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The Effects of Insulin on Hepatic Glucocorticoid Receptor Content in the Diabetic Rat

Jeffrey John Yourick

THE EFFECTS OF INSULIN ON HEPATIC GLUCOCORTICOID RECEPTOR CONTENT IN THE DIABETIC RAT

by

Jeffrey John Yourick

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THE EFFECTS OF INSULIN ON HEPATIC GLUCOCORTICOID RECEPTOR CONTENT IN THE DIABETIC RAT

Jeffrey John Yourick, M.S.

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The streptozotocin-induced diabetic rat liver was analyzed for glucocorticoid receptor (GCR) content by a saturation and Scatchard analysis. A preliminary study was performed on the stabilization of the cytoplasmic GCR in a frozen cytosol preparation. The addition of 10% glycerol (V/V), 10 mM sodium molybdate, and 0.2 mg/ml trypsin inhibitor resulted in an approximate 20% sparing of GCR content upon freezing.

The hepatic GCR content of diabetic rats was significantly decreased from a control level of 0.1667 ± 0.0142 pmol/mg protein to 0.1094 ± 0.007 pmol/mg protein. Insulin replacement therapy to the diabetic rat dramatically increased the hepatic GCR content. The insulin treated diabetic rats had liver levels of GCR of 0.2602 ± 0.022 pmol/mg protein as compared to the diabetic value of 0.1094 ± 0.007 pmol/mg protein.

The results suggest insulin may have a possible role in the regulation of hepatic GCR content.

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INTRODUCTION

A hormone is a chemical mediator in a communication system between a regulatory organ and effector cells. The association of the hormone with the target cell is facilitated by a specialized binding protein referred to as a "hormone receptor". Each hormone has a specific receptor at the cellular level, which selectively binds at K_a values in the range of 10^{-9} molar. Specific steroid receptors have been well characterized for estrogen, progesterone, androgens, and glucocorticoids (Beato and Feigelson, 1972) as well as for insulin (House and Weidemann, 1970). This study deals with glucocorticoid receptor (GCR) concentrations in rat liver as affected by blood insulin levels.

Glucocorticoids are major regulatory hormones that exhibit control over such processes as the growth of new tissue, anti-inflammatory processes, and gluconeogenesis. The initiation of the cellular response to glucocorticoids is mediated by the GCR.

The observation of a cortisol binding factor in target cells was made by Hollander and Chui (1966) using a mouse lymphosarcoma. Later studies noted specific glucocorticoid receptor binding in rat thymus cells (Munck and Brinch-Johnsen, 1968; Schaumberg and Bojesen, 1968), HeLa cells (Melnyhovych and Bishop, 1969), mouse fibroblasts (Hackney

et al., 1970), and in rat liver cell cultures (Baxter and Tomkins, 1970). A complete characterization of the GCR was accomplished in rat liver cytosol by Beato and Fiegelson (1972).

All target organs for glucocorticoids have been found to contain a specific receptor. The association constant for the receptor in adrenalectomized rat brain, kidney, liver, thymus, lungs, and spleen was in the range of 10^{-8}M, with a binding capacity of between 0.6 to 4.0 ng per gram organ weight. Such tissues contain the receptor specific for glucocorticoids within cytosol fractions (Volchek et al., 1979). Other tissues known to contain a glucocorticoid receptor include the rat pancreas (Svec and Rudis, 1981) and the rat hippocampus (Wrange, 1979).

The physiochemical properties of the GCR have been derived from rat liver cytosols specifically binding either ^{3}H-dexamethasone or ^{3}H-triamcinolone. These characteristics differ slightly according to the methods and steroid used. The receptor consists of one polypeptide chain, and it seems the receptor contains only one hormone binding site per molecule (Wrange et al., 1979). At physiologic ionic strengths, the sedimentation coefficient is 4s. At low ionic strengths, the receptor has a sedimentation coefficient of about 7s. This difference in sedimentation coefficient related to the ionic strength is responsible for the variability in values reported in the literature.

Proteases have been used to further characterize the GCR structure. Cytosol receptor preparations are particularly vulnerable to proteolysis by trypsin and other proteases. After performing electrofocusing studies on partially purified receptor preparations, which were retained on ice for an extended time period, the presence of a double protein peak was observed (Wrange et al., 1979). The two peaks have a molecular weight of about 89,000 and 45,000. The 89,000 dalton protein is the receptor molecule. The 45,000 dalton protein retains steroid binding capacity, but it is believed to be one of two proteolytic fragments of the whole receptor.

It appears that the receptor is a phosphoprotein. Dephosphorylation is one means of destroying the binding capacity of the free GCR. The steroid bound GCR does not seem to be affected by this dephosphorylation inactivation (Nielsen et al., 1977). The cytoplasm to nucleus translocation of the hormone and receptor is dependent on the presence of ATP. The dephosphorylation mechanism, regulated by ATP, may become a mechanism which regulates effective intracellular concentrations of the receptor (Rousseau and Baxter, 1979). Other enzymes, mainly proteases, such as chymotrypsin, trypsin, papain, and pronase have the ability to inactivate the GCR binding capacity. This property will be discussed later in terms of stabilization of the receptor.

Only recently have the methods for the isolation and purification of the GCR been developed to attain a high degree of homogeneity from rat liver cytosol. One recent study claims that the receptor was purified to 85% homogeneity using SDS-gel electrophoresis (Wrange et al., 1979) and another study claims a 60,000-fold purification from the crude rat liver cytosol (Westphal and Beato, 1980). The methods are similar, so only the protocol of Westphal and Beato (1979) will be described here in detail. The purification scheme is based on the observation that only the activated (ability to bind to nuclear chromatin) form of the receptor will bind to a column of phosphocellulose. To begin, rat liver cytosol is incubated with ^{3}H-triamcinolone and then passed onto a phosphocellulose column. The eluate, which contains the inactive form of the GCR, is heat activated for 30 minutes at 25^{o}C. This is then passed onto a DEAE-cellulose column and the eluate is precipitated with ammonium sulfate. The ammonium sulfate yield is passed through another phosphocellulose column. The phosphocellulose columns were eluted with a NaCl gradient of from 0.0-0.6 M. The activated GCR eluate is concentrated by passage through a DNA-cellulose column and then is precipitated by ammonium sulfate. The final step is centrifugation in a sucrose density gradient, where the activated receptor has a sedimentation coefficient of 3.0. The above scheme claims a 60,000-fold purification from rat liver cytosol.

The mechanism of action of the GCR has been partially described. A portion of the mechanism is based upon analogies from other steroid receptors, such as the receptors for progesterone. The following discussion will summarize the mechanism by which the glucocorticoid-receptor complex elicits its cellular response.

The basis of the response is that the glucocorticoid-receptor complex is responsible for regulating specific mRNA concentrations by an association with nuclear chromatin. Glucocorticoid administration to HTC cell cultures produced increased levels of mRNA even in the presence of protein synthesis inhibitors (Peterkofsky and Tomkins, 1968). In lymphoid cells, where glucocorticoids are inhibitory, substances which block RNA synthesis also blocked the inhibitory effects of glucocorticoids (Roberts, 1969). It seems that in cultured hepatoma cells and cultured pituitary cells, less than 2% of the expressed genes of the cell are involved in the GCR stimulated increase in mRNA synthesis (Johnson et al., 1979). This fact indicates the specificity of the response to glucocorticoids. These same increases in specific mRNA levels are also exhibited by the estrogen-estrogen receptor complex (Palmiter, 1973).

Two mechanisms have been proposed for mRNA synthesis regulated by glucocorticoids. They consist of a "catalytic" and a "stoichiometric" model. The catalytic mechanism involves considering the steroid-receptor complex as: 1) an enzyme;

2) a complex which has the ability to activate an enzyme; or 3) a regulatory molecule causing an alteration in chromatin structure. The enzymatic stimulation of mRNA expression may come from the receptor itself or from a secondary effect of the receptor on another enzyme which binds directly to the chromatin. It may also act by opening up the structure of the chromatin, which would allow easy access for replicative enzymes. The theory behind the catalytic mechanism of glucocorticoid action has mainly been derived by analogies with bacterial cell gene regulation.

At present, the most generally accepted mechanism is the stoichiometric model. This model states that the glucocorticoid-receptor complex controls the transcription of specific mRNA by binding directly to certain regulation sites on the chromatin. It is still not known how the receptor complex recognizes its specific binding site on the DNA. There is evidence for two possible modes of action. The first theory suggests that the receptor-steroid complex associates with specific sequences on the DNA. The second possibility is that the receptor binds to specific chromatin proteins at the regulation site on the DNA (Johnson et al., 1979).

Eukaryotic chromatin consists of histone proteins, nonhistone proteins, and DNA. The histone and nonhistone proteins have the property of being phosphorylated. It is also known that the histones may act as a site recognition signal for protein kinases. In rapidly dividing liver cells,

the phosphorylation of histone H_1 is greatly increased as compared to nonreplicating tissue (Balhorn et al., 1971). Histone H_1 is selectively phosphorylated by protein kinases whose activity may be regulated by glucocorticoids. After a single dose of cortisol, rat liver nuclear proteins show an increase in phosphorylation (Allfrey et al., 1973) and these have a greater ability to stimulate mRNA transcription (Kleinsmith et al., 1976).

The acetylation of certain histones may also represent a factor in the catalytic mechanism. The four histones (H_{2a}, H_{2b}, H_3, and H_4) which comprise the nucleosome structure of the nucleus may be highly acetylated under certain conditions. The crystalline structure of the nucleosome is maintained by the histones. The acetylation of the histones causes an opening in the crystalline structure, which allows greater polymerase access to DNA (Johnson et al., 1979). Acetylation of chromatin seems to increase RNA synthesis in the presence of RNA polymerase (Marushige, 1976). In rat liver, after an injection of glucocorticoids, there is an increase in histone acetylation (Libby, 1973). Within lymphocytes, where glucocorticoids are inhibitory, cortisol inhibits the acetylation of histones (Allfrey et al., 1966). It is possible that both phosphorylation and acetylation of chromatin histones are necessary for glucocorticoid-stimulated increases in mRNA transcription.

