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Factors regulating the synthesis and secretion of corticosteroid-binding globulin (CBG) in the young pig

Jeonghoon Heo

To the Graduate Council:

I am submitting herewith a thesis written by Jeonghoon Heo entitled "Factors regulating the synthesis and secretion of corticosteroid-binding globulin (CBG) in the young pig." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

H.G. Kattesh, Major Professor

We have read this thesis and recommend its acceptance:

J. D. Godkin, T. T. Chen

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the council:

C. W. Minkel

Associate Vice Chancellor and
Dean of the Graduate School

FACTORS REGULATING THE SYNTHESIS AND SECRETION OF CORTICOSTEROID-BINDING GLOBULIN (CBG) IN THE YOUNG PIG

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Jeonghoon Heo

December, 1996

DEDICATION

This thesis is dedicated to my beloved wife, Eunji, and my parents and wife's parents, who have given me the encouragement to accomplish this educational goal

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ABSTRACT

This study examined the in vitro production of corticosteroid-binding globulin (CBG) by liver collected from pigs at various ages following birth, the effects of various hormone treatments on the in vitro hepatic release of CBG, and the relationship between the hormone effects and postnatal development. Liver was collected from pigs (n=20) immediately following euthanasia on 3, 10, 20, 30 or 40 days of age. Liver slices (200 mg) were incubated for 12 and 24 hours with either cortisol (10^{-7} M), estradiol (10^{-8} M), progesterone (10^{-7} M), or thyroxine (10^{-7} M) prepared in 0.5% ethanol. A single blood sample was collected upon euthanasia for determination of plasma CBG levels. The amount of CBG released per unit weight of liver slices and in circulation was measured using an enzyme-linked immunosorbent assay (ELISA) procedure. The percentage of cell's dying and the amount of total protein released were measured to compare with CBG concentration. Cell death was higher ($P<0.05$) on days 3, 10, and 20 than on days 30 and 40. Addition of 0.5% ethanol to the media did not affect the release of total protein or CBG. The release of total protein did not change ($P>0.10$) with age. Plasma CBG levels increased ($P<0.05$) on day 30 and CBG levels measured in media from liver slices decreased ($P<0.01$) on day 20. Hormone treatment had no apparent effect on the release of

CBG by the cultured liver. Consequently, the release of CBG per unit weight of liver decreased ($P<0.01$) on day 20. CBG release from hepatic tissue may be regulated in a different manner from that of other proteins. The absence of a hormone treatment effect on the release of CBG from liver slices may indicate that regulation of CBG release from the liver may be primarily related to age.

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CHAPTER I

INTRODUCTION

During the period from birth to weaning the young pig encounters various environmental and management-related stressors (Curtis, 1974; England, 1974; Mersmann, 1974). The adrenal glucocorticoid, cortisol, plays an important role in the pig's ability to adjust to these stressors as well as in the maturation process (Baxter and Rousseau, 1979). Cortisol exists in plasma in either the unbound or protein-bound forms. The major cortisol binding proteins in plasma are corticosteroid-binding globulin(CBG) and albumin (Ballard, 1979). CBG binds cortisol with remarkably high affinity and specificity (Slaunwhite, Jr. and Sandberg, 1959). As a result, CBG not only transports cortisol but also modulates the bioavailability of cortisol to target cells (Siiteri et al., 1982).

The plasma levels of CBG are determined by its hepatic synthesis, metabolism, and transfer to extravascular spaces. The amount of CBG in plasma varies with age (Henning, 1978; Dalle et al., 1980; Kattesh and Robert, 1993), gender (Dalle et al., 1980; Mataradze et al., 1992), and physiological conditions including pregnancy (Rosenthal et al., 1969; Cohen et al., 1990; Martin and Silberzahn, 1990; Scott et al., 1990) and inflammation (Hammond et al., 1990). CBG is

mainly produced by the hepatocyte (Scrocchi et al., 1993) and its production is regulated by various hormones (Feldman et al., 1979; Elfahime et al., 1992; Smith and Hammond, 1992). CBG production in response to these hormones may vary with age (D'agostino and Henning, 1981, 1982).

The objective of this study was to investigate the relationship between age and hormonal regulation of in vitro hepatic secretion of corticosteroid-binding globulin in the young pig.

CHAPTER II

REVIEW OF LITERATURE

Environmental influence on the development of young pig

During the period from birth to weaning the young pig must cope and respond to a changing environment. Before birth the piglet is provided predigested nutrients, oxygen, a warm environment and antibodies by the sow. However, following birth the piglet must perform digestion, respiration, thermoregulation and antibody production by itself (Curtis, 1974; Wilson, 1974).

It is not uncommon for a swine producer to experience a 20 to 30% pig death loss between birth and weaning. This poor survival rate may be attributed to several aspects related to physiological immaturity such as low levels of phosphorylase, defective gluconeogenic capacity, a deficient hepatic mitochondrial number, low body fat stores, and defects in amino acid metabolism (Mersmann, 1974). Environmental stressors encountered by the piglet following birth (i.e. temperature change) make it more difficult for the animal to maintain homeostasis (Curtis, 1974).

Roles of Cortisol

Hormones serve as regulators of various physiological functions in maintaining homeostasis and participating in

the maturation process. It is known that adrenal glucocorticoids, primarily cortisol in the pig, play an important role in adaptation to environmental changes (Baxter and Rousseau, 1979), inflammation (Fauci, 1979) and the initiation of parturition (First and Bosc, 1979).

Cortisol production results from activation of the hypothalamic-pituitary-adrenal axis (HPA). The hypothalamus produces corticotrophin releasing hormone (CRH) which stimulates the production of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. The adrenal cortex depends on ACTH for the production of cortisol (Baxter and Rousseau, 1979).

The effects of cortisol on glucose and protein metabolism are grossly anabolic in the liver and catabolic and anti-anabolic in certain other tissues. Cortisol increases glucose production in the liver through induction of gluconeogenic enzymes and increased availability of substrates. Cortisol induces the breakdown of both protein and nucleic acids and decreases protein synthesis in muscle (Exton, 1979). In adipose tissue, cortisol stimulates lipolysis and promotes the lipolytic effect of epinephrine and increases the production of lactate (Baxter and Rousseau, 1979). Thus, cortisol acts to mobilize energy sources during prolonged inaccessibility to an immediate energy substrate, especially glucose (Baxter and Rousseau, 1979).

Cortisol is important for the maintenance of normal blood pressure through direct influence on cardiac contractility, vascular reactivity and vascular tone. Cortisol may directly influence the glomerular filtration rate and therefore increase the clearance of water (Baxter and Rousseau, 1979).

Cortisol can directly act on the central nervous system. Patients with Cushing's syndrome resulting from hypersecretion of cortisol by adrenal cortex may appear euphoric with an increase in appetite. Similarly, Addison's syndrome with cortisol deficiency by gradual destrution of adrenal cortex due to an auto immune process or tuberculosis is associated with depression and a general lack of feeling well (McEwen, 1979).

Cortisol may profoundly influence the course of infections and the inflammatory and immunologic responses. Cortisol can suppress virtually every phase of the immunologic and inflammatory response. The effects are very selective and there are marked species differences in sensitivity (Fauci, 1979).

Distribution of cortisol in circulation

Cortisol exists in plasma in both unbound and protein-bound forms (Ballard, 1979). It is generally believed that the protein-bound form is biologically inactive and only the unbound steroid can gain access to target tissues and

initiate actions. The major cortisol binding proteins in plasma are corticosteroid-binding globulin (CBG; transcortin in humans) and albumin. Although CBG is present in plasma at low concentrations, CBG is a major binder of cortisol due to its high affinity for this particular ligand. In contrast, albumin is present at a high concentration and has a much lower affinity (Siiteri et al., 1982). The normal concentration of CBG in humans is 0.71 uM with an association constant for cortisol at 3.0×10^7 L/mol. In contrast, albumin plasma levels are 550 uM with an association constant for cortisol at 0.5×10^4 L/mol. In spite of the large concentration difference between CBG and albumin in plasma, the percentage of cortisol bound to CBG is significantly higher than that bound to albumin (78% vs 15%; Ballard, 1979).

Gayrard et al.(1996) calculated unbound, CBG-bound, and albumin-bound cortisol fractions from the CBG-binding parameters and measured plasma cortisol levels in several species to evaluate the potential availability of cortisol to target tissues. Plasma cortisol concentrations varied from 0.02 to 0.05 uM (ewe, dog, cow), 0.1 to 0.6 uM (horse, human, cynomolgus monkey), and 1.6 uM (squirrel monkey). The CBG maximal capacity was 3 (1.7 to 5.2) times more than the plasma cortisol levels, with the cow, dog and ewe exhibiting the lowest and the cynomologus monkey exhibiting the highest capacity. The distribution of unbound(6 to 14%), CBG-bound

(67 to 87 %) and albumin-bound (7 to 19%) fractions of cortisol were similar between species.

This pattern of cortisol distribution may vary due to age. In the newborn guinea-pig the unbound, CBG-bound and albumin-bound fractions were 20, 62 and 18 %, respectively (Dalle et al., 1980). By day 20 of age the unbound fraction was 6 %, the CBG-bound fraction was 88 % and the albumin-bound fraction was 6%. Kattesh et al.(1990) reported that in the pig unbound, CBG-bound, and albumin-bound cortisol fractions were 30%, 40%, and 30% at birth, respectively and 15%, 54%, and 31% on day 42, respectively. Unbound cortisol appears to be high at birth and decreases with age. This change in distribution of cortisol may have an important role in the maturation and adaptation of the animal during early postnatal development.

CBG Structure

The CBG molecule is associated with the alpha-globulin area in the electrophoretic separation of human plasma proteins (Slaunwhite and Sandberg, 1959) and is characterized as a 50-60 kDa monomeric glycoprotein with a single steroid binding site that interacts preferentially with biologically active glucocorticoids (Hammond et al., 1991). Human CBG is composed of 383 amino acids, and has a polypeptide molecular weight of 42,646 (Hammond et al., 1987). There are two cysteine residues and six consensus

sites for N-glycosylation. A number of CBG complementary DNAs (cDNA) from human liver and lung libraries have been isolated by production of specific antisera against human CBG and used to isolate cDNAs for CBG from other species (Hammond et al., 1987). Phylogenetic comparisons of their primary structures reveal conserved domains that may be structurally and functionally important (Hammond, 1990).

Smith and Hammond (1989) isolated CBG cDNA from a lamda-gt liver cDNA library and determined the primary structure of rat CBG. The protein contained 374 amino acids (Mr = 42,196) and six consensus sites for N-glycosylation. There was a 60% identity over 383 residues to that of human CBG. Furthermore, the single cysteine in rat CBG corresponded to one of two cysteines in human CBG. Northern analysis revealed 1.8 kilobase CBG mRNA in the rat liver and Southern analysis suggested the presence of a single gene for CBG. Similarly, Seralini et al. (1990) demonstrated that the cDNA-deduced primary structure of rabbit CBG contains 383 amino acids(molecular weight = 42,326), including three cysteine residues and four sites for N-glycosylation. It is primarily the product of a 1.68-kilobase hepatic mRNA.

Kattesh and Roberts (1993) isolated and purified porcine CBG from serum using affinity chromatography and HPLC-DEAE anion exchange techniques. SDS-PAGE analysis of porcine CBG revealed that the protein consists of two polypeptides of approximately 54 and 59 kDa and both having

significant amino acid homology (>50%) to that of rabbit , human and hamster CBG for the first 38 amino acids.

