, and insulin sample buffer followed procedure described by Kekow et al. (1988). For measurement of insulin, rabbit anti-guinea pig antiserum (1 μg in 100 μL coating buffer) was added to each well and dried at 33-35 °C overnight. Wells were rinsed with washing buffer three

times. Seventy-five μL of anti-insulin antibody (10 RIA-tube quantity / mL of insulin sample buffer) was added to each well and incubated for 2 d at 4 °C. After three washes, samples or insulin standards (75 μL) were added and incubated at 37 °C for 50 min followed by addition of peroxidase-labeled insulin (0.4 μg in 75 μL sample buffer). After another 40 min at 37 °C, plates were washed three times. ABTS, substrate for peroxidase, was then added in citrate buffer containing hydroperoxide (4 mg ABTS in 1 mL water added to 10 mL citrate buffer containing 10 μL 30% hydroperoxide). Color was developed for 60 min at ambient temperature before the optical density were measured at 405 nm.

For measurement of NPY, goat anti-rabbit NPY antiserum (5 μg in 200 μL coating buffer) was added to each well and dried at 33-35 °C overnight. Wells were rinsed three times with washing buffer. Rabbit anti-NPY IgG (0.2 μg in 0.1 mL NPY sample buffer-0.15 M phosphate saline buffer with 0.1% Tween-20 and 1% BSA, pH 7.4) were incubated for 2 d at 4 °C. After three washings, blocking buffer (NPY sample buffer plus additional 1% BSA) was added and incubated at 37 °C for 1 h to minimize nonspecific binding. After three washings, samples or NPY standard (100 μL in NPY sample buffer) were incubated at 4 °C overnight and biotinylated rat NPY (50 ng in 50 μL NPY sample buffer) was subsequently added. Plates were incubated at 4 °C for overnight, followed by six washings. Avidin-peroxidase conjugate (200 ng in 100 μL NPY

sample buffer) was then added and incubated at 37 °C for 1 h, followed by seven washings. Substrate for peroxide, ABTS, was then added (same as for insulin ELISA). Color was developed for 30 min at room temperature. Optical density was measured at 405 nm. The sensitivity of this assay was 5-10 pg. The correlation of variations were 4.3% (n=10) and 4.0% (10 duplicate assays) for inter- and intra-assays, respectively.

Dissection of brain regions and micropunches of discrete hypothalamic regions. Mice were decapitated 30 min after icv injections. Heads were immediately placed in ice until the hypothalamus, brain stem and hippocampus were dissected and placed in 1.0 ml of 0.1 N HCl containing a protease inhibitor-aprotinin. The rationale for selection of these brain regions is presented earlier in the experimental design.

The micropunch procedure is modified from Dube et al. (1992). Brains were removed as soon as the mice were killed and a cut perpendicular to the midline of the mid-hindbrain was made before the brain was frozen on dry ice. The frozen brain was mounted to a specimen block, placed in the cryostat (Cryostat 1800, Reichert-Jung) at -10 °C for at least 30 min before it was sliced coronally (400 μm sections). The sections were mounted onto conventional glass slides and were frozen on dry ice immediately. The triangle formed by the subfornical organ (SF) and the

bilateral anterior commissure (ac) was used as the landmark for beginning of the hypothalamus. Discrete hypothalamic regions including medial preoptic area (MPO), suprachiasmatic nucleus (SCN), anterior hypothalamus (AH), lateral hypothalamus (LH), paraventricular nucleus (PVN), dorsomedial nucleus (DMH), ventromedial nucleus (VMH), and arcuate nucleus (ARC) were micropunched under a magnifying lens with fabricated 20- or 24-gauge stainless steel needles (Figure 5). The stereotaxic parameters were determined according to Burton et al. (1975). Micropunches were placed in 0.1 mL of the 0.1 N HCl-aprotinin solution.

Extraction of NPY. Dissected hypothalamus, brain stem and hippocampus were extracted for 30 sec via an ultrasonic homogenizer. Aliquots of the homogenate were removed for protein assay with the modified Lowry method (Markwell et al. 1981). The remaining solution were centrifuged at 10,000 g for 10 min at 4 °C. Supernatants were stored at -20 °C until analysis. Recovery of added NPY was verified and found to be 98 ± 2 % (n=2) with this extraction method.

Micropunches from each region were sonicated for 10 sec and centrifuged at 10,000 g for 10 min at 4 °C. The protein content in the pellet, instead of an aliquot of the homogenate as employed for the brain regions, was determined because of the small amount of tissue available for assays. The protein precipitate was determined with a micro-protein assay (Bio-Rad Lab., Hercules, CA).

