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thesis entitled

Binding of cytosolic glucocorticoid receptors in obese (ob/ob) mice

presented by

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has been accepted towards fulfillment
of the requirements for

M.S. degree in Human Nutrition

Major professor

Date 11-7-90

# BINDING OF CYTOSOLIC GLUCOCORTICOID RECEPTORS IN OBESE (ob/ob) MICE

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1990

ABSTRACT

BINDING OF CYTOSOLIC GLUCOCORTICOID RECEPTORS IN OBESE (ob/ob) MICE

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Adrenalectomy prevents development of obesity in ob/ob mice. Replacement studies have shown that these mice exhibit hypersensitivity to corticosterone. Binding of corticosterone to glucocorticoid or mineralcorticoid receptors is the first intracellular step in corticosterone action. This study was conducted to determine if the numbers or binding affinity of glucocorticoid or mineralcorticoid receptors were altered in ob/ob mice. Cytosolic glucocorticoid receptor numbers, measured by a ligand receptor binding assay, were lower by 26% in liver, 23% in brain, and 26% in brown adipose tissues of 8-wk-old male ob/ob mice when compared with lean mice. But, cytosolic glucocorticoid receptor numbers were similar in liver and brain of 4-wk-old lean and ob/ob mice, indicating that lower receptor number in 8-wk-old ob/ob mice is likely secondary to elevated plasma corticosterone concentrations in the older ob/ob mice.

Next, 8-wk-old mice were adrenalectomized to allow up-regulation of cytosolic glucocorticoid receptors. Cytosolic glucocorticoid receptor numbers in liver and brain increased significantly within 2 h after adrenalectomy only in ob/ob mice. Two d after adrenalectomy, cytosolic glucocorticoid receptor numbers in liver and brain of ob/ob mice became similar to those in lean mice. To examine down-regulation of cytosolic glucocorticoid receptor, 0.5 or 5 ug dexamethasone /g body weight, was injected ip into adrenalectomized mice. Cytosolic glucocorticoid receptor numbers in liver and brain were equally lowered in ob/ob and lean mice 1 h after dexamethasone injection. Glucocorticoid receptor binding affinity was slightly lower in ob/ob mice than in lean mice. This lower receptor binding affinity cannot explain the hypersensitivity to corticosterone in ob/ob mice. Mineralcorticoid receptor numbers and responses to dexamethasone were similar in lean and ob/ob mice. These results indicate that the site responsible for hypersensitivity to corticosterone in ob/ob mice is not at receptor binding kinetics.

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# INTRODUCTION

Genetically obese (ob/ob) mice characteristically exhibit hyperphagia, high energy retention efficiency, low thermogenesis, and high concentrations of circulating plasma corticosterone, insulin, and glucose (1-2). The rise of plasma corticosterone, at about 3 weeks of age, has been suspected to cause obesity in these mice (2), since Ohshima et al (3) and Saito et al (4) showed that adrenalectomy of ob/ob mice at 4-5 weeks of age was able to prevent most obesity symptomes. Tokuyama et al (5) demonstrated that 8-wk-old adrenalectomized ob/ob mice were more sensitive to corticosterone than were lean mice. When their adrenalectomized mice received replacement corticosterone, ob/ob mice started to develop obesity at physiological replacement plasma corticosterone concentrations whereas lean counterparts did not. This increased sensitivity to corticosterone may be due to changes in many possible factors, eg. higher rates of corticosterone uptake by tissues, changes in glucocorticoid receptor number or binding affinity, or changes in post-receptor binding event. I will focus on glucocorticoid receptors to determine whether the observed hypersensitivity to corticosterone in ob/ob mice might be explained by changes in the number or binding affinity of glucocorticoid receptors.

I first investigated the number of cytosolic glucocorticoid receptors and their binding affinity in

intact 8-wk-old lean and ob/ob mice. Adrenalectomy was then used to eliminate the difference of plasma corticosteone concentrations between lean and ob/ob mice and to determine how corticosterone affected the binding characteristics of glucocorticoid receptors. Upon binding to ligand in the cytosol, glucocorticoid receptors are transformed to an activated form (6-7) which in turn is translocated to the nucleus (7-8) and bound to deoxyribonucleic acid (DNA). Since the ligand-receptor binding assay cannot measure activated receptors (9), another purpose of adrenalectomy was to eliminate corticosterone so that the nuclear glucocorticoid receptors would recycle from the nucleus and become unactivated in the cytosol. With adrenalectomy, cytosolic receptor number, representing the total corticoid receptor number in the cell, was then determined. Finally, dexamethasone was injected into adrenalectomized mice to determine whether there was a difference in receptor activation rates between lean and ob/ob mice when exposed to glucocorticoid.

## LITERATURE REVIEW

### Adrenalendocrine regulation of glucocorticoid secretion

The mammalian adrenal consists of two different segments, the medulla and the cortex (13). These two segments have different embryologic origins and produce different hormones. The adrenal medulla produces catecholamine hormones. Release of catecholamines from the adrenal medulla is regulated by neural stimulation, which is a calcium dependent process (13). The adrenal cortex produces glucocorticoid, mineralcorticoid and androgenic steroids. Release of corticoids from the adrenal cortex is regulated by adrenocorticotropic hormone (ACTH), which in turn is regulated by corticotropin-releasing hormone (CRH) (13-15).

ACTH activates adenylate cyclase through the $G_s$ protein receptor system and increases cAMP levels in the cortex cell (13). cAMP dependent protein kinase (protein kinase A) is then activated by cAMP. An esterase is activated by protein kinase A and converts cholesterol ester to free cholesterol. Cholesterol is cleaved in the mitochondria by a cytochrome P-450 side chain cleavage enzyme (P-450scc) to form pregnenolone, which is the precursor of all the steroid hormones. Since there is little, if any, storage of steroid hormone in the adrenal cell, steroid hormones are released into the plasma as they are synthesized. Thus, ACTH increases the production and release of adrenal steroids by enhancing the conversion of

cholesterol ester to pregnenolone. Since the concentrations of plasma corticoids are primarily governed by release of corticoids from the adrenal cortex, plasma corticoid concentrations show a periodicity that follows the diurnal rhythm of ACTH levels.

ACTH is a 39 amino acid peptide synthesized from the proopiomelanocortin (POMC) gene (13-15). POMC peptide is synthesized mainly in the anterior and intermediate lobe of the pituitary gland as a precursor of about 285 amino acids. Cleavage of the POMC peptide gives rise to ACTH, beta-lipotropin and an N-terminal peptide in the anterior pituitary, but in the intermediate lobe, these peptides are further cleaved to form corticotropin-like intermediate lobe peptide (CLIP), melanocyte-stimulating hormone (MSH), and endorphins.