The administration of glucocorticoids to pituitary cells may lead to a net increase of up to 60% or to a total of

500,000 sites/nucleus in the number of polymerase binding sites (Johnson and Baxter, 1978). At steroid concentrations which saturate the receptor, only a calculated 40,000 glucocorticoid-receptor complexes enter the nucleus. This means that the glucocorticoid-receptor stimulation of initiation sites is not strictly a stoichiometric process, when compared to the number of glucocorticoid-receptor complexes which enter the nucleus.

Glucocorticoids are also inhibitors of activity in certain cell types. It seems that this effect may be mediated by the receptor complex causing decreased polymerase binding to DNA. Dexamethasone causes a decrease in chromatin template activity in cultured lymphoma cells. Receptor involvement is necessary, since a cell line (R^-), which has no receptor binding capacity, does not show a decrease in chromatin activity (Johnson et al., 1979). As we can see the glucocorticoid receptor complex may either stimulate or inhibit RNA transcription depending on the cell type.

Summary of the available data suggests a possible mechanism of action of glucocorticoids might be as follows: the binding of the glucocorticoid to the receptor opens a chromatin binding site on the receptor and nuclear translocation by diffusion occurs. The binding to the chromatin stimulates an enzyme reaction which increases the phosphorylation and acetylation of the histone and nonhistone proteins. This alteration in nuclear proteins facilitates RNA

polymerase access to the chromatin, which will stimulate transcription. In certain cells glucocorticoid treatment may increase the number of initiation sites for RNA polymerases to 500,000 per cell. The binding of the steroid to the receptor may also expose a DNA-binding site. The receptor complex may directly influence transcription by binding to the initiation sequence on the DNA or by binding to specific chromatin proteins at the regulation site. Since this is only a hypothesis of the mechanism, it is possible that all or only part of the above is essential to the mode of action of the GCR. Understanding the systemic regulatory role of glucocorticoids is important to the basis of GCR regulation.

The regulation of gluconeogenesis by glucocorticoids is an essential topic for this paper. The pathologic condition of diabetes will be utilized as a means to examine the regulation of GCR concentrations in rat liver. The liver and kidney are the two main organs which perform gluconeogenesis. The liver is the primary location with the kidney involved when the organism is under physiological stress. Regulation of gluconeogenesis is a complex system controlled by a number of hormones. Glucocorticoids, insulin, glucagon, and catecholamines control the rate of gluconeogenesis during periods of diabetes, starvation, and exercise. These various hormones act on the liver directly as well as on adipose and muscle tissue, which serve to release the precursors for gluconeogenesis.

Glucocorticoids partially affect gluconeogenesis by increasing the release of amino acids from muscle and adipose tissue (Smith and Long, 1967). The glucocorticoid-mediated release of amino acids from muscle involves a probable multi-faceted mechanism. A decrease in protein synthesis as well as removal of the inhibitory role of insulin on amino acid release from muscle by glucocorticoids has been suggested as a mechanism. Adrenalectomy greatly decreases the rate of release of amino acids from peripheral tissues, while glucocorticoid administration to adrenalectomized rats stimulates the release.

Catecholamine-induced release of lactate from muscle and glycerol release from fat cells may be inhibited by a lack of glucocorticoids. In adrenalectomized rats the response to epinephrine on glycogenolysis and lipolysis is reduced. This effect seems to work through a decrease in the cell's ability to produce cAMP in response to epinephrine and norepinephrine (Exton, 1979). Since there is an insensitivity to cAMP increases in the adrenalectomized rat, glucocorticoids seem to provide some permissive controls over gluconeogenesis.

Glucocorticoids also have the ability to reduce the sensitivity of certain tissues to insulin. The insulin-related enhancement of ^{14}C-glycine incorporation into protein may be altered by glucocorticoids. Adrenalectomy increases the amount of ^{14}C-glycine uptake stimulated by insulin, while cortisol treatment decreases the amino acid incorporation

(Manchester et al., 1959). The release of amino acids from peripheral tissues is a major source of gluconeogenic precursors. These facts indicate that glucocorticoids are antagonists to the actions of insulin, as well as being important to gluconeogenesis regulation.

Glucocorticoids play a role in the control of gluconeogenesis not only in peripheral tissues, but also within the liver. Adrenalectomy produces a decrease in the rate of gluconeogenesis in fasted and diabetic perfused rat livers (Exton et al., 1973). This effect could be reversed by the administration of cortisol to those animals. These findings indicate that glucocorticoids are necessary for changes in the rate of gluconeogenesis. In perfused diabetic rat liver, the increase in gluconeogenesis stimulated by glucocorticoids appears after one hour. This increase may be inhibited by transcriptional (cordycepin) and translational (cycloheximide) blockers. The metabolic steps in gluconeogenesis controlled by the glucocorticoids were found by measuring the concentrations of metabolic intermediates of gluconeogenesis in adrenalectomized, diabetic, perfused-rat livers. Two "cross-over" points in the metabolic intermediate chain were noted between pyruvate and phosphoenolpyruvate and glucose and glucose-6-phosphate. The lack of glucocorticoids caused a fall in glucose compared to glucose-6-phosphate and an increase in pyruvate compared to phosphoenolpyruvate.

The two enzymes responsible for the conversion of the gluconeogenic intermediates at these two steps are phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. The regulation of phosphoenolpyruvate carboxykinase by glucocorticoids functions by causing an increase in enzyme synthesis. The main control acts as a stimulator at the transcriptional site for the enzyme synthesis (Exton, 1979). A secondary control seems to be a steroid-induced increase in the amount of mRNA specific for phosphoenolpyruvate carboxykinase. This effect is mediated by increased cAMP levels which stimulate mRNA translation (Gunn et al., 1975). These two mechanisms suggest a dual regulation of phosphoenolpyruvate carboxykinase synthesis at both the transcriptional and translational levels by glucocorticoids.

The control of glucose-6-phosphatase is less well defined. Cortisone treatment increases the activity of hepatic glucose-6-phosphatase. In as much as actinomycin-D or puromycin are able to block the stimulation of new enzyme synthesis, protein synthesis seems to be required. However, studies indicate that the increased activity is due to activation of the enzyme. At present it seems that glucocorticoids regulate hepatic gluconeogenesis through stimulation of specific enzyme synthesis. This occurs at both the transcriptional level as well as at the translational level by involvement of cAMP. Activation of enzyme activity may be required in the case of glucose-6-phosphatase.

Glucagon and epinephrine-induced activation of hepatic gluconeogenesis is greatly reduced in adrenalectomized rats (Exton, 1979). The glucagon activation mechanism acts by way of cAMP-dependent phosphorylation of l-pyruvate kinase. The decrease of pyruvate breakdown would lead to an increase in the gluconeogenic precursors. Epinephrine activation is dependent upon α-adrenergic receptors. It has been shown that adrenalectomy can decrease the stimulatory effect of α-adrenergic agonists on gluconeogenesis (Chan, 1977). We can conclude that glucocorticoids function to control gluconeogenesis in several ways. They may affect: 1) the release of gluconeogenic precursors from peripheral tissues; 2) the synthesis of phosphoenolpyruvate carboxykinase and the activation of glucose-6-phosphatase; and 3) the permissive elements (amino acid release) of gluconeogenic stimulation by glucagon and epinephrine.

Beyond gluconeogenesis, glucocorticoids affect many other aspects of metabolism. Only those points pertinent to this study will be reviewed here. The variety of glucocorticoid-sensitive tissues and cells, as well as the effects of hormone action, have been characterized by Munck and Leung (1977). A prerequisite for glucocorticoid action is the presence of its specific cell-bound receptor. A GCR has been found in all target tissues including the thymus (Ranellette et al., 1980), liver (Beato and Fiegelson, 1972), heart (Gregory et al., 1976), brain (Wrange, 1979).

pancreas (Svec and Rudis, 1981), kidney (Feldman et al., 1978), spleen and lungs (Valchek et al., 1979). The consistent presence of the GCR in such a diverse group of tissues illustrates the complexity of glucocorticoid actions in mammals. This review will focus only upon the metabolic changes observed within liver, adipose tissue, muscle, and lymphoid tissue. Excluding liver, glucocorticoids stimulate mainly catabolic reactions. There is a decrease in glucose utilization, protein synthesis and nucleic acid synthesis. In muscle and lymphoid tissue, glucocorticoids cause an increase in protein catabolism. This allows for a greater release of amino acids into the bloodstream, which can be used as precursors for gluconeogenesis in the liver. There is also an increase in free fatty acid release from adipose tissue (Munck and Leung, 1977). These fatty acids are also used in glucose synthesis. This general increase in release of gluconeogenic substrates from the peripheral tissues helps to stimulate hepatic glucose production.

In the liver, which is the primary site for gluconeogenesis, we observe a multitude of metabolic changes due to glucocorticoids. The primary effects are an increase in amino acid uptake, glucose production, glycogen deposition, and protein and RNA synthesis (Baxter and Forsham, 1972). Secondary effects include an increase in ketone body formation and urea production. Many of the changes are due to an activation of numerous hepatic enzymes. The gluconeogenic

enzyme activities are elevated from 130% to 300% (Schulster et al., 1976), with the major regulatory enzyme activities (phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) being used the most. Since amino acids serve as a major precursor in glucose production, the amino acid metabolizing enzyme activities are also greatly enhanced. Enzymes such as alanine aminotransferase, tyrosine aminotransferase, and tryptophan pyrrolase exhibit activity increases of 1000%, 800%, and 600%, respectively (Baxter and Forsham, 1972; Schulster et al., 1976). All these increased enzyme activities contribute to the greatly enhanced rate of gluconeogenesis. From the overall scheme of glucocorticoid action, we can conceive of a process whereby the liver serves as a control center for glucose production. Muscle, adipose tissue, and lymphoid tissue serve to release the amino acids for the hepatic glucose production.

