BIOCHEMICAL STUDIES ON THE MODULATORS OF GLUCOCORTICOID RECEPTORS IN MICE

ABSTRACT

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

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ABSTRACT

Glucocorticoids are synthesized and secreted into the circulation by the zona fasciculata cells of the adrenal gland that play a key role in regulating a wide variety of biological responses in animals including human. Virtually all tissues in the body are target organs for glucocorticoids and can respond in one way or another (Borski 2000). In spite of the broad spectrum of biological effects induced by glucocorticoids, there is a set mechanism whereby target cells can respond to the hormonal signal, through the mediation of a cytoplasmic receptor protein called glucocorticoid receptor (GR) in most target cells (Carlstedt-Duke 1999). Binding of hormone to GR leads to generation of activated hormone-receptor complex, which then undergoes translocation into the nucleus. In the nucleus this complex binds to specific DNA sequence, termed as glucocorticoid response element (GRE) in the chromatin and modulates transcription of a gene, leading to changes in cellular physiology (Chen et al 2000). Modulation of GR by a number of factors is important in the control of signal transduction as it is now widely believed that GC action on target cells is regulated not only by the level of receptors, but also by endogenous modulators. Each of the steps in the steroid-receptor action mechanism, that is, steroid binding, activation, DNA binding and transcriptional regulations of specific genes, may be controlled by the receptor interactions with other proteins and modulators (Pratt and Toft 1997).

Now it is known that there is a single gene for GR in all vertebrates. Thus, there must be other mechanisms that modulate the action of GR to enable the myriad roles of glucocorticoid actions in animals, including humans. A number of reports indicate the presence of tissue-specific factors/proteins/enzymes that may specify and/or modulate the cellular role of GCs through GR (Bamberger et al 1996). These factors may act at a particular or distinct step of steroid action. A number of such factors such as pyridoxal 5-phosphate, free fatty acids, biotin, melatonin, parathymosin etc have been attributed under physiological conditions to regulate GR action. Apart from these, a number of signal molecules and tissue-specific transcriptional co-activators/co-repressors have also been demonstrated to regulate the GR mediated gene expression in target cells (Tronche et al 1998). Hence, GR action can be modulated by a number of factors, which may alter its tissue responsiveness. In view of the diverse role of GR and the susceptibility of its regulation by various agents, it was decided to study the modulatory effects of some endogenous and exogenous agents on hormone binding to receptor and its stabilization, activation, DNA binding and also the effects of diabetic state and senescence onto GR. Findings of the work done have been summarized below under different sections:

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Steroid binding to GR and stabilization of steroid-bound receptor

Dithiothreitol (DTT) significantly increased (maximally at 4-8 hr) hormone binding to hepatic GR compared to control. However, DTT did not show any significant increase in stabilization of hepatic hormone-bound GR. Mercaptoethanol and glutathione were ineffective in enhancing the steroid binding to hepatic GR compared to control, with no influence on hormone-bound receptor stabilization. Furthermore, none of these reducing agents could show any differences in steroid binding to GR and the stabilization of hormone-bound receptor in an age- and tissue-specific manner.

Activation modulation of GR

Both heat (25°C) as well as salt (20 mM Ca^{2+}) were able to activate the GR from the liver and kidney of mice. Interestingly, the activation of GR by heat is more pronounced in mature (120-day) animal's liver as compared to immature (15-day), without any such changes in the case of kidneys. This difference in heat activation of immature and mature hepatic GR was attributed to alterations in the receptor property as evident from cross-mixing experiments. Various exogenous and endogenous agents used to modulate the activation of receptor did modulate the activation process. Cadmium, selenite and arsenite were found to be inhibitors of heat activation of GR in liver and kidney. The potency of these modulators was cadmium > selenite > arsenite. Additionally, leupeptin and PUFAs were also found to be potent inhibitors of heat activation of GR. Among the PUFAs, both linoleic and arachidonic acid had greater potency (~70% at 160 μM) in inhibiting GR heat activation compared to oleic acid (38% at 40 μM). Interestingly, pyrophosphate (PPi), unlike other modulators, was found to significantly induce (~65%) activation of GR at 0°C from both liver and kidney. However, the magnitude of activation modulation by these modulators remains the same at the two ages studied, indicating that the mechanism(s) of activation modulation does not get altered during these ages of mice.

Acceptor binding modulation of GR

Various modulators of activated receptor binding to DNA inhibited the binding of GR to DNA. Pyridoxal phosphate (PLP) was found to be a potent inhibitor of activated receptor binding to DNA. Besides PLP as a physiological inhibitor of activated receptor binding to DNA, aurintricarboxylic acid (ATA), a synthetic triphenylmethane dye, and methyl methanethiosulfonate (MMTS) were also found to be effective inhibitors of GR binding to DNA. Among these, ATA exhibits strong inhibition followed by PLP and MMTS as evident from their IC_{50} values. These modulators do not yield any age- and tissue- specificity in inhibiting the activated receptor binding to DNA-cellulose and nuclear DNA.

Diabetes and GR modulation

STZ-induced diabetic mice exhibited a similar level of GR in the liver and kidney of immature (15-) and mature (120-day) animal as compared to control, without any change in the affinity (K_d) for the hormone. This shows that STZ-induced diabetes have no effect on modulating the level of GR and the affinity for the hormone in either liver or kidney at these two ages studied. However, STZ-induced diabetes decreased the heat activation of hepatic GR from diabetic animals in both the ages studied, with no such decrease in the kidney, thereby indicating tissue- specificity. Such decrease in activation of hepatic GR in diabetic mice is attributed to receptor specificity as judged by cross-mixing experiments. These observations indicate that the reduced hepatic GR activation during STZ-induced diabetes might play an important role in controlling glucose homeostasis in diabetic animals.

Aging and GR modulation

Aging and GR modulation studies have indicated changes in GR concentration, heat activation, activation modulation by PUFAs and chromatin organization during old age of mice. The level of GR is significantly reduced in the liver and kidney of older (120-week) mice as compared to young (4-week) ones, however, with no change in the affinity (K_d) for the hormone. Also, the magnitude of heat activation of GR was more pronounced in the liver and kidney of young mice than those from older ones. Polyunsaturated fatty acids (PUFAs), linoleic and arachidonic acid showed variable impact on activation inhibition of GR in an age-specific manner. Linoleic acid caused greater inhibition of GR heat activation in the liver and kidney of young mice as compared to old ones. Whereas, arachidonic acid exhibited greater inhibition of GR activation only in the liver of young mice as compared to old. In contrast, the inhibition of renal GR heat activation by arachidonic acid was age-independent. DNase I digestion of hepatic and renal nuclei from young and aged mice revealed significant higher digestion extraction of bound GR complexes from young animal tissues compared to old ones. These findings indicate more compact nuclear chromatin organization in old mice's tissues. Such alterations may contribute towards functional changes in glucocorticoid action and responsiveness in target tissues of senescent animals.

In conclusion, the findings summarized in this thesis indicate glucocorticoid receptor modulation by various endogenous/exogenous modulators and also by diabetic state and old age in mice. Such modulation of GR may in turn be responsible for tissue's responsiveness towards glucocorticoids during animal's health and diseases.

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Biochemical studies on the modulators of glucocorticoid receptors in mice

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Department of Biochemistry

Submitted in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Biochemistry

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This is being submitted to the **North Eastern Hill University** for the degree of **Doctor of Philosophy** in **Biochemistry**.

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ABBREVIATIONS

ATA	: Aurintricarboxylic acid
ADP	: Adenosine 5'-diphosphate
ATP	: Adenosine 5'-triphosphate
BSA	: Bovine serum albumin
CPM	: Counts per minute
Ci	: Curie
Cys	: Cysteine
DMSO	: Dimethylsulfoxide
DNA	: Deoxyribonucleic acid
DNA-cellulose	: Deoxyribonucleic acid Cellulose
DNase I	: Deoxyribonuclease I
DTT	: DL-Dithiothreitol
DBD	: DNA binding domain
EDTA	: Ethylenediaminetetraacetic acid
fmol	: Femto mol
g	: Centrifugal field
GSH	: Glutathione
GC	: Glucocorticoid
GR	: Glucocorticoid receptor
GRE	: Glucocorticoid responsive element
GRU	: Glucocorticoid responsive unit
GME	: Glucocorticoid modulatory element
HBD	: Hormone binding domain
Hsp	: Heat shock protein
kDa	: Kilo Dalton
mM	: Millimolar
mg	: Milligram
Met	: Methionine
MMTS	: Methyl methanethiosulfonate
nM	: Nano molar
PUFA	: Polyunsaturated fatty acid
PLP	: Pyridoxal 5-phosphate
RNA	: Ribonucleic acid
STZ	: Streptozotocin
Tris	: Tris(hydroxymethyl)-amino methane
v/v	: Volume/volume
w/v	: Weight/volume

INTRODUCTION

In multicellular organisms, elaborate signaling mechanisms enable their cells to communicate with one another so as to coordinate their behavior for the benefit of the organism as a whole. Each cell in a multicellular animal is programmed during development to respond to a specific set of signals that act in various combinations to regulate the behavior of the cell and to determine whether the cell lives or dies and whether it proliferates or stays quiscent. These signals or signaling molecules include proteins, peptides, steroids, amino acids and their derivatives, fatty acids, retinoids etc.

The term hormone refers to a Greek verb horman, meaning "to stir up or excite". They are chemical messengers released from specialized group of cells to elicit response by interacting with cognate receptors in target cells. A class of hormones, called endocrine hormones, that arise in one tissue or gland and travel a considerable distance through circulation to reach a target cell having cognate receptors. Paracrine hormones on the other hand arise from a cell and travel a relatively small distance to interact with their cognate receptors on neighboring cell. Autocrine hormones, alternatively, are produced by the same cell that functions as the target cell for those hormones (Hardie 1990; Norman and Litwack 1997).

Regardless of the nature of hormone, the target cell responds by means of a specific protein called **receptor** (Levitski 1984). Receptor specifically binds the signaling molecule and then initiates a response in the target cell. The specific way by which a cell reacts to its environment varies according to the set of receptors that a cell possesses through which it is tuned to detect a particular subset of the available signal and also according to the intracellular machinery by which the cell integrates and interprets the information that it receives. Thus a single signaling molecule often has different effects on different target cells.

For any given hormone, an incredible array of biological responses can be modulated depending upon the phenotype of the target cell that possesses the cognate receptor. In any given target cell, only a small subset of genes will have their chromatin in an active or "open" configuration. Thus, while a hormone may modulate as many as 300 genes in a given organism, in a specific target cell perhaps only a few genes will be available for regulation (Bamberger et al 1996; Norman and Litwack 1997). Furthermore, each cell is bombarded with chemical signals that regulate diverse physiological responses. In general, these signals bind to cell-surface receptors that activate several intracellular signaling pathways, so that the intracellular signals generated from different receptors will interact with one another in many complex ways. An important consequence of this cross-talk is that it will not be easily disrupted by removing or changing a single signaling element in one of these pathways. In most cases the receptors are transmembrane proteins on the target cell surface; when they bind an

extracellular signaling molecule, they become activated so as to generate a cascade of intracellular signal that alter the behavior of the cell (Kahn 1976). Additionally, in some cases, receptors are inside the target cell and the signaling ligand has to enter the cell to activate them.

Signaling through cell surface receptors

All water-soluble signaling molecules (including neurotransmitters, protein/peptide hormones, and growth factors) bind to specific receptors on the surface of the target cells. Most cell-surface receptors belong to one of the two classes; enzyme-linked cell-surface receptors and G-protein-coupled receptors (Nishizuka 1992; La Marco and Vivanco 1996).

Enzyme-linked cell-surface receptors

Enzyme-linked cell-surface receptors (ELCSRs) are transmembrane proteins with their ligand-binding domain on the outer surface of the plasma membrane (La Marco and Vivanco 1996). Their cytosolic domain has either an intrinsic enzyme activity or associate directly with an enzyme. One of the best understood cell-surface receptor with intrinsic enzyme activity is the receptor tyrosine kinases (RTKs). The RTKs are a family of more than 50 different transmembrane polypeptides with in-built tyrosine kinase activity domain towards cytoplasmic side. Binding of cognate hormone results in diverse cellular responses (Geer et al 1994). All the members of the RTK family share common structural and functional domains Majority of growth factor receptors are RTKs. These include the receptors for epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin, insulin-like growth factor (IGF-1) and nerve growth factor (NGF). The insulin receptor belongs to the RTK family, with extramembrane α subunits those bind to insulin and the membrane spanning β subunits having RTK towards cytoplasmic side of the subunits (Hubbard et al 1994). Insulin is an important hormone not only in regulating glucose metabolism but also it plays diverse role, influencing cellular growth and development in animals.

G-protein-coupled receptors

G-protein-coupled receptor (GPCR) were discovered in the 1970s by Rodbell and Gilman and since then many hormones have been shown to transduce signals through these receptors. GPCRs are the largest family of cell-surface receptors, with more than 100 members have already been defined in mammals. Cloning studies have revealed several hundred GPCRs that are structurally and functionally related, with all displaying the seven-transmembrane helical (heptahelical, serpentine) motif. GPCR and their endogenous hormones are involved in

regulating a number of important physiological phenomena in almost every major tissues and organs.

Signaling through Intracellular receptors

Steroid hormones and the related lipophilic molecules, retinoids, vitamin D_3 and thyroid hormones, diffuse through the cell membrane and bind to specific cytosolic (corticosteroids) and nuclear (gonadal steroids, Vitamin D_3 and thyroid hormones) receptors. The hormone-receptor complexes then modulate the expression of target genes in the chromatin thus regulating cellular responses. Additionally, a number of receptors ('orphan receptors') have been identified for which the specific ligand has not been identified as yet.

Orphan receptors

Orphan receptors (ORs), discovered in 1988, represent a class of putative, ever growing class of intracellular nuclear receptors, whose defining characteristic is the lack of identifiable physiological regulatory ligands (LaMarco and Vivanco 1996). ORs are found in every metazoan species and were identified by applying low stringency hybridization screening and genetic and molecular cloning techniques. Some ORs do interact with some novel ligands, while others may represent constitutive activators/repressors or factors whose activities are modulated by post translational modifications (Mangelsdorf et al 1995; Mangelsdorf and Evans 1995; Enmark and Gustaffson 1996). Many ORs have structural domains similar to that of members of steroid/nuclear receptor superfamily. Several ORs have been characterized in recent years, some of which have been suggested to control vital physiological and developmental processes.

Vitamin D receptors

The vitamin D metabolite, 1,25-dihydroxy vitamin D_3 (calcitriol), regulates cell growth and differentiation, immune function and myriad cellular activities associated with mineral metabolism. These actions of vitamin D3 are mediated by its cognate receptor in the nucleus called vitamin D receptor (VDR) (Pike and Sleator 1985). VDR belongs to the nuclear receptor superfamily. The ligand activated VDR can bind to DNA as homodimer or heterodimer (with RXR or RAR) in the direct repeat response element called vitamin D response element (VDRE), which may be a positive or negative regulator of cognate gene expression (Koszewski et al 2000).

Retinoid receptors

The retinoids [vitamin A (retinol) and its natural and synthetic derivatives] are essential for diverse biological processes, including, vision, reproduction, differentiation, metabolism, bone

development, hematopoiesis, and pattern formation during embryogenesis (Sporn et al 1984; Gudas et al 1994). The myriad roles of retinoids are mediated by retinoid receptors, which bind *all-trans* - and 9-cis-retinoic acid (RA). The retinoic acid receptor (RAR) (Petkovitch et al 1987; Giguere et al 1987) proteins belong to the steroid/nuclear receptor superfamily. Three different subtypes of RAR have been identified, RARα, RARβ and RARχ in mammals, birds and amphibians (Chambon 1994). Another nuclear receptor termed the retinoid X receptor (RXR), has been identified (Mangelsdorf et al 1990) which also exists in three different subtypes, RXRα, RXRβ and RXRχ. The RXR has a unique ability to form heterodimer with RARs, THRs, VDRs and an increasing number of ORs, indicating their potential physiological functions.