CRH and glucocorticoids are the major factors controlling release of ACTH from the anterior pituitary gland (13). CRH works through a cAMP-mediated $G_s$ protein receptor system to signal the release of ACTH. However, it is not clear whether CRH can increase the mRNA abundance of PMOC gene. Glucocorticoids on the other hand can inhibit the release of ACTH and reduce the transcription rate of POMC gene, ie. negative feedback control (16). Release of ACTH is also controlled by neural input from a number of sites. Neural input from the suprachiasmatic nucleus drives the diurnal rhythm that controls release of CRH, and therefore release of ACTH and corticoids. Neural input from

the amygdala nuclei mediates the ACTH response to emotional stress, apprehension, fear and anxiety, whereas fibers from the spinothalamic pathway and reticular formation mediate the response to pain. These responses are able to override both the diurnal rhythm and negative feedback control on ACTH.

CRH is a 41 amino acid neural peptide synthesized in the hypothalamus (17). Synthesis and release of CRH from the hypothalamus is under a sophisticated regulation. Glucocorticoids are able to regulate the mRNA abundance for CRH in the hypothalamus (negative feedback control). Adrenalectomy increases mRNA abundance for CRH, while replacement corticosterone decreases mRNA for CRH in adrenalectomized rats (18). Release of CRH is regulated by neural inputs; acetylcholine and norepinephrine have stimulatory effects on release of CRH from hypothalamus (19-20). The response to acetylcholine is mediated through a muscarinic receptor while the response to norepinephrine is mediated through a beta-adrenergic receptor. Release of CRH is under feedback control of downstream hormones and has been shown in vitro to be inhibited by CRH, ACTH, and glucocorticoid (20).

Effects of glucocorticoids on nutrient metabolism

As the name implies, glucocorticoids are corticoid hormones that are important in the regulation of glucose metabolism and maintenance of plasma glucose homeostasis (13, 21). Glucocorticoids maintain plasma glucose

concentrations by enhancing gluconeogenesis in liver and, to a smaller extend, in kidneys. Glucocorticoids also increase degradation of proteins and mobilization of triacylglycerol in extrahepatic tissues as the carbon and energy sources for gluconeogenesis; at the same time glucocorticoids inhibit utilization of glucose in these tissues. Glucocorticoids increase gluconeogenesis and amino acid degradation by increasing the amount of enzymes, eg. phosphoenolpyruvate carboxykinase in gluconeogenesis, and alanine aminotransferase, tyrosine aminotransferase, and tryptophan oxygenase in amino acid degradation. The increase in enzyme protein is due to an increase in transcription rate, which is enhanced by the interaction of glucocorticoid receptors with the hormone responsive elements on DNA (22).

Glucocorticoids also have permissive and synergistic effects on the action of glucagon and catecholamines, two other hormones involved in nutrient metabolism (13,21). In the presence of glucocorticoids, actions of glucagon and catecholamines are optimized (permissive effect). Combined treatment with glucagon, catecholamines and glucocorticoids increases glucose production to a much greater extend than the sum of treatments with each individual hormone (synergistic effect). On the other hand, in the absence of glucocorticoids, eg. adrenalectomy, functions of glucagon and catecholamines are attenuated.

Since glucocorticoids have a net effect of increasing plasma glucose concentrations, they indirectly potentiate

secretion of insulin from the pancrease via hyperglycemia (21). Glucocorticoids may also enhance insulin secretion from pancreas via a glucose-independent pathway since injection of glucocorticoid into intracerebral ventricles increases plasma insulin concentrations without increasing plasma glucose concentrations (unpublished observation, Cho-Walker).

Glucocorticoids have dual effects on insulin receptors: glucocorticoids increase the number of insulin receptors within 1-2 days by reducing the inactivation of insulin receptors, whereas with longer exposure to glucocorticoids (3-7 days), insulin receptor number decreases (21). This decrease in insulin receptor number is at least partially secondary to glucocorticoid-induced hyperinsulinemia and contributes to insulin resistance.

Glucocorticoid and mineralcorticoid receptors

There are two subtypes of corticoid receptors, the glucocorticoid receptor and the mineralcorticocoid receptor (23-24). Both glucocorticoid and mineralcorticoid receptors are members of the steroid receptor superfamily (25). They are very similar to other steroid receptors in structure and the molecular functioning mechanism.

The glucocorticoid receptor, which is also referred as type 2 corticoid receptor, has high affinity for glucocorticoids, eg. cortisol and corticosterone (26). The glucocorticoid receptor has been found in most organs, eg. liver (27-28), muscle (29-30), adipose tissue (31-32),

pancreas (33) and brain (23-26). Functions of the glucocorticoid receptor in peripheral tissues reflect the hormonal effects of glucocorticoids. Functions of the glucocorticoid receptor in brain are not fully understood, but one of the known functions is to mediate the feedback control of glucocorticoids (16,26). The glucocorticoid receptor has a wide distribution in brain, but it is concentrated in neurons and glial cells of the paraventricular nucleus, site of CRH synthesis, and the n. tractus solitarii, site of blood pressure regulation (26). These receptors are responsible for the feedback control on CRH production and blood pressure.

The mineralcorticoid receptor which is also referred as type 1 corticoid receptor has very high affinities for aldosterone and corticosterone, but relatively lower affinity for glucocorticoids (26). The mineralcorticoid receptor in the kidney regulates mineral homeostasis, whereas the mineralcorticoid receptor in cerebral circumventricular organs regulates salt appetite; in hippocampal and septal neurons, it mediates tonic influence of corticosterone and responds with stringent specificity to corticosterone (26).

To distinguish a glucocorticoid receptor from a mineralcorticoid receptor, specific agonists or antagonists for corticoid receptors, which have minimum cross binding reactivity to the other subtype of receptor, are needed (24). Dexamethasone is a synthetic glucocorticoid agonist

that has a high affinity for the glucocorticoid receptor, and a moderate affinity for the mineralcorticoid receptor. RU28362 is a synthetic glucocorticoid antagonist which has a very high affinity for the glucocorticoid receptor, and a very low affinity for the mineralcorticoid receptor.

There are several approaches to determine glucocorticoid and mineralcorticoid receptor numbers. One can use labeled dexamethasone to measure glucocorticoid plus mineralcorticoid receptor number, and use labeled dexamethasone in the presence of RU28362, a glucocorticoid antagonist, to measure mineralcorticoid receptor number (23-24). The number of glucocorticoid receptor is then calculated as the difference between these two measurements. One can also use labeled RU28362 to measure glucocortiocoid receptor number directly, and labeled aldosterone in the presence of RU28362 to measure mineralcorticoid receptor number directly (23-24).