As was suggested earlier the majority of metabolic effects produced by glucocorticoids are opposed by insulin. Insulin is generally considered an anabolic hormone. Insulin increases glucose and amino acid influx into cells, stimulates protein and glycogen synthesis in non-hepatic tissues, prevents lipolysis, decreases hepatic protein production, and inhibits the stimulation of gluconeogenic enzymes (Schulster et al., 1976). This means that insulin is able to decrease amino acid and free fatty acid release from non-hepatic tissues. It may also increase glucose utilization and inhibit gluco-

neogenesis (Ashmore, 1964). The antagonistic behavior exhibited between insulin and glucocorticoids is an essential point pertaining to this study and it will be examined in greater detail in the following sections.

Glucocorticoids have been shown to exhibit a wide range of effects in mammals. The metabolic action of glucocorticoids is initiated by the binding of the steroid to its specific cytoplasmic receptor. Without this receptor binding, glucocorticoid action is inhibited. It is of great importance then to have an understanding of the mechanisms which regulate the concentrations of GCR at the cellular level.

The ability to study glucocorticoid receptors, in terms of quantification, in different cell types and under specific metabolic conditions is hampered by a high rate of receptor degradation in cell-free preparations. In kidney, heart, and thymus cytosol receptor preparations, the half-life of glucocorticoid receptor binding capacity is only 2 to 4 hours at $0^{o}C$. In liver the half-life is approximately 15 to 25 hours (Nielsen et al., 1977). Glucocorticoid receptors vary in their binding capacity depending upon activation. The process is not well understood. Only recently have the activation mechanisms for receptors been investigated. The term "activation" has two different meanings. First, a receptor is "activated" once it has undergone a biochemical change to display enhanced affinity of the receptor for the nucleus and chromatin. Secondly, "activation" may only

involve an increased rate of receptor synthesis or a decrease in receptor degradation. The distinction is not clear; "activation" always appears experimentally as an increase in binding capacity. This phenomenon is seen with glucocorticoid as well as estrogen and progesterone receptors.

The GCR in an unbound state is particularly vulnerable to inactivation or degradation. Cell-free preparations yield the GCR susceptible to a multitude of degradative enzymes. It is because of this fact that GCR preparations are used immediately upon preparation. It would be ideal to have the ability to store a cytosol preparation which could be used at the investigators convenience. At this time, this is not possible without losing a certain amount of binding capacity.

The inactivation caused by heating cytosol receptor preparations to 25^{o}C, can be attenuated by 10 mM molybdate (Nielsen et al., 1977; Maki et al., 1980; Noma et al., 1980). The protective action of molybdate occurs also with the estrogen receptor (Krozowski and Murphy, 1981; Noma et al., 1980) and the androgen receptor (Noma et al., 1980). Molybdate also protects unbound receptors from inactivation by high salt concentrations and ammonium sulfate (Leach et al., 1979). Molybdate has the ability to inhibit temperature-dependent transformation of GCR complexes to a DNA-binding state (Leach et al., 1979). Leach and others propose that molybdate reversibly binds directly with the GCR at the

location of the receptor's phosphate group. This may induce a conformational change in the receptor which increases stability (Maki et al., 1980). It has also been suggested that molybdate may inhibit the phosphatase enzymes which are present in cell-free preparations, thus helping to stabilize the receptor (Nielsen et al., 1977; Leach et al., 1979). Nielsen and others (1977) reported that a membrane-bound phosphatase may be in part responsible for the activation of unbound receptor complexes. This "low molecular weight inhibitor" may itself be inhibited in the presence of molybdate (Noma et al., 1980).

The usefulness of other phosphatase inhibitors such as fluoride and glucose-1-phosphate have been examined (Nielsen et al., 1977; Leach et al., 1979). Both inhibit heat-dependent receptor inactivation, but not to the extent of molybdate's action. Molybdate can inhibit the heat-dependent activation of the GCR to a DNA-binding state, whereas fluoride and glucose-1-phosphate cannot. This points to the fact that more than one mechanism is functioning in the inactivation of the receptor.

Glucocorticoid receptors may also be stabilized by physically preserving the structure of the receptor from hydrolysis. Degradation may be slowed by adding sulfhydryl-group protective agents, EDTA, and glycerol to assay buffers (Nielsen et al., 1977). The combination of these agents with molybdate offers a system which helps to preserve the freshly isolated receptor.

The activation process also seems to involve ATP. It has been shown that non-activated glucocorticoid receptors may be transformed into an activated form which will bind to nuclei by addition of ATP at 4°C (John and Moudgil, 1979). The activation ability of ADP was less than ATP, and AMP did not have any capacity for activation. The mechanism by which ATP contributes to receptor activation is not known, but many theories have been proposed. It is possible that an energy-dependent reaction is responsible for the nuclear uptake of steroid-receptor complexes by cells. Another role of ATP may involve a phosphorylation-dephosphorylation reaction which regulates the concentration of activated receptors. The theory presented by John and Moudgil (1979) suggests that ATP binds to a nucleotide binding site on the receptor resulting in a binding site for the nucleus becoming exposed.

The last topic to be discussed on receptor stabilization is that of specific protease inhibitors. Especially in cytosol preparations, proteases are released and pose an immediate problem to steroid receptors. Some activity of the proteases may be depressed by maintaining experimental conditions at 0°C. However, over long periods of time, receptor degradation occurs even at cold temperatures. This is why the addition of protease inhibitors to cytosol preparations helps to inhibit the degradation of steroid receptors. The destruction of the estrogen receptor in cow uterus and

the progesterone receptor in hen oviduct is reduced by the addition of 0.5 mM leupeptin or 0.5 mM antipain to assay buffers. Leupeptin and antipain are inhibitors of thiol and serine proteases (Hazato and Murayama, 1981). Trypsin inhibitor was moderately effective with approximately 50% inhibition of inactivation for the progesterone receptor. Glucocorticoid receptors seem to be affected by two different type proteases. One enzyme-type was inhibited by 1 mM antipain or leupeptin. The other proteases were inhibited by 1 mM phosphoramidon or 10 mM sodium molybdate (Hazato and Murayama, 1981).

Many factors seem to be responsible for variations in tissue levels of glucocorticoid receptors. The most basic control is imposed by glucocorticoids themselves. In AtT-20 mouse pituitary tumor cells, the administration of glucocorticoids to the incubation media results in a decrease in the number of glucocorticoid receptors (Svec and Rudis, 1981). The opposite effect on receptor concentration may be exhibited by adrenalectomy. The removal of endogenous glucocorticoids causes a significant increase in hepatic glucocorticoid receptor levels within 3 days (Rouse et al., 1975). Receptor populations may also be altered in the rat hepatic cytosols by stress (Agarwal, 1977; Golikou et al., 1981), diet (Varma and Mulay, 1981), and obesity (Olefsky et al., 1976). The ability of hormones, other than the glucocorticoids, to regulate GCR levels is also of importance.

The interrelationship between hormones and receptor regulation may prove to be fundamental to certain human diseases in terms of treatment and their etiology. Glucocorticoid receptors are implicated in certain disease states such as human leukemias and lymphomas, breast tumors, ovarian tumors, fetal disorders, and hypercortisolism (Kontula,1980). Insulin has been shown to affect the levels of the cytoplasmic estrogen receptor in a human breast cancer cell line (Moore, 1981), inasmuch as high levels of insulin in the incubation media significantly decrease the concentration of the estrogen receptor.

Insulin and glucose also have an effect on the GCR concentration in adrenalectomized rat liver. The administration of glucose sufficient to raise blood glucose levels to 226 mg/100 ml decreased specific dexamethasone binding to rat liver cytosol by 34% after 1.5 hours (Rouse et al., 1975). Simultaneous glucose and insulin (10 units/kg) treatment to rats maintained the level of specific dexamethasone binding near that of control cytosols. From these results we may conclude that glucose, insulin, or both may have a direct or indirect effect on glucocorticoid binding. In a recent study by Grunfeld et al. (1981), glucocorticoids enhanced insulin resistance evidenced by a decreasing amount of 2-deoxyglucose uptake in the rat adipocyte. The steroid also decreased insulin binding to its receptor. Part of the glucocorticoid effect was due to a decrease in affinity of the receptor for insulin.

In previous work from this laboratory, a GCR has been characterized in the liver of the Chinese hamster (Gibson, 1979). This same work revealed a GCR concentration difference between the diabetic Chinese hamster as compared to normal Chinese hamsters. The intent of the present work is to investigate the differences in GCR concentrations in diabetic rat livers as compared to controls. The metabolic conditions existing within a diabetic animal is of particular interest, since insulin and glucocorticoids are antagonistic in action. The animal model to be used is that of the streptozotocin-induced diabetes in rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing between 275 and 310 grams, were used in the experiments. The rats were maintained on a 12 hour light/dark cycle at 22 ± 1°C with free access to Purina rat chow and water.

Preparation of Cytosol

The preparation of the cytosol was based on the methods of Beato and Feigelson (1972). The rats were sacrificed by cervical dislocation between 3:00 PM and 6:00 PM. The liver was perfused *in situ* through the portal vein with 12 ml ice cold nonstabilized buffer. The nonstabilized homogenization buffer contained: 20 mM Tris-HCl (Sigma), 100 µM dithiothreitol (Sigma), 1 µM EDTA (potassium salt) adjusted to pH 7.4. The "stabilized" buffer had the following additions: 10% glycerol (V/V), 10 mM sodium molybdate, and 0.2 mg/ml of trypsin inhibitor (Soybean Type I-S, Sigma). The liver was removed and minced at 0°C and then washed twice with an excess of nonstabilized homogenization buffer. The liver tissue to buffer ratio was maintained at 1:2 (W/V) for homogenization. The minced liver was homogenized with a motor driven teflon pestle and glass homogenizer for 20 seconds with either "stabilized" or "nonstabilized"

homogenization buffer. All preparation steps were strictly maintained at 0-4°C. The homogenate was first centrifuged at 10,000g for 10 minutes (Sorvall RC2-B). The supernatant was then centrifuged at 135,000g for 2 hours (Beckman Ultracentrifuge L2-50). The floating lipid layer was removed and the remaining supernatant was used as the cytosol receptor preparation. The cytosol was either used immediately or stored at -70°C.