When human CBG was compared with other known protein sequences, there was an unexpected finding that the protein is structurally related with several members of the serine protease inhibitor (SERPIN) superfamily (Hammond et al., 1987). It has been demonstrated that neutrophil elastase cleaves the CBG molecule in a position that is closely aligned with the proposed elastase binding site on alpha1-proteinase inhibitor (A1-PI) resulting in a dramatic reduction in CBG steroid binding activity (Pemberton et al., 1988).

Biosynthesis of CBG

The liver has been identified as the major site of CBG biosynthesis in guinea pig (Perrot-Applanat et al., 1981), human (Khan et al., 1984), rat (Smith and Hammond, 1989), rabbit (Seralini et al., 1990), hamster (Lin et al., 1990), fetal sheep (Jacobs et al., 1991), and mouse (Scrocchi et al., 1993). Elfahime et al.(1992) examined enriched populations of hematopoietic and parenchymatous cells prepared from fetal liver for their abilities to accumulate CBG mRNA and showed that the homogeneous hematopoietic cell fraction (virtually free of hepatocytes) failed to accumulate CBG mRNA while the parenchymatous cell fraction did express the CBG gene. Also, CBG has been successfully

produced and secreted by hepatocytes in culture (Khan et al., 1984; Rosner et al., 1984).

The nature of CBG-producing cells was studied in guinea-pig liver by immunocytochemistry. CBG-stained hepatocytes were more numerous in the peripheral regions of the lobules and around the portal space. The rough endoplasmic reticulum was identified as the site of CBG synthesis in the guinea-pig hepatocytes (Perrot-Applanat et al., 1981). Khan et al.(1984) demonstrated that a human hepatoma cell line synthesized and secreted a protein that was functionally, as well as physically and immunologically, the same as CBG.

The transcription unit responsible for CBG mRNA in the human liver has been characterized revealing the presence of a highly conserved promoter region (Underhill and Hammond, 1989). The CBG gene promoter contains a consensus binding sequence for the hepatocyte-specific transcription factor, HNF-1. This factor is probably important in the development of the liver phenotype and therefore is an essential part of the transcriptional apparatus responsible for the expression of the CBG gene in the liver (Hammond et al., 1991).

The availability of species-specific cDNAs for CBG allowed for the detection of CBG mRNA by Northern blot analysis of spleen and ovary RNA from adult rabbits (Seralini et al., 1990) and kidney and pituitary RNA from fetal sheep (Berdusco et al., 1995). The levels of CBG mRNA

in these tissues are relatively low when compared to adult liver samples and, therefore, only a small proportion of cells within these extra-hepatic tissues may express the CBG gene (Seralini et al., 1990).

Roles of CBG in circulation

The generally accepted model of steroid hormone action suggests that unbound steroid diffuses passively through target cell membranes and binds to a soluble intracellular receptor. The steroid-receptor complex apparently moves into the nucleus where it modifies the chromatin transcriptional activity which results in altered levels of protein synthesis (Siiteri et al., 1982). A variety of extracelluar binding proteins enhance the solubility of steroid hormones in biological fluids and facilitate their transport from steroidogenic tissues to target cells (Hammond, 1990). CBG binds glucocorticoids with remarkably high affinity and specificity. As a result, CBG not only transports glucocorticoids but also modulates their bioavailability (Siiteri et al., 1982) and may participate directly in the delivery of steroids to certain cells by interaction with plasma membrane receptors (Rosner, 1990).

The role that CBG serves in regulating the bioavailability of cortisol can be demonstrated in the human during pregnancy. Although the cortisol level rises considerably during pregnancy, the percentage of cortisol

distributed among CBG, albumin and the unbound compartments remains almost constant throughout pregnancy and is very similar to those in normal women (Rosenthal et al., 1969). It appears that the concomitant rise of CBG during pregnancy keeps the physiologically available cortisol level low enough so as not to produce Cushing's syndrome.

CBG serves primarily as a relatively inert reservoir in which corticoids are protected from metabolism and excretion, but still available by dissociation to provide sufficient hormone when steroid production is increased (Ballard, 1979). Lepper et al.(1988) demonstrated that the increase in plasma corticosterone concentration in the rat during the third week of life resulted from a decrease in metabolic clearance rate(MCR) for corticosterone. The decrease in MCR was due to a decline in the apparent volume of distribution for corticosterone in plasma which is affected through the increasing concentration of CBG because of high affinity of CBG for corticosterone. On the other hand, the authors also showed that the half-life of bound corticosterone was less than that of free corticosterone and suggested that bound corticosterone may be preferentially metabolized by a mechanism for the internalization of CBG through receptor-mediated endocytosis in the target tissues. Specific binding sites for CBG were detected on membranes perpared from rat spleen (Singer et al., 1988).

It has been known that CBG responds as an acute phase negative protein dramatically decreased in serum during inflammation and that serum CBG levels drop rapidly during artificially induced inflammation (Savu et al., 1980). During inflammation, activation of the neutrophils results in the production of large amounts of superoxide radicals and serine proteinases, specifically elastase (Hammond et al., 1991). The production of superoxide radicals destroy the ability of alpha1-proteinase inhibitor(A1-PI) to inhibit elastase and thereby allows this serine proteinase to function more efficiently in a local context. On the other hand, CBG is not influenced by the superoxide radicals and retains its ability to interact with neutrophil elastase, which is associated with the surface of these cells once they have been activated (Hammond et al., 1991). The CBG is then rapidly cleaved by elastase and provides a mechanism for the delivery of relatively large amounts of glucocorticoids to the activated neutrophils (Hammond et al., 1990). At this stage in the inflammatory reaction, the glucocorticoids down-regulate neutrophil activity by decreasing the production of chemostatic factors (Fauci, 1979).

Since it has been suggested that serine proteinases play an important role in the normal process of tissue remodeling and development, it is possible that CBG may promote the delivery of glucocorticoids to sites where rapid

growth and tissue remodeling are occurring as a result of normal development (Hammond et al., 1991).

Factors affecting CBG production

The fetal rat has a relatively high concentration of CBG until approximately 4 days before birth. At this time circulating CBG levels begin to fall and rapidly reach low values that are maintained through the first 9 days after birth. During the second postnatal week, serum CBG concentrations begin to rise again. This increase continues until a plateau is reached on approximately day 24 (Henning, 1978; D'Agostino and Henning, 1981). Smith and Hammond (1991) compared the levels of serum CBG and hepatic CBG mRNA from day 15 of gestation until 12 weeks of age after birth. The hepatic CBG mRNA concentration in day 15 fetuses was 55.1 pg CBG mRNA/ug RNA and decreased progressively to 1.4 pg CBG mRNA/ug RNA by day 21 of gestation. At 1 week of age after birth, the level of CBG mRNA was 0.4-0.8 pg CBG mRNA/ug RNA and increased at least 5-fold by 2 weeks of age after birth. After this time, CBG mRNA values doubled reaching adult levels by 3 weeks of age. Although adult CBG mRNA levels were attained by 3 weeks of age, serum CBG concentrations did not reach adult values for an additional 3 weeks. Also, the half-life of CBG in infants (6.9 hours) was consistently less than that in adults(14.5 hours). The authors suggested that variations in CBG biosynthesis and

clearance may influence glucocorticoid action during fetal and postnatal development (Smith and Hammond, 1991).

Gender differences in the circulating concentration of CBG have been noted. Serum CBG level in the adult female rat were 2.5 fold higher than that in the male(Jansson et al., 1989; Mataradez et al., 1992). Likewise, hepatic CBG mRNA content in the adult female was 3-fold higher than that of the adult male (Smith and Hammond, 1989). This sexual dimorphism in CBG gene expression may be attributed to sex differentiation of growth hormone secretion induced by the secretion of androgens and estrogen, but also by androgen imprinting of the neonatal male rats (Jansson et al., 1984; Jansson et al., 1989).

Measurement of CBG binding capacity is an indirect method for measurement of CBG in plasma, based upon its ability to bind radioactive cortisol (Siiteri et al., 1982). Both age and sex-related changes in the binding capacity of CBG for cortisol occur in the guinea pig during development which parallel the changes in plasma cortisol concentration (Dalle et al., 1980). CBG binding capacity for cortisol in plasma was highest at birth (male: 2.15 umol/l; female: 2.20 umol/l) followed by a consistent and significant decrease by day 10. On day 20, CBG binding capacity in the male(0.74 umol/l) was not different from that measured on day 10. However, female guinea-pigs measured on day 20 had significantly higher CBG binding capacity(1.6 umol/l) than

that found on day 10 (0.7 umol/l), which was significantly higher than that of male animals at the same age.

Kattesh and Roberts (1993) documented the changes in plasma CBG concentrations in the pig measured at various ages from 3 to 126 days of age using enzyme-linked immunosorbent assay (ELISA) procedures. The CBG concentration decreased from day 3 (227.2 ug protein/l) to day 7 (195.6 ug protein/l). The concentration of CBG was significantly higher on day 28 (271.6 ug protein/l) reaching a plateau on day 42 (351.4 ug protein/l). The increase in CBG levels around 28 days of age reflect corresponding changes in the percent distribution of cortisol among protein bound and free forms in the pig as documented earlier (Kattesh et al., 1990).

Hsu and Kuhn(1988) demonstrated a diurnal variation in the level of CBG similar with that in cortisol, with the highest levels detected at the beginning of the dark or active period in the rat. The circadian variation in serum CBG was the result of changes in the distribution of CBG between plasma and interstitial fluid, and the relative distribution of CBG is determined by permeability of the capillaries to CBG which is decreased by corticosteroid (Ottenweller et al., 1979).

Species-specific variations in CBG concentration have been noted in the gestating female. In the women (Rosenthal et al., 1969), rabbit (Seralini et al., 1990) and hamster

(Lin et al., 1990), plasma CBG is considerably elevated during pregnancy. In the pregnant rat, CBG levels remain almost unaltered(Smith and Hammond, 1989). In contrast, the mare shows a significant decrease in CBG binding capacity in plasma throughout pregnancy and this decrease may be due to an increase in CBG uptake by the tissues (Martin and Silberzahn, 1990).

Hormonal regulation of CBG production

Many studies have demonstrated that various hormones are involved in the regulation of circulating concentrations of CBG. The responses of CBG to these hormones may vary with age.

During postnatal development in the rat, serum concentrations of corticosterone begin to rise in parallel with serum CBG levels (Henning, 1978), but corticosterone apparently has no effect on this developmental rise of CBG (D'Agostino and Henning, 1982). In the adult male rat, dexamethasone (25 ug/100g body weight) injection for 2 days decreased CBG production rate by liver slices in culture (Feldman et al., 1979) and serum CBG and hepatic CBG mRNA levels were also decreased after twice daily dexamethasone (25 ug/100g body weight) injection for 3 days (Smith and Hammond, 1992). Fetal sheep infused with the synthetic glucocorticoid dexamethasone had increased plasma CBG levels, hepatic CBG mRNA and the CoA binding forms, and

adult sheep treated with dexamethasone showed a significant decrease in plasma CBG levels and hepatic CBG mRNA (Berdusco et al., 1993). Glucocorticoid treatment exerted opposite effects on CBG biosynthesis in fetal and adult sheep. The authors concluded that the response of the CBG gene to glucocorticoid may be developmentally regulated.