Thyroid hormone receptors

Thyroid hormones (T_3 and T_4) are known to be important modulators of developmental processes in humans and several other organisms. Thyroid hormones also play a crucial role in the development and maturation of the nervous system, however, very little is known about the role of this hormone in adult brain (Calza et al 1997). The physiological actions of thyroid hormones are carried out by specific nuclear localized receptors called thyroid hormone receptors (THRs). T_3 binds to THR with higher affinity (10 times higher) than T_4 and hence, is more biologically potent. The THRs have been isolated from brain, pituitary, lung, liver, pancreas and testes. THR belongs to the nuclear receptor superfamily and several isoforms of this receptor have been identified, THRα1, THRα2, and THRß1, and THRß2 (Glauser and Barakat 1997).

Steroid receptors

The steroids, glucocorticoid, mineralocorticoid, androgen, estrogen and progesterone act at the cellular level through the mediation of their respective cognate intracellular receptors. The glucocorticoid receptor (GR), androgen receptor (AR), estrogen receptor (ER) and progesterone receptor (PR) all belong to the nuclear receptor superfamily.

Androgens play critical role in several stages of development and maintenance of male phenotype (Brinkmann et al 1999). The biological action of androgens are mediated by the AR, a 110 kDa phosphoprotein, which upon ligand binding, leads to transcriptional activation of androgen responsive genes (McPhaul 1999).

Estrogens are the principle feminizing hormones involved in regulation of physiological functions, such as, secondary sexual development, maintenance of female phenotype and pregnancy. The wide range of action is mediated by the estrogen receptors (ERs). Two isoforms of ER are known, ERα and ERβ (Cowley and Parker 1999), with similar transcriptional activities. ER has been described to be present in a number of tissues, such as, ovary, prostrates, cerebral cortex and hippocampus.

Progesterone through the mediation of PR has principal targets on the uterus and ovary, where it has a central role in reproduction, being involved in ovulation, implantation and pregnancy. Associated with this is the involvement of progesterone in regulation of uterine function during the menstrual cycle. Other tissues showing responsiveness towards progesterone are breasts and brain.

Glucocorticoids exert most of their effects on target cell through the mediation of glucocorticoid receptor (GR). GR is the most thoroughly studied steroid receptor from the nuclear receptor superfamily. The detailed knowledge of GR structure and function including recent developments are given below:

Glucocorticoid receptors

The study of glucocorticoid receptor (GR) assumes great significance on account of the myriad physiological and biochemical role of the glucocorticoid (GC) hormones, influencing almost each and every tissues and organs. GR is a housekeeping protein and functionally acts as a transcriptional regulatory protein, and hence, most of the known actions of GCs ultimately culminate in regulating target gene expression in responsive tissues. Thus, changes in GR functions may automatically modulate the expression of GR responsive genes (Tronche et al 1998; Kellendonk et al 1999).

Tremendous pace of work on the GR-mediated gene expression during the last 10 years, reveal the ever-growing complexity of this process. It is now substantially clear that, the GR requires numerous other basal transcriptional factors to co-regulate gene expression (Bamberger et al 1996; McNally et al 2000). Also, some of the effect of GCs through GR on target genes may be through the process of cross-talk (Cella et al 1998). Recent advances have also suggested that for some responsive genes, the GRs need not interact physically with the DNA and that the GR binding to DNA is not essential for survival (Tronche et al 1998).

Glucocorticoids and physiology

GCs are synthesized and released into the circulation by the zona fasciculata of the adrenal cortical cells. Upon binding to its cognate receptor, they act on a variety of different target cells and regulate normal metabolic and other functions leading to homeostasis, differentiation and growth of animal tissues through modulation of gene expression. Increased circulating level of GCs lead to several pathological consequences, such as Cushing's syndrome in humans. Therefore, GC level is precisely controlled by an endocrine cascade, the hypothalamo-pituitary-adrenal axis (HPA) (Fink 1997). GCs control their production via feedback, exerted at the levels of hypothalamus and pituitary. GCs are an absolute necessity for the maintenance of homeostasis and their coordinate actions allow the body to respond to internal and external alterations.

In liver, GCs are primarily gluconeogenic, where they stimulate transcription of genes for enzymes like phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase and tyrosine aminotransferase (TAT). In kidney, they enhance reabsorption of Na^+ and increase K^+ and H^+ elimination. In adipocytes, they stimulate lipolysis by facilitating the action of epinephrine and norepinephrine and in peripheral tissues they inhibit glucose uptake. In cardiovascular system, they influence vascular permeability, maintain blood pressure and increase hemoglobin and number of RBCs. In the brain, GCs have been suggested to influence emotions and cognitive processes like learning and memory (Sapolsky 1996; Kellendonk et al 1999; Welberg and Seckl 2001). They also regulate sleep patterns and neuronal firing in different groups of animals. However, elevated GC levels appear to impair brain functions. On the immune system, they are involved in the supression of inflammation and immune responses, by inhibiting the action of lymphokines and acting as lymphotoxic (Barnes and Adcock 1993).

GCs are not only involved in controlling adult physiology, but also have been shown to influence critical developmental processes. Important roles are suggested, for example, lung maturation, chromaffin cell differentiation and erythroblast proliferation (Haagsman 1991; Wessely et al 1997).

The physiological effects of glucocorticoids are mediated by a receptor of approximately 94 kDa molecular mass, mainly localized in the cytoplasm of the target cells, called the glucocorticoid receptor (GR). GR is present in all tissues that are targets of glucocorticoid action. Tissues that lack functional receptors, or are receptor deficient, failed to respond to the normal circulating levels of hormones. The identification of intracellular receptors was made possible by the use of radiolabelled hormones by Jensen (1960) that could specifically bind to its cognate receptor. The intracellular localization of GR was uncertain before its discovery in the rat thymic cytosol (Munck 1961). It is now well established that they are predominantly present in the cytoplasmic compartment, however, there is a constant nucleo-cytoplasmic shuttling of the receptor even in hormone unbound form (Scherrer et al 1990). The GR phyllogenetically belongs to the steroid/nuclear receptor superfamily. This superfamily is the single largest class of eukaryotic transcription factors, and has been divided into three types. Type I represent the GR, MR, ER, AR, PR and THR localized in the cytoplasm or nucleus. Type II includes the VDR, RAR, and RXR, whereas the type III represents the so-called "orphan receptors", for which most of the ligands are not yet fully characterized. The GR is the most thoroughly studied receptor of this superfamily, and its structure, function and mode of action is best understood.

The 9S GR

The presence of unliganded GR as 9S receptor complexes was first reported in the rat hepatoma cell cytosol (Baxter and Tomkins 1971). This observation was rapidly confirmed for

GR in the cytosol of rat liver (Beato and Feigelson 1972), brain (Chytil and Toft 1972), and mammary gland (Gardner and Witliff 1973). Although, it was known that cytosolic GR existed in the 9S complex and that the temperature-transformed GR extracted from nuclei with salt was 4S, investigators did not focus on the dissociation of the 9S complex as the transforming event. Rather, they focussed on the other half of the coin, i.e., the acquisition of DNA/nuclear binding activity. The unliganded 9S GR is a part of a multiprotein complex that consists of a 94 kDa steroid binding protein, 2 molecules of Hsp90, one Hsp70 and one Hsp56 (Pratt 1993; Hutchison et al 1994; Prima et al 2000). In addition, other less-well characterized proteins have been found to be involved in this complex. In the absence of hormone, this oligomeric complex undergoes constant cycles of dissociation and ATP and Hsp70-dependent reassociation (Hu et al 1994).

The 9S GR is much less stable in cytosol preparations compared to the 9S forms of ER and PR. The instability of the 9S GR complex, however, turned out to be an advantage. The GR-Hsp90 complex is required to maintain the high affinity competence of the steroid-binding pocket. Dissociation of Hsp90 from the unliganded receptor results in the loss of cytosolic steroid binding activity (Bresnick et al 1989). It was the study of this instability of cytosolic glucocorticoid-binding activity that led to the discovery of agents that prevents the loss of steroid binding activity, most notably, molybdate and some other metal oxyanions (Nielsen et al 1977a,b). Molybdate has been widely used for the purification of untransformed GR and for identification of Hsp90 component of the 9S complex.

The GR isoforms

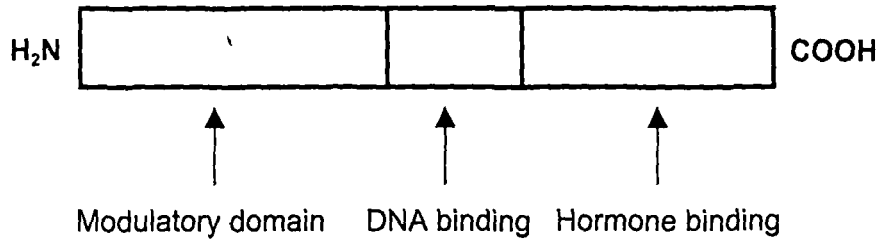
GR structure polymorphism has been extensively studied in human. The two isoforms of GR discovered were termed hGR alpha (hGRα) and hGR beta (hGRß), that are generated by alternative splicing of human GR pre-mRNA (Hollenberg et al 1985; Encio and Detera-Wadleigh 1991). The hGRα is the predominant form while the hGRß is a minor variant (Rivers et al 1999). These two protein isoforms have the first 727 amino acids in common and thus, contain both the transactivation and the DBD. The hGRß is the non-hormone binding splice variant that differs from the wild-type hGRα only at the carboxyl terminus. The carboxyl terminus of hGRß contan a unique 15 amino acid sequences with replacement of the last 50 amino acids of hGRa (Hollenberg et al 1985). This alteration renders hGRß unable to bind glucocorticoid hormone. The hGRß mRNA and protein were shown to be physiologically relevant (Bamberger et al 1995; Castro et al 1996). Indeed, in human multiple cell types, hGRß was found to inhibit the hGRα-mediated activation of the MMTV promoter (Oakley et al 1999). In the absence of glucocorticoids, hGRß binds to the GRE containing DNA with a greater affinity than hGRα. Also glucocorticoid treatment was found to increase hGRα but not hGRß binding to DNA. It was also demonstrated that hGRα and hGRß can physically associate with

each other in a heterodimer. It is now clear that the dominant negative activity of hGRß resides within its unique 15 amino acid carboxyl-terminal sequence.

Another novel variant of human GR has been recently reported through analysis of cDNA from different tissues. This variant, termed hGR gamma (hGRχ), which is generated as a result of alternative splicing where three bases are retained from the intron separating exons 3 & 4 (Rivers et al 1999). These three bases code for an additional amino acid (arginine) in the DBD of the receptor. This novel isoform of hGR have been found to be expressed at a relatively high level (4-9% of total GR) in different tissues. Otto et al (1997) reported the absence of hGRß in mice using reverse transcriptase PCR. However, the role of hGRß and hGRχ in the mediation of glucocorticoid actions remains uncertain.

Modular structure of GR

Molecular cloning and structure/function analyses have revealed that the members of the steroid/nuclear receptor superfamily have a characteristic common functional domain structure. GR was the first member of the superfamily whose characteristic three-dimensional model was described (Bamberger et al 1996). This includes a variable N-terminal domain (modulatory domain), often important for transactivation of transcription; a central well conserved DNA binding domain (DBD), crucial for recognition of specific DNA sequences and protein-protein interactions; and at the C-terminal end, a ligand/hormone binding domain (LBD/HBD), important for hormone binding, protein-protein interactions and additional transactivation activity.



A schematic domain structure of glucocorticoid receptor

The ligand-binding domain

Glucocorticoids, being lipophilic hormones, cross the plasma membrane of target cells and interact with the high affinity intracellular cytoplasmic GR. The domain which bind the hormone is referred to as ligand binding domain (LBD) or hormone binding domain (HBD), which extends for approximately 250 amino acids located at the C-terminal end of the receptor. Extensive studies using partial proteolysis have revealed that hormone binding induces a

unique conformational change at the C-terminus of the receptor (Allan et al 1992; Vegeto et al 1992). HBD is very complex and participates in diverse functions (Simons 1989). It possesses the crucial property of hormone recognition and binding, and ensures both specificity and selectivity of physiologic response. The HBD is thought of as a "molecular switch" that, upon hormone binding, shifts the receptor to a transcriptionally active state (Mangelsdorf et al 1995). The conformational competence of HBD is maintained by its association with multiple chaperone protein system, most notably two Hsp90, one each of Hsp70, and Hsp56 (immunophilin) and p23 (an acidic protein) (Biola and Pallardy 2000). It has been reported that at least three other proteins are required for formation of active Hsp90-steroid receptor complex (Prima et al 2000). In addition, Hsp90 maintains the receptor in an inactive form in the absence of ligand (Kang et al 1999). The HBD is rich in cysteine and methionine and plays important role in hormone binding to the pocket. Several of these amino acids have been characterized in human (Cys638 and 665) and rat (Cys640, 656,661,674 and met622) (Yu et al 1995).

The DNA-binding domain

GR is in many ways indistinguishable from other eukaryotic transcription factors that regulate gene expression. In most cases, GR selectively binds to DNA, primarily as dimers. Moreover, they posses identifiable activation functions (AFs) that confer transactivation potential to the DNA binding domain (DBD). The structure of DBD was first revealed by NMR studies and later by X-ray crystallography (Hard et al 1990; Luisi et al 1991;Schwabe et al 1993). The ~70 amino acid DBD can be expressed in bacteria as a functional recombinant protein fragment. The isolated domain contains two Zn^{2+} atoms tetrahedrally coordinated by conserved cysteine residues (zinc fingers), exhibits sequence specific binding to GREs and contains amino acids necessary for dimerization (La Marco and Vivanco 1996). The structure of the DBD obtained by NMR spectroscopy indicates that this domain exists as a monomer in solution (Hard et al 1990). The DBD is a globular structure and can be subdivided into two modules: the first module contains both the zinc finger motifs for DNA binding, the second module is involved in phosphate contacts and receptor dimerization. Both these modules contain loop structure referred as P-loop and D-loop, respectively. The ability to dimerize depends upon the D-loop, a stretch of five amino acids. Several contacts made by the D-loop residues at the dimerization interface stabilize receptor dimers and thereby allowing cooperative DNA binding (Dahlman-Wright et al 1991; Luisi et al 1991). Mutations within the D-loop have a lethal effect on cooperative binding to GREs, but do not completely abolish DNA binding (Dahlman-Wright et al 1991).

The N-terminal domain

The N-terminal end of GR is highly variable in sequence and length (~439 amino acids) and contains the transcription activation function (AF1 or tau1) motif. This motif along with other motif, AF2 in the C-terminal end, presumably modulate target genes by interacting with coactivators or other transactivators (Hollenberg and Evans 1988). AF1/tau1 has been shown to be critical for target gene specificity (Dahlman-Wright et al 1995). AF2, present at the distal C-terminal end of the HBD has been shown to be important for hormone binding and hormone-dependent transactivation (Lanz and Rusconi 1994). The role of AF2 in transcriptional activation by GR has been extensively studied and reveals that it undergoes a conformational change upon hormone binding. This enables the receptor to bind to a series of coactivator proteins, such as steroid receptor coactivator-1 (SRC-1) and CREB-binding protein (CBP) (Glass et al 1997).

Glucocorticoid action mechanism

At the cellular level, most known effects of glucocorticoids are mediated by a ~94 kDa intracellular phosphoprotein, the GR. In the hormone-bound state, the GRs specifically bind to and modulate the activity of target gene promoters and are, therefore known as ligand-regulated transcriptional regulatory protein. Glucocorticoids act on the target cells with a mechanism that may be summarized in the following steps:

i) Free glucocorticoids enter the target cell by passive diffusion through the plasma membrane and bind non-covalently to the high affinity cytoplasmic GR to form hormone-receptor complex.

ii) The hormone-receptor complex undergoes activation/transformation, a process that involves conformational changes leading to dissociation of chaperone proteins and subsequent nuclear translocation of the activated hormone-receptor complex.

iii) Inside the nucleus, the activated hormone-receptor complex interacts with the specific DNA sequences in the chromatin called glucocorticoid response elements (GREs) of the target gene.

iv) The modulation of gene expression by protein-protein interactions and ultimately, generating cellular response(s).