Binding Assay

The cytosols were frozen for 4.5 days then removed and thawed at room temperature for use in the binding assay. The binding assay follows the procedure described by Beato and Feigelson in 1972. Aliquots of 100 µl of cytosol were added to 50 µl of ^{3}H-dexamethasone (50 Ci/mmol, New England Nuclear) at concentrations of 3 nM, 6 nM, 10 nM, 15 nM, and 30 nM (in nonstabilized homogenization buffer). Also present in the incubation mixture was 25 µl of ethylene glycol in the total-bound and total count assay tubes to substitute for the unlabeled dexamethasone. Non-specific binding was measured by the addition of a 1000-fold excess of unlabeled dexamethasone (Sigma) dissolved in 25 µl of ethylene glycol to the nonspecific bound assay tubes. The tubes were briefly vortexed and then incubated at 0-4°C for 2 hours to allow maximum steroid-receptor binding. After the incubation, 50 µl of a dextran-coated

charcoal solution was added to adsorb any unbound steroid. The solution contained dextran (Sigma, 60,000-90,000 MW) and Norit-A charcoal (Sigma) in a ratio of 1:10, made up fresh for each assay as a 5% charcoal solution in 10 mM Tris-HCl (Sigma) at pH 8.0. Each tube was then vortexed for 10 seconds, incubated for 10 minutes at 0-4°C, and centrifuged (0-4°C) at 3000g for 10 minutes. A 100 µl sample of supernatant was removed from each assay tube and placed in 4 ml of scintillation fluid (6 g PPO, 150 mg POPOP, 850 ml toluene, 150 ml Bio-Solv/liter). The radioactivity was measured in a Searle ISOCAP/300 scintillation counter. All measurements at each concentration were performed in triplicate.

The specific binding of the steroid to the receptor was obtained by subtracting the nonspecifically bound radioactivity from the total bound radioactivity on a per tube basis. The total amount of radioactivity present at each concentration was determined by substitution of 100 µl of homogenization buffer for the cytosol. The total count measurement was made by substitution of 50 µl of 10mM Tris-HCl for the charcoal mixture. Quenching during scintillation counting was corrected for by the use of the external standard channel ratio method. A Scatchard analysis (Scatchard, 1949) was used to determine association constants and concentration of the GCR.

Determination of Protein Concentration

The measurement of cytosol protein concentrations was performed according to the methods of Lowey et al. (1951). Bovine serum albumin was used for the standard.

Induction of Diabetes in Rats

Just prior to the injection of the rats, streptozotocin (Sigma, mixed anomers) was dissolved in 0.1 M citrate buffer (0.1 M citric acid and 0.1 M sodium citrate) at pH 4.5. The streptozotocin was injected intraperitoneally in a single dose at 80 mg/kg body weight (Stearns et al., 1979; Khandelwal et al., 1979). After 4 days an initial determination for glucosuria was made using Tes-Tape^{R}. After 7 days, an animal was considered diabetic with a Tes-Tape^{R} response of at least +3 or greater than ½% glucose in the urine. The occurrence of polyuria and polydipsia (100ml/day/rat) also accompanied glucosuria. Rats that had been diabetic for at least 14 days were used in the binding studies.

Insulin Treatment

A total of 7 rats that were diabetic 7 days after streptozotocin treatment were used in the insulin-treated diabetic group. Beginning on the eighth day, insulin injections were started. Each rat was monitored daily for glucosuria with Tes-Tape^{R} and serum glucose levels

were measured periodically with a Dextro-stix (Miles Laboratories). Each daily subcutaneous insulin (Lilly) injection was adjusted according to morning Tes-Tape^{R} measurements which would reduce glucosuria to zero. A "typical" insulin injection consisted of 10 units of Regular Iletin^{R} and 5-7 units of NPH Iletin^{R} or 5-7 units of Lente Iletin^{R}. After 8 days of insulin treatment to the diabetic rats, they were sacrificed along with the controls. The cytosols were prepared as previously described.

Measurement of Glucose

Blood samples were collected from each rat at the time of sacrifice. The serum was isolated and frozen for later use. Blood glucose was measured by the Glucostat method (Ortho Chemical Co.).

Analysis

Statistical comparisons were made by use of the Student's t-test on the data from the Scatchard plots. All measurements are expressed as the mean ± standard error of the mean. The regression coefficients on Figures 1, 2, and 3 were analyzed according to 95% confidence intervals for the line slopes.

RESULTS

Optiminization of Assay Procedures

Addition of molybdate, glycerol, and trypsin inhibitor had no effect on receptor concentration, if the binding assay was performed immediately after preparation of the cytosol (Figure 1 and 2). In Figure 1, the addition of the stabilization factors did not change the K_a significantly from a control value of 2.89×10^{-9} M to 2.25×10^{-9} M. The GCR concentration remained essentially constant at values of 0.0875 and 0.0855 pmol/mg protein. After freezing at $-70^{o}C$ for 6 days, the K_a value remained unchanged, however freezing caused a decrease in receptor concentration from 0.0875 to 0.0575 pmol/mg protein. This represents approximately a 34% loss of receptor activity due to freezing, even in the presence of the stabilization factors.

Figure 2 depicts slightly different results on essentially the same experiment as performed in Figure 1. The cytosol with the stabilization factors exhibited a nonsignificant increase in the K_a from 0.486×10^{-9} M to 0.863×10^{-9} M as compared to control cytosol. In this case there was a nonsignificant decrease in the GCR binding affinity after the addition of stabilization factors. There was also an increase in the number of receptors

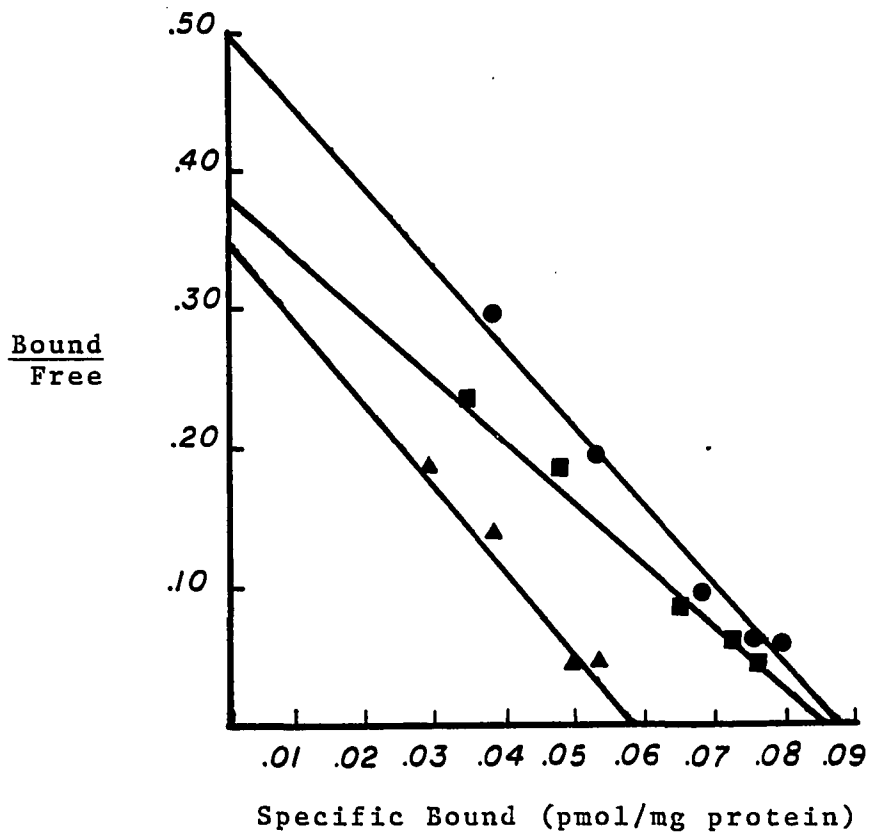

Figure 1. The addition of stabilization factors to the cytosol preparation: effect on glucocorticoid receptor content after freezing at -70°C. This represents the results from a single animal cytosol preparation which was divided into 3 aliquots. A Scatchard analysis was performed to determine receptor content. The receptor assay performed immediately following cytosol preparation (●) has a receptor content of 0.0875 pmol/mg protein and a K_a of 2.89 x 10^{-9} M. The receptor assay performed immediately following cytosol preparation (▲), but with the following addition to the 20 ml of homogenate of 10% glycerol (V/V), 10 mM sodium molybdate, and 0.2 mg/ml trypsin inhibitor, had a receptor content of 0.0855 pmol/mg protein and a K_a of 2.25 x 10^{-9} M. The receptor assay performed after storage at -70°C for 6 days, with the above additions to the homogenate (■), had a receptor content of 0.0575 pmol/mg protein and a K_a of 3.03 x 10^{-9} M. All differences in values are nonsignificant.