Since much more research on molecular mechanisms of corticoid receptor action has been done with the glucocorticoid receptor than the mineralcorticoid receptor, the functioning of the glucocorticoid receptor will be discussed here as an example. There are two major domains involved in functioning of the glucocorticoid receptor, the steroid binding domain and the DNA binding domain (6,25). The steroid binding domain has specificity for the ligand, though it may exhibit low affinity for closely-related steroids. The DNA binding domain is composed of two

zinc-finger structures, which have been proposed to interact with the hormone responsive elements on DNA, and regulate transcription frequency of the gene downstream (25).

The glucocorticoid receptor has a unique characteristic distribution in cells. Unbound free receptor is located in the cytosol, and associates with heat shock protein 90 (HSP90) forming a receptor-HSP90 complex (25). HSP90 is thought to stablize the receptor, and prevent it from binding to the glucocorticoid responsive element (GRE) on the DNA.

When ligand binds to the cytosolic glucocorticoid receptor, the interaction between these two molecules triggers a conformational change in the receptor (34). This ligand bound receptor is then activated. During activation, the HSP90 dissociates from the receptor-HSP90 complex, and exposes the DNA binding site on the receptor. This change in receptor structure and size is also referred to as transformation (34). Activated receptor is then translocated to the nucleus where it binds to the glucocorticoid responsive element on DNA, and consequently either stimulates or inhibits transcription of the gene downstream. Therefore, like other steroid receptors, glucocorticoid receptors exert action at the transcription level.

Besides transcriptional effects, there is accumulating evidence suggesting that glucocorticoids may have certain exonucleus effects independent of transcription.

Dexamethasone is able to stimulate protein synthesis in enucleated human fibroblasts (35). Kanazir et al (36) reported that cortisol enhanced protein synthesis and histone phosphorylation rates significantly within 10 minutes which was shorter than the time required for cortisol to exert its function through transcription and translation. Therefore, glucocorticoids may function via a exonucleus pathway that does not require transcription or translation. The molecular mechanism of this exonucleus pathway is, however, not clear.

Cushing's syndrome

Cushing's syndrome is a human disease characterized by high concentrations of plasma cortisol (37-38). The causes of Cushing's syndrome are usually due to excessive circulating cortisol or ACTH, either exogenously adminstered or endogenously over-secreted. In the ACTH-dependent types of Cushing's syndrome, ACTH is over-secreted by the pituitary gland or by an ectopic source. In some cases, CRH is over-secreted by an ectopic source which leads to over-secretion of ACTH by the pituitary gland. In the ACTH-independent types of Cushing's syndrome, excessive cortisol is secreted by adrenocortical tumors or by autonomous nodular hyperplastic glands.

Common symptoms of Cushing's syndrome include 1) truncal obesity; 2) hyperglycemia; 3) signs of protein catabolism; 4) osteoporosis; 5) purple striae; 6) hypertension; 7) hirsutism; 8) hyperpigmentation, and 9)

psychiatric manifestation. All of these symptoms are attributable to excessive corticoids and androgen secretion by the adrenal cortex; symptoms can be reversed by adrenalectomy or removal of ectopic tissues. Of these symptoms, truncal obesity is most common among Cushing's syndrome patients (38). About 95% of Cushing's syndrome patients develop obesity. Obese (ob/ob) mice provide a good animal model to study the mechanism of obesity development in Cushing's syndrome, since high concentrations of corticoids have been directly or indirectly linked to development of obesity in these animals (2-4).

Corticosterone in obese mice and rats

Corticosterone is the main glucocorticoid found in rodents (13). Several obese rodents models have been reported to have higher levels of plasma corticosterone than their lean counterparts (1, 2, 40). Among these rodents, the obese (ob/ob) mouse has been most widely studied. The rise of plasma corticosterone concentrations in ob/ob mice occurrs at about 3 weeks of age, and the plasma corticosterone concentrations remain high for most of the life span (1-2). Other animal models, eg. fa/fa rats and db/db mice have been also reported to have higher concentrations of plasma corticosterone, though the rise of corticosterone concentrations are at different ages (2). Some researchers have not observed elevated corticosterone concentrations in fa/fa rats (12).

In ob/ob mice, the higher concentrations of

corticosterone are not caused by an impaired degradation of plasma corticosterone, but rather by elevated ACTH levels (2, 39). The cause of elevated ACTH levels is not clear in ob/ob mice, however, in fa/fa rats, it has been demonstrated that the hypothalamo-pituitary-adrenal axis has increased activity with increased hypothalamic CRH and anterior pituitary ACTH contents and hypertrophic zona fasciculatea in adrenal cortex (40-41).

Role of corticosterone in the development of obesity in animals

It is generally thought that the higher concentration of plasma corticosterone is not the primary factor causing development of obesity in rodents, because obesity starts to develop in these animals before the rise of plasma corticosterone concentrations (2, 5). However, the presence of corticosterone is essential for development of obesity, since removal of corticosterone by adrenalectomy prevents development of obesity in these animals, and effects of adrenalectomy are abolished by replacement corticosterone (2). Interestingly, adrenalectomy has minimum effects on body composition in lean counterparts of the obese rodents.

When adrenalectomized animals receive replacement corticosterone, genetically obese rats and mice start to develop obesity at physiological replacement plasma corticosterone concentrations whereas lean counterparts do not (5, 42). These data indicate that obese rodents not only need corticosterone to develop obesity, but that they

are also more sensitive to replacement corticosterone than lean counterparts.

There are several possible alterations that might account for higher sensitivity to corticosterone in obese rodents. Uptake of corticosterone by the cell, receptor binding affinty, receptor number, binding of receptor to DNA and post-receptor events are all possible sites where the effectiveness of glucocorticoid might be altered to enhance the sensitivity. Since we have already observed that there are no differences in uptake of corticosterone by liver and brain in genetically obese (ob/ob) mice versus lean counterparts (unpublished observation, Warwick et al), I focused on the possibility that corticosterone receptor number and binding affinity might be altered in obese (ob/ob) mice, causing hypersensitivity to corticosterone.