Statistics. Data are expressed as means ± SE. One-way ANOVA and post-hoc Dunnett's test were used to determine treatment effect in experiment 1 where only ob/ob mice were used. Experiment 2 and 3, 2-way factorial ANOVA were used to analyze the main effect of phenotype, treatment and interation of these two factor. Treatment effect within the phenotype were determined with post-hoc Least Significant Difference test. Effects were considered significant when $P<0.05$. All data were analyzed with Systat for Macintosh (v. 5.2).

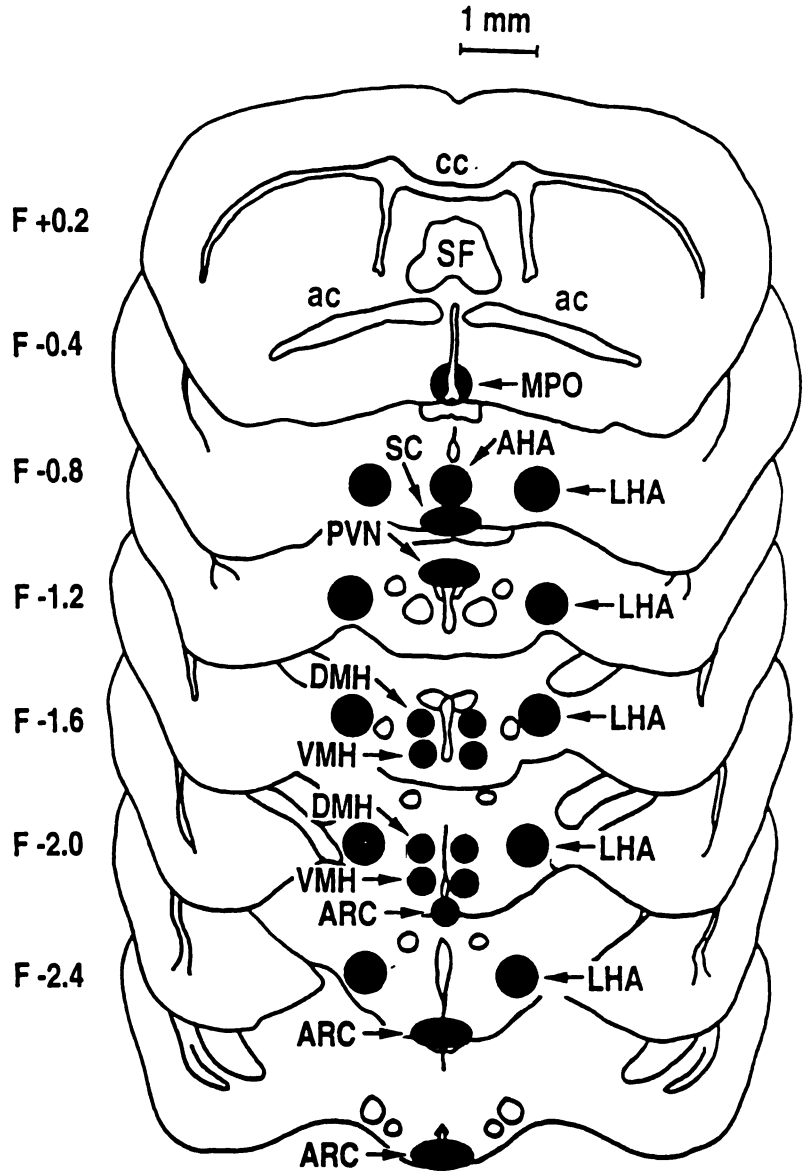

Figure 5. Eight hypothalamic regions that were micropunched with 20- or 24-gauge needles. The numbers following F represent the relative positions (in mm) to bregma (positive number indicates anterior to the bregma and vice versa). Abbreviations of these regions are: MPO, medial preoptic area;SCN, suprachiasmatic nucleus; AH, anterior hypothalamus; LH, lateral hypothalamus; PVN, paraventricular nucleus; DMH, dorsomedial nucleus; VMH, ventromedial nucleus; ARC, arcuate nucleus. The triangle formed by the subfornical organ (SF) and the bilateral anterior commissure (ac) was used as the landmark for beginning of the hypothalamus. cc stands for corpus callosum.