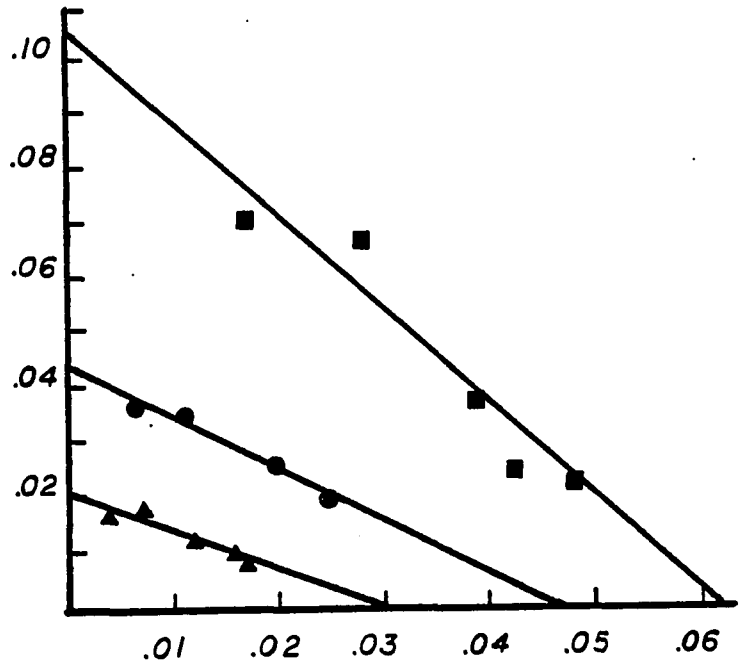

Figure 2. Scatchard analysis of the effect of stabilization factors and freezing on glucocorticoid receptor content. The cytosol extract from a single animal was divided into 3 portions and assayed as follows: the receptor assay performed immediately following cytosol preparation (●) without additions had a receptor content of 0.046 pmol/mg protein and a K_a of 0.486 x 10^{-9} M. The receptor assay (■) performed immediately following cytosol preparation, with the additions of 10% glycerol (V/V), 10 mM sodium molybdate and 0.2 mg/ml trypsin inhibitor, had a receptor content of 0.062 pmol/mg protein and a K_a of 0.863 x 10^{-9} M. The receptor assay (▲) performed after storage at -70°C for 6 days, with the identical additions as above, had a receptor content of 0.029 pmol/mg protein and a K_a of 0.346 x 10^{-9} M.

present in the cytosol with additions as compared to the normal cytosol. The receptor concentration increased from 0.046 to 0.061 pmol/mg protein, which is an increase of 33%. The freezing of the cytosol with additions again exhibited a K_a close to that of the normal cytosol, but here there was a decrease in receptor concentration. The receptor levels fell from 0.046 to 0.029 pmol/mg protein, which is a decrease of 37%. This decrease due to freezing compares well with the result from Figure 1, with a mean loss upon freezing of 36%. To optimize the assay conditions on frozen cytosol, the "stabilizers" glycerol, molybdate, and trypsin inhibitor were added to the cytosol preparations. After freezing at -70°C for 4.5 days, the binding assay was performed. This procedure allowed better experimental group organization, since multiple animals could be sacrificed on a single day.

Freezing of Unstabilized Cytosols

Freezing of cytosol without the addition of stabilizers leads to a decrease in receptor concentration of 46% after 3 days of storage at -70°C (Figure 3). After being frozen for a total of 9 days the receptor population was reduced by 56%. Thus, the addition of cytosol receptor stabilizers (Figures 1 and 2) reduced the loss due to 4.5 days of freezing to only 36%.

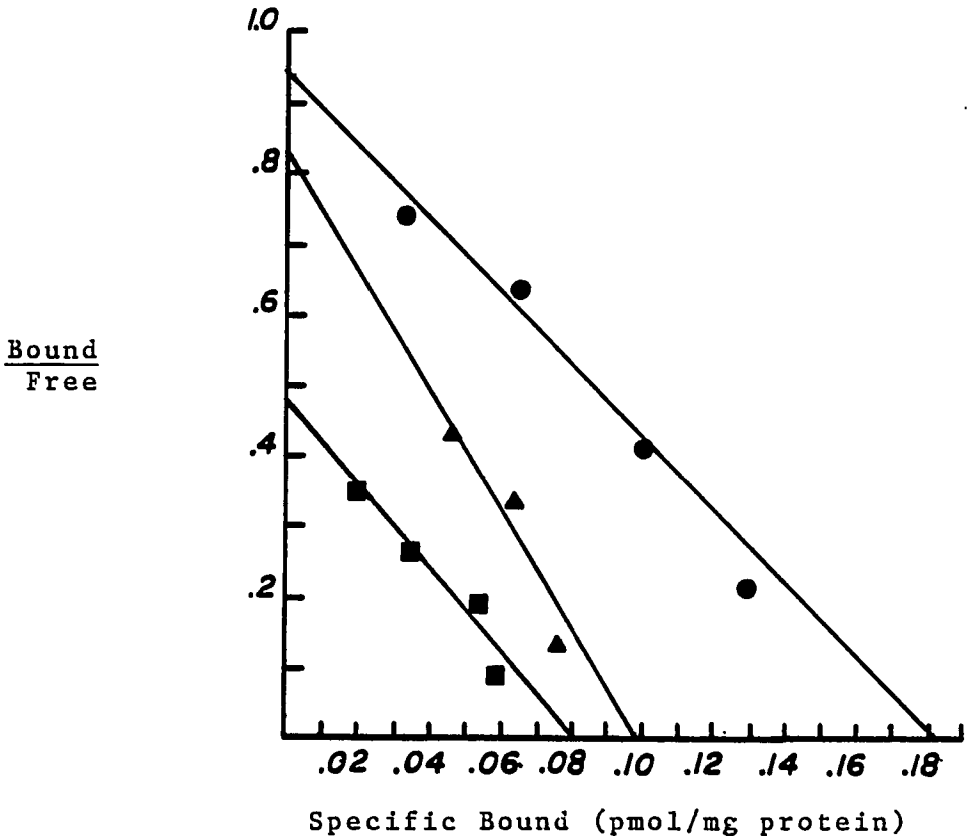

Figure 3. The effect of freezing on glucocorticoid receptor content in normal unstabilized cytosol. The cytosol (●) assayed immediately following preparation exhibited a receptor content of 0.182 pmol/mg protein. After 3 days of storage at -70°C, the cytosol (▲) dropped to a receptor content of 0.099 pmol/mg protein. This was a loss in content of 46%. After freezing for 9 days at -70°C (■), the receptor content further decreased to 0.0805 pmol/mg protein, which is a loss of 56% in receptor content. This graph is the result of one experimental trial.

Interpretation of Saturation Analysis

The graph presented in Figure 4 represents the typical results expected from a binding system which contains one receptor (Scatchard, 1941). This may be determined by subtracting the amount of nonspecific binding from the total binding at each concentration. This produces a measure of specific binding, which above a certain steroid concentration is saturable.

Serum Glucose Levels

All serum glucose values reported are from blood samples taken at the time of sacrifice (Figure 5). The glucose levels in the control rats were 165 ± 23.5 mg/dl. This is slightly higher than the reported value of 120-125 mg/dl for a nonfasted rat between meals (Steffens, 1969). It is however impossible to know when the experimental animals last ate, since food was available ad libitum. The average serum glucose concentration for the diabetic rat group was 598 ± 17.5 mg/dl. This was accompanied with urine glucose levels of at least 2% by the end of the experimental period. The long-term (110 days post-streptozotocin injection) group had an average serum glucose of 573 ± 33 mg/dl. This is in good agreement with the short-term (14 days post-streptozotocin injection) diabetic rat glucose values. The insulin-treated diabetic group had

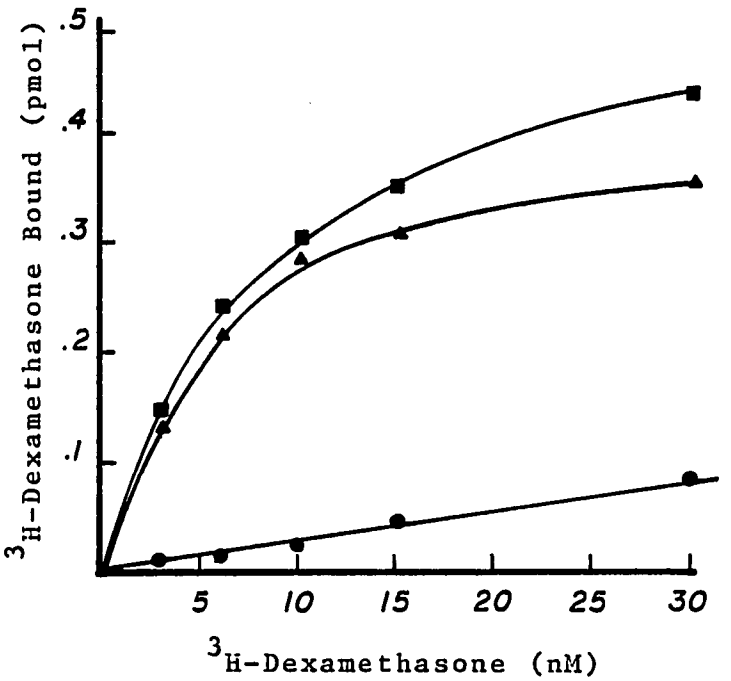

Figure 4. Saturation analysis using ^{3}H-dexamethasone and a charcoal adsorption technique. This graph represents the data from one animal in the insulin replacement therapy group. The top line (■) represents the total binding of dexamethasone to the receptor. The bottom line (●) is the amount of nonspecific binding in the presence of a 1000x excess of unlabelled dexamethasone. The middle line (▲) represents the amount of specific ^{3}H-dexamethasone binding to the receptor. This is determined at each concentration by subtracting the nonspecific binding from the total binding.