Glucocorticoid receptors in obese animals

The concentrations and binding affinities of the cytosolic glucocorticoid receptor in obese animals have been measured by several researchers. Svec (10) reported that the glucocorticoid receptor number and binding affinity in liver and kidney of 12-week-old male ob/ob mice were not significantly different from those of their counterparts. These results are surprising since the higher concentrations of circulating plasma corticosterone in ob/ob mice would be expected to cause down-regulation of the cytosolic glucocorticoid receptor numbers (27-28). Shargill et al (12) observed a lower liver cytosolic glucocorticoid

receptor binding in 6-week-old female fa/fa rats than their lean counterparts, even though the plasma corticosterone concentrations were similar in fa/fa and lean rats. Interestingly, adrenalectomy increased the receptor bindings in both fa/fa and lean rats to a similar level. However, the binding affinity of glucocorticoid receptor was not determined. Webb et al (11) reported that mdb/mdb mice have lower cytosolic glucocorticoid receptor binding in liver and several brain regions. However, the age and gender of their mice were not mentioned specifically. Whereas in another trial, the glucocorticoid receptor numbers were determined in brain and liver of male or female mdb/mdb mice between 1-10 months of age. It appeared that at some ages glucocorticoid receptor numbers were lower in mdb/mdb mice than in lean mice. Unfortunately, each observation eas determined with a pool of 3-5 mice, therefore the difference between lean and mdb/mdb mice could not be confirmed with statistics. Since their mdb/mdb mice did not survive adrenalectomy, they were unable to obtain information about receptor bindings after adrenalectomy. The receptor binding affinity in mdb/mdb mice was not determined, either.

Despite these reports on glucocorticoid receptors in obese animals, it is still not clear if the glucocorticoid receptor is involved in obesity development, and what causes hypersensitivity to corticosterone in obese rats and mice. In addition, concentrations of the mineralcorticoid receptor in brain have not been reported in obese animals. To answer

these questions, it is necessary to investigate the binding kinetics of the glucocorticoid receptor and compare responses of receptors to adrenalectomy and replacement glucocorticoid in lean and obese animals.

## EXPERIMENTAL

### Animals

Lean (+/?) and obese (ob/ob) male mice, weighing approximately 25 g and 38 g, respectively, were obtained from our breeding colony (C57BL/6J-ob/+) and used at 4 or 8 weeks of age. Littermate lean and ob/ob mice were weaned at 3 weeks of age and housed in plastic boxes containing wood chips for bedding. Room lights were on between 0700-1900 h, and room temperature was maintained at 22-25° C. Mice were allowed ad libitum access to stock diet (Wayne Rodent Blox, Bartonville, IL), and water. Mice were killed between 1000-1100 h by cervical dislocation.

Some 8-wk-old mice were anesthetized with ether and bilaterally adrenalectomized 2, 24, 48, or 72 h before they were used. Adrenalectomized mice were given 0.9% saline solution to drink. Adrenalectomized mice were used only if plasma corticosterone concentrations were less than 1 ug/dl. Some adrenalectomized mice received an intraperitoneal injection of dexamethasone (0, 0.5, or 5.0 ug/g body weight) 1 h before cervical dislocation. Dexamethasone solution was prepared by dissolving 5 mg dexamethasone in 1 ml of 100% alcohol; this solution was then diluted to 50 or 500 ug/ml with saline.

### Materials

[6,7-$^{3}$H] Dexamethasone (50 Ci/mmole), [1,2,6,7-$^{3}$H] aldosterone (75 Ci/mmole), [1,2-$^{3}$H] corticosterone (58 Ci/mmole) and RU28362, a glucocorticoid antagonist, were

purchased from New England Nuclear (Boston, MA). Dexamethasone, aldosterone, corticosterone, Tris HCl, Tris base, EDTA, molybdate ($Na_2MoO_4$), dithiothreitol (DTT) and activated charcoal were purchased from Sigma (St. Louis, MO). Dextran was obtained from Pharmacia (Piscataway, NJ).

Cytosol preparation

Mice were killed by cervical dislocation; the brains, intrascapular and subscapular brown adipose tissue depots, and livers were excised and washed in a cold buffer containing 20 mM Tris, 1 mM EDTA, 10 mM molybdate (TEM buffer) pH 7.4 at 25° C. Tissues were homogenized with a teflon pestle in a glass tube (10 strokes). All tissues were homogenized with 3 volumes (w/v) of a cold buffer containing 20 mM Tris, 1 mM EDTA, 10 mM molybdate, 5 mM DTT (TEMD buffer) pH 7.4 at 25° C, except for brown adipose tissue depots of ob/ob mice which were homogenized with 1.5 volumes (w/v) of cold TEMD buffer because of the lower concentration of cytosolic protein in brown adipose tissue of ob/ob mice. Homogenates were centrifuged at 81,000 x g at 1° C for 1 h. Supernatants were used immediately in the receptor binding assays.

Cytosolic glucocorticoid receptor binding assay

For Scatchard analysis of binding affinity and maximum binding sites of the receptors, 100 ul of cytosol, containing approximately 3 mg protein, was incubated in final concentrations of 3.75, 7.5, 15, 30, 60 or 120 nM [$^3$H] dexamethasone in the absence or presence of 15 uM

(final concentration) dexamethasone for total and non-specific binding determinations, respectively. When the receptor binding assay was conducted at a single ligand concentration, 30 nM [$^3$H] dexamethasone was used. Binding of brain glucocorticoid receptor was determined by incubating brain cytosol with 30 nM [$^3$H] dexamethasone in the absence or presence of 3 uM (final concentration) RU28362 for glucocorticoid plus mineralcorticoid receptors and mineralcorticoid receptor, respectively. Binding of brain glucocorticoid receptor was then calculated as the difference between these two measurements. TEMD buffer was added to adjust final incubation volumes to 250 ul for liver cytosol or 150 ul for brain and brown adipose tissue cytosols.

Cytosol preparations were incubated for 5 h at 0-4$^{o}$ C. At the end of the incubation, an equal volume of cold dextran-charcoal (3.75 g activated charcoal and 0.375 g dextran/100 ml TEM buffer) was added to the incubation media to absorb unbound dexamethasone. After 10 min, the mixture was centrifuged at 3,000 rpm for 5 min to pellet the dextran-charcoal mixture. A 200 ul aliquot of supernatant was then removed for determination of bound [$^3$H] dexamethasone in a liquid scintillation counter. Specific binding was obtained by subtracting non-specific binding from total binding.

Cytosolic mineralcorticoid receptor binding assay

Brain cytosol was incubated in a final concentration of 10 nM [$^3$H] aldosterone in the presence of 1 uM (final concentration) RU28362 for total binding or 2 uM (final concentration) aldosterone for non-specific binding. Incubation and separation conditions were the same as those for the glucocorticoid receptor binding assay.