Thyroxine(T4) exhibits developmental profiles similar to CBG and its administration results in a precocious rise in serum CBG during postnatal development (D'Agostino and Henning, 1981, 1982). In the adult rat, T4 administration twice daily for 3 days increased serum CBG and hepatic CBG mRNA by 66% and 39%, respectively, but did not influences the rate of CBG gene transcription. Thus, the elevated hepatic CBG mRNA in these rats are probably mediated by increased CBG mRNA stability (Smith and Hammond, 1992). The magnitude of the T4 effect on CBG binding capacity during the neonatal period is considerably greater than that seen in adult rats (D'Agostino and Henning, 1981). Daily administration of T4 to adults males for 7 days resulted in a two fold elevation of CBG. In contrast, neonatal rats subjected to a 7-day period of hyperthyroidism caused a 24-fold increase in CBG concentrations. D'Agostino and Henning(1982) demonstrated that the response of CBG to T4 increased with advancing age during development. Elfahime et al.(1992) examined the effects of both T3 and rT3 on CBG mRNA in liver from neonatal rats and showed that only T3 was

able to enhance the level of the messenger. This suggests that in the fetus, in which rT3 is the predominant form of circulating thyroid hormone, liver CBG mRNA may be regulated differently in neonates compared to adults.

Estrogen also shows developmental profiles similar to CBG and is known to be involved in the regulation of adult CBG concentrations. The administration of estrogens to nonpregnant women resulted in a significant rise in CBG concentration similar to that in noninjected pregnant women (Slaunwhite and Sandberg, 1959). The adult male rat injected with estrogen showed a 100% increase in CBG secretion by the perfused liver system (Fieldman et al., 1979). However, in the postnatal rat, the developmental rise in the binding capacity of CBG for cortisol in plasma is independent of estradiol and estrone. This lack of response to estrogen could be due to an insufficient number of receptors for estrogen or lack of interaction of estrogen with the hypothalamus because of secondary effect of estrogen on the increase of thyroxine production through thyroid-stimulating hormone (D'Agostino and Henning, 1981).

Progesterone injection (25 ug/100g body weight for 3 days) to adult male rats did not alter serum CBG levels (Smith and Hammond, 1992), but much higher doses(~5 mg/100g body weight for 10 days) increased CBG levels in male rats by 90% (Gala and Westphal, 1965). This may be indirectly related to a reduction in circulating corticosterone levels

because the markedly elevated progesterone levels play some part in displacing cortisol from CBG as part of the competition for binding sites on CBG (Rosenthal et al., 1969) and, therefore, much higher doses probably mimic the postadrenalectomy rise in serum CBG levels (Smith and Hammond, 1992).

Castration of mature rats led to a 40-50% increase in CBG content and testosterone treatment led to a decrease by 40-50 % compared with vehicle-treated rats. However, the CBG levels in immature castrated rats treated with testosterone did not differ from that in vehicle-treated rats (Mataradze et al., 1992). Jansson et al.(1989) demonstrated that neither androgen nor estrogen treatment affected the serum CBG levels in hypophysectomized rats. However, continuous infusions of growth hormone increased serum CBG levels in both hypophysectomized male and female rats. They suggested that the sex differentiation of growth hormone pulsatility induced by testicular androgens and ovarian estrogens in the adult rat mediate the effect of gender and gonadal steroids on the sex difference in serum CBG levels of normal rats.

CHAPTER III

MATERIALS AND METHODS

Animals

Twenty crossbred female pigs (Landrace, Duroc and Hampshire breeding), born naturally and reared conventionally, were used. Pigs were left with the sow (n=7) in a conventional farrowing crate throughout the experiment. All pigs were provided an 18% crude protein-pelleted creep feed beginning on day 14 of age and water was provided ad libitum. Four pigs each was randomly allotted for study on days 3, 10, 20, 30, and 40 following birth.

Plasma collection and Tissue cultures

At the prescribed age a single pig was removed from its litter, anesthetized by intramuscular injection of a ketamine (15 mg/kg body weight) and acepromazine (0.5 mg/kg body weight) solution. The animal was placed under general anesthesia by closed circuit halothane administered by a gas inhalation machine.

For determining plasma CBG level at each age studied, a 4 ml blood sample was collected from the anterior vena cava of each anesthetized pig and the sample placed in a heparinized tube. Plasma was collected by centrifugation at

3,000 rpm for 10 minutes at 4°C and stored at -20°C for later CBG analysis.

Two lobes of liver were quickly removed from the anesthetized pig and perfused with ice cold phosphate buffer saline (PBS) solution (pH 7.4). Each lobe was sliced with a double edged razor blade in cold HBSS solution (pH 7.4, Sigma chemical Co., MO) and rinsed with cold HBSS solution. Slices of uniform thickness (~0.5 mm) were blotted on sterilized gauze and weighed. For each treatment approximately 0.2 g of sliced liver were placed in dishes containing 4 ml of Williams' medium E (pH 7.4, Gibco BRL, NY) and pre-incubated in a humidified 5% CO_2, 45% O_2, and 50% N_2 atmosphere at 37°C for 3 hours to remove blood from the liver slices.

After pre-incubation, the samples were washed with warm HBSS solution and 5 ml of treatment medium containing the specific hormone concentration was added. Six samples of each liver were incubated in triplicate in a humidified 5% CO_2, 45% O_2, and 50% N_2 atmosphere at 37°C. A 1 ml aliquot of the culture media was collected after 12 hours incubation and the remaining culture media was collected after 24 hours incubation. The collected culture media was stored at -20°C for the determination of CBG concentration.

Hormone preparations

Hydroxycorticosterone, L-thyroxine, 17 beta-estradiol, and progesterone (Sigma Chemical Co., MO) were initially prepared in absolute ethanol. The working concentration of hormone used was 10^{-7} M (cortisol, thyroxine, and progesterone) and 10^{-8} M (estradiol) prepared in 0.5% ethanol-media solution. The control group contained 0.5% ethanol-media. An additional control group was made with William's medium E alone. Liver slices from each pig were subjected to five treatments examined in triplicate.

Cell Viability

The leakage of lactate dehydrogenase (LDH) from liver slices into medium during incubation was used as a reliable indicator of cell viability in previous study (Smith et al., 1985). LDH activity in liver slices or medium was measured with a kit (catalog No.500, Sigma Chemical Co., MO; see appendix A). The rate of cell death was expressed as the percentage of cytoplasmic LDH activity leaked from liver slices into medium compared to liver slices and medium activity combined. LDH activity was measured at 12 and 24 hours of incubation for two pigs of each age.

Total protein measurement

The total protein released from liver slices during incubation was measured with Coomassie Blue G-250 Reagent

solution (Pierce Chemical Co., KC) by a micro coomassie protein assay procedure (see appendix B). The amount of total protein was expressed as microgram of protein released per milligram of liver. Intraassay and interassay coefficients of variation for 16 sets of triplicates from serum pool of pig were 1.8 and 6.8%, respectively.

Enzyme-linked immunosorbent assay (ELISA) for CBG

The amount of CBG in plasma and synthesized by cultured liver was measured by an ELISA for porcine CBG (pCBG) developed by Kattesh and Roberts(1993) as outlined in appendix C. Intraassay and interassay coefficients of variation for 20 sets of duplicates from a serum pool of pig plasma were 6.9% and 14%, respectively

Statistics

The results were analyzed using General Linear Mixed Models (GLMM) procedures (Blouin and Saxton, 1990) in order to determine differences in CBG concentrations with regard to hormone treatment and age effect. Least square means and standard errors were calculated for all variables. Significant differences among means were partitioned using Duncans procedures.

CHAPTER IV

RESULTS

Body weight. Mean (± SEM) pig body weight by age is illustrated in figure 1. Body weight for each of the five age groups increased ($P<0.01$) 10.5 kg in a linear fashion from day 3 to day 40, with the greatest increase occurring from days 30 to 40.

Cell death rate in liver slices during incubation. Cell death rate was based on the percentage of cytoplasmic enzyme lactate dehydrogenase (LDH) activity released from liver slices into media after 12 and 24 hours incubation compared to tissue and media activity combined. The percentage of cell death following 12 hours of incubation (44.9 ± 6.1%; Figure 2) was approximately 19.4% less than that following 24 hours of incubation (64.3 ± 5.5%; Figure 3). The 12 hour cell death percentage in liver slices taken from pigs on days 30 and 40 was 26.1 and 35.6%, respectively which was lower ($P<0.05$) than that from pigs on days 3, 10, and 20.

Total protein concentration in medium with and without 0.5% ethanol. To determine whether ethanol affects the release of total protein from liver slices during incubation, an ethanol treatment group (0.5% final concentration of ethanol in media) was compared to medium

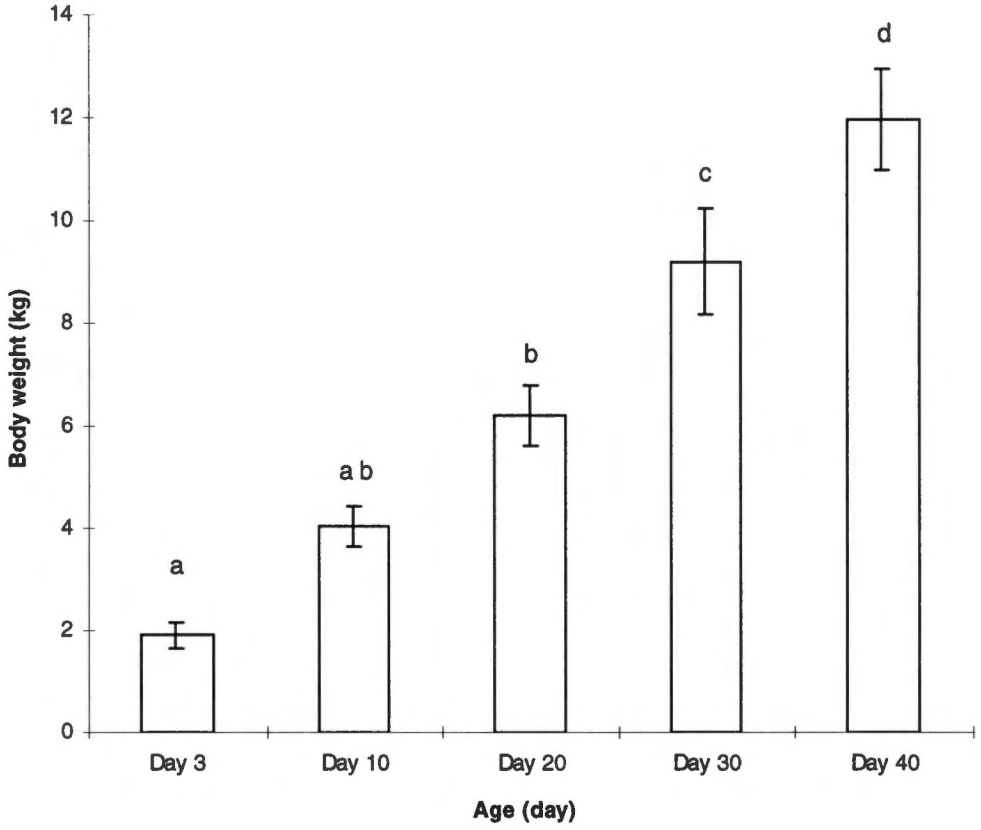

Figure 1. Body weight of pigs at each age studied. Each bar represents the mean ± SE (n=4). Columns with different letters are significantly ($P<0.01$) different.