Binding of hormone to GR

The adrenal cortex synthesizes and releases glucocorticoids into the blood circulation under the precise control of HPA-axis (Miller and Tyrrel 1995). In plasma, these hormones are bound to corticosteroid binding globulin (CBG), also known as transcortin. The bound form functions as a circulating reservoir of hormones that keeps a supply of free hormones available to tissues. Glucocorticoids, being liposoluble, cross the plasma membrane of target cells by

passive diffusion and bind to its high affinity cognate receptor in the cytoplasm. The unbound GR exists as an inactive hetero-oligomeric complex in the cytoplasm due to its association with receptor associated proteins (RAPs) or chaperones (two Hsp90 & one Hsp70), co-chaperones (Hop & Hsp40) and several other polypeptides such as immunophilin (Cyp40 & FKBP59) and p23 to achieve a high affinity hormone-binding state (Prima et al 2000; Biola and Pallardy 2000). This complex dissociates in response to hormone binding into a holo-GR that translocates to the nucleus, and regulates the activity of glucocorticoid responsive genes (Sarlis et al 1999).

Activation of hormone-bound GR complex

It is obligatory for the hormone action that GR must be able to assume at least two states- one that is active and one that is inactive- with the binding of the hormone promoting the activation/transformation from the inactive to the active form. Transformation of steroid receptors has been described to occur under physiological conditions (Munck and Foley 1979). As mentioned earlier, it is the inactive form of GR that binds to the hormone, which subsequently causes the associated proteins to dissociate from the steroid receptor complex under physiological intracellular condition, a process termed as **activation** or **transformation** (Tsai and O'Malley 1994; Pratt and Toft 1997). The existence of GR as inactive complex in the cytoplasm in association with Hsp's is to facilitate the folding of the HBD into a high-affinity steroid binding conformation. The activation of GR is a crucial step in GC action, as it is a rate-limiting step for nuclear or chromatin binding. There is an absolute requirement of hormone binding to GR to allow the activation to occur and the same was subsequently demonstrated by genomic footprinting that hormone was required for receptor binding to specific response elements in intact cells (Bamberger et al 1996; Pratt and Toft 1997).

In vitro, activation can be made to occur by several artifactual transforming conditions. Thermal activation at 25°C of cytosol containing hormone-bound GR causes the dissociation of bound chaperones and subsequent activation (Milgrom et al 1979). Salt such as ammonium sulfate, dialysis, gel filtration and elevated pH promoted GR transformation in vitro. The transformed 4S receptor exhibits increased binding affinity for isolated nuclei, chromatin, DNA-cellulose, phosphocellulose and ATP-Sepharose. Salt at 0°C also causes dissociation of Hsp 90 from the receptor and is accompanied by concomitant and proportional generation of the DNA-binding state (Meshinchi et al 1990). Transformation of hepatic GR from rat by ammonium sulfate at 0°C also increased the nuclear binding ability (Dahmer et al 1981; Sanchez et al 1987), by dissociating Hsp90 from the GR and subsequent precipitation (at ~30% saturation). It was later demonstrated by Western blotting that, hormone-free GR may also be transformed to a state that binds DNA-cellulose (Sanchez et al 1987). Dilution, gel filtration and dialysis all transform cytosolic GR by reducing the concentration of, or elimination of, a small heat stable cytocolic factor that, like molybdate, stabilizes the 9S GR complex (Bailly et al 1977). Cake et

al (1976) showed that passage of cytosol through gel filtration column transformed the GR. Both dilution and gel filtration of cytosol facilitated GR activation by elimination of low molecular weight cytosolic inhibitor (Sato et al 1980). Later, Bodine and Litwack (1988), purified an active factor from rat liver as a novel ether aminophosphoglyceride which inhibited activation of (Litwack 1988).

Models of GR activation/transformation

Numerous diverse mechanisms for GR transformation have been proposed, including a conformational change in the receptor (Atger and Milgrom 1976; Bailly et al 1980), dissociation of receptor oligomer (Holbrook et al 1983; Vedeckis 1983), dissociation of macromolecular or low molecular weight inhibitor (Sato et al 1980; Sekula et al 1981), and receptor dephosphorylation. Milgrom et al (1973) suggested that GR transformation consisted of a simple change in the conformation of 4S receptor molecule induced by hormone, in his 'equilibrium model of transformation'. However, now we know that during the transformation process, the HBD undergoes a conformational change, and the process is reversed only by a complex protein-folding reaction involving Hsp90, Hsp70, and other proteins. Milgrom's equilibrium model of transformation turned out to be under cloud. Another observation by Atger and Milgrom (1976), where they have examined the energy changes that accompany the binding of hormone to the receptor and the subsequent heat transformation (at 25°C), and found that binding of hormone to the receptor requires a moderate thermodynamic activation energy. Moreover, the complex corresponds to a striking lower level of free energy. Therefore, a high energy of activation is required for receptor transformation, however, the transformed receptor is at a level of free energy similar to that of untransformed receptor. They concluded that mainly the binding of hormone drives this overall reaction to the receptor, which is accompanied by a large change in free energy. It is now clear that the Hsp90-bound HBD is in the high affinity steroid-binding conformation, and that an important energy barrier that must be overcome in transformation of the receptor is provided by the non-covalent bonds responsible for the protein-protein interaction between GR and Hsp90.

The actual process of GR transformation in vivo is still unclear and complex. There seems to be little doubt that the models of receptor transformation based on purely cytosolic observations will be simplistic. However, it is only through examining hormone-mediated dissociation of more purified receptor heterorocomplexes and through studying the reversal of this transformation with purified Hsp chaperone system, that eventually a correct molecular model describing how the steroid hormone causes receptor transformation could be developed.

In vitro modulation of GR activation

Nishigori and Toft (1980) first reported inhibition of PR transformation by molybdate. Subsequently, both heat and salt transformation of cytosolic GR were inhibited by molybdate (John and Moudgil 1979; Chong and Lipman 1981-82). Tungstate and vanadate were also active in causing inhibition of transformation (Murakami 1982). The effect of molybdate was reversible, and it was effective only when added before transformation; that is, addition of molybdate after transformation did not influence DNA binding of activated receptor. Studies by Raaka et al (1985) on the effect of molybdate on receptor transformation in intact cells revealed reduced nuclear accumulation of GR after steroid treatment. Various activators of *in vitro* GR transformation have also been identified which include the nucleoside triphosphates such as ATP, GTP, CTP etc.

Nuclear translocation

Activated GR is ultimately destined to migrate to the nucleus, where they modulate target gene expression upon binding to chromatin. Nuclear translocation is now understood to be a complex process, which probably utilizes the nuclear localization signals (NLS) sequences in the receptor itself (Picard and Yamamoto 1987), and bidirectional shuttling of the receptor into and out of the nucleus occurs constantly (DeFranco et al 1995). The NLS, NL1 overlaps with the C-terminal end of the receptor DBD (Tsai and O"Malley 1994) and additional NLS, called NL2 (a ligand-dependent NLS) has been identified in the HBD, whose sequence has not been delimited. Nuclear localization also appears to be dependent in large part on the nuclear matrix (van Steensel 1995), and other nuclear components (van Steensel 1995). In the 'heterocomplex model of receptor translocation', Pratt et al (1992) proposed that the receptor migrates to the nucleus in association with Hsp90 and the immunophilin (Cyp40) acting as a protein transport unit or 'transportosome' (Pratt 1993). This model of receptor migration was further supported by the work of Kang et al (1994), in which Hsp90 was targeted to the nucleus by fusion to the nucleoplasmin NLS. Recent work by Kang et al (1999), revealed that GR, after in vivo activation, was still able to reassociate with Hsp90, suggesting that this interaction plays a role in intact cells, probably in translocation and receptor recycling. Also, it has been proposed that an intact cytoskeleton is required for nuclear translocation. Use of a fused chimera of green fluorescent protein (GFP) and GR, under physiological conditions to test the notion that Hsp90 is required for the activated GR translocation along the intact cytoskeletal tract has yielded positive results (Galigniana et al 1998), indicating that the GFP-GR complex utilizes the Hsp90 activity. Geldanamycin, a Hsp90-binding benzoquinone ansamycin, inhibits the activated receptor complex migration to the nucleus from the cytoplasmic compartment, indicating that, a possible interaction of Hsp90 with the activated receptor is required for nuclear translocation (Czar et al 1997). However, in cells without intact cytoskeletal system, the GFP-GR complex was reported to migrate through the cytoplasm by diffusion.

Modulation of nuclear translocation

Numerous endogenous factors have been identified, which indicate that they modulate the nuclear migration of activated GR. Pyridoxal 5-phosphate (PLP) an active form of vitamin B_6, was one of the early identified agent, which has been implicated to be an inhibitor of GR nuclear translocation (Milgrom and Atger 1975; Goidl et al 1977). Rats deficient in PLP, were found to have higher rate of migration of receptor from the cytoplasmic compartment to the nuclear, and under opposing condition of elevated PLP concentration, a decreased nuclear migration was observed (Maksymowych et al 1990). Possible mode of such inhibition may be the influence of PLP with the NLS domain of the receptor (Allgood et al 1990). Recently, an endogenous protein factor termed macromolecular-translocation inhibitor (MTI), has been identified in rat hepatocytes, with some role in modulating the nuclear translocation of GR. Three species of MTI (MTI-I, MTI-II & MTI-III) have been separated, out of which MTI-II from rat liver is a 11.5 kDa Zn^{2+}-binding acidic protein (ZnBP, or parathymosin) (Okamoto and Isohashi 2000), and an inhibitor of activated GR translocation to the nucleus. Interleukin-1 alpha was also demonstrated to inhibit dexamethasone-induced GR migration in cell lines (Pariante et al 1999). Retinoic acid was shown to increase the nuclear translocation of activated rat hepatic GR (Chambon 1994; Audouin-Chevallier et al 1995). Nuclear translocation has been found to increase in cells treated with Hsp56 binding drug FK506 (Hutchison et al 1993; Ning and Sanchez 1993) and a similar observation was found with an antibiotic, cyclosporin A (Renoir et al 1995). Geldanamycin, has been found to impede the glucocorticoid-bound receptors to the nucleus in L-cells as measured by indirect immunofluorescence with anti-receptor antibody and by a shift of specifically bound [^{3}H]triamcinolone acetonide from the cytosolic to the nuclear fraction. However, the exact mechanism of its action is not completely understood. In cell-free experiments, it has been shown that geldanamycin prevents the association of the p23 component of the heterocomplex assembly system with Hsp90 (Johnson and Toft 1995).

DNA binding

Once the activated GR complex reaches the nucleus, the final effect culminates in its binding to specific DNA sequences (GRE) in the chromatin, ultimately leading to modulation of responsive gene transcription. Under physiological conditions, the occupancy of the HBD is thought to promote the binding of receptor to DNA, as deletions within the HBD in the human estrogen receptor failed to induce transcription, indicating that the HBD is indispensable for DNA binding (Pratt and Toft 1997). It has been observed that hormone-free GR binds specifically to MMTV-LTR promoter and therefore it was suggested that the function of hormone in vivo could be to modulate nuclear partitioning of the receptor (Willmann and Beato 1986). The interaction of GR with the GRE of a target gene was retarded in the absence of hormone, indicating that the hormone requirement is absolute (Becker et al 1986).

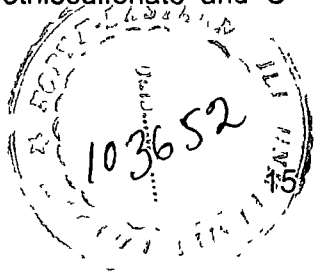
Mutational analysis of GREs revealed them to be a 15 base pair (bp) palindromic sequence [5' GGTACANNNTGTTCT 3'] composed of 6 bp half sites, separated by a 3 bp spacer (Beato et al 1995) This structure of GRE suggests that GR binds to it as a dimer. However, dimerization occurs only after binding to palindromic GREs (Dahlman-Wright et al 1991).

Modulation of DNA binding

The interaction of GR with chromatin is modulated by a number of parameters, such as state of phosphorylation of the receptor and other factors, which vary in response to activation of other signal transduction pathways. Cytosolic GR, being a phosphoprotein, is said to be hyperphosphorylated upon hormone binding (Orti et al 1993). However, the mechanism by which hyperphosphorylated GR promotes its ability to bind to DNA is not clear. The cAMP/protein kinase A (PKA) pathway stimulation has been reported to influence GR binding to its target GRE (Rangarajan et al 1992; Reisfeld and Vardimon 1994). Footprinting studies in hepatoma cells revealed that GR binding to GRE of the tyrosine aminotransferase (TAT) gene promoter requires the activation of PKA (Espinas et al 1995), and the effect of PKA is not on the receptor itself, but, perhaps on the phosphorylation of factors interacting with the receptor (Moyer et al 1993).

Various endogenous factors have also been identified, which suggest their role as modulators of DNA binding of activated GR. PLP has been identified as an inhibitor of DNA binding in vivo, since, it leads to ~50% decrease in glucocorticoid-induced transactivation (Allgood et al 1993; Tully et al 1994). ATP-stimulated translocation promoter (ASTP), a 93 kDa histone-binding protein, isolated from rat hepatocytes, has been reported to raise the nuclear binding of activated GR in vitro (Martin et al 1993), which could, perhaps, stimulate the same in intact cells. Also, recent finding that MTI-II (a Zn^{2+} binding acidic protein or parathymosin), isolated from rat liver could modulate DNA-binding in vivo, as, in vitro studies have shown that it inhibits activated GR binding to DNA containing GRE and nuclei (Okamoto and Isohashi 2000). Another report recently revealed that melatonin, inhibits the binding of activated ER to ERE in the DNA (Rato et al 1999), with the possibility of similar action on GR binding to DNA sequences (Asainz et al 1999).

Numerous chemical agents have been identified, which modulate the binding of activated GR to DNA as assessed by using DNA-cellulose, isolated nuclei, ATP-Sepharose etc. in vitro. Methylxanthines like theophylline, aminophylline and caffeine have been shown to greatly enhance the binding of activated GR complex from rat liver cytosol to DNA-cellulose and isolated nuclei (Cake and Litwack 1978), with similar effect of GR from kidney and thymus. Covalent binding of biotin to purified GR decreases the nuclear binding capacity by as much as 50% (Hapgood and Holt 1987). Various other agents also block the activated GR binding to DNA-cellulose and nuclei, like aurintricarboxylic acid, methyl methanethiosulfonate and *O*-phenanthroline (Moudgil et al 1984).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Glucocorticoid response elements

Cloning and analysis of the MMTV genome and the human metallothionein IIA gene, led to identification of first hormone response elements (HREs), in this case called the glucocorticoid response elements (GREs), present in the 5'-regulatory region of the gene, which serve as GR binding sites and inducible enhancer elements (Yamamoto 1985; Beato et al 1995). Transactivation by the GR requires binding of receptor dimers to specific palindroimic sequences in the *cis*-regulatory region of target genes called GREs. The ability to dimerize (post-binding dimerization) depends on the D-loop, a stretch of five amino acid residues located in the DBD. It has been found that mutations within this region have a lethal effect on cooperative binding to palindromic GREs (Dahlman-Wright et al 1991). In the classical model, GR transactivates transcription by binding to the classical or positive GRE, as in TAT and PEPCK genes (Beato 1995), however, it was found that some genes are negatively regulated by the GR and do not contain a classical GRE (Reichardt et al 1998). Subsequently, it was proposed that the transrepression action of GR are mediated by utilizing different classes of response elements, namely, negative, composite, and tethering GREs (Diamond et al 1990; Miner et al 1991). The negative GREs involve direct DNA binding of the GR as exhibited in the POMC gene (Drouin et al 1993). The composite elements, where the GR contacts the DNA together with another transcription factor, as in case of proliferin gene (Diamond et al 1990), whereas in the tethering elements, repression is facilitated by protein-protein interaction without direct GR binding to the DNA, for example, in genes regulated by AP-1 (Schreiber et al 1995) and NFkB (Auphan et al 1995).