Protein and corticosterone assays

Protein concentrations were determined by the method described by Lowry with bovine serum albumin as the standard. Plasma corticosterone concentrations were determined by radioimmunoassay (Endocrine Science, Tarzana, CA).

Statistics

Linear regression analysis was employed to obtain $K_d$ and $B_{max}$ values from Scatchard analysis data. The Student's two-tailed t-test was used to detect significant differences between lean and ob/ob mice. Multiple treatment effects were analyzed by analysis of variance (ANOVA). If heterogenous variance was significant, the data were logistically transformed before ANOVA. The Bonferroni two-tailed t-test was employed to detect post-hoc differences between treatment and control groups.

## RESULTS

General characteristics of 8-wk-old lean and ob/ob mice are shown in table 1. All variables, except brain protein content, of ob/ob mice were significantly different from those of lean mice.

The dissociation constant ($K_d$) of the cytosolic glucocorticoid receptors for dexamethasone in each organ of intact 8-wk-old ob/ob mice examined was higher than that of intact lean mice, indicating that the glucocorticoid receptors from ob/ob mice had a weaker binding affinity for dexamethasone (table 2). Dissociation constants were similar among the three organs examined within each phenotype.

There were fewer maximum binding sites, $B_{max}$ (fmol/mg protein), for cytosolic glucocorticoid receptors in intact ob/ob mice than in intact lean mice in each organ examined (table 2). $B_{max}$ values in liver and brain were more than twice as high as those in brown adipose tissue within each phenotype. Maximum binding sites of the whole organ, $B_{max}$ (pmol/organ), was higher in livers of ob/ob mice than in livers of lean mice (table 2) because ob/ob mice had more hepatic cytosolic protein (table 1).

Since 8-wk-old ob/ob mice had higher plasma corticosterone concentrations, the specific binding of dexamethasone to liver and brain glucocorticoid receptors was measured in 4-wk-old mice, at which time plasma corticosterone concentrations are similar in lean and ob/ob

Table 1. Characteristics of lean and ob/ob mice.

| | Phenotype | |
|---|---|---|
| | Lean | ob/ob |
| Body Weight, g | | |
| | 25±1 | 38±1* |
| Plasma Corticosterone, ug/dl | | |
| | 4±1 | 36±6* |
| Organ Weight, mg | | |
| Liver | 1660±90 | 3520±80* |
| Brain | 473± 4 | 437± 2* |
| BAT | 195± 7 | 394±16* |
| Cytosolic Protein Content, mg/organ | | |
| Liver | 161.3±4.8 | 323.5±7.8* |
| Brain | 15.8±0.3 | 15.2±0.3 |
| BAT | 5.4±0.4 | 3.8±0.4* |

Values are means ± S.E. for 8-wk-old mice; body weight and plasma corticosterone values are means of 6 mice; organ weight and protein content are means of 10, 15 , or 30 mice for liver, brain, and BAT, respectively.
BAT indicates intrascapular and subscapular brown adipose tissue depots.

* Significantly different from lean at $p<0.05$ with Student's two-tailed t-test.

Table 2. Dissociation constants ($K_d$) and maximum binding sites ($B_{max}$) of glucocorticoid receptor.

| | Organ | | |
|---|---|---|---|
| | Liver | Brain | BAT |
| $K_d$, nM | | | |
| Lean | 9.5±1.0 | 9.7±0.7 | 9.4±0.9 |
| ob/ob | 15.3±1.1* | 16.6±2.3* | 14.4±1.9* |
| $B_{max}$, fmol/mg protein | | | |
| Lean | 329±20 | 290± 8 | 129±11 |
| ob/ob | 243± 6* | 224±13* | 96± 4* |
| $B_{max}$, pmol/organ | | | |
| Lean | 52.9±2.5 | 4.58±0.15 | 0.71±0.06 |
| ob/ob | 78.8±4.0* | 3.39±0.19* | 0.36±0.03* |

Values are means ± S.E. of 5 observations in 8-wk-old mice; each observation is a pool of 2, 3, or 6 mice for liver, brain, and BAT, respectively.
BAT indicates intrascapular and subscapular brown adipose tissue depots.
* Significantly different from lean at $p<0.05$ with Student's two-tailed t-test.

mice (table 3). Specific bindings of dexamethasone to glucocorticoid receptors in livers or brains of 4-wk-old ob/ob mice were similar to those values in lean counterparts (table 3).

Adrenalectomy slightly decreased liver cytosolic protein content in 8-wk-old obese mice, but not in lean mice, by 24 h after the surgery (figure 1). Adrenalectomy had minimal effects on brain cytosolic protein content (figure 1).

The time-course of increases in dexamethasone binding (30 nM) to cytosolic glucocorticoid receptors in livers and brains, and aldosterone binding (10 nM) to brain cytosolic mineralcorticoid receptors, after adrenalectomy is shown in figure 2 (left panels). Increases in specific binding to glucocorticoid receptors occured more rapidly after adrenalectomy in ob/ob mice than in lean mice. Specific binding of dexamethasone to glucocorticoid receptors increased 38% in livers and 110% in brains of ob/ob mice within 2 h after adrenalectomy, while specific binding increased only 26% in livers and 14% in brains of lean mice within 24 h after adrenalectomy. Specific binding to glucocorticoid receptors in livers, but not in brains, of ob/ob mice remained slightly lower than that of lean mice 48 h after adrenalectomy (figure 2, left panels). The lower binding of dexamethasone to receptors in intact ob/ob mice combined with the rapid increase of binding after adrenalectomy caused a significant interaction of phenotype

Table 3. Characteristics and cytosolic glucocorticoid receptor specific binding in 4-wk-old mice.

| | Phenotype | |
|---|---|---|
| | Lean | ob/ob |
| Body Weight, g | 13.3±0.3 | 13.2±0.4 |
| Plasma Corticosterone, ug/dl | 7.0±0.9 | 9.5±0.5 |
| Cytosolic Protein Content, mg/organ | | |
| Liver | 101±4 | 117±9 |
| Brain | 21±1 | 20±1 |
| Specific Binding[1], fmol/mg protein | | |
| Liver | 330±49 | 303±34 |
| Brain | 116±14 | 87± 7 |

Values are mean ± S.E. of 6 mice.

[1] Specific binding of dexamethasone to cytosolic glucocorticoid receptors determined with 30 nM dexamethasone, see method.
There were no significant differences between lean and ob/ob mice ($p>0.05$) with Student's two-tailed t-test.