4. Results

Mice were studied 2-3 wk after ADX. At this time many of the metabolic characteristics of intact ob/ob mice are not evident. Daily food intake in the 3-d period prior to the experiment averaged 3.7 ± 0.2 g/d (n=6) for ADX ob/ob mice and 3.6 ± 0.2 g/d (n=6) for ADX lean mice. Body weights of ADX ob/ob and ADX lean mice averaged 22.5 ± 0.6 (n=42) and 22.0 ± 0.5 (n=40) g, respectively. The ADX ob/ob mice were not hyperinsulinemic, plasma insulin concentrations averaged 0.85 ± 0.08 ng/ml (n=21) in ADX ob/ob mice and 0.83 ± 0.06 (n=17) ng/ml in ADX lean mice. Plasma glucose concentrations were also comparable in these mice with glucose; average value were 210 ± 12 mg/ml (n=21) in ADX ob/ob mice and 210 ± 11 (n=17) mg/dl in ADX lean mice. Therefore, treatment effects were not confounded by baseline differences in food intake, body weight, or plasma insulin concentrations, as usually occurs in comparisons of intact 6-7 wk old ob/ob and lean mice.

A single injection of DEX rapidly (within 30 min) suppressed brown adipose tissue thermogenic activities by 29% and increased plasma insulin concentrations by about 70% in ADX ob/ob mice (Figure 6), in agreement with previous studies (Walker and Romsos 1992, 1993). A single injection of PYX-2, the NPY receptor antagonist administered in this study, did not influence GDP binding and plasma insulin concentration when compared with the saline-injected group (Figure 2). Coadministration of PYX-2 with DEX abolished

DEX-induced decreases in GDP binding and increases in plasma insulin concentrations (Figure 6). Plasma glucose concentrations were not affected by treatment with DEX (222 ± 9 mg/dL), PYX-2 (223± 8 mg/dL) or the combination of PYX-2 with DEX (208 ± 8 mg/dL) when compared with the saline-injected group (219 ± 8 mg/dL), suggesting that the DEX-induced increase in plasma insulin concentration was not secondary to changes in plasma glucose. These results indicate that NPY participates in the rapid-onset DEX-induced metabolic changes in ADX ob/ob mice. Thus, the possibility that DEX increases NPY secretion was examined.

A single icv injection of DEX rapidly lowered the hypothalamic NPY concentration by 35% in ADX ob/ob mice when compared with the saline-injected counterparts (Figure 7), suggesting that icv DEX increased NPY secretion within the hypothalamus. DEX did not influence NPY concentrations in the brain stem or hippocampus in ADX ob/ob mice (Figure 7). In agreement with the ineffectiveness of DEX to modulate BAT activity or plasma insulin concentration in ADX lean mice (Walker and Romsos 1992), icv injection of DEX also failed to influence the NPY concentrations in any of these three regions examined in ADX lean mice (Figure 7). NPY concentrations were higher in the hypothalamus than in the other regions examined, and were unaffected by phenotype in saline-injected ADX mice (Figure 7).

Aldosterone-induced activation of type I glucocorticoid receptors fails to affect BAT metabolism or plasma insulin

in ADX ob/ob mice (Chen and Romsos 1994). If increased NPY secretion is a critical component of the DEX-induced actions in ob/ob mice, then aldosterone administration should not stimulate NPY secretion. Consistent with this, a single icv injection of aldosterone (637 pmol) did not influence NPY concentrations in the hypothalamus, brain stem or hippocampus in ADX ob/ob mice or in lean mice (Figure 8). Plasma insulin and glucose concentrations were not enhanced by icv injection of aldosterone either (data not shown).

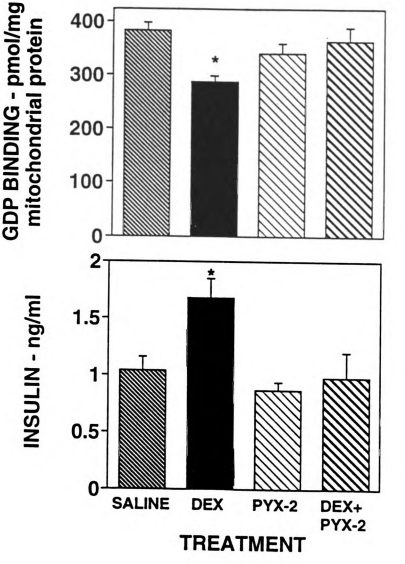

Figure 6. Effects of PYX-2, a NPY receptor antagonist, on DEX-induced changes in GDP-binding to BAT mitochondria, plasma insulin concentrations. Each bar represents means ± SE of 5-7 ADX ob/ob mice. * indicates significant difference ($P < 0.05$; Dunnett's test) from saline-injected mice.