GRE Consensus sequence

MMTV	TGGTTT GGTATC AAA TGTTCT GATCTG
hGH	CCTTTG GGCACA ATG TGTCCT GAGGGG
TO	CTCATA TGCACA GCG AGTTCT AGTGAG
TAT	CTCTGC TGTACA GGA TGTTCT AGCTAC

GGTACANNNTGTTCT

MMTV: mouse mammary tumor virus
hGH: human growth hormone
TO: tryptophan oxygenase
TAT: tyrosine aminotransferase

There have been reports on the existence of the so-called glucocorticoid modulatory elements (GMEs) upstream of the GREs (Simons et al 1992; Szapary et al 1992). The GME of the rat TAT gene is located at 3.6 kbp to 1 kbp upstream of GREs. The GME has the unique transcriptional properties of modulating GR action, and it has been found that the expression of

GME activity involves the binding of two unique proteins, GME-1 and GME-2 (Zeng et al 2000). It was previously reported that GME and GRE mediate transcriptional activation synergestically, however, recent findings have revealed that the mechanisms of expression of GME and GRE activation probably utilize parallel, rather than common pathways.

Regulation of gene expression

In the classical model of gene regulation, GR homodimers transactivate transcription by binding to GREs in the promoter region of glucocorticoid responsive genes (Beato 1989). The role of activated GR is to recruit and maintain a preinitiation complex at the promoter of target genes. Transactivation is probably mediated through interaction of DNA-bound GR homodimers with basal transcriptional machinery coactivators and other transcription factors (Beato et al 1995) directly as in the case of transcription factor IIB (TF IIB). Various independent lines of evidence have suggested that liganded nuclear receptors are capable of directly contacting basal transcription factors, many of which appear to be cell-specific in their expression patterns (Jacq et al 1994; May et al 1996). The characterization of a 170 kDa GR-associated protein (GRIP-170), which stimulated GR transactivation in vitro, suggested that these endogenous cofactors were functionally limiting (Eggert et al 1995). The interaction of GRE-bound GR homodimers may be indirect, as in the case of steroid receptor coactivator (SRC-I) and CREB binding protein (CBP) (McKenna et al 1999). This interaction is presumably sufficient to stabilize the preinitiation complex on the promoter and, thus, to stimulate transcription by RNA polymerase II (Tsai and O'Malley 1994).

In addition, binding of activated GR homodimer to the GRE can induce a rearrangement of the chromatin structure in the respective promoter region, then allowing access to other transcription factors to interact to the previously inaccessible DNA (Li and Rosen 1994; Truss et al 1995). Furthermore, eukaryotic genes are required to exist in configurations that maximize access to the promoter regions with which DNA binding transcription factors specifically interact. These genes are organized into structurally repressed nucleosomes, which allow strict access of transcriptional proteins to key regions of genes, thereby allowing precise regulation of such genes. Covalent modifications (like acetylation) of nucleosomal histones have been suggested to reduce the affinity for DNA and to be an important preface to transcriptional activation *in vivo*. The link between chromatin disruption and transcriptional activity is now well established. CBP, p300 etc contain histone acetyltransferase activity, which indicates that GRs might function in part by recruiting these proteins and directing nucleosomal modification at their target promoters (Bannister and Kouzarides 1996; Yang et al 1996).

Promoters of many glucocorticoid responsive genes contain negative GREs (nGREs) (Tsai and O'Malley 1994). nGREs are also specific DNA sequences that bind activated GRs and lead to inhibition of transcription of responsive genes. A classic case is the nGRE in the pro-opiomelanocortin (POMC) gene promoter (Drouin 1993). It has been reported that instead of

binding as GR homodimer to nGRE, three molecules of GR interact with nGRE leading to inhibition of POMC gene expression.

Interestingly, in the last decade, several groups found that GR also regulates transcription by protein-protein interaction without directly binding to DNA (Jonat et al 1990). This is true for the antiinflammatory/immunosupressive effects of glucocorticoids, where activating protein-1 (AP-1) regulated genes like collagenase-3, whose transcriptional regulation by proinflammatory cytokines can be repressed by GR. However, the interaction between GR and AP-1 is direct or needs an intermediary factor is unknown and perhaps might vary from gene to gene (Kamei et al 1996). Activated GR has also been reported to be involved with the interference of functions of other transcription factors such as NF-κB, CREB etc (Caldenhoven et al 1995; Stocklin et al 1996). Also, the protein-protein interaction between GR and other transcription factors is not always a negative regulation, but can also synergize with other factors as in the case of jun homodimers or STAT-5 (Pearce and Yamamoto 1993; Stocklin et al 1996).

The emerging scenario indicates several possible interactions of GR with GRE and transcription factors, and also with a large number of intermediary factors. These molecular events are only partly understood. It now appears that transcriptional control by GRs is a multistep process, a fact reflected in the diversity of coregulators with which GR interacts in myriad manner.

Is the DNA binding essential for survival?

The presence of intact GR is essential for survival, as experiments involving generation of GR-deficient mice ($GR^{-/-}$) have demonstrated (Cole et al 1995). In humans, several GR mutations have been described, suggesting that GR may be indispensable for life (Reichardt et al 1998). The generation of two independent mutant mouse lines, one involving the insertion of neomycin resistance cassette into exon II (GR^{hypo}), and the other involving deletion of exon III, which encodes the first zinc finger of the DBD (GR^{null}). The GR^{null} leads to complete inactivation of GR. Interestingly, all the $GR^{null/null}$ mice die shortly after birth, whereas 20% 0f $GR^{hypo/hypo}$ survived until adulthood, suggesting the indispensability of DNA binding. As mentioned earlier, genes like TAT, PEPCK etc are positively regulated by the classical GREs, where the GR binding to DNA is an absolute necessity. To study the in vivo relevance of DNA binding, a point mutation was introduced into the mouse GR gene. This mutation was generated by substitution of alanine458 by a threonine (A458T) in the D-loop (Reichardt et al 1998). This substitution resulted in a dimerization-defective GR (GR^{dim}), that could no longer bind cooperatively to palindromic GREs (Heck et al 1994). It was subsequently shown by Reichardt et al (1998) that the $GR^{dim/dim}$ mice could not respond to GC with regard to induction of TAT mRNA. Also, it was shown that, there was a large decrease in the inducibility of the MMTV-based promoter, however, the residual induction observed could be due to the fact that GR^{dim} can bind non-cooperatively to the multiple GREs in the MMTV promoter.

The $GR^{dim/dim}$ mice, however, appeared relatively normal and healthy under standard laboratory conditions and strikingly, survived. The most notable conclusion was that the GRE-mediated gene activation is not essential for development and survival. Unlike the $GR^{dim/dim}$ mice, the $GR^{-/-}$ mice die shortly after birth due to respiratory failure because of immature lungs. However, the $GR^{dim/dim}$ mice showed normal differentiation of lungs (Reichardt et al 1998), indicating that the GRE-mediated gene induction is not the basis for GC-induced lung maturation. This indicates that the DNA-binding of GR is not essential for survival. Moreover, the $GR^{dim/dim}$ mice did not express gluconeogenic enzymes, indicating that these enzymes are not necessary for survival under standard laboratory conditions. However, induction of gluconeogenic enzymes may be required for viability under stressful conditions. Furthermore, the AP-1-GR complex-mediated transrepression was intact in the $GR^{dim/dim}$ mice, indicating that the AP-1 inducible genes are regulated by protein-protein interactions.

Glucocorticoid sensitivity determinants

GRs are essential for glucocorticoid-induced changes to occur, but hormonal sensitivity is not guaranteed simply by the presence of the receptors. There is, in general, a good correlation between the level of GRs in a cell and cellular sensitivity to glucocorticoids. However, other factors may modulate glucocorticoid sensitivity, including the presence of nonfunctional or modified receptors and other cellular factors that modify receptor function. Glucocorticoid sensitivity basically refers to the extent to which an already glucocorticoid responsive system responds to glucocorticoids. In general, sensitivity of tissues to steroid hormones may be important in both physiologic and pathologic conditions. In the case of glucocorticoids, changes in tissue's sensitivity may participate in the maintenance of resting and stress-related homeostasis (Chrousos 1995). The level of intracellular hormone available is an important parameter, since, they, in appropriate concentration must bind to the specific receptor to be able to transduce signal in the target cells. Kidney cells are primarily mineralocorticoid responsive, but glucocorticoids are also able to enter kidney cells to exert their effects. However, the intracellular glucocorticoids in renal cells are metabolized to an inactive derivative, rendering the kidney specifically mineralocorticoid responsive even though they contain functional GRs. This inactivation of glucocorticoids is achieved by 11ß-hydroxysteroid dehydrogenases (11ß-HSD) (Funder et al 1988; Albiston et al 1994), which exists in at least two isoforms, 11ß-HSD 1 & 11ß-HSD 2. 11ß-HSD 1 is also reported to be expressed in tissues other than the kidney, where they may modulate glucocorticoid sensitivity.

The magnitude of GR-mediated cellular response is also dependent on the intracellular level of GR (Vanderbilt et al 1987). The expression level of GR varies in a tissue- and age-specific manner. GRs were to be expressed in a number of tissues including brain, liver, kidney, skeletal muscle etc., with the thymus, expressing in highest number. Tissue-specific level of GR may be dependent on the presence of various factor(s) which modulate the expression of

GR mRNA and/or the stability of the receptor protein itself. Glucocorticoids themselves cause down-regulation of the receptor, as shown in cells from intact animals (Burnstein et al 1991; Burnstein and Cidlowski 1992). At least, there are three mechanisms that take part in glucocorticoid-mediated down-regulation of GR. At the transcriptional level, glucocorticoid seems to repress the expression of GR gene by interference with AP-1- and /or AP-2-mediated pathway of GR gene expression (Vig et al 1994; Nobukuni et al 1995). It was also reported that the binding of activated GR to sequences within the structural gene, rather than within the consensus GREs, which are absent in the GR gene, may modulate its expression (Encio and Detera-Wadleigh 1991; Nobukuni et al 1995). Also, the presence of glucocorticoids may reduce the half-life of GR (Mcintyre and Samuels 1985). Other than glucocorticoids, estrogen was shown to repress the expression of GR in the anterior pituitary (Peiffer and Bardin 1987). Age-dependent alteration in GR level is also an important regulatory mechanism for glucocorticoid sensitivity. In most animals, especially in rodents, GR level was found to increase with increasing age, reaching a maximum in adults and then a gradual decline is observed. This variation in GR level may be due to the change in the endogenous modulators of GR itself (Kalimi et al 1988).

Untransformed GR in the cytoplasm is a phosphoprotein, which has been reported to become hyperphosphorylated upon hormone binding and dissociation of chaperone proteins (Bamberger et al 1996). However, the role of GR phosphorylation in determining its biological function is not clearly established, and there seems to be a consensus that GR phosphorylation status codetermines its subcellular location rather than its gene regulatory activity.

How GRs search for a target site in the genome?

GRs functions as transcription factors by binding to specific DNA sequences (GREs), generally upstream of transcription initiation sites. In order to bind these DNA elements, the receptor must first locate these sites in the genome. In vertebrates, this search entails locating a small fraction of functional binding sites from billions of base pairs of DNA, requiring this receptor to sample an immensely vast number of possible binding sites in a very short period of time. Nevertheless, GRs are able to bind their specific binding sites very rapidly. However, there is no unanimous agreement for the exact mechanism employed to locate specific binding sites in the chromatin. Given below a brief discussion on some of the models proposed to explain this search mechanism.

One model, the cycling model (Berg and Hippel 1985), proposes that GRs bind target elements by repeated cycles of association and dissociation, until, a high affinity site is found. This mechanism is commonly assumed to be the search tactics employed by site-specific DNA-binding proteins. This model predicts that the search will be controlled solely by the intrinsic rate of receptor dissociation from nonspecific DNA sites. However, the explanation made by this model was not very satisfactory. A second model, the sliding model, involves the receptor

sliding along the DNA chain and conducting a one-dimensional search until it encounters a specific binding site. This model has an advantage that, it is highly efficient for locating a binding site over a limited distance along the DNA molecule. However, the sliding model indicates that this mechanism may not be an efficient strategy for a long distance search. The third model, the intersegment transfer mechanism proposes that GRs search for target site in the genome by intersegment transfer (Lieberman and Nordeen 1997). In this model, receptor dimers bind nonspecific DNA sequences and search for a target site by binding to a second strand of DNA before dissociating from the first. This has an advantage that high concentration of DNA favors, rather than hinders, the search, by increasing the apparent dissociation rate of the receptor, unlike the cycling and sliding model, in which rate of receptor dissociation is intrinsic and not affected by exogenous DNA. Using the purified DBD fragment of rat GR, it was found that receptor dissociation from DNA was highly dependent on the concentration of DNA in solution, supporting the intersegment transfer model (Lieberman and Nordeen 1997). However, this model is yet to be studied with full-length GR, nonetheless, it is an attractive idea for the search of GR to its specific binding site.

Nucleocytoplasmic trafficking of steroid-free GR

The binding of hormone to GR is a transient process, and the loss of hormone from the receptor leads to recycling of the receptor into the Hsp-containing oligomeric complex (Scherrer et al 1990). When liganded GR are shuttling proteins that traffic continuously between nucleus and cytoplasm (Madan and DeFranco 1993). Steroid receptors are differentially localized in the cell in absence of cognate ligand. Unliganded ER and PR are nuclear, whereas GR, MR, and AR are cytoplasmic. The molecular basis of these differences in localization is not well understood. The predominant localization of naive GR in cytoplasm is probably due to the masking of one of its NLS, NL1 by Hsp90, which somehow prevents its translocation to the nucleus (Pratt 1993; Czar et al 1995). This explanation is further supported by a study demonstrating that Hsp association of GR prevents the binding of an NL1-specific antibody (Hache et al 1999). However, other studies do not favor this simple model, for example, over expression of GR in many cell lines results in the nuclear translocation of the Hsp-associated receptor without apparent change in other properties (Sanchez et al 1990). Studies involving nucleocytoplasmic trafficking of hormone-free GR prior to hormone treatment and following hormone withdrawl have yielded encouraging results (Hache et al 1999), indicating that Hsp-associated GR complexes are not sufficient to prevent the trafficking across the nuclear membrane. Hache et al (1999) observed that following the withdrawl of its endogenous ligand, cortisol or the hormone antagonist RU486, GRs were able to rapidly recycle into the Hsp-associated, hormone-responsive complexes. However, the redistribution of GR to the cytoplasm, upon cortisol withdrawl was very slow, with absolutely nil in the case of RU486. The reason attributed was not due to the defect in export machinery, since in both the

instances, the complexed nuclear GRs migrated normally between heterologous nuclei (heterokaryon) in cell fusion experiments. Furthermore, the fusion of a heterologous protein (nuclear retention signal) to the N-terminus of GR stimulated the transfer of latent receptor to the nucleus in the absence of ligand. These studies strongly suggest that the localization of GR to the cytoplasmic compartment is attributed by precise regulation of the rates of transfer of GR across the nuclear membrane and/or by active retention that occurs independently from the association of GR with Hsp's.