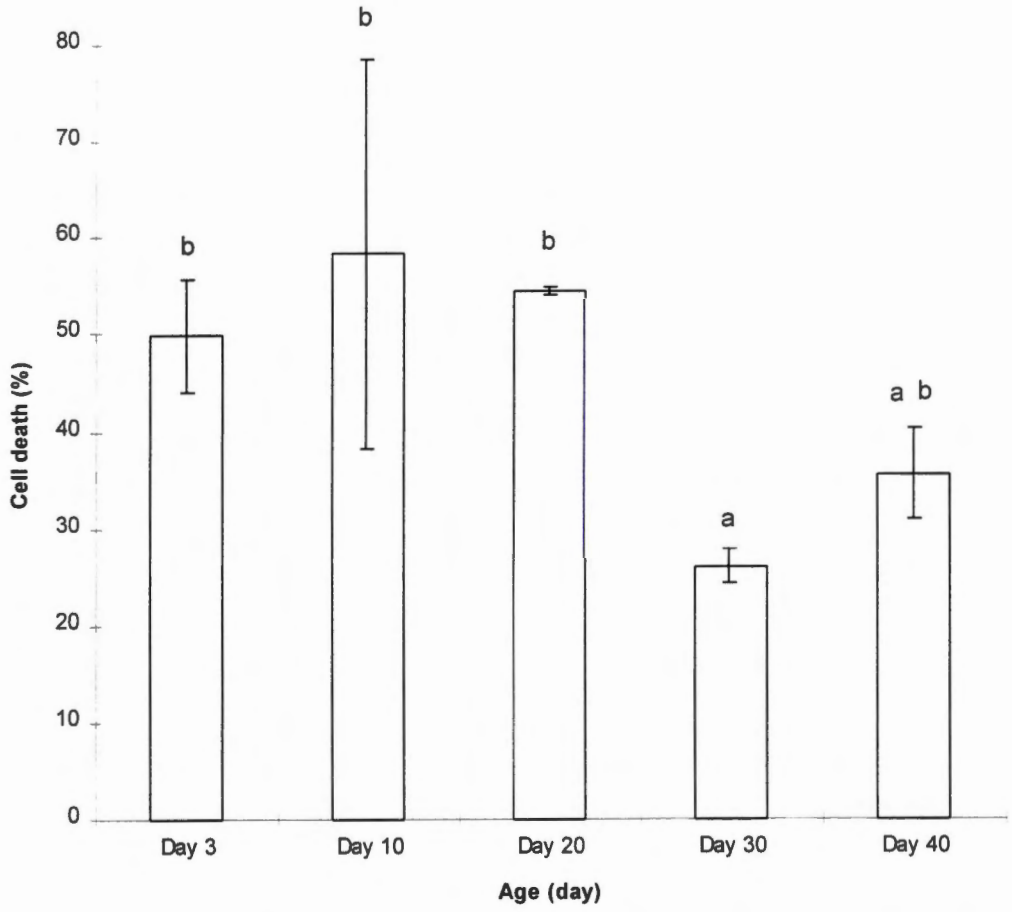

Figure 2. The percentage of cell death in liver slice cultures following 12 hours of incubation at each age studied. Each bar represents the mean ± SE (n=2). Columns with different letters are significantly ($P<0.05$) different.

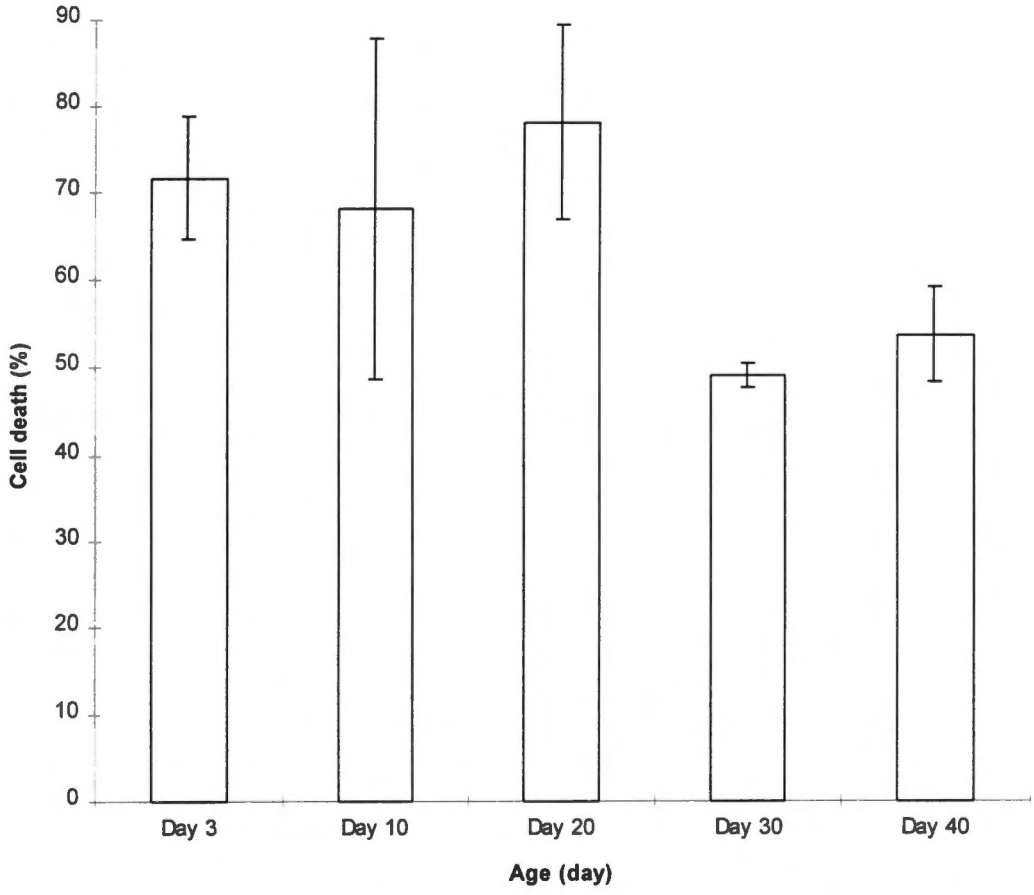

Figure 3. The percentage of cell death in liver slice cultures following 24 hours incubation at each age studied. Each bar represents the mean ± SE (n=2).

alone. There was no significant difference (P>0.10) between the ethanol control group and medium alone (Figure 4).

Total protein concentration released from liver slices during incubation. Figure 5 shows the amount of total protein released per unit weight of liver slices in medium following 12 and 24 hour incubation for each age. The amount of total protein released in medium was not different (P>0.10) between time of incubation or age of pig studied.

Plasma CBG concentration. The concentration of CBG in plasma from pigs sampled on days 3 and 10 was lower (P<0.05) than that measured on day 30 (Figure 6). CBG increased (P<0.05) from 1.38 ± 0.06 ug/ml on day 10 to 2.18 ± 0.29 ug/ml on day 30.

CBG concentration from liver slices cultured in medium alone by age. Figures 7 and 8 show the concentration of CBG released per unit weight of liver slices in medium following 12 and 24 hour incubation, respectively. Culture medium from liver slices incubated for 12 hours contained less (P<0.05) CBG than those incubated for 24 hours (2.91 ± 0.39 ng/mg vs 3.91 ± 0.31 ng/mg, respectively). The concentration of CBG released from liver slices following 12 hours incubation was higher (P<0.01) on day 10 than that measured on the other days. The concentration of CBG released from liver slices following 24 hours incubation did not change from day 3 to day 40.

The percentage of CBG to total protein To compare the

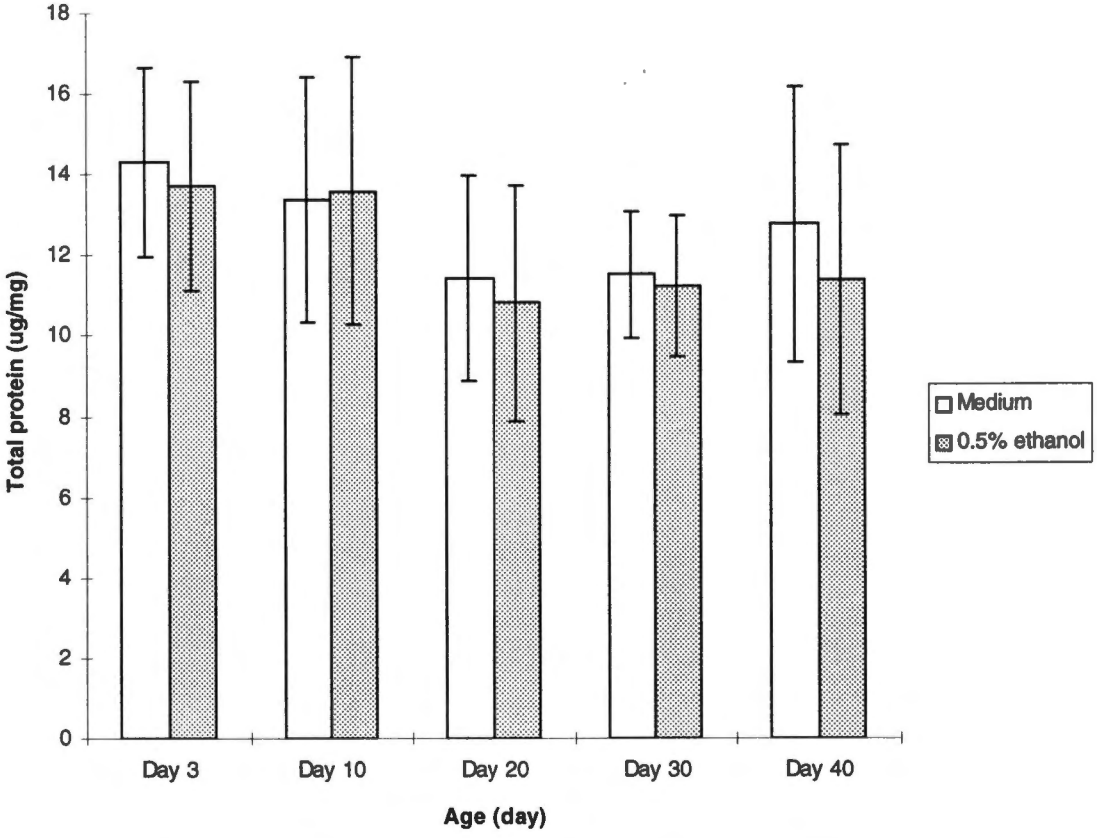

Figure 4. Total protein concentration from liver slices with and without 0.5% ethanol following 24 hours incubation at each age studied. Total protein concentration is expressed as microgram of total protein released per unit weight of liver slice (ug/mg). Each bar represents the mean ± SE (n=4).

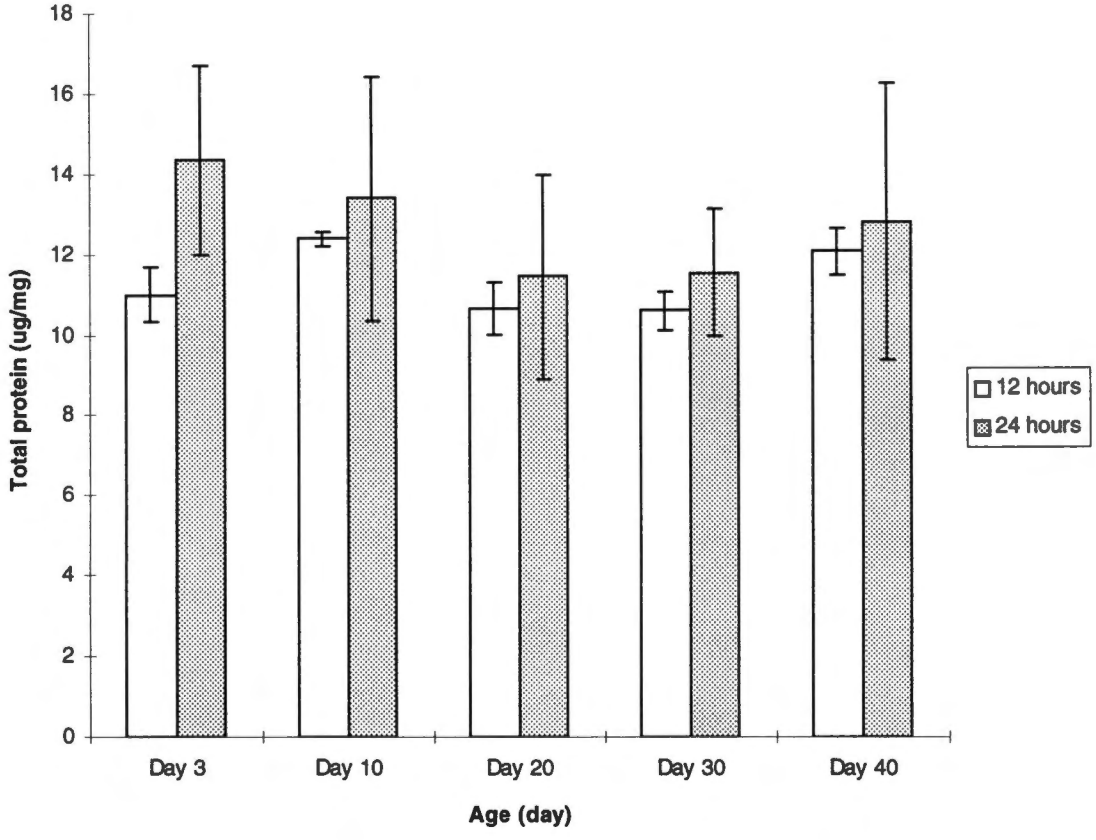

Figure 5. Total protein concentration from liver slices following 12 and 24 hours incubation in medium alone. Total protein concentration is expressed as microgram of total protein released per unit weight of liver slice (ug/mg). Each bar represents the mean ± SE (n=4).