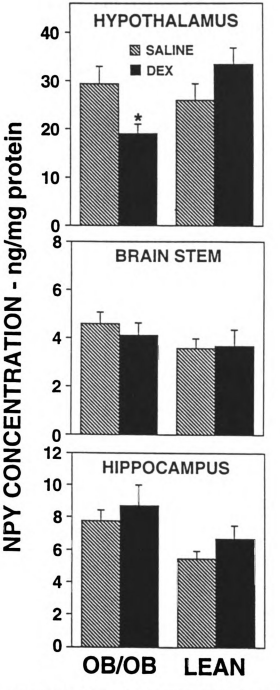

Figure 7. Effects of icv DEX (637 pmol) on NPY concentrations in the hypothalamus, brain stem and hippocampus. Each bar represents means ± SE of 7-12 ADX mice. * indicates significant effect ($P < 0.05$; LSD testwithin phenotype). The hypothalamic NPY concentration exerted significant phenotype and treatment interaction as analyzed as 2-way ANOVA.

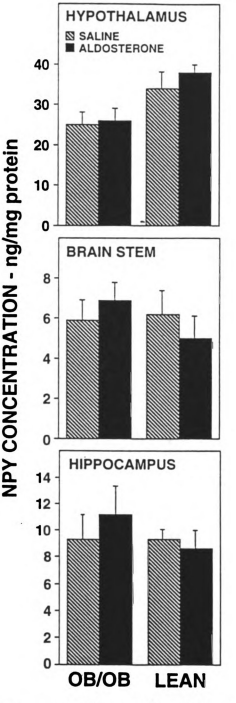

Figure 8. Effects of icv aldosterone (637 nmol) on NPY concentrations in the hypothalamus, brain stem and hippocampus in ADX ob/ob and lean mice. Each bar represents means ± SE of 5-9 mice. Two-way factorial ANOVA indicated that none of phenotype, treatment and interactions of these two factors were significant except phenotype effect in the hypothalamic NPY concentration.

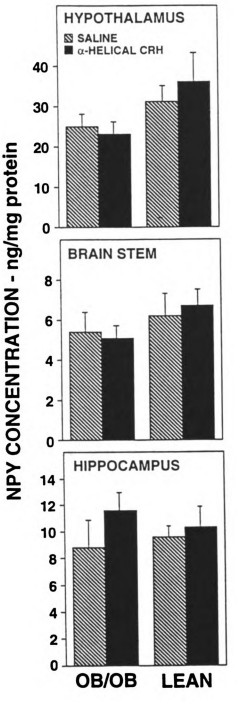

Figure 9. Effects of icv α-helical CRH (2.67 nmol) on NPY concentrations in the hypothalamus, brain stem and hippocampus in ADX ob/ob and lean mice. Each bar represents means ± SE of 5-9 mice. Two-way factorial ANOVA indicated that none of phenotype, treatment and interactions of these two factors were significant except phenotype effect in the hypothalamic NPY concentration.

Alpha-helical CRH has been shown to partially mimic DEX effects on plasma insulin concentration in ADX ob/ob mice 1 h after icv injection (Walker and Romsos 1992). A single icv injection of α-helical CRH did not influence NPY concentrations in any regions examined in this study within 30 min of injection in either ADX ob/ob or lean mice (Figure 9).

To further localize site of the DEX-induced decrease in hypothalamic Hypothalamic NPY concentrations in ADX ob/ob mice, discrete hypothalamic regions were micropunched. NPY concentrations in most discrete hypothalamic regions examined in this study were also similar between saline-injected ADX ob/ob and lean mice (Table 1). Two exceptions occur, post-hoc test indicated that NPY concentrations in the arcuate nucleus and dorsomedial nucleus of saline-injected ADX ob/ob mice were greater than those in the ADX lean littermates, respectively (Table 1). Icv administration of DEX to ADX ob/ob mice rapidly decreased NPY concentrations in the arcuate nucleus, dorsomedial nucleus, ventromedial nucleus, medial preoptic area, and suprachiasmatic nucleus by 70%, 59%, 41%, 43%, and 66%, respectively (Table 1). NPY concentration in the paraventricular nucleus, lateral and anterior hypothalami in ADX ob/ob mice did not decrease 30 min after icv injection of DEX. Icv-administered DEX did not alter NPY concentrations in any of the discrete hypothalamic regions in ADX lean mice (Table 1).

Table 1.
NPY concentrations in discrete hypothalamic areas of icv-injected ADX ob/ob and lean mice (ng NPY/mg protein precipitate)[1,2]

Treatment	ARC	DMH	VMH	SC	MPO	PVN	AH	LH
ADX ob/ob								
Saline	172±25†	104±21†	59±8	153±34	189±31	177±28	114±23	45±11
DEX	52±9*	43±6*	35±5*	51±8*	108±16*	123±20	72±11	40± 5
ADX lean								
Saline	65±14	52±14	60±25	101±24	181±68	152±29	151±17	39±7
DEX	107±25	86±14	111±19	101±16	134±16	177±41	102±25	39±8

[1]NPY concentration of each region represents the means ± SE of 5-10 mice.