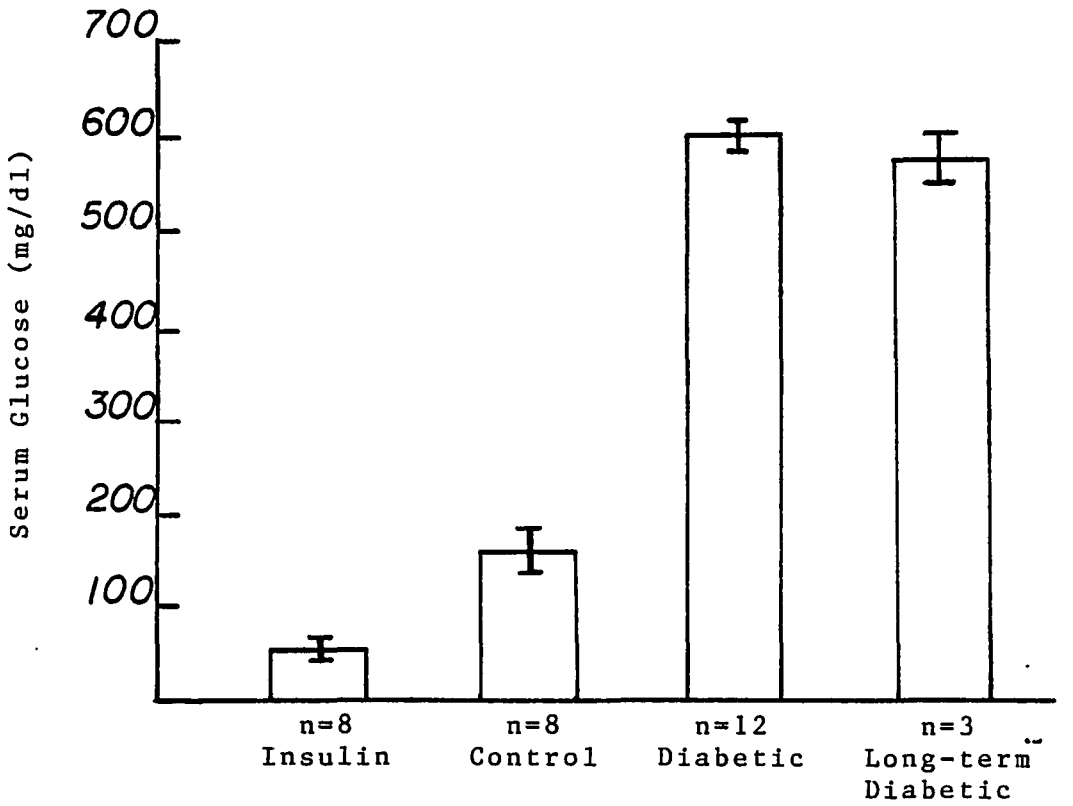

Figure 5. Serum glucose level comparisons between experimental groups. Blood samples were collected at the time of sacrifice for each animal. The plasma was isolated and frozen for later use. The control animals had serum glucose levels of 165 ± 23 mg/dl as compared to the diabetic animals of 602 ± 17 mg/dl. The insulin-treated group exhibited even lower glucose levels (58 ± 8.3 mg/dl) than controls. The long-term diabetic group had glucose levels of 573 ± 32.8 mg/dl.

a mean glucose of 58 ± 8 mg/dl which is 50% of the control group.

Receptor Content of Diabetic and Nondiabetic Rat Liver

The first study measured hepatic receptor concentrations in control animals as compared to diabetic animals. The results from Figure 6 were obtained from liver cytosols to which no stabilizing factors were added and the cytosols were not frozen. The receptor assay was performed immediately after preparation of the cytosol on the day of sacrifice for each rat. The receptor concentration for the control rat livers was 0.164 ± 0.017 pmol/mg protein. The receptor concentration for the diabetic hepatic cytosols were significantly lower with 0.051 ± 0.007 pmol/mg protein.

The second study, as depicted in Figure 7, was performed on control and diabetic liver cytosols to which stabilization factors were added. The experimental groups were processed on two successive days and the cytosols then frozen. The cytosol storage procedure allows for stricter limits upon treatment schedules and experiment duration. The control hepatic cytosol had a receptor concentration of 0.1667 ± 0.014 pmol/mg protein. The diabetic cytosol contained a significantly lower receptor level of 0.1094 ± 0.007 pmol/mg protein.

A significant difference was noted between control animals and the diabetic animals. This was true whether

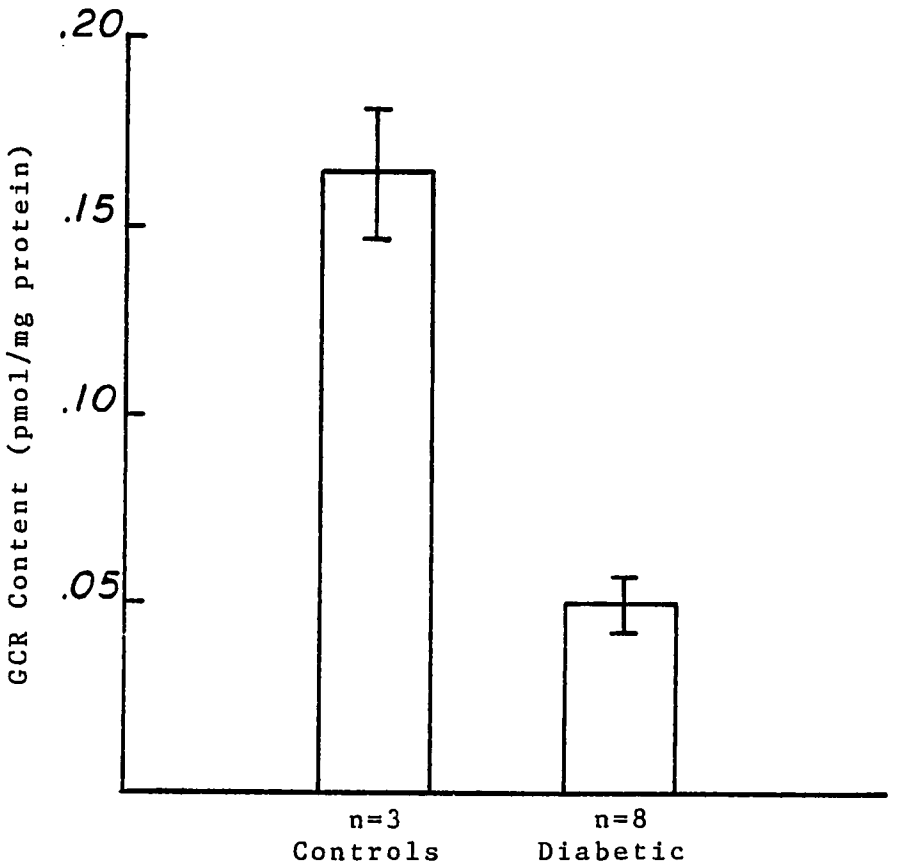

Figure 6. Glucocorticoid receptor content of diabetic and control rat livers in unfrozen, nonstabilized cytosols. This graph represents a composite of the results from the Scatchard analysis performed on diabetic (n=8) and control (n=3) rat hepatic unfrozen, nonstabilized cytosols. The receptor assay was performed immediately following cytosol preparation. There was a significant ($p<0.1$) decrease in GCR content in the diabetic (0.051 ± 0.007 pmol/mg protein) as compared to the controls (0.164 ± 0.017 pmol/mg protein) hepatic cytosols.

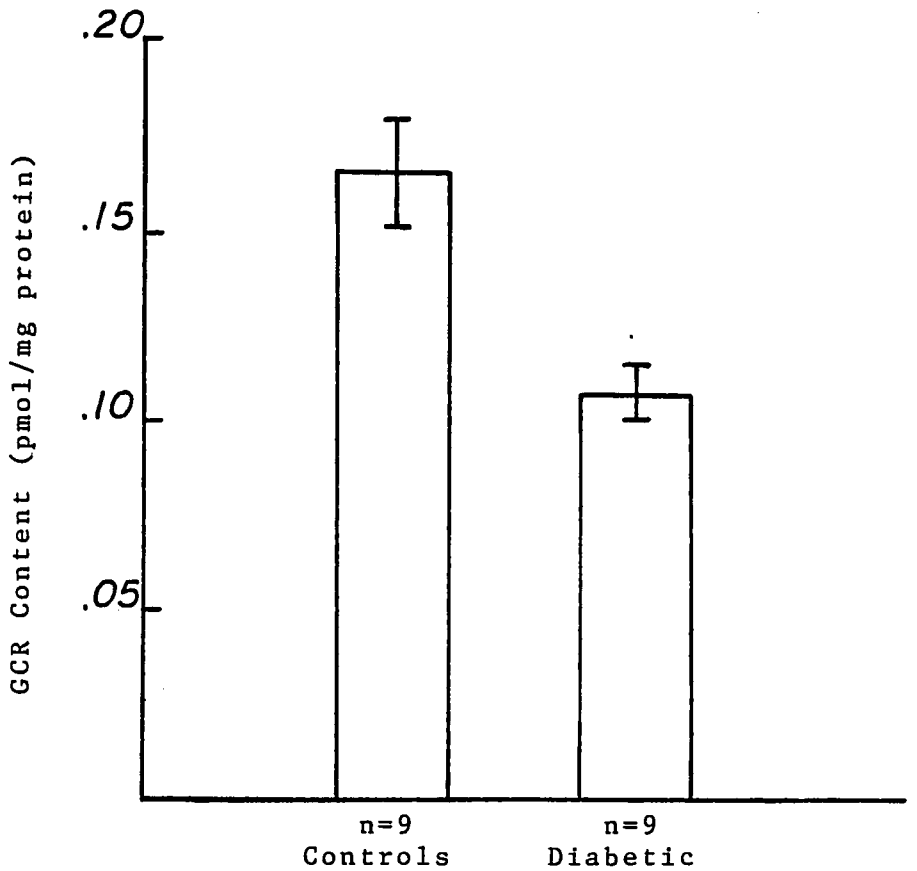

Figure 7. GCR content of stabilized, frozen diabetic cytosols. The homogenate was prepared in the presence of 10% glycerol, 10 mM sodium molybdate, and 0.2 mg/ml trypsin inhibitor. The cytosol fraction was isolated and frozen at -70°C. After 4.5 days the cytosols were thawed and the binding assay was performed. The control value was 0.1667 ± 0.0142 pmol/mg protein of GCR. The diabetic animals have a significantly ($p<0.05$) lower receptor content of 0.1094 ± 0.007 pmol/mg protein.

the cytosol was treated and frozen or not. The diabetic cytosols contained a significantly lower concentration of the GCR.