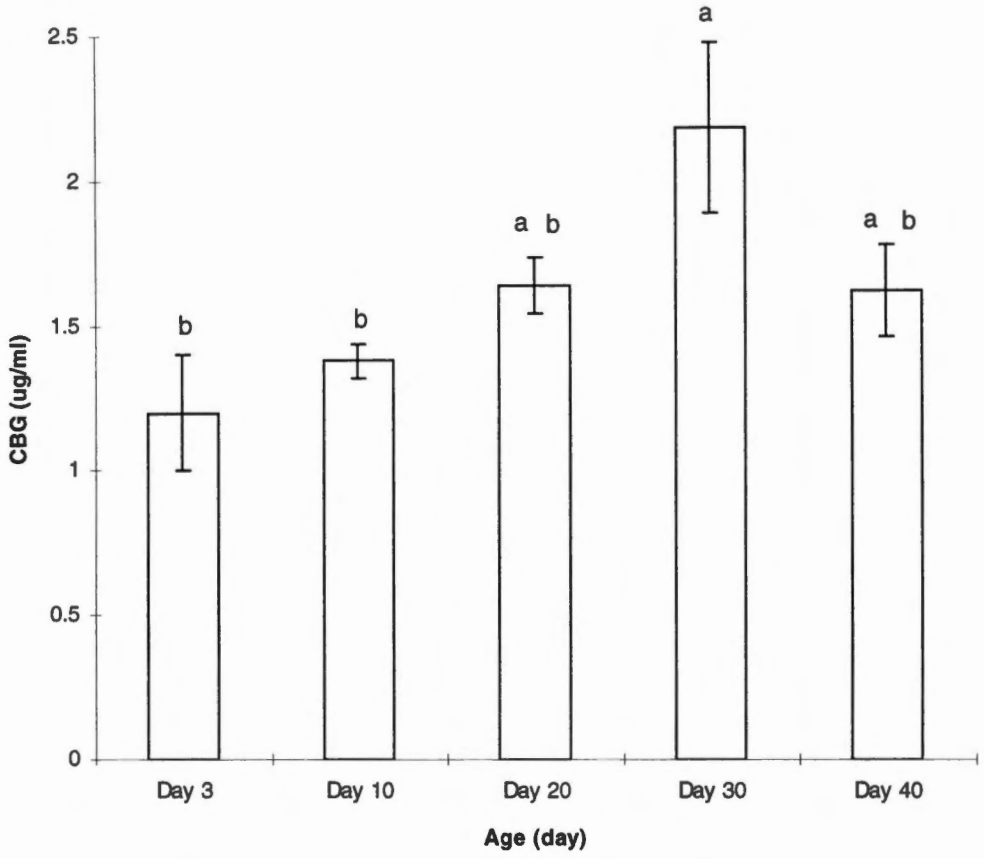

Figure 6. Plasma CBG concentration at each age studied. CBG concentration is expressed as microgram per milliliter of plasma (ug/ml). Each bar represents the mean ± SE (n=4). Columns with different letters are significantly ($P<0.05$) different.

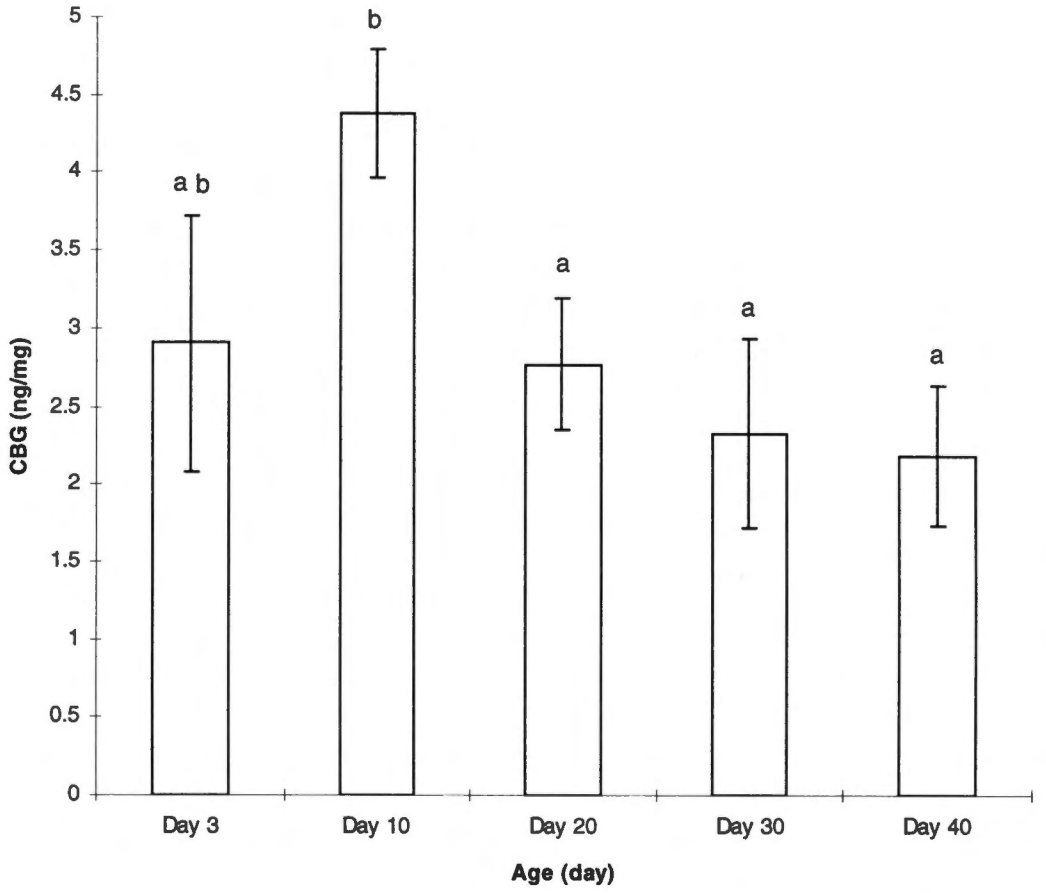

Figure 7. CBG concentration from liver slices in medium alone following 12 hours incubation. CBG concentration is expressed as nanogram of CBG per unit weight of liver slice (ng/mg). Each bar represents the mean ± SE (n=4). Columns with different letters are significantly ($P<0.01$) different.

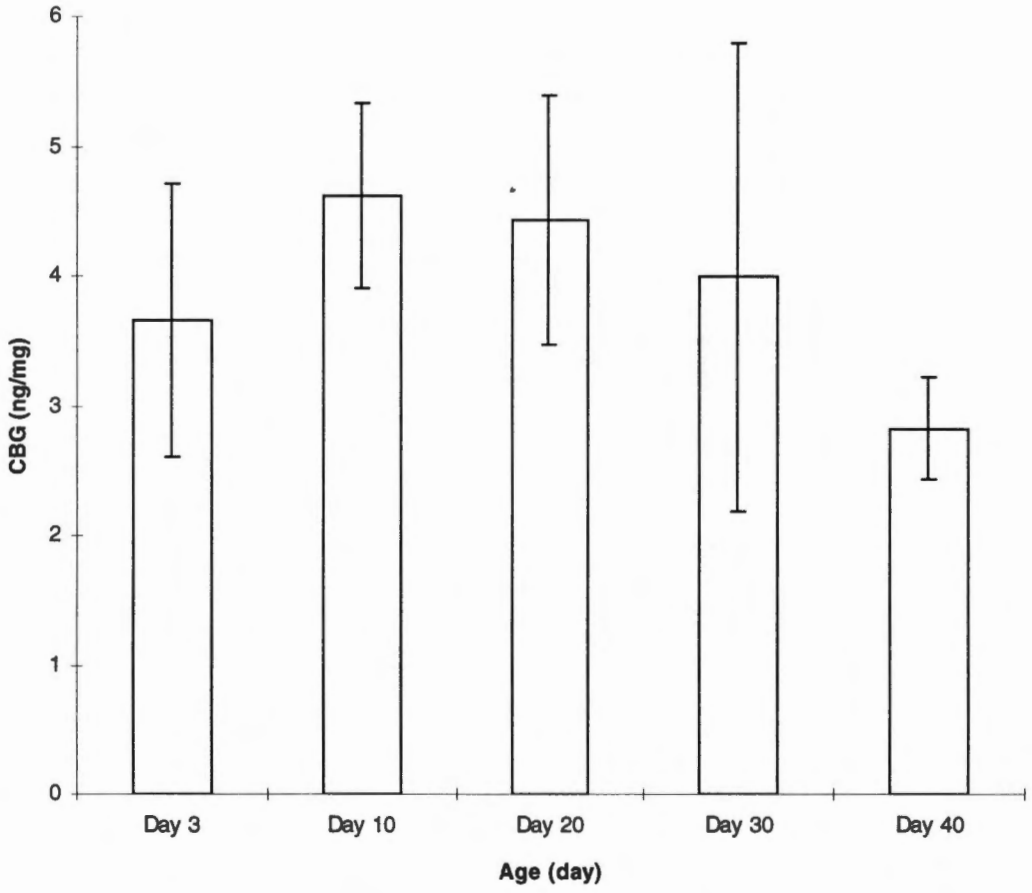

Figure 8. CBG concentration from liver slices in medium alone following 24 hours incubation. CBG concentration is expressed as nanogram of CBG per unit weight of liver slice (ng/mg). Each bar represents the mean ± SE (n=4).

release of CBG relative to that of total protein, the percentage of CBG to total protein was calculated for each age studied. The overall percentage of CBG to total protein following 24 hours incubation (0.042 ± 0.006%; Figure 10) on days 20, 30, and 40 was approximately two-fold higher than those following 12 hours incubation (0.022 ± 0.002%; Figure 9). The percentage of CBG to total protein on day 10 (0.036 ± 0.0023%) was higher ($P<0.01$) than that measured on days 20, 30, and 40 (0.026 ± 0.003%, 0.022 ± 0.005%, and 0.018 ± 0.004 %, respectively). This pattern was similar to that seen for CBG release from liver slices following 12 hours incubation.