[2]Abbreviations: ARC, arcuate nucleus; DMH, dorsomedial nucleus; VMH, ventromedial nucleus; SC, suprachiasmatic nucleus; MPO, Medial preoptic area; PVN, paraventricular nucleus; AH, anterior hypothalamus; LH, lateral hypothalamus. See Figure 5 for anatomical locations.

*Significant effect when compared DEX effect within phenotype with LSD test ($P < 0.05$).

†Significant effect when compared saline-injected ADX ob/ob and ADX lean mice with LSD test ($P < 0.05$).

5. Discussion

Results obtained in the present study provide evidence that the rapid-onset, DEX-induced suppression of BAT metabolism and stimulation of insulin secretion in ADX ob/ob mice are mediated by stimulation of NPY secretion in specific hypothalamic regions via a type-II like glucocorticoid receptor.

We first demonstrated that NPY actions are indeed required for DEX to exert rapid-onset actions in the CNS of ADX ob/ob mice. To do so, PYX-2, a NPY receptor antagonist (Tatemoto et al 1992) known to block NPY-induced feeding (Leibowitz et al. 1992) was used. Administration of PYX-2 alone did not alter BAT activity and plasma insulin concentration, suggesting that basal secretion of NPY under this experimental condition is not involved in regulation of BAT thermogenesis and insulin secretion in ADX ob/ob mice. Coadministration of DEX and PYX-2 abolished the rapid effects of DEX in ADX ob/ob mice. There are a family of NPY receptors including Y1 and Y2 receptors (Wahlestedt et al. 1990, Larhammar et al. 1992). PYX-2 seems to act on both Y1 and Y2 receptors of NPY (Tatemoto et al 1992). Studies have suggested that Y1 and Y2 receptors belong to the G-protein coupled superfamily of receptors (Wahlestedt et al. 1990). Y1 receptors are coupled to increases in Ca^{2+} influx, and decreases in cAMP (Wahlestedt et al. 1990). In contrast, Y2 receptors are related to increase in cAMP and decrease in Ca^{2+} influx (Wahlestedt et al. 1990). DEX-induced NPY

secretion in different region may activate different subtype of NPY receptors which decrease sympathetic drive to the BAT, but increase vagal drive to the pancreatic β cells. However, the subtype of NPY receptors involving in rapid DEX actions remains to be investigated.

We examined the DEX-induced changes in NPY content within the 30 min period during which DEX-induced BAT thermogenesis and plasma insulin concentration occur. The rapid DEX-induced decrease in hypothalamic NPY concentration in ADX ob/ob implies abrupt NPY secretion because glucocorticoids are known to increase, instead of decrease, NPY synthesis (Corder et al. 1988, McKibbin et al. 1992, White et al. 1990). NPY concentration in the brain stem was not altered in either ADX ob/ob or lean mice, suggesting that DEX did not increase transport of NPY from the brain stem within the time frame (30 min) examined in this study. Thus, the DEX-induced NPY release is possibly from the NPY neurons originating from the arcuate nucleus of the hypothalamus.

We demonstrated in our previous study that a type II-like glucocorticoid receptor mediates rapid effects of DEX on BAT activity and plasma insulin concentration (Chen and Romsos 1994). If NPY regulates these metabolic parameters downstream of DEX, then DEX-induced changes in the hypothalamic NPY concentration in ADX ob/ob mice should also be mediated by type II-like glucocorticoid receptors in the CNS. DEX is a type II glucocorticoid receptor agonist with

some binding affinity to the type I receptor (Krozowski and Funder 1983). Thus, we further defined the subtype of glucocorticoid receptors that is responsible for the regulatory role of DEX on hypothalamic NPY release. We demonstrated in this study that aldosterone, a type I receptor agonist, did not alter NPY concentration in the hypothalamus, brain stem or hippocampus. This ineffectiveness of aldosterone excluded the involvement of type I glucocorticoid receptor in mediating the rapid changes in NPY concentrations induced with icv DEX injection and supports the suggestion that DEX possibly modulated NPY content through type II glucocorticoid receptor in the hypothalamus of ob/ob mice.

The hippocampus served as a negative control in this study because it contains rich amounts of glucocorticoid receptors but lacks NPY neurons projecting to the hypothalamus. Again, the result from the present study confirms that NPY concentrations in the hippocampus did not respond to icv-administered DEX, aldosterone or α-helical CRH in either phenotypes.