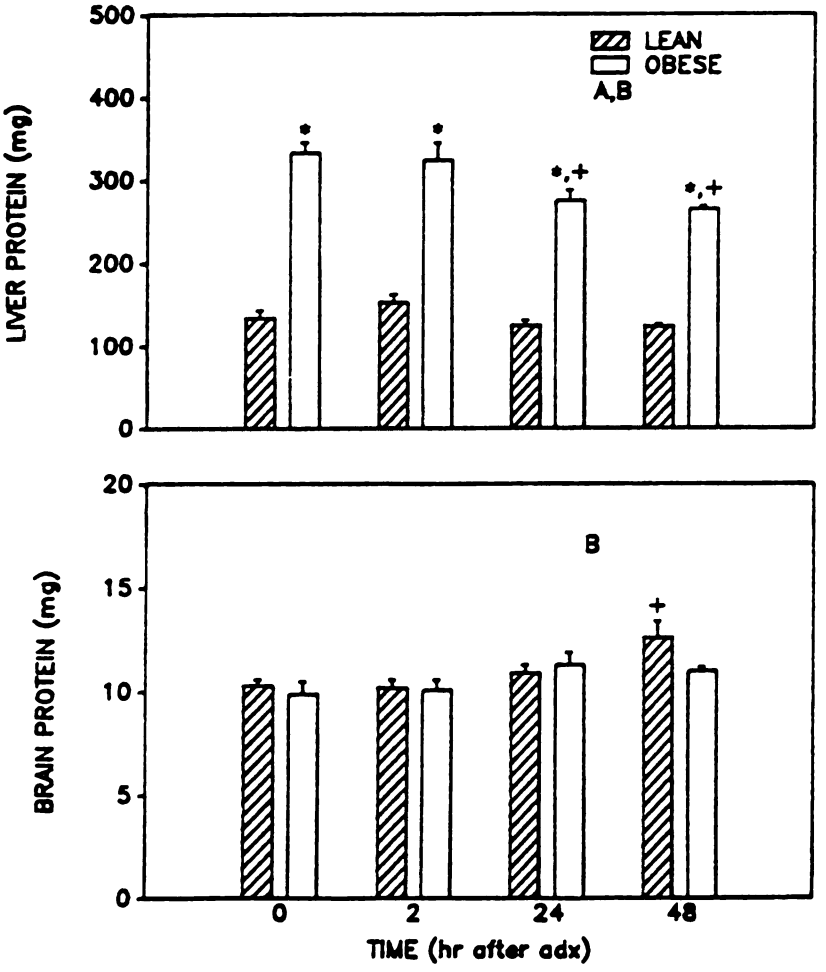


Figure 1. Effect of adrenalectomy on liver cytosolic protein content (upper panels) and brain cyotosolic protein content (lower panels). Each bar represents a mean of 6 8-wk-old mice. Letters in the upper portion of each panel indicate the significant effects of phenotype (A), adrenalectomy (B), or interaction (AB).

* Significantly different from corresponding lean mice at $p<0.05$.

\+ Significantly different from corresponding 0-time group at $p<0.05$.

adx: adrenalectomy.