CBG concentration from liver slices cultured in medium with different hormones added. There was no apparent effect of hormone treatment on the release of CBG from liver slices following 12 or 24 hours incubation (Figures 11 and 12). Thyroxine treatment on day 10 decreased ($P<0.01$) the release of CBG from liver slices following 12 hour incubation when compared to the other treatment groups of the same age (Figure 11).

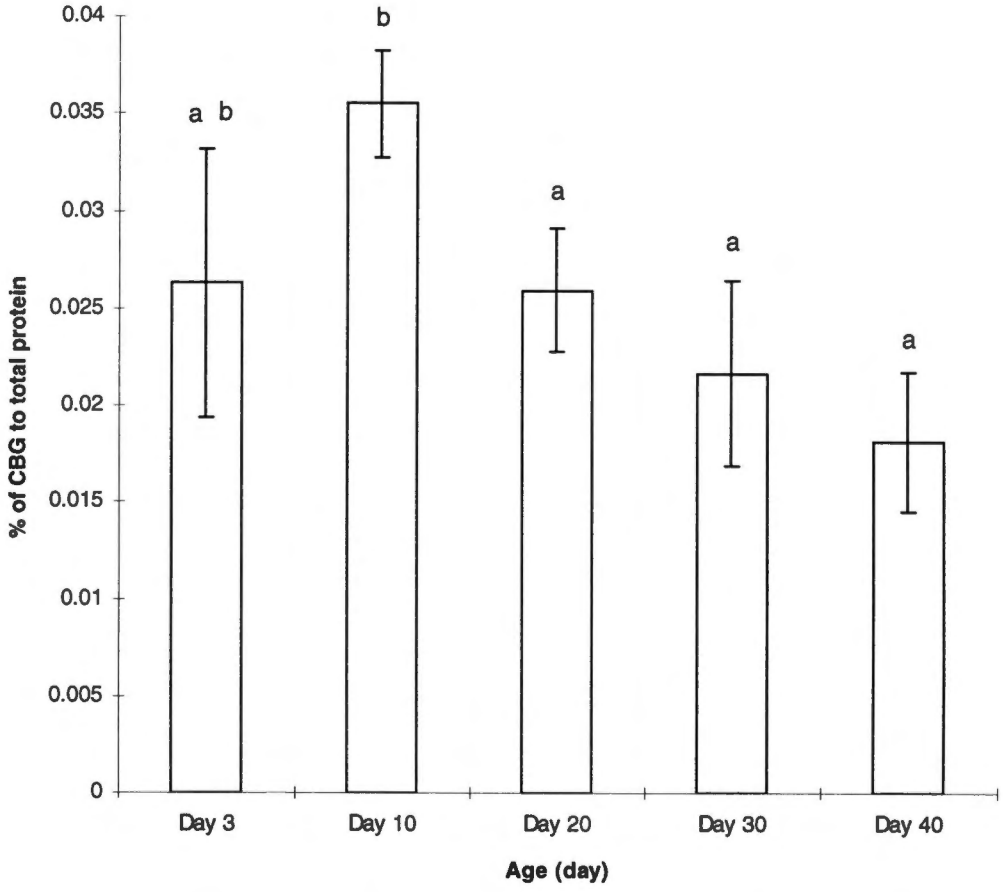

Figure 9. The percentage of CBG to total protein released from liver slices in medium alone following 12 hours incubation. Each bar represents the mean ± SE (n=4). Columns with different letters are significantly ($P<0.01$) different.

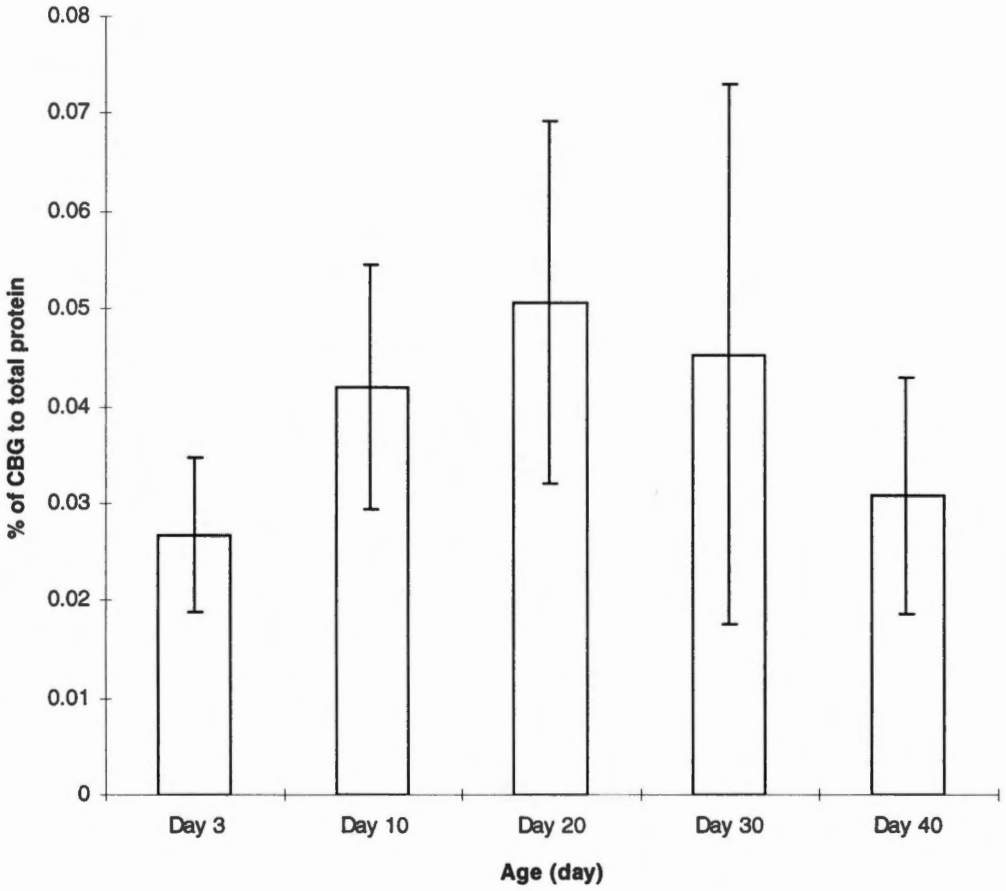

Figure 10. The percentage of CBG to total protein released from liver slices in medium alone following 24 hours incubation. Each bar represents the mean ± SE (n=4).

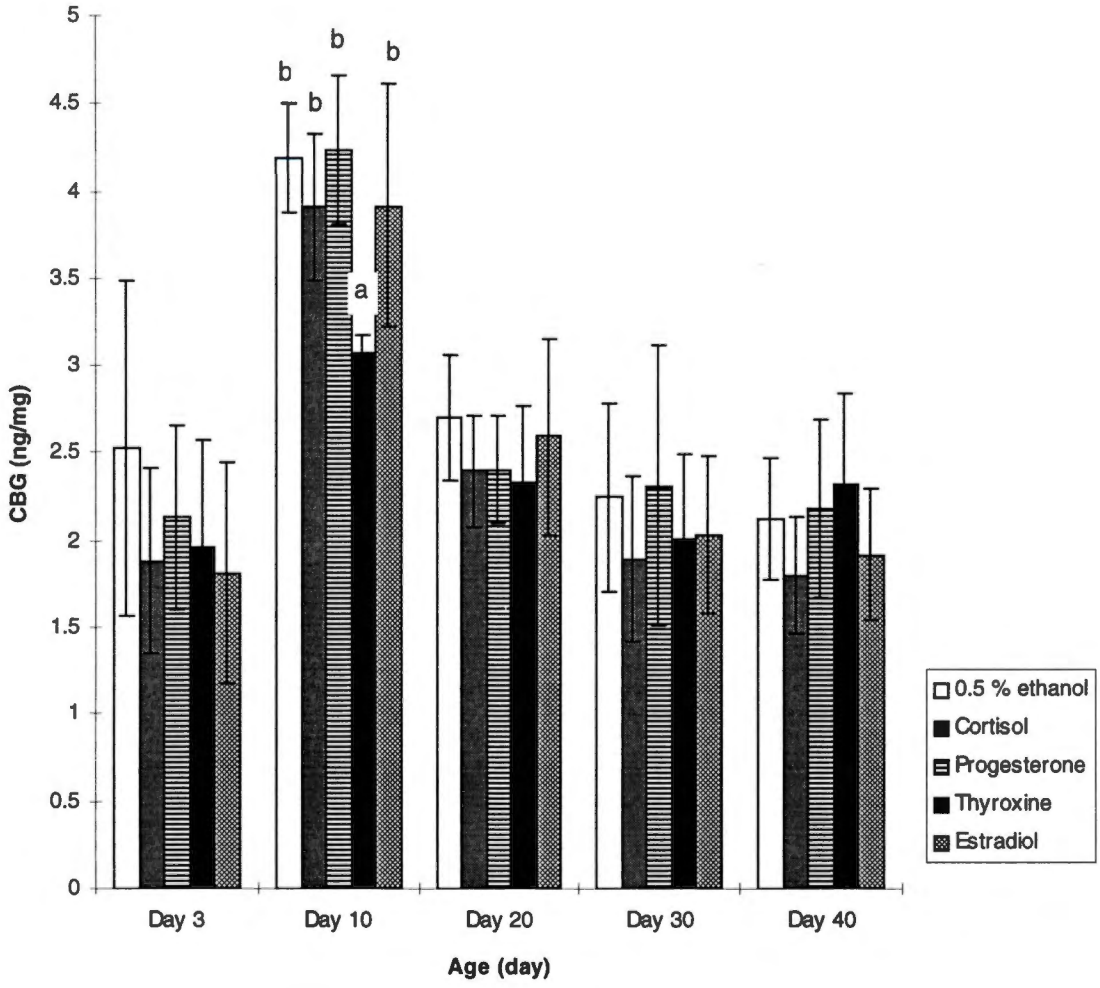

Figure 11. The effects of hormone treatment on the release of CBG from liver slices following 12 hours incubation. CBG concentration is expressed as nanogram of CBG per unit weight of liver slice (ng/mg). Each bar represents the mean ± SE (n=4). Columns with different letters are significantly ($P<0.01$) different.