Cross-talk with other signaling pathways

Biological regulation is generally exerted through combinatorial events. Interaction of regulatory pathways with individual transcriptional regulatory protein culminates in cell specific gene expression. The regulation of GR function is impinged upon by numerous other signaling pathways making it a complex, multifaceted event. By modulating GR signal pathway, cross-talk mediators may participate in defining the sensitivity of a cell to glucocorticoids either in a tissue-specific or generalized fashion. There is no consensus in how hormone binding influences the various steps in GR action and on whether hormone binding is the only way of activating the receptor. It seems that nuclear receptors can bind to target DNA sequences even in the absence of ligand or when complexed with antagonistic ligand. Recently it was demonstrated that GR is activated in the absence of hormone through signals originating from cell-surface receptors (Tsai and O'Malley 1994). These membrane signaling can modulate the activity of the GR complex by mechanism possibly involving phosphorylation/dephosphorylation. The biochemical modulation of GR is suggested to be achieved by phosphorylation (activation) and dephosphorylation (inactivation) at seven different phosphorylatuion sites (Orti et al 1992; Hu et al 1994; Webster et al 1997). Glucocorticoids being anti-inflammatory agents is frequently administered on human subjects suffering from asthma. In asthma, treatment regimen containing glucocorticoids and $ß_2$-agonists results in better symptom control. These clinical observations suggest an interaction of both classes of drugs at a molecular level. GR being a cytoplasmic receptor, the $ß_2$-adrenergic receptor ($ß_2$-AR) is a cell-surface G-protein-coupled receptor (GPCR), and transducing signal through adenylate cyclase that elevates the concentration of cAMP. Recently, ligand-independent activation of GR by the $ß_2$-AR agonists, such as salbutamol and salmeterol in primary lung fibroblasts and vascular smooth muscle cells has been reported (Eickelberg et al 1999). Treatment of cells with the above drugs resulted in increased translocation of hormone-free GR into the nucleus, and binding to GRE, as revealed by histochemical and Western blotting experiments. The effects of salbutamol and salmeterol were mediated upon binding to the $ß_2$-AR, because blocking $ß_2$-AR with propranol abrogated GR activation. However, the molecular mechanism of this activation is not demonstrated, but it is assumed that $ß_2$-AR activation leads to increase in

the level of cAMP, protein kinase A (PKA) and calmodulin (CaM) (Della-Rocca et al 1997; Eickelberg et al 1999). The pathway involving CaM, a cytosolic calcium binding protein, has gained considerable importance, as CaM directly activated GR with a mechanism suggested to be the phosphorylation of specific tyrosine residues in GR (Ning and Sanchez 1995). These demonstration opens out the possibilities of cross-talk between signal transduction pathways involving PKAs, CaM and steroid hormone action.

The idea of cross-talk between the intracellular steroid action cascade and the cell-surface protein/peptide hormone action cascade arose and visualized the inter-relation among the protein/peptide and steroid hormone actions (Sharma 1993, 1999). The protein/peptide hormone modifiers can modulate steroid hormone action. It has been reported earlier that the protein kinase C activators and inhibitors modulate the glucocorticoid-dependent regulation of TAT and TO in cultured rat hepatocytes (Sharma et al 1990; Sharma 1991). Several others have also observed that the protein kinases are central to these cross-talks, as most of the steroid receptors are phosphoproteins and their phosphorylation might control the activation and affinity of these receptors to DNA response elements. Selected steroid receptors can be activated in a ligand-independent manner by a membrane agonist. Dopamine has been reported to mimic the action of progesterone in activating the progesterone receptors while 8-bromo-cAMP has been demonstrated to mediate progesterone receptor-dependent transcription in the absence of progesterone (Denner et al 1990; Power et al 1991).

More recently, in a significant deviation, the receptors for steroid hormones were also found to be located on the membrane surfaces of certain cell types such as spermatozoa, oocytes, endometrial cells and granulosa cells (Revelli et al 1998; Sharma 1999). The non-genomic effects of 17β-estradiol, progesterone, testosterone and androstenedione on these reproductive cell types are well-documented (Revelli et al 1998; Sharma 1999). Grazzini et al (1998) have shown that progesterone inhibits oxytocin signaling by binding to the membrane-bound oxytocin receptor and changing the conformation such that oxytocin does not interact efficiently to its own receptor.

A number of cell-surface receptors are also known to activate transcription factors, but act through an enzymatic mechanism. A classic case is the mode of action of cytokines, which upon binding to the transmembrane cytokine receptors activate its inbuilt tyrosine kinase activity, which in turn phosphorylate a latent cytosolic transcription factor known as Stat (signal transducers and activators of transcription) (Stocklin et al 1999). Phosphorylated Stat monomers dimerize and translocate to the nucleus and assume the ability to bind to specific DNA sequences in target gene promoters. Stat5, a unique Stat molecule is activated by several essential cytokines, and has been demonstrated to functionally interact with the GR, giving rise to cooperation between GRs and Stat mediated pathways (Cella et al 1998). Stat5 acts both as a coactivator and corepressor of GR-mediated pathway of target gene expression modulation. The ß-casein gene promoter expression requires GR, which acts as transcriptional activator for

Stat5 and enhances Stat5 dependent transcription of this gene promoter, but independent of a GRE (Cella et al 1998). Conversely, Stat5 molecule act as a corepressor of GR, since its binding to GR diverts the protein-protein complex from binding to GRE and therefore interferes with gene transcription, as in the case of MMTV-LTR transcription (Stocklin et al 1996; Stocklin et al 1997). This interaction between glucocorticoid- and cytokine-mediated transcriptional pathways may suggest tissue- and cell-specific activity of these extracellular signals.

Glucocorticoids, being immunosupressive agent, are known to trigger apoptosis in T cells through GRs (Helmberg et al 1995). However, recently it was demonstrated that this apoptotic activity of glucocorticoids is blocked by the activation of T cell antigen receptor (TCR) (Jamieson and Yamamoto 2000), that suggests cross-talk between these two distinct signal transduction pathways. It was shown that the TCR activation of mitogen-activated protein kinase kinase cascade (MAPKK) via Ras protein is involved in inhibition of GR-mediated apoptosis in T cell lines. Also, the activation of various components (TCR, Ras and MAPKK1) changes the GR-mediated transcription. These findings reveal the importance of the convergence of the signal transduction pathways.

The above information's give an insight into the role of GR *in vivo* and help decipher the molecular mechanisms underlying its action. There is a clear role for agents that modulate GR function. The emerging picture shows different modulators as important agents that regulate GR-mediated signaling pathways. This thesis displays a biochemical attempt to study the modulation of GRs in mice using different endogenous/exogenous agents and also by diabetic and senescent state.

The entire work was performed with the following objectives:

i) To study the effects of DL-dithiothreitol (DTT), 2-mercaptoethanol (ME) and reduced glutathione (GSH) on hormone binding to glucocorticoid receptor (GR) and on the stabilization of hormone-receptor complexes as a function of time.

ii) Study the activation process of GR by heat and salt, and its modulation by cadmium, selenite, arsenite, leupeptin, polyunsaturated fatty acids (PUFAs) and pyrophosphate (PPi).

iii) Modulate the activated GR complexes binding to acceptor sites by pyridoxal 5-phosphate (PLP), aurintricarboxylic acid (ATA) and methyl methanethiosulfonate (MMTS).

iv) Induce diabetes by streptozotocin (STZ) and study the modulatory role of diabetes, on GR level, affinity and activation (by heat and salt) in diabetic and control animals.

v) To study the modulatory effect of aging on GR level, affinity, activation (by heat), activation modulation by PUFAs, and DNase I digestion in young and old animals.

MATERIALS

Animals

Swiss albino mice (balb/c strain) maintained under standard laboratory conditions (24 ± 2°C; 12 h light/dark cycle) were used in all the experiments. The animals were caged in husk layered polycarbonate cages and fed with standard food pellets (Amrut laboratories Pune, India) and tap water *ad libitum*. Male mice of two different postnatal ages (15- and 120-day old) were used for experiment wherever mentioned, including young (4-) and old (120-week) male mice for aging studies.

Chemicals and reagents

All chemicals and reagents used were of highest analytical grade and purity. The following companies have supplied the different chemicals and reagents:

Amersham Biotech, England: [1,2,4,6,7-^{3}H]dexamethasone (specific activity, 89 and 91 Ci/mmol).

Sigma Chemical Company, St. Louis, USA: Nonradioactive dexamethasone, DNA-cellulose, tris, glutathione (reduced), 2-mercaptoethanol, sodium molybdate, calcium chloride, EDTA, activated charcoal, dextran T-70, bovine serum albumin (BSA), calf thymus DNA, dimethyl sulfoxide (DMSO), magnesium chloride, cadmium chloride, pyridoxal phosphate, sodium arsenite, sodium selenite, leupeptin, aurintricarboxylic acid, methyl methanethiosulfonate, DL-dithiothreitol, Triton X-100, oleic acid (C18:1), linoleic acid (C18:2), arachidonic acid (C20:4) [all free acids], streptozotocin and deoxyribonuclease I (DNase I).

Whatman, England: Qualitative filter papers.

Sisco Research laboratories, India: Scintillation cocktail-T.

Merck, India: Acetaldehyde.

Glaxo, India: sucrose, glycerol, sodium chloride, sodium citrate, sodium hydroxide, acetic acid, hydrochloric acid and orthophosphoric acid.

Bengal Chemicals and Pharmaceuticals, India: Absolute ethanol.

Instruments and apparatus

Tissues were homogenized in Remi tissue homogenizer (model RQ-127A). Centrifugations were carried using Hitachi (himac CR20B2 model) high-speed refrigerated centrifuge. All spectrophotometric analyses were carried out utilizing Hitachi U-2000 double beam UV/visible spectrophometer. Tissues were stored at -80°C in Heto ultra freezer until use. pH meter was from Control Dynamics, India. All radioactive countings (CPM) were performed in Beckman LS 1801 and Wallac 1409 liquid scintillation counters having an efficiency of 65% and 68%, respectively. Pipettings were done using Gilson pipettes.

Buffers

All buffers were prepared in double distilled water and the pH set at room temperature. Buffers were kept under refrigeration at 2-4°C until use. All experiments were performed at 0-4°C, unless otherwise mentioned.

A. *Steroid binding and stabilization studies:* 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.5

B. *Activation-modulation experiments* :

i) 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6; **ii)** 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6/ 0.5% (v/v) Triton X-100

C. *Radioreceptor assays* :

0.25 M sucrose/ 10 mM Tris-HCl, pH 7.5/ 1 mM EDTA/ 10 mM sodium molybdate/ 10 % (v/v) glycerol/ 1 mM DL-dithiothreitol/ 10 mM NaCl

D. *DNase I digestion experiments* : 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6/ 4.2 mM $MgCl_2$

METHODS

Studies on hormone binding to GR and its stabilization

Cytosol preparation

Male mice of two different postnatal age groups (15- and 120-day old) were sacrificed by cervical dislocation at a fixed time of the day (10:00h). Their livers and kidneys were quickly removed, washed in chilled normal saline (0.9% NaCl) and blotted dry and stored under deep

freezer (-80°C) until use. For the steroid binding and stabilization experiments, stored tissues were properly thawed and minced. A 20% (w/v) homogenate was prepared in buffer A, using motor driven Potter-Elvejham homogenizer in ice bucket for 1-1.5 min at 800-1000 rpm. Homogenates thus obtained were subjected to centrifugation at 27,500 *g* for 1 h at 2°C. Clear cytosols, free of fatty layer were then used for [^{3}H]dexamethasone (^{3}Hdex)binding to receptor.

[^{3}H]dexamethasone binding studies

The stabilization studies were performed according to the method of Kalimi et al (1983) with minor modifications. Aliquots (100 µl) of cytosol were incubated with 20 µl 40 nM [^{3}H]dex alone and in addition, consisted of 10 µl stabilizers [DL-DTT, ME and GSH] separately to a final concentration of 1 mM, prepared as 14 mM stock in buffer A and 10 µl of buffer A alone, which gave the **total binding**. For **non-specific binding,** the reaction mixture consisted of the same as total binding, but with 10 µl of 500-fold excess nonradioactive dexamethasone instead of buffer A. The whole reaction mixture was incubated for 1-24 hr at 0°C with regular gentle vortexing to get the maximal saturable binding at each time intervals. At the end of each incubation period, 50 µl dextran-coated charcoal (4% activayted charcoal + 0.4% dextran T-70) prepared in buffer A was added to each tube to remove any unbound hormone. The charcoal particles were kept in suspension by gentle vortexing for 10 min and finally pelleted at 2000 *g* for 10 min. 100 µl of clear cytosols were then pipetted to scintillation vials and further 5 ml of scintillation cocktail-T was added; the contents were thorougly mixed and bound radioactivity (CPM) was counted. **Specific binding** of [^{3}H]dexamethasone was obtained by subtracting the radioactivity bound in the presence of 500-fold excess unlabeled dexamethasone (**non-specific binding**) from that bound in the presence of labeled dexamethasone alone (**total binding**). Results were finally expressed as specific [^{3}H]dexamethasone bound to receptor (CPM).

Activation studies

Preparation and activation of glucocorticoid-receptor complexes

Mice (15- and 120- day old) were sacrificed by cervical dislocation at a fixed time of the day (10:00h), their livers and kidneys were quickly removed, washed in chilled normal saline (0.9% NaCl) and blotted dry. A 20% (w/v) homogenate of these tissues were prepared in buffer B (i) and centrifuged at 2000 *g* for 10 min at 2°C to sediment nuclei and other cellular debris. The crude nuclear pellet was further processed to give purified nuclei. The resulting supernatant was further centrifuged at 27,500 *g* for 60 min at 2°C to give clear cytosol. Finally, the clear, fat-free cytosol (2 ml) was incubated with 40 nM [^{3}H]dexamethasone (final concentration) for 4 hr

at 0°C with regular vortexing, during which maximal saturation binding occurred. 500 µl of ice-cold DCC (4% activated charcoal + 0.4% dextran T-70) prepared in buffer B (i) was then added and incubated at 0°C for 10 min with regular vortexing to keep the charcoal in suspension. After 10 min, the charcoal particles were pelleted at 2000 *g* for 10 min to give the bound hormone-receptor (H-R) complexes.

Bound H-R complexes in the cytosol were then heat (25°C) and salt (20 mM Ca^{2+}) at 0°C activated for 45 min to give the activated H-R complexes as described by Borbhuiya and Sharma (1995a,b). Aliquots of cytosol were also kept at 0°C without any to yield unactivated H-R complexes. The magnitude of heat and salt activation was then assessed by allowing the binding of such H-R complexes to DNA-cellulose and purified nuclei.

DNA-cellulose binding assay

The method of Kalimi et al (1975) was employed for DNA-cellulose binding assay. Commercially available DNA-cellulose (containing 3 mg DNA/g DNA-cellulose) was incubated for 24 hr at 2°C in buffer B (i). The mixture was then gently vortexed to give homogenous suspension, from which aliquots (100 µl) containing 50 µg DNA were pipetted to 1.5 ml microcentrifuge tubes and further 0.5 ml buffer B (i) was added. Cellulose suspension was pelleted at 2000 *g* for 10 min and the supernatant was discarded. Equal aliquots of unactivated and activated H-R complexes were then added separately in duplicate to cellulose pellets and the mixture gently vortexed and incubated for 60 min at 0°C. The reaction mixtures were regularly vortexed to keep the cellulose in suspension. Binding was stopped by addition of 1 ml ice-cold buffer B (i) and centrifugation at 2000 *g* for 10 min. Pellet thus obtained was further washed twice with the same buffer. To the final pellet was added 0.5 ml cocktail-T and transferred to scintillation vials, to which 3.5 ml cocktail was again added and the content thoroughly mixed and counted for the bound radioactivity. The result were expressed as [^{3}H]dex-R complex bound to DNA-cellulose (CPM/100 µg DNA).

Nuclear binding assay

For the nuclear binding assay, crude nuclei obtained as above were further purified (Eberhardt et al 1978). 1 ml of ice-cold buffer B (ii) was added to the crude nuclear pellet and the content gently homogenized at low speed and subsequently pelleted at 2000 *g* for 10 min. Pellets thus obtained were washed thrice in buffer B (i) followed by centrifugation. Finally the pellets were suspended in the buffer B (i) to give a homogenous suspension. Aliquots of which containing 100-150 µg DNA were transferred to 1.5 ml microcentrifuge tubes and further 1 ml of buffer B (i) was added. Tubes were then subjected to centrifugation at 2000 *g* for 10 min to give the final nuclear pellets and the supernatants were decanted. Equal aliquots of activated H-R

complexes were then added, in duplicate to the nuclear pellets and the content gently vortexed to keep the pellet in suspension for better interaction. For the **cross-mixing experiments**, heat-activated hepatic H-R complexes from 120-day old mice were allowed to bind with the nuclei of 15-day old mice and *vice versa*. Binding of activated H-R complexes was allowed to continue for 60 min at 2°C, with regular vortexing for proper interaction. Subsequent processing of pellets and counting of bound radioactivity was performed as indicated for DNA-cellulose binding assay. The concentration of DNA in the nuclear pellet was estimated according to the diphenylamine method of Burton (1956, 1968) (appendix III). Finally, the results were expressed as [^{3}H]dex-R complex bound to nuclei (CPM/100 μg DNA).