Insulin Replacement Therapy

The insulin replacement therapy to the diabetic rats significantly increased the receptor concentration above that of the diabetic group (Figure 8). The receptor level of the insulin-treated rats was 0.2602 ± 0.007 pmol/mg protein. Another comparison may also be made between the control and insulin-treated diabetic group. The receptor concentration was also significantly increased in the insulin-treated group as compared to the control animals (0.1667 ± 0.142 pmol/mg protein). Figure 8 shows that the amount of insulin given possibly increased receptor concentration beyond that of the control rats. This was unexpected, but compared well with the glucose level of 58 mg/dl in the insulin-treated as compared to 165 mg/dl in the control group (Figure 3).

Long-term Diabetes

It seems that the receptor concentration in the diabetic liver cytosols may be dependent upon the amount of time spent in the diabetic state. After 14 days in the diabetic state the GCR level is significantly lower than the paired-control group (Figure 7). Measurement of the

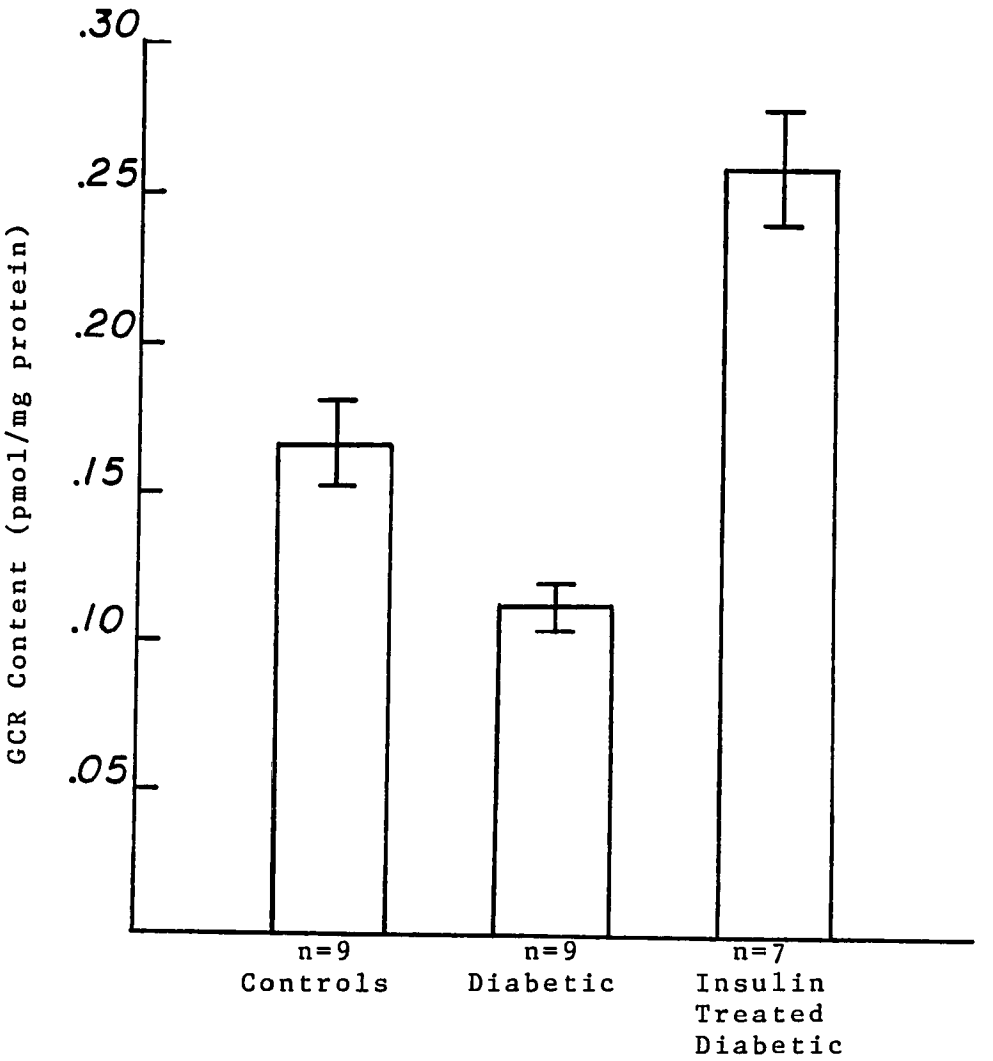

Figure 8. GCR content from diabetic and insulin-treated diabetic hepatic cytosol. The cytosols were stabilized (10% glycerol, 10 mM sodium molybdate, and 0.2 mg/ml trypsin inhibitor) and frozen at -70°C for 4.5 days. At that time the binding assay was performed. The nondiabetic hepatic cytosols had a receptor content of 0.1667 ± 0.0142 pmol/mg protein. The diabetic cytosols had a significant decrease ($p<0.05$) in receptor levels to 0.1094 ± 0.007 pmol/mg protein. The insulin-treated diabetic cytosols exhibited a significant increase in receptor content (0.2602 ± 0.022 pmol/mg protein) over that of the diabetic or nondiabetic cytosols.

diabetic receptor concentration after 25 days (Figure 9) yielded a level of 0.1807 ± 0.014 pmol/mg protein with the 14 day diabetic cytosol content of 0.1094 ± 0.007 pmol/mg protein. The 25-day diabetic GCR content is essentially the same as the 14-day control level. After 110 days in the diabetic state the GCR level increased significantly, further above that of the 25-day diabetic level to 0.2667 ± 0.033 pmol/mg protein (Figure 9). Restraint should be used in drawing conclusions from these results, since no paired-control values were measured at 25 and 110 days. This may suggest some type of long-term accommodation mechanism regulating receptor content.

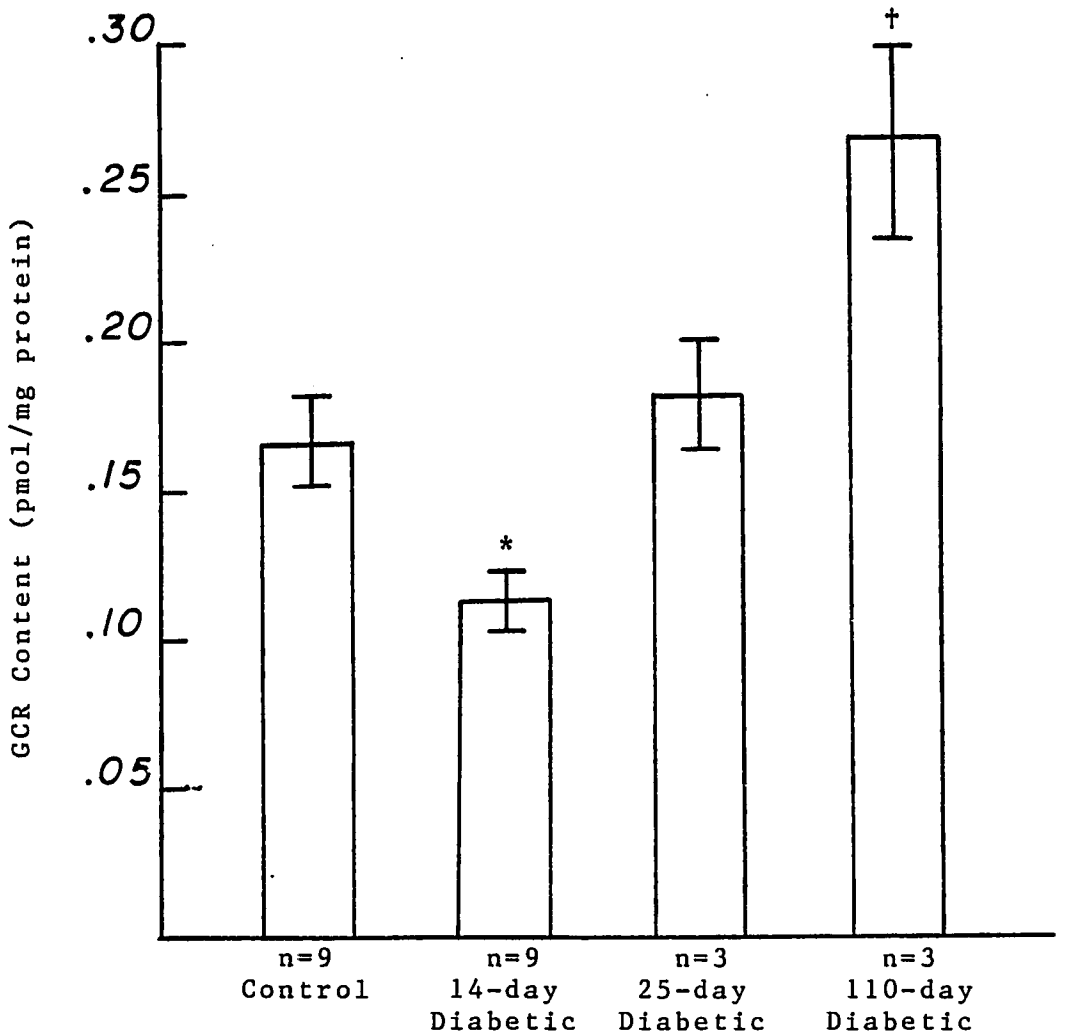

Figure 9. Time course study of GCR content in the diabetic rat liver. The cytosol preparations were stabilized (as previously described) and frozen at -70°C for 4.5 days after the rats were killed. The GCR concentrations for the 14-day diabetic, 25-day diabetic, and 110-day diabetic were 0.1094 ± 0.0142 pmol/mg protein, 0.1807 ± 0.019 pmol/mg protein and 0.2667 ± 0.033 pmol/mg protein, respectively.