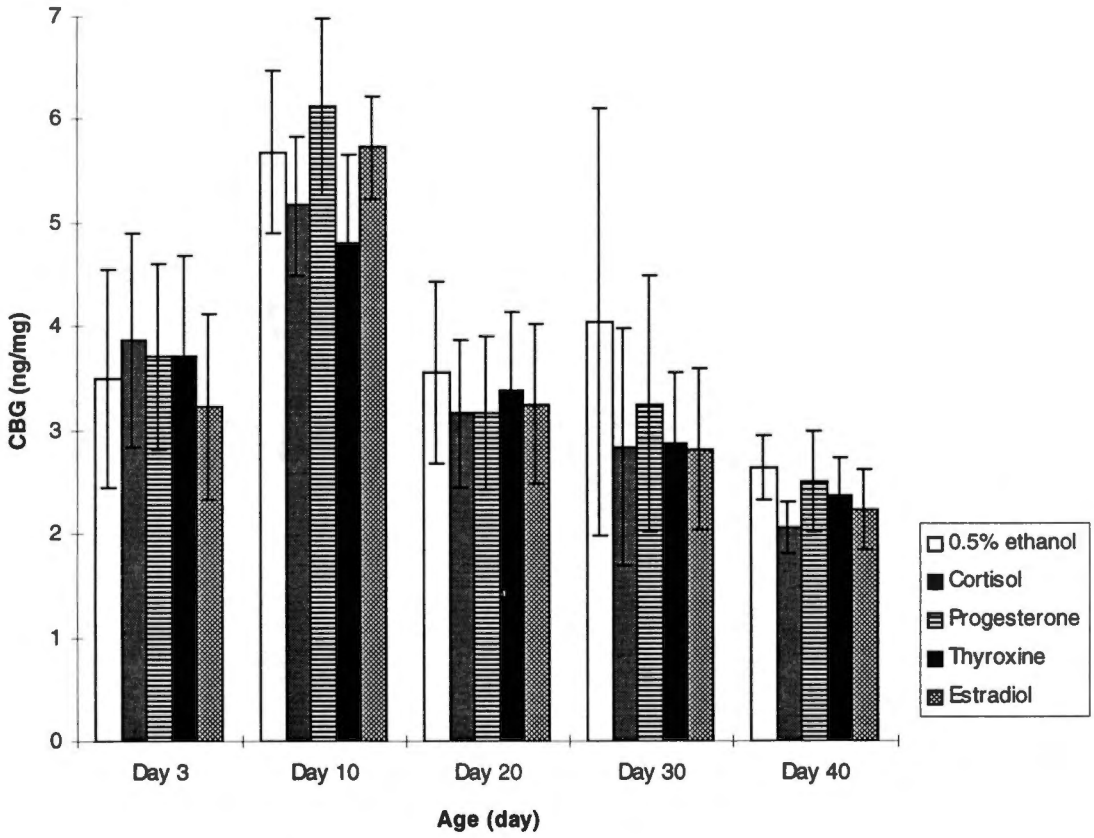

Figure 12. The effects of hormone treatment on the release from liver slices following 24 hours incubation. CBG concentration is expressed as nanogram of CBG per unit weight of liver slice (ng/mg). Each bar represents the mean ± SE (n=4).

CHAPTER V

DISCUSSION AND IMPLICATIONS

DISCUSSION

Cell death is classically evaluated by the quantification of plasma membrane damage. The quantification of plasma membrane damage is based on the measurement of cytoplasmic enzyme activity released by the damaged cell. Cytoplasmic enzyme leakage into the surrounding culture medium provided a useful tool in the evaluation of cytotoxicity (Dujovne et al., 1972; Acosta, et al., 1978). In essence, this index of cytotoxicity assesses the stability of the plasma membrane and its ability to retain important intracellular enzymes (Anuford, et al., 1978).

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme in all cells. It is rapidly released into the cell culture supernatant upon plasma membrane damage. The leakage of LDH from the cells into the reaction medium was used previously as an estimate of cellular damage (Medzihradsky and Marks, 1975; Anuford, et al., 1978; Mitchell et al., 1980). Medzihradsky and Marks (1975) showed that the pattern of LDH release into the medium was markedly similar to that of uptake of trypan blue and dead cell numbers. LDH leakage was used as an indicator of parenchymal cell integrity in preparing hepatic parenchymal cells from the livers of male

rats (Brown and Bidlack, 1988). They showed that LDH leakage from parenchymal cells increased with the decrease of trypan blue dye exclusion following a 2 hour incubation.

Sawyer et al.(1994) used LDH leakage to assess cell viability when examining the in vitro toxicity of ethanol using the precision-cut rat liver slice. They showed that exposure of liver slices to 0.5% ethanol had no effect on cell viability regardless of the duration of exposure. Liver slices exposed to 8% ethanol for 12 hours caused 35% loss of cell viability. Results of the present study indicated approximately 20 ~ 55% cell death following 12 hours of incubation. The percentage of cell death on days 3, 10, and 20 were 20% higher than that on days 30 and 40. This high rate of cell death during incubation may be due to variables such as developmental maturity of liver or damage during the preparation of liver slices. Also, there were no significant differences in total protein or CBG in medium containing 0.5% ethanol compared to medium alone.

The total protein released from liver slices during incubation was measured at each age to examine whether the high cell death rate, incubation time or animal age affects the protein synthesis of liver slices. The amount of total protein released from liver slices showed little difference between 12 and 24 hours incubation, regardless of age. The percentage of cell death on days 30 and 40 significantly decreased, but the amount of total protein released from

liver slices remained unchanged. The release of total protein from liver slices may not be influenced by incubation time or animal age. However, the difference may be masked by the large release of total protein due to reduced cell viability, which is at least 20 % on day 30.

In several species it has been shown that serum or plasma levels of CBG change two to four weeks after birth (Henning, 1978; Dalle et al., 1980; D'agostino and Henning, 1981; Kattesh and Roberts, 1993). In the rat, plasma CBG levels are very low during the first 9 days after birth and significantly increase between days 9 and 12 until a plateau is reached on day 24 (Henning, 1978). In the female guinea pig, CBG binding capacity for cortisol is high at birth, significantly decreases by day 10 and then increases on day 20 (Dalle et al., 1980). Kattesh and Roberts (1993) documented the changes in plasma CBG concentrations in the pig measured from 3 to 126 days of age. The plasma CBG concentration decreased from day 3 to day 7, but significantly increased between 21 and 28 days of age. The present study showed that plasma CBG levels were unchanged by day 10, but increased 19% on day 20 and 33% on day 30 compared to day 10 values.

Liver has been identified as the major site of CBG biosynthesis in the guinea pig (Perrot-Applanat et al., 1981), human (Khan et al., 1984), rat (Smith and Hammond, 1989), rabbit (Seralini et al., 1990), hamster (Lin et al.,

1990), fetal sheep (Jacobs et al., 1991), and mouse (Scrocchi et al., 1993). Plasam CBG is primarily produced by liver (Smith and Hammond, 1991; 1992).

The present study used the liver slice model because a liver slice retains the normal microarchitecture so that cell-cell communication is not disrupted (Smith et al., 1985) and remains viable for 48 hours in culture using LDH leakage and tetrazolium compound reductive capacity as indicator of cell viability (Sawyer et al., 1994). Results of the present study showed that the amount of CBG released from pig liver slices during 12 hours incubation decreased on day 30 in spite of a decrease in the rate of cell death, while plasma CBG levels began to increase on day 20.

The liver slice model has been used in the rat to examine the synthesis and secretion of CBG (Weiser et al., 1979) and the hormonal regulation of hepatic CBG production (Feldman et al., 1979; D'agostino and Henning, 1982). CBG production by liver slices increased linearly from 309 ± 32 fmol/mg of protein at 0 minute to 579 ± 86 fmol/mg protein after 240 minutes in a time-dependent manner (Weiser et al., 1979).

Generally liver consists of 60-65% parenchyma cells, which are essentially hepatocytes, and 40% sinusoidal cells, which consist of 44% endothelial, 33% Kupffer, 22% lipocytes, and 1% pit cells (Berry et al., 1991; Phillips et al., 1987). In the rat this percentage changes slightly with

increasing liver weight and age from about 30% sinusoidal cells at 200 mg of body weight to about 38% sinusoidal cells at 440 mg of body weight (Aterman,1963).

In studies examining the localization of CBG biosynthesis in the guinea pig liver, CBG was detected in the cytoplasm of hepatocytes and was not detected in Kupffer or bile duct cells (Perrot-Applanat et al., 1981). A parencymatous-enriched cell population expressed CBG mRNA while the homogenous hematopoietic cell population failed to accumulate CBG mRNA (Elfahime et al., 1992). CBG secretion by hepatocytes in culture has also been demonstrated (Khan et al., 1984; Rosner et al., 1984).

Smith et al (1991) demonstrated that although adult rat CBG mRNA levels were attained by 3 weeks of age, serum CBG concentrations did not reach adult values for an additional 3 weeks, and that the half-life of CBG in 3 week old infants (6.9 hours) was consistently less than that in adults (14.5 hours). Therefore, the age-related differences in the clearance of CBG may be responsible for age-related difference in secretary profiles of CBG from liver slices and subsequent plasma CBG concentrations.

A positive relationship between liver weight and body weight was reported in three different breeds of pigs by Herpin et al. (1993). They showed that at birth, the absolute weight of the liver in a Chinese breed of pig (23.6 g, liver weight; 814 g, body weight) was lower than that in

a European breed (38.4 g, liver weight; 1,289 g, body weight) as well as a composite line highly selected for lean tissue growth (34.3 g, liver weight; 1,451 g, body weight). From the present data, although the release of CBG per unit weight of liver tissue decreased, the amount of CBG released from the entire liver may be increased by the increase of liver weight associated with increasing body weight. This may have contributed to the increase in plasma CBG levels.

Elfahime et al (1992) examined the difference between plasma CBG levels and hepatic CBG mRNA contents in neonatal rats. They showed that hepatic CBG mRNA levels and plasma CBG concentrations increased in parallel from postnatal day 10 to day 15, but hepatic CBG mRNA levels decreased on day 20 while plasma CBG levels continued to increase. They suggested that difference between plasma CBG levels and hepatic CBG mRNA content may be due to an increase in CBG half-life in circulation, which was about 3 days. This observation was similar to the result of the present study in that plasma CBG levels increased on day 30 while CBG release from liver slices decreased on day 20.

Therefore, an increase in CBG half-life (Smith et al., 1991), an increase in liver weight (Herpin et al., 1993) and a decrease in hepatic CBG mRNA levels (Elfahime et al., 1992) associated with increasing age may be responsible for the age-related difference in the profiles of the release of CBG per unit weight of liver tissue and plasma CBG

concentrations in the present study. Furthermore, although the amount of CBG released from liver slices following 12 hours incubation increased on day 20, the amount of total protein remained unchanged over the ages studied. This suggests that regulation in CBG release by liver may be working independent from that in the release of other proteins.

The hormonal regulation of the ontogenic rise in serum CBG in the rat was investigated by D'agostino and Henning (1981). They examined serum CBG binding capacity on day 8 after daily injections of estrogen or thyroxine on postnatal days 2 through 7 and suggested that the developmental rise in the binding capacity of CBG was independent of estradiol and estrone and instead was elicited by the rising concentrations of circulating thyroxine that normally occurs in the early postnatal period. To examine the temporal characteristics of the CBG response to thyroxine, D'agostino and Henning(1982) gave n-propylthiouracil (PTU) -induced hypothyroid rats a single injection of thyroxine (0.1 ug/g body weight) on postnatal days 5, 6, or 7. They demonstrated that only animals treated on day 7 showed a significant increase in serum CBG levels and suggested that the response of CBG to thyroxine was a function of the age of the animal.

Research by Feldman et al. (1979) examined hormonal regulation of hepatic CBG production using liver slices from adult male rats. They showed that glucocorticoid (25 ug/100g

body weight) administration for 2 days reduced the rate of CBG production and secretion and estradiol (15 ug/100g body weight) stimulated the rate of CBG production. Their experiment suggested that there is multihormonal regulation of the rate of CBG synthesis by liver and these hormonal treatments alter circulating CBG levels. Smith and Hammond (1992) examined hepatic CBG mRNA levels in adult male rats following twice daily administration of steroids or thyroxine for 3 days. They showed that dexamethasone (25 ug/100 g body weight) treatment decreased hepatic CBG mRNA synthesis and thyroxine (10 ug/100 g body weight)increased CBG mRNA stability, while estradiol (25 ug/100 g body weight), corticosterone (25 ug/100 g body weight), and progesterone (25 ug/100 g body weight) did not affect hepatic CBG mRNA levels.

All of above results came by studies examining the effects of in vivo hormone administration. In an in vitro experiment, a human hepatic cell line exhibited increased secretion of testosterone-estradiol-binding globulin (TeBG) but not CBG after 48 hours in culture with physiological concentrations (5×10^{-6} M) of thyroxine (Rosner et al., 1984). Estradiol (5×10^{-6} M) treatment did not influence the levels of TeBG or CBG . They concluded that the effect of thyroxine is specific and the known effects of estradiol in increasing plasma CBG are either indirect or function by retarding the clearance of CBG from plasma. Likewise, the

present data showed that there were no apparent effects of in vitro hormone treatment on the release of CBG by liver slices in the pig. These results suggest that the hepatic secretion of CBG in the young pig is not influenced by hormones of the kinds and amounts used in this study.