Activation modulation studies

For activation modulation studies, hormone-receptor complexes were prepared from the liver and kidney of immature (15-) and mature (120-day) old mice as described above. The magnitude of activation modulation was assessed using DNA-cellulose and purified nuclei. H-R complexes were subjected to heat activation (25°C) for 45 min in the presence of cadmium (0-4 mM), selenite (0-8 mM), arsenite (0-8 mM), leupeptin (0-40 mM), polyunsaturated fatty acids (oleic, linoleic and arachidonic acids) (0-200 μM) and tetrasodium pyrophosphate (0-16 mM) separately. These modulators were prepared as stocks in buffer B (i), except for PUFAs (prepared in dimethylsulfoxide, DMSO) and added to aliquots of H-R complexes to give the final concentrations as indicated. Control tubes received the buffer (minus modulators) and DMSO (instead of PUFAs). The binding assays, subsequent processing of the DNA-cellulose and nuclear pellets and the determination of bound radioactivity were performed as described above. Results were expressed as % inhibition. Controls (minus modulators) were attributed 0% inhibition. In case of PPi, results were expressed as [^{3}H]Dex-R complexes bound to DNA-cellulose/nuclei (CPM/ 100 μg DNA).

Acceptor (DNA) binding modulation studies

For acceptor binding modulation studies, hormone-receptor complexes were prepared from the liver and kidney of immature (15-) and mature (120-day) old mice as described above. Hormone-receptor complexes were subjected to heat activation (25°C) for 45 min to obtain activated H-R complexes. The magnitude of acceptor binding modulation was assessed using DNA-cellulose and purified nuclei. Pyridoxal 5-phosphate (PLP) (0-10 mM) and methyl methanethiosulfonate (MMTS) (0-8 mM) were prepared as stocks in buffer (i), whereas, aurintricarboxylic acid (ATA) (0-0.7 mM), was prepared in dimethylsulfoxide (DMSO), were added separately in duplicate to aliquots of heat-activated H-R complexes to the indicated final

concentrations. The reaction mixture was then incubated for 30 min at 0°C with gentle vortexing at regular intervals. The control tubes received appropriate volume of buffer (i) and DMSO (in place of ATA) instead of modulators. After the incubation period, PLP, MMTS and ATA treated H-R complexes were incubated in duplicate with pre-washed pellets of DNA-cellulose (50 μg DNA) and purified nuclei (100-150 μg DNA) separately for 60 min at 0°C with regular vortexing to keep the pellets in suspension for proper interaction and binding. Subsequent processing of the DNA-cellulose and nuclear pellets and the determination of bound radioactivity was performed as described above. Results were expressed as % inhibition. Controls (minus modulators) were attributed 0% inhibition.

Diabetes and GR modulation studies

Streptozotocin (STZ) treatment

Overnight fasted mice (15- and 120- day old) were injected intraperitoneally with a single dose of STZ (20 mg/ 100 g body weight) in ice-cold 0.1 M sodium citrate buffer, pH 4.5. Control mice received the vehicle of STZ. The animals were allowed food and water *ad libitum* and the blood glucose level was determined routinely. When the blood glucose level was increased to three fold or more compared to control values, the mice were sacrificed on day 7 after STZ treatment.

Blood glucose estimation

Blood glucose level was routinely determined using commercially available Glucometer (Ames type) according to user's guide with minor modifications. Blood was collected from the tail of mice and a drop was applied to the pad of the glucose stripes. After 20 seconds, the pad surface was carefully blotted dry and inserted into the test slot of the Glucometer. The values of glucose levels, as indicated in the display screen were recorded. Wherever needed, the glucose concentration was also measured using *O*-toluidine method (Sigma technical bulletin 1980). Blood glucose level was expressed as mg/dl.

Assay of glucocorticoid receptors

Male mice of two different postnatal age groups (15- and 120-day old) were sacrificed by cervical dislocation at a fixed time of the day (10:00h). Their livers and kidneys were quickly removed, washed in chilled normal saline (0.9% NaCl), blotted dry and stored under deep freezer (-80°C) until use. For the radioreceptor assay experiments, stored tissues were properly thawed and minced. A 20% (w/v) homogenate was prepared in buffer C, using motor driven Potter-Elvejham homogenizer in ice bucket for 1-1.5 min at 800-1000 rpm Homogenates thus

obtained were subjected to centrifugation at 27,500 *g* for 1 h at 2°C. Clear cytosols, free of fatty layer, were then used for glucocorticoid receptor assay.

Liver and kidney of 4-5 animals of each age group were used for determining the concentration of glucocorticoid receptors. The method of Kalimi et al (1983) was followed for the assays performed in microcentrifuge tubes of volume 1.5 ml. For Scatchard analyses (Scatchard 1949), aliquots of cytosols (100 µl) were incubated at 0°C for 4 h in duplicate with 20 µl [^{3}H]dexamethasone of increasing concentrations (1-120 nM) and 20 µl buffer C and were gently vortexed at regular intervals to ensure proper binding of hormone to the receptor; this gave the **total binding**. For **nonspecific binding,** 100 µl of cytosol was simultaneously incubated under identical conditions with 20 µl [^{3}H]dexamethasone (1-120 nM) and 500-fold excess of nonradioactive dexamethasone to that of respective [^{3}H]dexamethasone concentration. 50 µl chilled dextran-coated charcoal (DCC) (4% activated charcoal + 0.4% dextran T-70) prepared in buffer C, was then added to remove any unbound hormone and incubated at 0°C for 10 min with gentle vortexing at regular intervals and finally centrifuged at 2000 *g* for 10 min at 2°C. 100 µl of charcoal free supernatant was then pipetted into scintillation vials and further, 5 ml of scintillation cocktail-T added. The vial contents were vigorously shaken and bound radioactivity (CPM) determined in the liquid scintillation counter. Specific binding was calculated as described earlier and expressed as fmol/mg protein. Details of conversion factor are given in appendix II. Total cytosolic protein in the final DCC treated reaction mixture was estimated according to the dye-binding method of Bradford (1976) (appendix I). Specific binding, $[S]_{bound}$ (i.e., total binding - nonspecific binding) was determined for each concentration of [^{3}H[dexamethasone used. Total hormone concentration, $[S]_{total}$ added to each tube was also determined. Free hormone concentration, $[S]_{free}$ was obtained by subtracting $[S]_{bound}$ from $[S]_{total}$ and the ratio $[S]_{bound}/[S]_{free}$ was plotted against specific bound ($[S]_{bound}$) to give a linear regression plot. The intercept of the plot on the X-axis gave the maximal specific binding sites (B_{max}) and the slope gave the dissociation constant (K_d).

Activation studies

For activation studies, hormone-receptor complexes were prepared using B (i) from the liver and kidney of immature (15-) and mature (120-day) old mice as described above. Aliquots of hormone-receptor complexes were then subjected to heat (25°C) and salt (20 mM Ca^{2+} at 0°C) activation for 45 min (Borbhuiya and Sharma 1995a,b). Aliquots of H-R complexes were also incubated at 0°C for 45 min to give the unactivated complexes. The magnitude of activation was assessed using DNA-cellulose and purified nuclei. Binding assays, subsequent processing of the DNA-cellulose and nuclear pellets and the determination of bound radioactivity were

performed as described above. Results were expressed as [^{3}H]dex-R complex bound to DNA-cellulose/nuclei (CPM/ 100 μg DNA).

Aging and GR modulation studies

Assay of GRs

Male mice of two different age groups, 4- week (young) and 120- week (old) were used to assay the GR concentration in the liver and kidney according to Kalimi et al (1983) and Borbhuiya and Sharma (1995a,b). Tissue removal and further processing were performed as mentioned earlier. Scatchard analyses of binding data were performed as described by Scatchard (1949) to determine the maximal specific binding sites (B_{max}) and the dissociation constant (K_d), as described above.

Activation studies

Activation studies were performed for the GR from the liver and kidney of young and old mice. Preparation of H-R complexes, heat (25°C) activation and DNA-cellulose and nuclear binding assays were performed to reveal age-related difference in the magnitude of activation of GR in these tissues, as described above. Results were finally expressed as [^{3}H]dex-R complex bound to DNA-cellulose/nuclei (CPM/ 100 μg DNA).

Activation modulation by polyunsaturated fatty acids (PUFAs)

Activation modulation by PUFAs (linoleic and arachidonic acid) was studied in the liver and kidney of young and old mice. Preparation of H-R complexes [in buffer B (i)] was done as mentioned above. Aliquots of H-R complexes thus obtained were heat (25°C) incubated for 45 min in the presence or absence of linoleic (C18:2) and arachidonic acid (C20:4) (prepared as 1 mM stock in dimethylsulfoxide, DMSO) to a final concentration of 160 μM. Control tubes received DMSO only, without the PUFAs. The magnitude of inhibition was assessed using DNA-cellulose and purified nuclei. The binding assays, further processing and determination of bound radioactivity were performed as given above. Results were expressed as [^{3}H]dex-R complex bound to DNA-cellulose/nuclei (CPM/ 100 μg DNA).

DNase I digestion extraction studies

DNase I digestion experiments were performed on the purified nuclei obtained from the liver and kidney of young and old mice, according to Borbhuiya and Sharma (1995a,b). Heat (25°C) activated [^{3}H]dexamethasone-receptor complexes from both the tissues and ages, were allowed to interact with their respective purified nuclei, as described above. After washing off the unbound complexes, the pellets were incubated with DNase I (prepared in buffer D) at a

concentration of 100U/100 μg DNA for 45 min at 2-4°C; control tubes received the buffer D only (Borbhuiya and Sharma 1995a,b). The digestion was stopped by the addition of 1 ml of buffer B (i), followed by centrifugation at 2000 *g* for 10 min. The pellet was processed and the remaining bound radioactivity determined (expressed as % [^{3}H]dexamethasone-receptor bound to nuclei) as described earlier. Controls prior to DNase I digestion were taken as 100% bound.

Results obtained from the experiments performed are plotted as line and bar diagrams. Each data point represents the mean value ± standard deviation. In some cases, results are presented in a tabular form. Results obtained from two sets of data were statistically analyzed according to Student's *t*-test, with $P< 0.05$ taken as significant.

Studies on hormone binding to GR and its stability by modulators:

Hormone binding to GRs and stability of hormone-receptor (H-R) complexes, after attaining maximal saturable binding with 40 nM [^{3}H]dexamethasone, [^{3}H]dex, at 0°C in the absence (control) or presence of stabilizers such as DTT, ME and GSH were examined as a function of time (1-24 hr). Pilot experiments performed in the liver of 120-day old mice revealed that the maximal saturable binding of GR by [^{3}H]dex reached a peak at about 4 hr without any change up to 8 hr and then sharply declined in control (Fig. 1A). However, in the presence of DTT (1mM) there was a significant higher (37%) specific binding of hormone to the receptor at 4 hr compared to control, and that was maintained for up to 24 hrs of incubation (Fig. 1A). This shows that DTT enhances the maximum specific binding of hormone to the receptor as compared to control. Interestingly, mercaptoethanol (Fig. 1B) and glutathione (Fig. 2) had no influence on the hormone binding to hepatic GR at 4 hr as compared to their respective controls

In terms of stability of GR after attaining maximal saturable binding at 4 hr, there was a similar pattern of decline of H-R complexes up to 24 hr in both control and DTT incubated cytosols (Fig. 1A). This shows that DTT is ineffective in stabilizing the H-R complexes after maximal saturable binding. Also, both mercaptoethanol (Fig. 1B) and glutathione (Fig. 2) had no stabilizing effect on H-R complexes after maximal saturable binding as compared to control. Hence, results indicate that DTT, a potent reducing agent enhances the hormone binding to receptor, however, both mercaptoethanol and glutathione had no influencing role on this process. In contrast, none of these were able to stabilize the H-R complexes as a function of time.

Next, it was decided to employ these modulators to find out differences, if any, on steroid binding to GR and stabilization of hormone-bound GR from the liver and kidney of immature (15-) and mature (120-day old) mice to reveal age- specific difference, if any. Our data from control group show greater (33%) maximal specific binding of hormone to the hepatic receptor at 4 hr from immature mice as compared to mature and that was maintained up to 16 hrs of incubation (Fig. 3A), with similar observation in the kidneys (Fig. 3B). However, there was no observed difference in the stability of hepatic (Fig 3A) and renal (Fig 3B) GR complexes of control in immature and mature mice after maximal specific saturable binding is attained In the

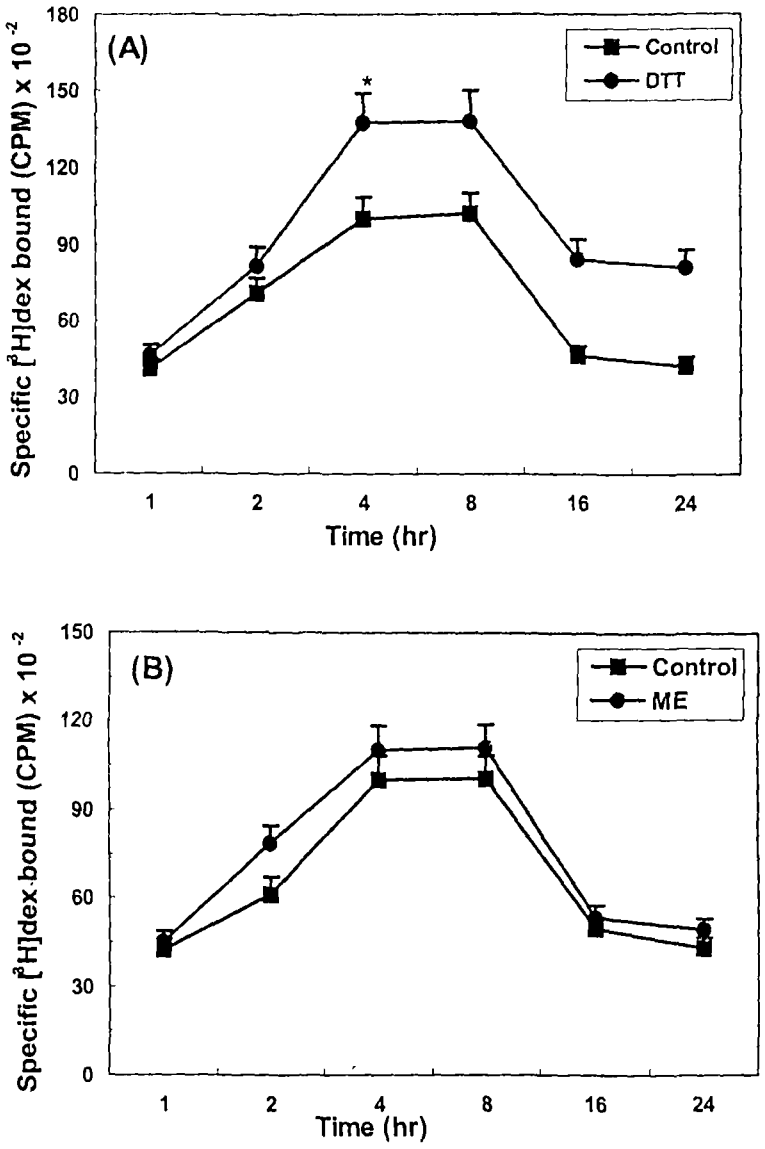


Figure 1. Effect of dithiothreitol (DTT) **(A)** and 2-mercaptoethanol (ME) **(B)** on the binding of [^{3}H]dexamethasone to **hepatic glucocorticoid receptors** and stabilization of hormone-receptor complexes. Aliquots of freshly prepared cytosol (100 µl) were incubated with 40 nM [^{3}H]dexamethasone ± 500-fold excess nonradioactive dexamethasone, and in the presence or absence(control) of 1 mM DTT or ME for 1-24 hr at 0°C. Unbound steroids were removed by dextran-coated charcoal (DCC) treatment as indicated in the materials and methods section. The values obtained from subtraction of non-specific binding from total binding represented the specific [^{3}H]dexamethasone bound to the receptor. Each data point represents mean ± standard deviation of 4 separate experiments performed in duplicate from 4-5 mice. *Statistically significant compared to control at 4 hr.