We have suggested that rapid DEX actions on BAT thermogenesis and on plasma insulin concentration in ADX ob/ob mice are independent of DEX-induced protein synthesis and support nongenomic role of glucocorticoid (Chen and Romsos 1994). Progesterone, also a steroid hormone, exerts rapid effects on release of LHRH from the hypothalamus and neurotransmitters including acetylcholine and dopamine

(Ramirez and Dluzen 1987, Meiri 1986, McEwen 1991). Glucocorticoids also exert rapid effects (within sec) on ion channel conductance, membrane potential and membrane resistance in hypothalamic brain slice preparations and in isolated celiac ganglion (Chen et al 1991, Hua and Chen 1989, Kasai and Yamashita 1988). The rapidity of these glucocorticoid actions suggest nongenomic effects on plasma membrane of neurons.

Recent evidence suggests that corticosterone acts on outer plasma membrane of synaptosomes isolated from the rat brain to enhance calmodulin binding by increasing affinity and decreasing dissociation (Sze and Iqbal 1994a). A further study also suggests that corticosterone, DEX, cortisol, and triamcinolone increase depolarization-dependent Ca^{2+} uptake (Sze and Iqbal 1994b). Calcium channel blocker, nitrendipine and nifedipine inhibit glucocorticoid-induced Ca^{2+} intake, suggesting that the dihydropyridine site of calcium channel is associated with this effect (Sze and Iqbal 1994b). Therefore, it is possible that glucocorticoids acts on membrane receptor-like proteins to enhance depolarization-dependent Ca^{2+} influx by promoting calmodulin-dependent modulation of Ca^{2+} channels. Membrane glucocorticoid receptors have not yet been explored in the mammal CNS although it has been demonstrated in the amphibian neurons (Orchinik et al. 1991).

Glucocorticoids exert feedback control on CRH production and secretion (Antoni 1986, Taylor and Fishman

1988, Suda et al. 1985). An increase in CRH production may mediate the inhibitory effects of ADX on obesity development since CRH administered icv activates the sympathetic nervous activity in rats (Arase et al 1988, Rothwell 1990). Conversely, icv injection of α-helical CRH, a CRH antagonist, increased plasma insulin concentration by 40% with no effect on BAT thermogenesis in ADX ob/ob mice (Walker and Romsos 1992). Thus, I examined if α-helical CRH participates in the rapid action of DEX on NPY secretion. The icv-administered CRH antagonist failed to modulate hypothalamic NPY concentration within 30 min of injection in both ADX ob/ob and lean mice (Figure 9).

We found most pronounced decreases in NPY concentration in the arcuate region where NPY cell bodies are located (Table 1). These findings suggest that DEX may induce rapid transport of NPY from cell bodies in the arcuate nucleus to the terminal regions for secretion in ADX ob/ob mice. Terminal regions including dorsomedial nucleus, ventromedial nucleus, suprachiasmatic nucleus, and medial preoptic area of the hypothalamus also exhibited pronounced decreases in NPY concentration in response to the icv administration of DEX (Table 1). The transport of NPY from the arcuate nucleus to terminals coupled with decreased NPY concentration in terminals suggests very rapid and pronounced NPY release induced by DEX.

It has been suggested that dorsomedial hypothalamus provides input to the dorsal motor nucleus of the vagus

(Bray 1991) and ventromedial hypothalamus has input into the sympathetic nervous system through the periaqueductal gray in the medulla (Bray 1991). Therefore, the observed DEX-induced NPY release in ob/ob mice may affect insulin secretion and brown adipose tissue activity via neuronal pathways involving dorsomedial and ventromedial hypothalami. The NPY concentrations in the suprachiasmatic nucleus and medial preoptic area also decreased with DEX treatment. NPY in the suprachiasmatic nucleus responds to circadian rhythm (Sahu et al. 1988). However, specific function of NPY in this region remains to be investigated. The role of medial preoptic area is contradictory. Microinjection of NPY into preoptic-anterior hypothalamic area increases the sympathetic input to the BAT (Egawa et al. 1991). However, perfusion of NPY in the preoptic area causes hypothermia in rats (Roscoe and Myers 1991). This latter finding suggests that the increase in NPY secretion in the medial preoptic area may cause suppressed total energy expenditure in ADX ob/ob mice, consistent with low metabolic rates in DEX-treated ADX ob/ob mice (Walker and Romsos 1992).

The paraventricular hypothalamus is well known for its role in NPY-induced food intake (Clark et al. 1984, 1985, Stanley et al. 1993). However, food intake of the DEX-injected ADX ob/ob mice did not increase when compared to the saline-injected counterparts, which is consistent with no change in DEX-induced NPY concentration in the PVN. Injection of NPY in the paraventricular hypothalamus also

decreases sympathetic nerve activity to the BAT in rats (Bray 1991). Thus, the present result suggests that the DEX-induced decrease in BAT activity probably is not mediated by the paraventricular nucleus, but by the ventromedial hypothalamus area.