IMPLICATIONS

This study was conducted to investigate the relationship between age and hormonal regulation of in vitro hepatic secretion of corticosteroid-binding globulin in the young pig. Plasma corticosteroid-binding globulin (CBG) level was low by day 10 after birth and increased on day 30. However, the release of CBG per unit weight of liver tissue was high on day 10 and decreased on day 20. Although the release of CBG per unit weight of liver tissue decreased on day 20, the increase of plasma CBG on day 30 may be due to the total increase of CBG released by the total liver and/or an increase of CBG half-life with age. The amount of total protein released per unit weight of liver tissue was not changed with age. Therefore, the secretion of CBG by liver tissue may be regulated in a different manner from that of other proteins. The absence of a hormone treatment effect on the release of CBG from liver tissue suggests that regulation of CBG release from the liver may be related primarily to age in the young pig.

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APPENDICES

APPENDIX A

PROCEDURE FOR CELL VIABILITY ASSAY

I. Preparation of reagents

A. 0.4 N NaOH solution

1.6 g NaOH anhydrous, 100 ml CO_2-free water.

1. Seal with paraffin film and mix gently.
2. Store at 4°C.

B. Pyruvate/NADH substrate (Sigma Chemical Co., MO)

1 ml Pyruvate substrate , 1 vial NADH

1. Incubate for a few minutes in 37°C deionized water bath just before use.

II. Preparation of samples

A. 0.2 g of sliced liver was incubated in 5 ml Williams' Medium E or 5 ml Williams' Medium E containing 0.5% ethanol.

B. The sample was incubated in a humidified 5% CO_2, 5% O_2, and 90% N_2 at 37°C for 12 or 24 hours.

C. 1 ml of the culture medium was taken off and stored at -70°C for LDH activity assay.

D. The remaining medium containing liver slice was homogenized with glass homogenizer and centrifuged

for 10 minutes at 3,000x g with Beckman J2-HS centrifuge (Beckman Instruments Inc., CA).

E. The supernatant was stored at -70°C for LDH activity assay.

III. Measurement of LDH activity

A. Dilute 1 part of sample with 4 parts of Williams' Medium E.

B. Put 10 ul of the sample in a microcentrifuge tube.

C. Add 100 ul of pyruvate/NADH substrate and gently mix.

D. Incubate for exactly 30 minutes in 37°C water bath.

E. Add 100 ul of Color Reagent (Sigma Chemical Co., MO) and mix well by swirling.

F. Allow to remain at room temperature for 20 minutes.

G. Add 1 ml of 0.4 N NaOH solution, cap and mix well by inversion

H. Wait at least 5 minutes after adding 0.4 N NaOH solution and transfer to cuvet.

I. Read an absorbance[A] at 442 nm on a Beckman DU 640 spectrophotometer (Beckman Instruments Inc., CA).

IV. Calibration curve for LDH assay

A. Label 6 test tubes and prepare mixture at below.

Tube Number	Pyruvate substrate	Deionized Water	LDH activity (B-B unit/ml)
1	100 ul	10 ul	0
2	80 ul	30 ul	28
3	60 ul	50 ul	64
4	40 ul	70 ul	104
5	20 ul	90 ul	153
6	10 ul	100 ul	200

B. Add 100 ul of Color Reagent.

C. Mix gently and let sit at room temperature for 20 minutes.

D. Add 1 ml of 0.4 N NaOH solution.

E. Wait at least 5 minutes after adding 0.4 N NaOH solution and transfer to cuvet.

F. Read the absorbance[A] on a Beckman DU 640 spectrophotometer.

G. Select a wavelength which will result in an absorbance of approximately 0.9 for tube 1, using water as reference.

H. Plot the absorbance[A] vs the corresponding LDH unit.

V. Calculation

A. Determine LDH activity of sample from calibration curve.

B. LDH activity of each sample is recalculated to Unit/mg of liver tissue.

C. Cell death rate is expressed as the percentage of the activity of LDH released from liver slices into medium compared to liver slices and medium activity combined.

APPENDIX B

PROCEDURE FOR PROTEIN QUANTIFICATION

I. Preparation of reagents

A. Dilution buffer

1.211 g Tris

1. Bring to 1,000 ml with deionized water
2. Adjust pH to 7.6
3. Store at 4°C

B. Bovine serum albumin (BSA) stock solution

1. Mix 40 ul of 2 mg/ml Pierce BSA (Pierce Chemical Co., KC) with 360 ml Dilution buffer.

C. Standard solution

1. Standard concentrations are:

Standard concentration	BSA stock (200 ug/ml)	Diluent buffer
25 ug/ml	100 ul	700 ul
20 ug/ml	80 ul	720 ul
15 ug/ml	60 ul	740 ul
10 ug/ml	40 ul	760 ul
5 ug/ml	20 ul	780 ul
1 ug/ml	4 ul	796 ul
0 ug/ml	0 ul	800 ul

II. Procedures

A. Dilute 1 part of sample with 39 parts of Diluent buffer.

B. Mix Coomassie Blue G-250 Reagent with standards, samples, or reference serum dilution (1/160) in a 1:1 ratio (vloume:volume) to a final volume of 640 ul in 12 x 75 mm glass tubes

C. Vortex immediately after adding reagent.

D. Load 200 ul of each mixture into the 96 well plate (Corning, NY).

E. Read the plate at 595 nm on a BioTek Automated Microplate Reader (Winooski, VT) between 5 and 60 minutes after adding reagent.

F. Calculate the results using Kineticalc software.

G. The amount of protein is expressed as microgram of protein per milligram of liver tissue.

APPENDIX C

PROCEDURE FOR ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

I. Preparation of Reagents

A. 10x Phosphate buffered saline (PBS) solution

80 g NaCl, 12.07 g Na_2HPO_4, 20 g KH_2PO_4, 2.0 g KCl, 2.0 g Sodium azide

1. Bring to near 1,000 ml with deionized water.
2. Adjust pH to 6.8.
3. Bring to 1,000 ml with deionized water.
4. Store at 4°C.

B. 10% Bovine serum albumin (BSA) solution

25 g bovine serum albumin (Fisher Biotech, NJ), 25 ml 10x PBS

1. Bring to 250 ml with deionized water.
2. Stir until dissolved at 4°C.
3. Sterilize with 0.2 nm filter.
4. Store at 4°C.

C. 10x Tris-buffered saline (TBS) solution

350.64 g NaCl, 96.88 g Tris, 8.0 g Sodium azide

1. Bring to near 4,000 ml with deionized water.
2. Adjust pH to 7.4.
3. Bring to 4,000 ml with deionized water.

4. Store at room temperature.

D. 10% Tween 20

50 ml Tween 20

1. Bring to 500 ml with deionized water.
2. Thoroughly rinse tween container to wash out the total 50 ml
3. Store at 4°C.

E. Glassware treatment solution

10 ml 10% BSA, 90 ml 10x PBS

1. Bring to 1,000 ml with deionized water.
2. Store at 4°C.

F. Wash buffer

400 ml 10x TBS, 20 ml 10% Tween 20

1. Bring to 4,000 ml with deionized water.
2. Store at room temperature.

G. 1x Diluent solution

100 ml 10% BSA, 90 ml 10x PBS, 5 ml 10% Tween 20, 5 mg Bromophenol blue

1. Bring to 1,000 ml with deionized water.
2. Store at 4°C.

H. 2x Diluent solution

200 ml 10% BSA, 180 ml 10x PBS, 10 ml 10% Tween 20, 300 ml 0.2 N Heps

1. Bring to 1,000 ml with deionized water.
2. Adjust pH to 7.4.
3. Store at 4°C.

I. Tris coating buffer solution

2.4422 g Tris, 5.84 g NaCl, 0.2 g Sodium azide

1. Bring to 1,000 ml with deionized water.
2. Adjust pH to 8.5
3. Store at 4°C.

J. 5% Na_2EDTA solution

25 g Na_2EDTA

1. Bring to 500 ml with deionized water.
2. Store at 4°C.

K. Standard solution.

1. For standards porcine CBG from pig serum pool was isolated and purified by affinity chromatography and HPLC-DEAE anion exchange technique and adjusted to 12.152 ng/ul by dilution with 1x PBS.
2. Standard stock solution was prepared in bulk and aliquoted with Williams' Medium E.

3. Standard concentrations are:

ng/well	ng/ul	pCBG stock (ul)	Williams' Medium (ul)
160	3.2	2,632.88	7,367.17
50	1	822.77	9,177.23
20	0.4	329.11	9,670.89
8	0.16	131.64	9,868.36
2.5	0.05	41.14	9,958.86
1.0	0.02	16.46	9,983.54
0.3	0.006	4.94	9,995.06

L. CBG antibody stock solution

1. Rabbit anti-pCBG was from a rabbit injected with CBG purified from porcine plasma.
2. A 1/1,000 dilution of antiserum was prepared with 1x Diluent solution.
3. Store in 250 ul aliquots in screw cap vial and frozen at -70°C.

M. CBG coating solution for 50 plates.

8,227.74 ul pCBG stock (12.154 ng/ul) solution,
491,772.26 ul Tris coating buffer.

1. Prepare immediately before use.

N. Substrate solution

1. For every 5 mls of substrate, mix 1 ml diethanolamine buffer(5x), 4 ml deionized water, and 1 tablet of p-nitrophenyl-phosphate

(Kirkegard & Perry Lab., MD).

2. Agitate gently to dissolve the tablet
3. Prepare the buffer just before use.

II. Procedure

A. Coat plate (Corning 96 well ELISA plate, NY) with purified porcine CBG (20 ng/well) by incubating at 4°C for 48 hours.

B. Concurrently, in a separate previously blocked plate, incubate samples (40 ul/well), reference porcine serum (5 ul/well), 2x Diluent control (50 ul/well) or standard porcine CBG (0.3 to 160 ng/well) with rabbit anti-pCBG serum at final dilution of 1/500,000 at 4°C for 48 hours.

C. Wash the pCBG coated plate with wash buffer using a Dynatech Ultrawash II Microplate Washer (Chantilly, VA),8-4-1-4 cycle and tap dry on paper towels.

D. Transfer the sample-antibody mixture (100 ul/well) to the corresponding wells of the coated plate and incubate in a humidified chamber at 30°C for 2 hours.

E. Wash the plate using 8-4-1-4 cycle on the plate washer and tap day.

F. Add 100 ul/well of anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co., MO) in a

1/12,000 dilution with 1x Diluent and incubate in a humidified chamber at 30°C for 2 hours.

G. Wash the plate using 8-4-1-4 cycle on the plate washer and tap day.

H. Add the phosphate substrate (100 ul/well) and incubate in a humidified chamber at 30°C for 1 hours.

I. Stop the reaction with an equal volume of 5% Na_2EDTA solution.

J. Read the plate at 405 nm on a BioTek Automated Microplate Reader (Winooski, VT).

K. Calculate the amount of CBG in the sample using a four parameter fit, kineticalc software (Bio-Tek Instruments, Inc., VT).

VITA

Jeonghoon Heo was born in Cheju city, Korea on August 19, 1964. In March of 1983 He entered the Kyung-Hee University and in February of 1987 received the degree of Bachelor of Science in Biology. The following March he entered the Kyung-Hee University and in February of 1989 received a Master of Science degree in Zoology. In August of 1994 he was enrolled in graduate school at the University of Tennessee, Knoxville in the Department of Animal Science and in December of 1996 received a Master of Science degree in Animal Science.