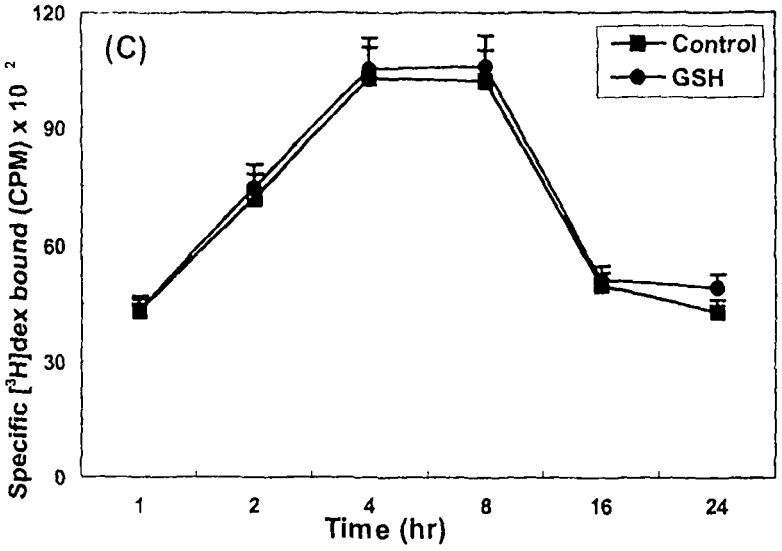


Figure 2. Effect of glutathione (GSH) on the binding of [^{3}H]dexamethasone to **hepatic glucocorticoid receptors** and stabilization of hormone-receptor complexes from mice. Cytosol preparation, hormone binding and further processing of the samples were performed as indicated in fig. 1. Results are mean ± standard deviation as given in fig. 1.

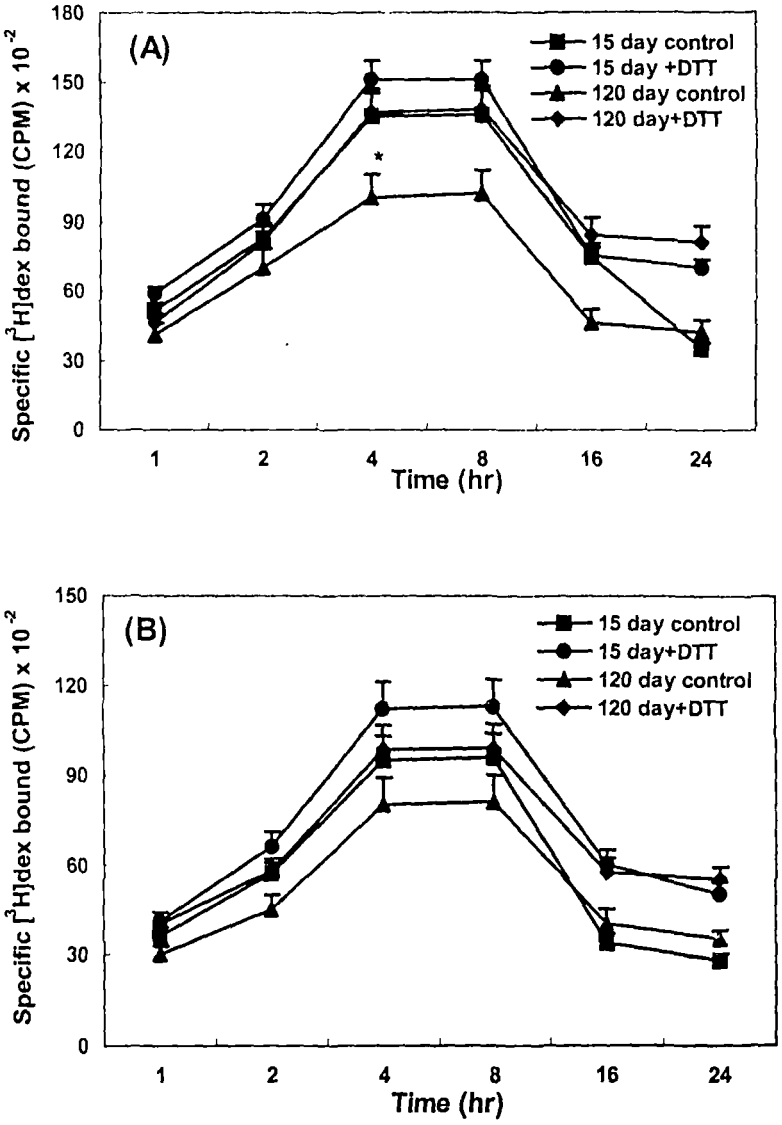


Figure 3. Effect of dithiothreitol (DTT) on the binding of [^{3}H]dexamethasone to glucocorticoid receptors and stabilization of hormone-receptor complexes from the liver **(A)** and kidney **(B)** of 15- and 120-day old mice. Cytosol preparation, hormone binding and subsequent processing of the samples were performed as indicated in fig. 1. Each data point represents mean ± standard deviation of 4 experiments performed each time in duplicate with 4-5 mice of each age group. *Statistically significant (P<0.05) compared to 15-day control.

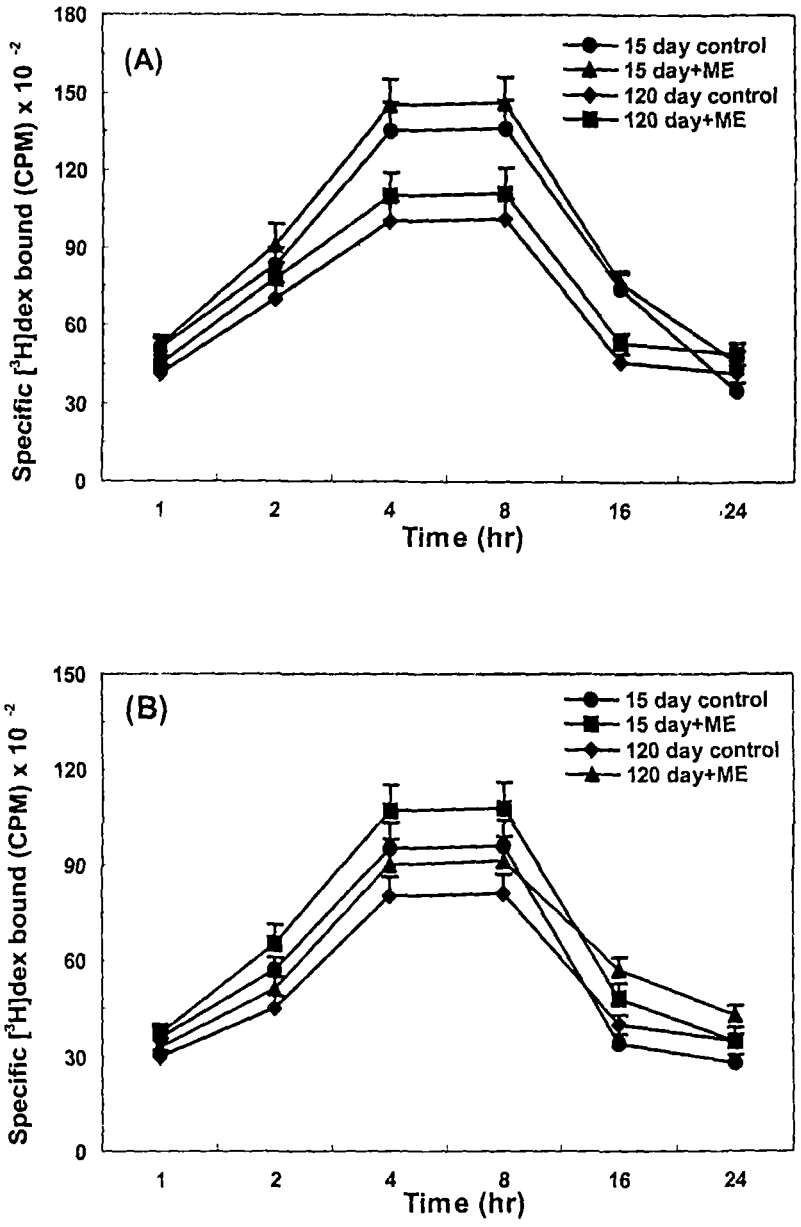


Figure 4. Effect of 2-mercaptoethanol (ME) on the binding of [^{3}H]dexamethasone to glucocorticoid receptors and stabilization of hormone-receptor complexes from the liver **(A)** and kidney **(B)** of 15- and 120-day old mice. Cytosol preparation, hormone binding and subsequent processing of the samples were performed as indicated in fig 1. Each data point represents mean ± standard deviation of 4 experiments performed each time in duplicate with 4-5 mice of each age group.

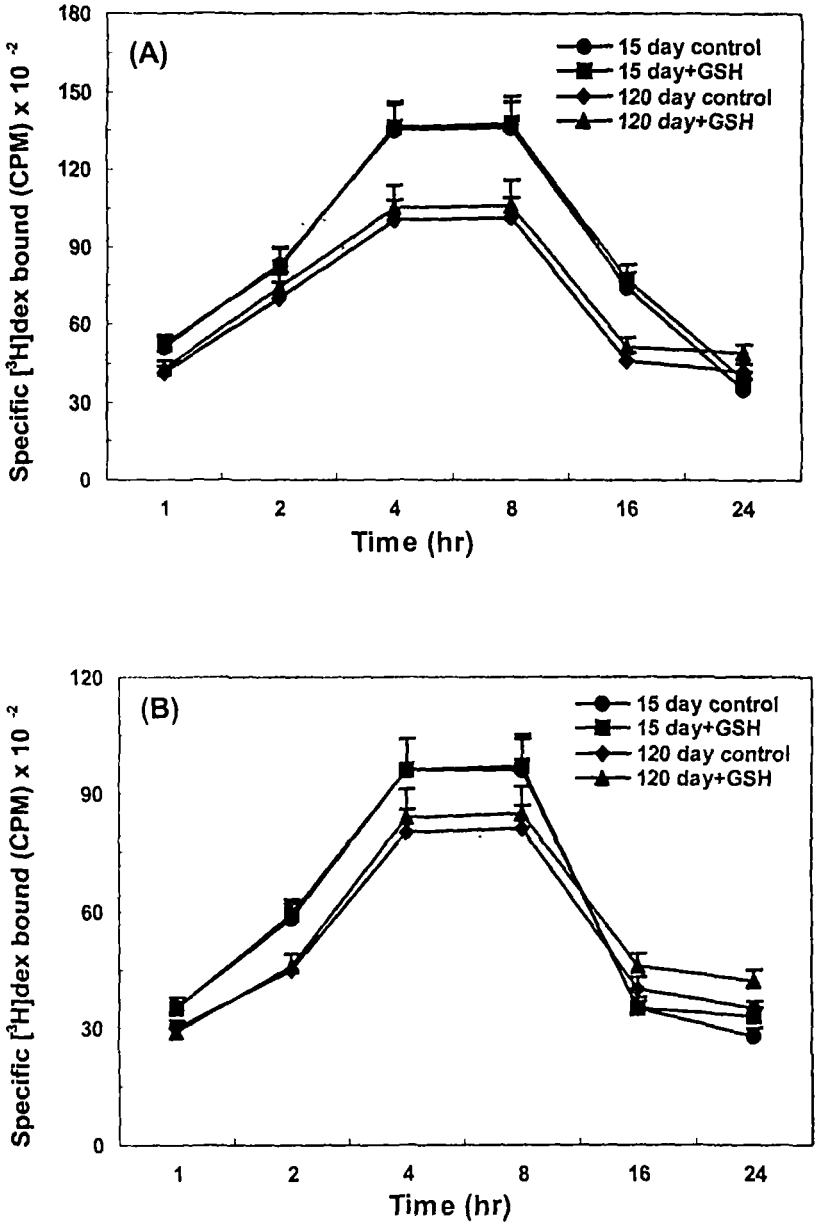


Figure 5. *Effect of glutathione (GSH) on the binding of [^{3}H]dexamethasone to glucocorticoid receptors* and stabilization of hormone-receptor complexes from the liver **(A)** and kidney **(B)** of 15- and 120-day old mice. Cytosol preparation, hormone binding and subsequent processing of the samples were performed as indicated in fig. 1. Each data point represents mean ± standard deviation of 4 experiments performed each time in duplicate with 4-5 mice of each age group.

presence of DTT, there were no apparent differences in the maximal specific binding and stabilization of liver (Fig. 3A) and kidney (Fig. 3B) H-R complexes of immature and mature animals. Similarly, both mercaptoethanol (Fig. 4A&B) and glutathione (Fig. 5A&B) also showed no alteration in specific binding of hormone to the receptor and the stabilization of H-R complexes from the liver and kidney of immature mice and mature mice. These studies therefore indicate no age- specific differences in hormone binding to GR and the stability of H-R complexes from both the tissues of immature and mature animals by these modulators.

Activation modulation of GR

Activation of GR was studied using heat and salt in the liver and kidney of 15- and 120- day old mice. DNA-cellulose and purified nuclei were utilized as acceptors of activated GR binding. Also, the modulation of hepatic GR activation process was studied in 120-day old mice using various endogenous and exogenous agents.

DNA-cellulose binding assays reveal that heat (25°C) significantly enhanced the activation of hormone-receptor (H-R) complexes from the liver (2.5-3.5 fold) (Fig. 6A) and kidney (Fig. 6B) (2.5 fold) of mice. Salt (20 mM Ca^{2+}) also enhanced the activation of H-R complexes at 0°C in the liver (Fig. 6A) (~3.3 fold) and kidney (Fig. 6B) (~3 fold). Since DNA-cellulose being a non-specific assay system, purified nuclei from both the tissues were utilized to mimic an *in vivo* assay system. Nuclear binding assay also exhibited increased activation of liver (Fig. 7A) and kidney (Fig. 7B) H-R complexes by heat and salt.

Experiments were also carried out in order to reveal any age- specific alteration in heat and salt activation of GR in the liver and kidney of immature (15-) and mature (120-day) mice. DNA-cellulose binding assay in liver (Fig. 6A) shows a higher magnitude (37%) of GR heat activation from mature animal as compared to immature ones, with no such differences in salt activation. Neither heat nor salt could reveal any age-related changes in GR activation in the kidney (Fig. 6B) of immature and mature mice. Nuclear binding assays also indicated a much higher (75%) degree of GR heat activation from mature mice's liver (Fig. 7A) as compared to immature ones, without any apparent differences in salt activation. In the kidneys, no alterations in the magnitude of GR activation by heat as well as salt were observed in immature and mature animals (Fig. 7B).

Hence, both DNA-cellulose and nuclear binding assays revealed a significant increase in heat and salt activation of GR from the liver and kidney of mice albeit, with a higher magnitude of heat activation from mature animal's liver compared to immature ones.