In conclusion, my study suggests that DEX exerts rapid effects on brown adipose tissue activity and on plasma insulin by increasing NPY release in specific regions of the hypothalamus in ADX ob/ob mice, but not in lean mice. It is proposed that this alteration in ob/ob mice is due to inactive ob protein product of defective ob gene (Zhang et al. 1994). The function of this ob protein in glucocorticoid actions remains to be investigated.

CHAPTER V. SUMMARY AND RECOMMENDATIONS FOR FUTURE RESEARCH

Genetic obese (ob/ob) mice display metabolic abnormalities including suppressed BAT thermogenesis and hyperinsulinemia (Bray and York 1979). Adrenalectomy generally normalize these alterations. Replacing glucocorticoids by an icv injection of DEX rapidly (within 30 min) suppresses BAT thermogenesis and increases plasma insulin concentration in ADX ob/ob mice, but not in ADX lean mice. I therefore examined the mode of rapid glucocorticoid actions in promoting obesity in ADX ob/ob mice.

I first examined the subtype of glucocorticoid receptors that mediate the rapid actions of DEX on peripheral metabolism in ADX ob/ob mice. Icv administration of aldosterone, a type I receptor agonist, did not affect BAT thermogenesis and plasma insulin concentration within 30 min of injection (Chapter III). Icv injection of RU-486, a type II receptor antagonist, abolished the rapid actions of DEX (Chapter III). These findings suggest that icv-administered DEX acts on the type II-like glucocorticoid receptor to rapidly suppress BAT activity and induce insulin secretion.

These DEX-induced suppressions in BAT activity and increases in plasma insulin in ADX ob/ob mice occurred

rapidly (within 30 min). This time frame seems incompatible with genomic mode of action. I therefore examined if these rapid-onset actions required protein synthesis. Peripheral administration of anisomycin, a translation blocker, nearly completely abolish cerebral protein synthesis (-92%) whereas rapid effects of DEX on BAT thermogenesis and on plasma insulin still occurred (Chapter III). This result indicates that these rapid effects of icv DEX on BAT thermogenesis and on plasma insulin likely occur through nongenomic mechanism, such as plasma membrane effects.

Steroids have been shown to exert rapid actions on release of hypothalamic hormone, such as LHRH, and neurotransmitters including dopamine and acetylcholine ((Ramirez and Dluzen 1987, Meiri 1986, McEwen 1991). Since a single icv injection of NPY exerts rapid effects on BAT thermogenesis and on plasma insulin in ADX ob/ob mice (Walker and Romsos 1993), I determined if NPY is a downstream mediator of rapid DEX actions in ADX ob/ob mice. Coadministration of PYX-2, a NPY receptor antagonist, abolished rapid actions of DEX in ADX ob/ob mice (Chapter IV). This indicates that NPY is a mediator of DEX actions on BAT thermogenesis and on plasma insulin concentration in ADX ob/ob mice. I thus examined if icv administration of DEX increased NPY secretion within 30 min of injection in ADX ob/ob mice and their lean littermates.

Icv injection of DEX into ADX ob/ob mice rapidly decreased NPY concentrations in the hypothalamus by 35%

(Chapter IV). The hypothalamus controls BAT activity and insulin secretion, suggesting that rapid release of hypothalamic NPY by DEX is an important component of the obesity-producing effects of DEX in ob/ob mice. NPY concentration in the brain stem does not change with icv DEX treatment in ADX ob/ob mice, suggesting that DEX did not induce rapid transport of NPY from the brain stem region (Chapter IV). The hippocampus served as a negative control since it is enriched in glucocorticoid receptors but lacks a NPY system projecting to the hypothalamus. The NPY concentration in the hippocampus was not altered with icv injection of DEX, in agreement with my hypothesis (Chapter IV). Icv injection of DEX failed to influence NPY concentrations in the hypothalamus, brain stem or hippocampus in ADX lean mice, which agreed with the observation that icv DEX did not affect peripheral metabolism in ADX lean mice (Walker and Romsos 1992).

The DEX-induced decrease in NPY concentration (or increased NPY secretion) is possibly mediated by type II-like glucocorticoid receptors because icv administration of aldosterone did not alter NPY concentration in any of the three brain regions examined in ADX ob/ob mice.