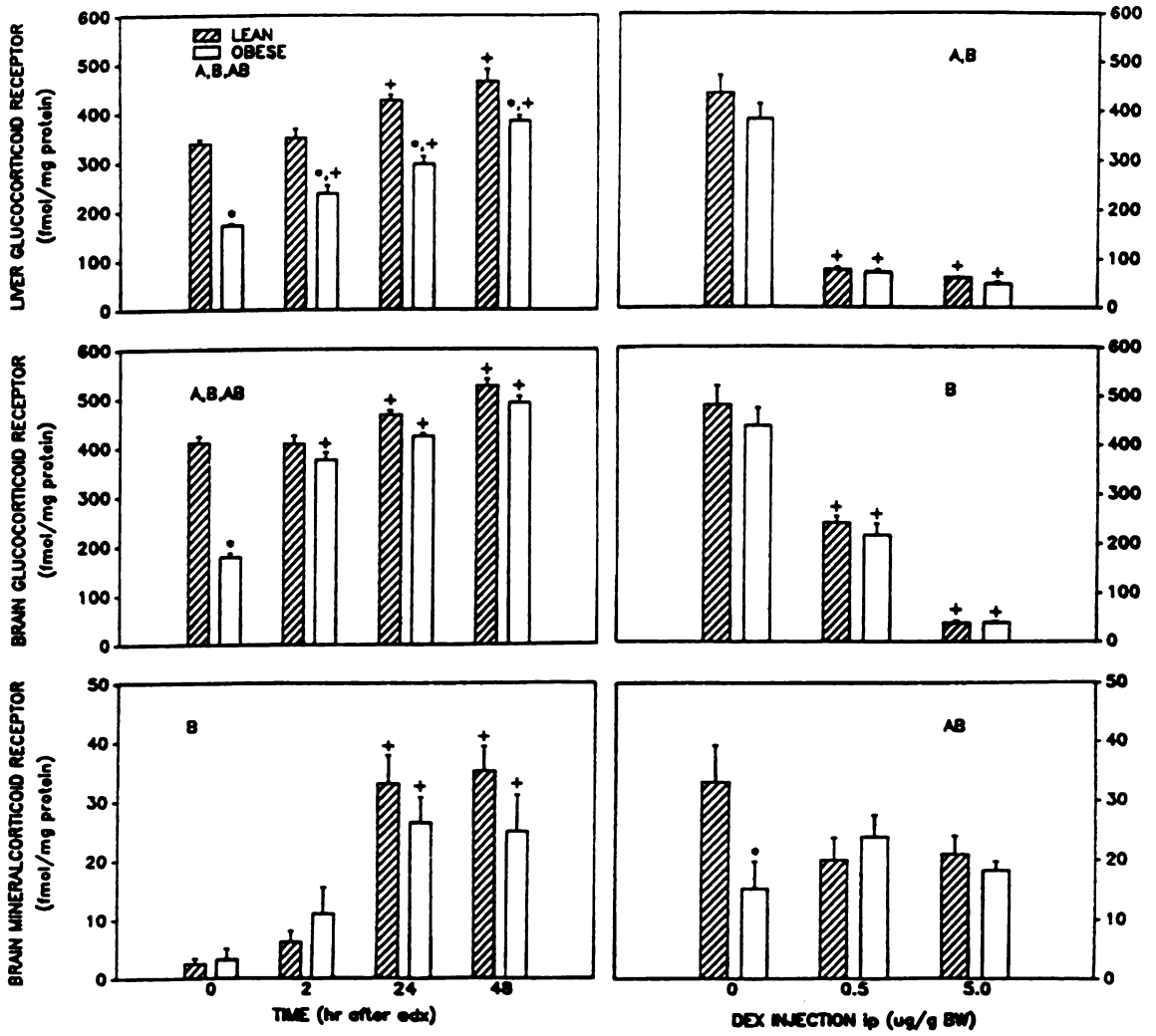


Figure 2. Effect of adrenalectomy (left panels) and dexamethasone injection, ip (right panels), on liver glucocorticoid receptor (upper panels), brain glucocorticoid receptor (middle panels), and brain mineralcorticoid receptor (lower panels). Each bar represents a mean of 6 (adrenalectomy effect) or 5 (dexamethasone injection) 8-wk-old mice. Letters in the upper portion of each panel indicate the significant effects of phenotype (A), adrenalectomy/dexamethasone (B), or interaction (AB). Glucocorticoid receptor bindings were measured with 30 nM of dexamethasone, and mineralcorticoid receptor bindings were measured with 10 nM of aldosterone, see methods for details. Receptor bindings was measured 1 h after injection of dexamethasone. Cytosolic protein content for mice in the adrenalectomy study are shown in figure 1, and those for dexamethasone injection study are 110 mg (lean liver), 210 mg (ob/ob liver), 11 mg (lean brain), and 12 mg (ob/ob brain), respectively.

* Significantly different from corresponding lean mice at $p<0.05$.

\+ Significantly different from corresponding 0-time or 0-dose group at $p<0.05$.

adx: adrenalectomy.

and adrenalectomy.

Specific binding of aldosterone to brain cytosolic mineralcorticoid receptors was relatively low in both intact lean and ob/ob mice (figure 2, lower left panel). Specific binding to mineralcorticoid receptors increased similarly in lean and ob/ob mice within 48 h after adrenalectomy.

Dissociation constants ($K_d$) and maximum binding sites ($B_{max}$) of hepatic cytosolic glucocorticoid receptor in intact and adrenalectomized (48 h) lean and ob/ob mice are shown in table 4. Adrenalectomy decreased the dissociation constants of liver cytosolic glucocorticoid receptors significantly ($p<0.05$) in ob/ob mice and slightly ($p<0.1$) in lean mice. Differences in $K_d$ values between lean and ob/ob mice, however, remained significant 48 h after adrenalectomy. $B_{max}$ values (fmol/mg protein) calculated from the Scatchard analysis showed similar responses as observed with the single ligand concentration assay (table 4 and figure 2). Since adrenalectomy had minimal effects on liver protein content (figure 1), maximum binding sites in the whole liver (pmol/liver) remained elevated in adrenalectomized ob/ob mice.

Responses of cytosolic glucocorticoid and mineralcorticoid receptors to injection of dexamethasone are shown in figure 2, right panels. Intraperitoneal injection of dexamethasone, 0.5 and 5.0 ug/g body weight, decreased binding of dexamethasone to hepatic glucocorticoid receptors in both lean and ob/ob mice to 18% of those in

Table 4. Effects of adrenalectomy on $K_d$ and $B_{max}$ of liver glucocorticoid receptor.

| | Treatment | | |
|---|---|---|---|
| | Intact | Adx[2] | ANOVA[1] |
| $K_d$, nM. | | | A,B |
| Lean | 7.5±0.8 | 5.3±0.2 | |
| ob/ob | 13.0±1.5* | 8.0±0.4*,+ | |
| $B_{max}$, fmol/mg protein. | | | A,B,AB |
| Lean | 404±13 | 570±39+ | |
| ob/ob | 242±23* | 560±27+ | |
| $B_{max}$, pmol/liver. | | | A,B,AB |
| Lean | 57±2 | 71± 6 | |
| ob/ob | 80±3* | 136±11*,+ | |

Values are means ± S.E. for 5 8-wk-old mice.

[1] Analysis of variance for 2 factors (A:phenotype effect, B: adrenalectomy effect) with interaction. Significant level is at $p<0.05$ on F-test.

[2] Adrenalectomized 48 hours before the receptor assay

* Significantly different from corresponding lean mice at $p<0.05$.

\+ Significantly different from corresponding intact mice at $p<0.05$.

adrenalectomized mice injected with saline. In brains, binding of dexamethasone to glucocorticoid receptors in both lean and ob/ob mice decreased to 50% of those of control group after injection of 0.5 ug dexamethasone/g body weight, and further down to 8% after injection of 5.0 ug dexamethasone/g body weight. Intraperitoneal injection of dexamethasone, however, had minimal effects on the binding of aldosterone to brain cytosolic mineralcorticoid receptors in adrenalectomized lean and ob/ob mice (figure 2, lower right panel).

## DISCUSSION

Cytosolic glucocorticoid receptor numbers in liver, brain, and brown adipose tissues were lower in 8-wk-old intact ob/ob mice than in lean mice (table 2). These finding are in agreement with the higher plasma corticosterone concentrations in 8-wk-old ob/ob mice (table 1), and the expected down-regulation of cytosolic glucocorticoid receptors induced by higher plasma corticosterone concentrations (27-28). It is unclear why Svec did not observe a significant depression in hepatic glucocorticoid receptor number in ob/ob mice (10), although his values tended to be lower in the ob/ob mice. Webb et al (11) reported that cytosolic glucocorticoid receptor numbers in liver and several regions of brain were lower in mdb/mdb mice than in lean mice. However, the gender and age of their animals were not mentioned. Whereas in another trial, glucocorticoid receptor numbers were determined in brain and liver of male or female mdb/mdb mice between 1-10 months of age. At some, but not all, ages receptor number appeared to be lower in mdb/mdb mice than lean mice. Unfortunately, each observation was determined with a pool of 3-5 mice, thus, differences between lean and mdb/mdb mice could not be confirmed with statistics. Shargill et al (12) reported that hepatic glucocorticoid receptor number was also lower in 6-wk-old fa/fa rat than in lean rat, even though plasma corticosterone concentrations were similar in lean and fa/fa rats. These observations indicated that obese animals

generally have lower cytosolic glucocorticoid receptor numbers. Nevertheless, the lower receptor numbers could have been secondary to the higher plasma corticosterone concentrations in obese animals.

To avoid the confounding influence of hypercorticosteronemia on cytosolic glucocorticoid receptor number, 4-wk-old mice were examined where plasma corticosterone concentrations were unaffected by phenotype. Under these conditions, cytosolic glucocorticoid receptor number was not altered in either liver or brain of ob/ob mice (table 3). Furthermore when 8-wk-old ob/ob mice were adrenalectomized, cytosolic receptor number in liver and brain of ob/ob mice increased to approximately parallel values observed in lean mice (table 4 and figure 2, left panels). In addition, Shargill et al (12) reported that adrenalectomy abolished the difference in hepatic glucocorticoid receptor numbers between 6-wk-old lean and fa/fa rats. These data suggest that the low cytosolic glucocorticoid receptor number in tissues of ob/ob mice is simply a secondary consequence to elevated corticosterone concentrations in these mice.