* indicates significant difference from controls ($p<0.05$).
† indicates significant difference from controls ($p<0.01$).

DISCUSSION

The results of the present study suggest that after the induction of the diabetic state in the rat; there is a significant decrease in the hepatic GCR content as compared to control liver cytosols. This decrease was apparent in nonstabilized, unfrozen cytosols as well as stabilized, frozen cytosols. The administration of insulin replacement therapy to the diabetic animals completely reversed the diabetes-induced decrease in receptor concentration. In fact, the insulin therapy correlated positively with an increase in GCR levels beyond that of controls. The increase in GCR content above that of controls seems to correlate with the lower than control serum glucose levels (58 ± 8 mg/dl) measured in the insulin replacement therapy group. This suggests a possible direct regulatory effect by insulin on the GCR.

The addition of the stabilization factors to the cytosols presented somewhat conflicting results in the two studies performed. It was not within the scope of the present project to deal in detail with defining a procedure for the utilization of the stabilization factors. The stabilization factors were used only for their "protective" action on the GCR during freezing. The GCR concentrations obtained in this study are in good

agreement with the value of 0.477 ± 0.062 pmol/mg protein reported by Endres et al. (1979) for nonadrenalectomized rat liver cytosols.

The long-term changes in GCR content observed over 110 days may suggest that there is some compensatory mechanism which will regulate receptor concentration. The results are from a small experimental group of animals without paired controls. The importance of the present data could only be determined by further experimentation.

There exist many different factors which may contribute to the cellular content of the GCR. The most obvious procedure that would alter GCR content in the hepatic cell would be to remove the endogenous source of glucocorticoids by adrenalectomy. It has been shown that 48 hours after adrenalectomy, there is a peak increase in the actual number of receptors present in both hepatic and cardiac cells (Gregory et al., 1976). This increase in the GCR content is due to a glucocorticoid absence within the organism. This inverse relation between plasma glucocorticoid levels and hepatic GCR content is further illustrated in protein-deficient rats. A low-protein diet causes a marked elevation of plasma glucocorticoid levels and a subsequent decrease in glucocorticoid receptor content (Varma and Mulay, 1981). Another condition known to decrease GCR content in liver is that of subjecting the rat to immobilization stress for 48 hours

(Golikou et al., 1981). The stress causes an elevation in plasma glucocorticoid levels which leads to the decrease in receptor content.

Hepatic GCR content is also dependent upon the sex of the animal. Lower receptor concentrations are found in females as compared with males. After adrenalectomy of both sexes, there exists no sex differences in the GCR content in liver cytosols. After ovariectomy, the thymus will exhibit an increase in GCR content (Endres et al., 1979). Ovariectomized rats which were adrenalectomized gave the same results as that of the adrenalectomized intact male rat. The response to ovariectomy in increasing GCR content is due to a lowering of plasma glucocorticoid levels. In the normal female rat, the ovaries seem to increase plasma glucocorticoids above that seen in the intact male rat. This increase in glucocorticoid level correlates with the lower GCR content of females. These facts suggest that estradiol has the ability to alter glucocorticoid plasma levels which will then inversely affect GCR content.

At this point it is only possible to discuss the role of insulin in regulating receptor content in non-glucocorticoid receptor systems; then by inference suggesting that insulin indeed plays a role in regulating GCR content. The results of this study suggest that the absence of insulin associated with the hyperglycemia,

is responsible for the reduction in GCR content in the liver. The correlation of reduced insulin levels with reduced receptor content has been demonstrated using the progesterone receptor. Ovariectomized rats exhibit elevated serum glucose levels and a significant decrease in insulin secretion from the islet of Langerhans (Seifi et al., 1981). Estradiol binding capacity in the islet was not altered by ovariectomy but progesterone binding was significantly decreased. Progesterone receptor binding is suggested to correlate with the release of insulin from the islet. In a different study utilizing a breast cancer cell line MCF-7, the amount of insulin present in the culture medium regulated the cytoplasmic estrogen receptor content. At high insulin concentrations (10 µg/ml) in the medium, the amount of estrogen receptor was greatly reduced (Horwitz et al., 1978). By using a lower insulin content (0.006 µg/ml) in the medium, the estrogen receptor concentration can be dramatically increased (Moore, 1981). The ability of insulin to decrease estrogen receptor content in a breast cancer cell line and the association of progesterone receptor binding and insulin release from the islet cells, illustrates the point of possible insulin regulation of steroid receptor content.

So far I have described how glucocorticoids themselves can regulate glucocorticoid content and the apparent

ability of insulin to regulate both the progesterone and estrogen receptor content in certain cells. Since insulin regulates these steroid hormone receptor contents, this may be important to the observed diabetogenic decrease in GCR content exhibited in this study. Little evidence exists to explain the direct lowering of GCR levels by the absence of insulin.

Glucocorticoids have been shown to alter insulin receptor binding. This may play a role in the relationship between insulin and the GCR. Excessive glucocorticoids cause glucose intolerance along with insulin resistance, even though hyperinsulinemia exists. The insulin resistance in this case is a result of the glucocorticoid excess causing a decrease of 33% in the insulin receptor affinity for insulin (Grunfeld et al., 1981). This occurs in rat adipose tissue and hepatic plasma membrane receptors. In a related study, the insulin binding capacity in rat fat cells was increased 37% after adrenalectomy as compared with controls (Haring et al., 1980). After administration of insulin or cortisol to the adrenalectomized rats, the serum insulin levels were increased above control values. With cortisol administration, even though plasma insulin levels were elevated, a decrease in insulin receptor binding affinity occurred. This again suggests a direct effect of glucocorticoids on the insulin receptor.

From the discussion it is realized that hormone receptor contents in various tissues are controlled by many different substances. The primary controlling factor for a specific hormone receptor is by the hormone specific to that receptor. The secondary factors seem to be a direct influence of another specific hormone regulating that receptor. An example of this is the regulation of the insulin receptor by glucocorticoids and of the progesterone receptor by insulin.

The results of this study suggest that insulin has the ability to regulate the GCR. There is evidence that glucocorticoids regulate the insulin receptor. It should not be surprising to find a reciprocal regulation of the insulin receptor by glucocorticoids and insulin and the GCR. Considering the actions of the hormones, insulin and glucocorticoids, on target organs and tissues, their actions often antagonize each other in the regulation of protein, lipid, and carbohydrate metabolism.

The following pages of this discussion on the inter-relationship of glucocorticoid and insulin actions are derived from a review by Ashmore (1964). He states that the involvement of the adrenal gland in the metabolic condition of diabetes mellitus was demonstrated by adrenalectomy of diabetic rats. A lowering of serum glucose was observed by removal of the adrenal gland. Insulin is mainly involved in the regulation of glucose

utilization by increasing glucose entry into cells and by increasing the glucokinase activity. Glucocorticoids have no effect on glucose entry into cells, but they are involved with glucokinase activity. Glucocorticoids decrease glucose phosphorylation as part of their ability to increase serum glucose. In the diabetic rat skeletal muscle there is a decrease in glucose phosphorylation, which is mediated by both an absence of insulin and the presence of glucocorticoids. This leads to elevated blood glucose levels.

The absence of insulin and the presence of glucocorticoids both are able to raise blood glucose levels. In a normal animal the blood glucose elevating effects of the glucocorticoids are antagonized by insulin. In the diabetic animal the glucocorticoids may act unopposed by insulin to further increase blood glucose. This effect is utilized in a clinical manner for prediabetes testing. In a normal individual a loading dose of glucocorticoids will be fully antagonized by insulin to maintain glucose tolerance. In an individual with a decreased insulin supply, the glucocorticoids may act partially unopposed which will be exhibited by a decrease in glucose tolerance.

The massive production of glucose observed in the diabetic state is due to glucocorticoid action. The rate of gluconeogenesis is elevated in the diabetic animal and may be decreased by adrenalectomy of the diabetic

animal. The hepatic activities of both fructose diphosphatephosphatase and glucose-6-phosphatase are elevated in the diabetic animal. This is a possible cause for the rise observed in the rate of gluconeogenesis. A more important increase in enzyme activity is that of phosphoenolpyruvate carboxykinase which is a major control point of the glucocorticoids on gluconeogenesis. Hepatic phosphoenolpyruvate carboxykinase activity was increased by 400%, after the induction of a diabetic state in a rat. The rate of gluconeogenesis is increased by glucocorticoids, while insulin's action is to decrease gluconeogenesis. The substrates for gluconeogenesis are partially provided by protein catabolism. Glucocorticoids stimulate protein catabolism, while insulin acts to inhibit protein breakdown.

Lipid metabolism is also under the control of insulin and glucocorticoids. Insulin stimulates fatty acid synthesis in adipose tissue, while in diabetes there is a decrease in fatty acid synthesis. Lipogenesis is decreased by glucocorticoids in the liver. Free fatty acid release from adipose tissue is inhibited by insulin and stimulated by the glucocorticoids. In diabetes there is an increased release of free fatty acids to be utilized for hepatic gluconeogenesis.

The actions of the glucocorticoids and insulin are antagonistic in nature as observed in the processes of

blood glucose maintenance. The induction of a diabetic state within an organism abolishes the opposition of glucocorticoid actions by insulin. In the examples cited there was a general shift in metabolism towards increasing blood glucose levels in the diabetic animal. Since there is a relative increase of glucocorticoid action in the liver, evidenced by an elevated rate of gluconeogenesis, it is possible that within the hepatic cytosol there is a "down regulation" of the GCR. We noted that protein deficient diets and immobilization stress caused an increase in plasma glucocorticoid levels which lead to a decrease in GCR contents.

Presented here is evidence that glucocorticoids regulate insulin receptor binding affinities. By inference it may be possible to suggest that insulin has a direct regulatory role on GCR content. The absence of insulin may contribute to the decrease in GCR content observed in the diabetic rats of this study.

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