I further examined the specific hypothalamic regions where the icv DEX-induced NPY secretion occurred within 30 min of injection. NPY concentration in the arcuate nucleus decreased by 70% indicating that DEX induced rapid NPY transport from this cell body region to terminal regions

within the hypothalamus (Chapter IV). NPY concentrations in the terminal regions such as dorsomedial hypothalamus, ventromedial hypothalamus, suprachiasmatic nucleus, and medial preoptic area decreased by 60%, 40%, 66% and 40%, respectively (Chapter IV). This rapid secretion of NPY in the dorsomedial and ventromedial hypothalami may function to modulate vagal nerve and sympathetic nerve activities, respectively, to suppress BAT activity and insulin secretion. The function of rapid NPY release in the suprachiasmatic nucleus in this study remains to be demonstrated. Perfusion of NPY into the medial preoptic area causes hypothermia in rats which may relate to decreased whole body energy metabolism. The notable unchanged NPY concentration in the paraventricular nucleus coincides with unchanged food intake during 30 min of post injection period (Chapter IV). NPY concentrations in the anterior and lateral hypothalamus did not change with a icv injection of DEX, indicating that the rapid effects of DEX on NPY secretion are site-specific (Chapter IV).

Finally, my present study suggests that glucocorticoid exerts rapid effects via the type II glucocorticoid receptor in the CNS to modulate NPY secretion that leads to suppressed BAT thermogenesis and increased insulin secretion in ADX ob/ob mice.

To continue searching for action mechanisms of glucocorticoid in obesity development in ob/ob mice, I suggest the following studies.

1. Where does DEX act in the NPY systems to increase NPY secretion within the hypothalamus?

In the present studies, central injection of DEX rapidly decreased NPY concentrations in the arcuate nucleus where cell bodies are located and several terminal regions including dorsomedial hypothalamus, ventromedial hypothalamus, suprachiasmatic nucleus and medial preoptic area. However, the primary site of DEX action remains to be explored. I suggest to examine the time course (10, 20 min after icv injection) of changes in NPY concentrations in the five regions that have responded to DEX within 30 min of icv injection. To be more specific, the arcuate nucleus can be further divided to subregions. The NPY concentration may be determined with ELISA or semiqualified with immunocytochemistry (commercial kit is available). The site that first responds to icv injection of DEX may be the primary site of DEX action.

I also suggest using an <u>in vitro</u> system to determine the primary site of DEX action. I have already determined the effects of a depolarization reagent (45 mM KCL) and of DEX on <u>in vitro</u> hypothalamic NPY secretion in ADX ob/ob and lean mice.

KCL increased NPY secretion by 70% and 125% in ADX ob/ob and lean mice, respectively, when compared to the NPY secretion prior to the KCL stimuli (unpublished data). ADX ob/ob mice were more resistant to KCL stimuli than ADX lean mice (unpublished data). I suspect the presence of site-specific negative input to the hypothalamic NPY neurons in ADX ob/ob than in lean mice. The characteristics of this inhibitory input remains to be investigated. I suggest to examine the effect of this inhibitory input in specific regions.

DEX alone did not stimulate NPY secretion in either phenotype (unpublished data). Combination of DEX and 45 mM KCL potentiated NPY secretion by 245% compared to the secretion 15 min earlier in ADX ob/ob mice (unpublished data). Therefore, combination of DEX and KCL should stimulate NPY secretion in the primary site of DEX action in ADX ob/ob mice faster than in the secondary site(s).

2. Since actions of icv-administered DEX are rapid, I propose that DEX exerts membrane actions via membrane receptor(s) in ADX ob/ob mice. This present study suggests that effects of DEX in ADX ob/ob mice are mediated through type II-like glucocorticoid receptor. Thus, I suggest to determine if there are type-II like glucocorticoid receptors on the neuronal membrane at the sites of DEX actions with a binding assay.

3. Determine the cellular mechanism of DEX actions in the hypothalamus. I suggest to determine 1) the role of calcium channels that have been shown to mediate membrane actions of steroid hormones (Sze and Iqbal 1994a,b). One could employ Ca channel blocker with the combination of DEX and KCL to explore the role of Ca channel in rapid effect of DEX in ADX ob/ob mice. 2) changes in the cAMP content, a possible messenger system for rapid effects of glucocorticoids (Harrelson et al. 1987).

4. I suggest to examine the possible interaction of the recently cloned ob protein with DEX in development of obesity in ob/ob mice. Ob gene is expressed in white adipose tissue of normal lean mice and presumably acts in the CNS to inhibit development of obesity (Zhang et al. 1994). Since ob/ob mice do not produce a functional ob protein and are more sensitive to the obesity-promoting effects of glucocorticoids, I postulate that ob protein may antagonize effects of glucocorticoid on BAT activity, energy expenditure and insulin secretion.

In order to explore the role of ob protein, I suggest to determine the dose response and time course of icv-injected ob protein on BAT thermogenesis, oxygen consumption and insulin secretion in intact ob/ob mice. If administration of ob protein increases BAT activity, oxygen consumption or decrease plasma insulin concentration in intact ob/ob mice, one could determine if ob protein blocks the effects of glucocorticoid in ADX ob/ob mice.

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