Removal of plasma corticosterone by adrenalectomy caused much more rapid increases in cytosolic glucocorticoid receptors in liver and brain of ob/ob mice than lean mice (figure 2, left panels). The most likely explanation for these rapid increases (within 2 h) in cytosolic glucocorticoid receptor number is the translocation of

receptor from the nucleus in the absence of corticosterone. This suggests that under physiological conditions there are more activated glucocorticoid receptors in the nucleus in ob/ob mice than in lean mice. Since glucocorticoid receptors function by interacting with glucocorticoid responsive elements in the nucleus, the greater number of glucocorticoid receptors in the nucleus of intact ob/ob mice would be expected to elicit a greater corticosterone responsiveness in these mice.

Injection of dexamethasone reduced cytosolic glucocorticoid receptor number in livers and brains of adrenalectomized lean and ob/ob mice to a similar extend. These data suggest that activation of glucocorticoid receptor is equally sensitive to glucocorticoids in lean and ob/ob mice. The higher dose of dexamethasone required to suppress glucocorticoid receptor number in brain than in liver is probably a function of site of dexamethasone injection (ip) and the relative availability of dexamethasone to brain and liver.

Based on Scatchard analysis, cytosolic glucocorticoid receptors in ob/ob mice have a slightly lower binding affinity than those in lean mice (table 2). Two days after adrenalectomy, there was an apparent increase in receptor binding affinity in ob/ob mice, though the binding affinity was still lower than that in lean adrenalectomized mice (table 4). The mechanism(s) responsible for the lower binding affinity of cytosolic glucocorticoid receptors for

dexamethasone in ob/ob mice have not been identified, although activated receptors have been reported to have much lower affinity for dexamethasone (43). Regardless of the mechanism, the lower binding affinity of glucocorticoid receptors in ob/ob mice is not consistant with the hypothesis that alterations in the glucocorticoid receptor contribute to the hypersensitivity of ob/ob mice to corticosterone.

Cytosolic mineralcorticoid receptor numbers in brain were low in both intact lean and ob/ob mice (figure 2, lower left panel). Unlike the glucocorticoid receptor, increases of cytosolic mineralcorticoid receptor number induced by adrenalectomy were parallel in lean and ob/ob mice, and were not evident until 24 hours after surgery. These data suggest that under physiological condition most of the mineralcorticoid receptor is activated in intact lean mice as well as intact ob/ob mice, and there are similar numbers of mineralcorticoid receptor in lean and ob/ob mice. Furthermore, the cytosolic mineralcorticoid receptor number in brain of adrenalectomized mice was not significantly affected by dexamethasone in either lean or ob/ob mice. Therefore, it appears that the mineralcorticoid receptor is not likely to be a factor contributing to the hypersensitivity to corticosterone in ob/ob mice.

In conclusion, despite the subtle difference in glucocorticoid receptor binding affinity, this study did not observe any difference in receptor number that would explain

the hypersensitivity to corticosterone observed in ob/ob mice. Since this study was limited to measuring the binding kinetics of receptor to ligand, it is still possible that other subtle difference in the receptor might contribute to the observed hypersensitivity to corticosterone. Webb et al (11) reported that there was no difference in glucocorticoid receptor DNA binding capability between lean and ob/ob mice or between lean and mdb/mdb mice. In addition, Shargill et at (12) also reported that similar observations in lean and fa/fa rats. Therefore, the cause of hypersensitivity to corticosterone observed in ob/ob mice probably is not directly associated with glucocorticoid receptors.

## FUTURE RESEARCH DIRECTIONS

To determine whether there are any structural differences in glucocorticoid receptors between lean and ob/ob mice, amino acid sequence and structure of the receptors should be compared. Since genes for human glucocorticoid and mineralcorticoid receptors have been cloned (44), cloning genes for glucocorticoid and mineralcorticoid receptors from lean and ob/ob mice is now more feasible. Amino acid sequences can then be obtained directly by reading the gene sequence. Alternatively, if cloning of the genes cannot be accomplished, corticosterone receptors can be purified from liver, kidney, or brain in quantity. The sequence can then be obtained by sequencing the purified receptor with traditional chemical approach. The structure of the receptor can then be predicted based on amino acid sequence (45-46). If there is enough crystallized receptor, the structure can be determined by X-ray crystallography.

Since gluccocorticoid and mineralcorticoid receptors regulate the transcription frequency of their target genes by binding to GRE upstream on DNA, it would be interesting to determine whether there are any genes that responsed differently to glucocorticoid or mineralcorticoid receptor regulation in lean versus ob/ob mice. It has been suggested that obesity in ob/ob mice is caused by an alteration in the central nervous system (47-48) rather than in metabolic enzymes which are also regulated by glucocorticoids. Thus,

attention should be given to glucocorticoid regulation of neuropeptides such as neuropeptide Y and CRH.

Other studies should focus on glucocorticoid regulation of enzymes which govern the formation of the neurotransmitters, acetylcholine, norepinephrine.

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## APPENDIX

Scatchard analysis (49) provides an approximate estimate of receptor and ligand binding affinity. The derivation of Scatchard analysis is as following:

[A] = concentration of agonist/ligand.

[R] = concentration of receptor.

[AR] = concentration of ligand and receptor complex.

Bmax = concentratoin of total receptor. = [R] + [AR].

When one receptor can bind to only one ligand and vice versa, the reaction of agonist and receptor binding can be expressed as:

$$[A] + [R] \Longleftrightarrow [AR]$$

At equilibrium, by definition the dissociation constant, Kd, can be expressed as:

$$Kd = [A] * [R] / [AR]$$

Substitute [R] with (Bmax - [AR]),

$$Kd = [A] * (Bmax - [AR]) / [AR]$$

Rearrange [A], [AR] and Kd,

$$[AR] / [A] = (Bmax - [AR]) / Kd$$

$$[AR] / [A] = (-1/Kd) * [AR] + (Bmax/Kd)$$

Let Y = [AR] / [A], and X = [AR], the equation above can be expressed as:

$$Y = (-1/Kd) * X + (Bmax/Kd)$$

If plotting ([AR] / [A]) against [AR], this will be approximately a linear line with:

slope = (-1/Kd)

intercept = (Bmax/Kd).