Cross-mixing experiments (i.e., binding of heat-activated H-R complexes from immature to nuclei of mature and *vice-versa*) were also carried out in the liver to reveal whether the observed increase in nuclear binding of heat-activated GR in mature mice's liver is due to

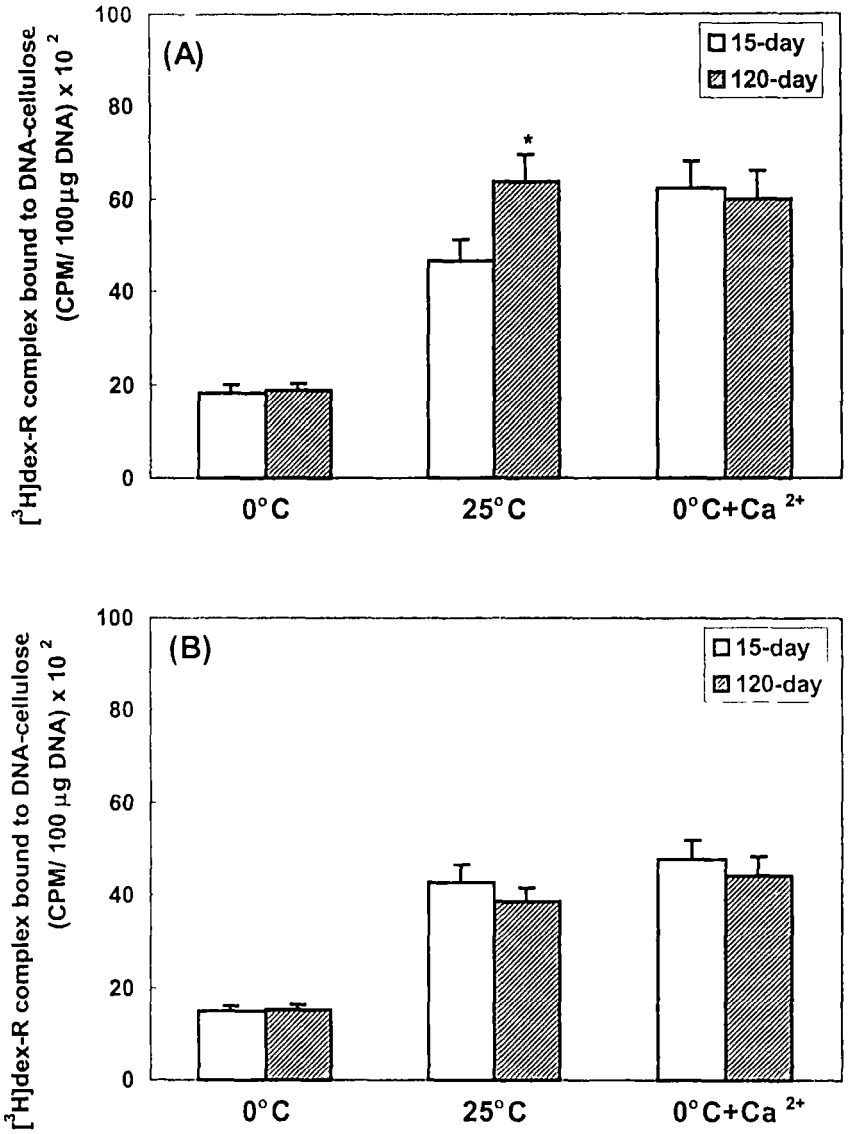


Figure 6 Binding of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes to **DNA-cellulose** in 15- and 120- day old mice Cytosols from these tissues were prepared in buffer B (I) and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C Activation conditions, DNA-cellulose binding and further processing of the pellets were performed as described in materials and methods The results are mean ± standard deviation of 4 experiments with 4-5 mice of each age group *Statistically significant (P<0 05) compared to 15- day old mice

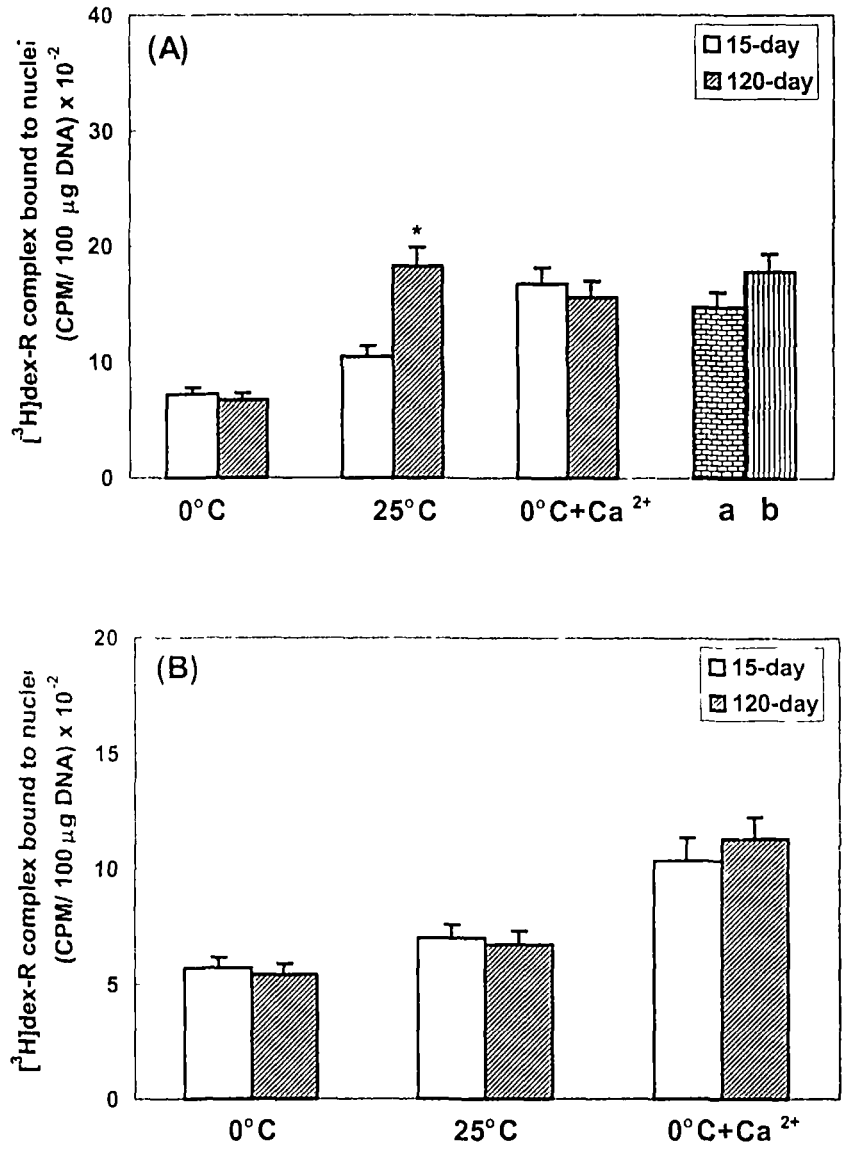


Figure 7 Binding of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes to **purified nuclei** in 15- and 120- day old mice. Purified nuclei of these tissues were used instead of DNA-cellulose for activation studies. Other experimental procedures are same as in figure 6. The **(a)** and **(b)** barograms in **(A)** represent cross-mixing experiments in which heat-activated hepatic GR complexes from 15-day old mice were incubated with nuclei of 120-day old **(a)** and heat activated GR complexes from 120-day mice with the nuclei of 15-day **(b)** The results are mean ± standard deviation of 4 experiments performed with 4-5 mice of each age group *Statistically significant (P<0.05) compared to 15- day old mice.

alteration(s) in the nuclear property. Result shows an increased nuclear binding of mature GR to the nuclei of immature animal (Fig. 7A). Hence, result obtained demonstrates that the higher magnitude of heat activation of mature mice's hepatic GR is due to alteration(s) in receptor property at this phase of life span.

Next, the activation modulation of GRs by utilizing various exogenous and endogenous agents was studied to see their modulatory effects in the liver of 120-day old mice. To examine the effect of cadmium (Cd^{2+}) on receptor activation by heat, a concentration of 0-4 mM Cd^{2+} was used. Result exhibited a dose-dependent inhibition (maximally 60% at 2 mM) of heat activation of H-R complexes from the liver of mice as assessed by binding to DNA-cellulose (Fig. 8A) and purified nuclei (Fig. 8B). Selenite (SeO_3^{2-}), a strong oxidant and a modifier of protein thiol groups, was used to reveal any modulatory effect on heat activation of hepatic GR, Results indicate that selenite when used at a concentration range from 0-8 mM, also inhibited the heat activation of hepatic GR, as assessed by binding to DNA-cellulose (Fig. 9A) and purified nuclei (Fig. 9B), to a magnitude of maximally 50% at 4 mM. Arsenite (AsO_2^-) was also utilized to see any modulatory effect on hepatic GR activation, as it was known that arsenite is a potent modifier of thiol groups in GR as well as in other proteins and enzymes. Arsenite (0-8 mM) caused a maximal inhibition (40%) of hepatic GR heat activation at 4 mM by DNA-cellulose (Fig. 10A) and nuclear binding assays (Fig. 10B).

Leupeptin, a bacterial peptide, is a potent inhibitor of ser/cys proteases and also a potent stabilizer of untransformed (non-DNA binding form) GR in cytosols. The effect of leupeptin on heat activation of GR was tested when used at a concentration range from 0-40 mM. Data revealed a significant inhibition of heat activation of hepatic GR by leupeptin, maximally (45-50%) at 20 mM by DNA-cellulose (Fig. 11A) and nuclear binding assays (Fig. 11B).

Polyunsaturated fatty acids (PUFAs) have also been used to study their modulatory role on *in vitro* receptor activation modulation. PUFAs such as oleic acid (C18:1), linoleic acid (C18:2) and arachidonic acid (C20:4) at a concentration range from 0-200 μM were used to study their modulatory effects on receptor activation in the liver of 120-day old mice. Interestingly, all the three PUFAs were found to be potential inhibitors of heat activation of hepatic GR. Oleic acid (C18:1) caused 38% maximal inhibition of heat activation at 40 μM as assessed by DNA-cellulose (Fig. 12A) and nuclear (Fig. 12B) binding assays. Linoleic acid (C18:2) also inhibited the heat activation of hepatic GR, albeit to a greater magnitude (~70% at 160 μM) compared to oleic acid (Fig. 13 A&B). Arachidonic acid (20:4) was also found to be a potential inhibitor of hepatic GR heat activation by both DNA-cellulose (Fig. 14A) and purified nuclei (Fig. 14B) binding assays, achieving a maximal inhibition of ~70% at 160 μM, similar to linoleic acid.

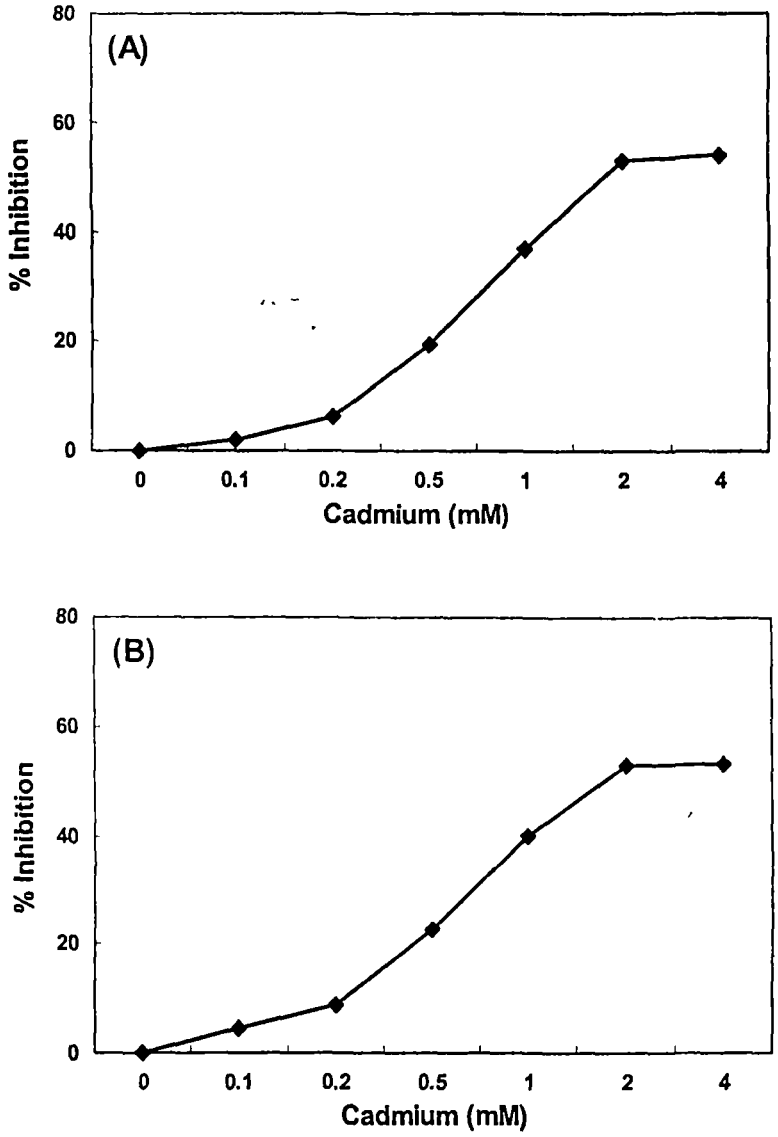


Figure 8. Effect of cadmium (Cd^{2+}) on the heat (25°C) activation of hepatic [^{3}H]dexamethasone-receptor complexes, as assessed by DNA-cellulose **(A)** and purified nuclear **(B)** binding assays. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of cadmium. DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Heat activation in the absence (control) of cadmium is taken as 0% inhibition.

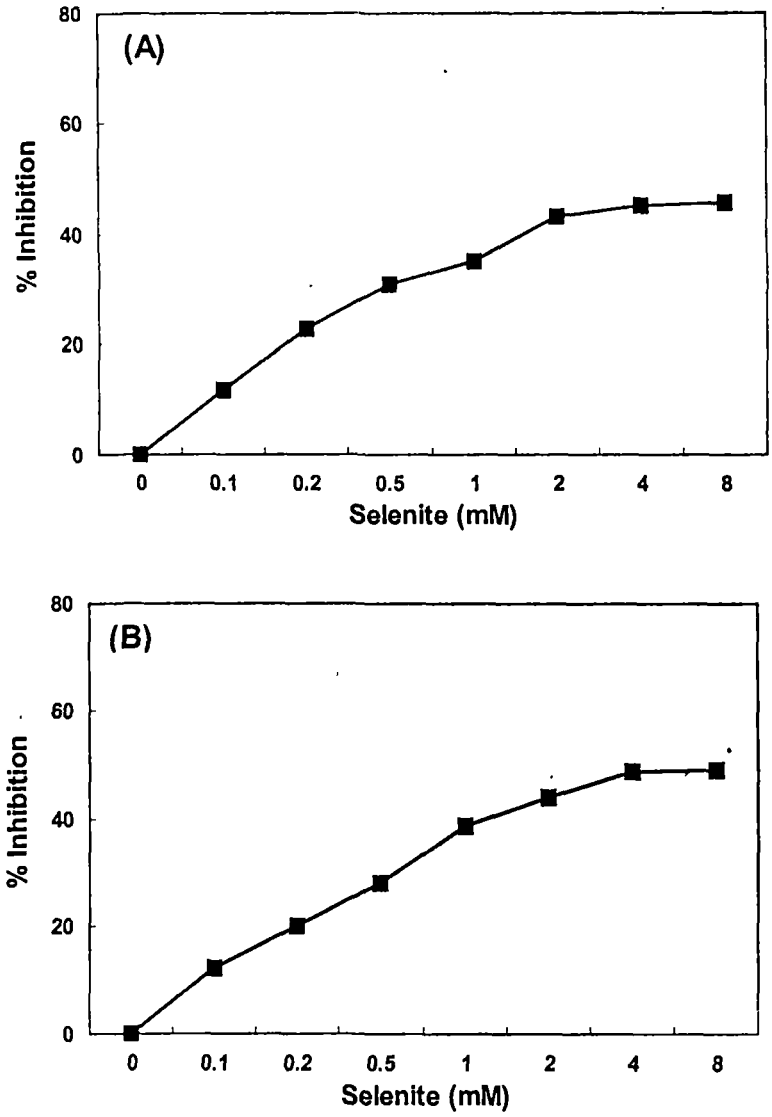


Figure 9. Effect of selenite (SeO_3^{2-}) on the heat (25°C) activation of hepatic [^{3}H]dexamethasone-receptor complexes, as assessed by binding to DNA-cellulose **(A)** and purified nuclei **(B)**. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of selenite. DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Heat activation in the absence (control) of selenite is taken as 0% inhibition.

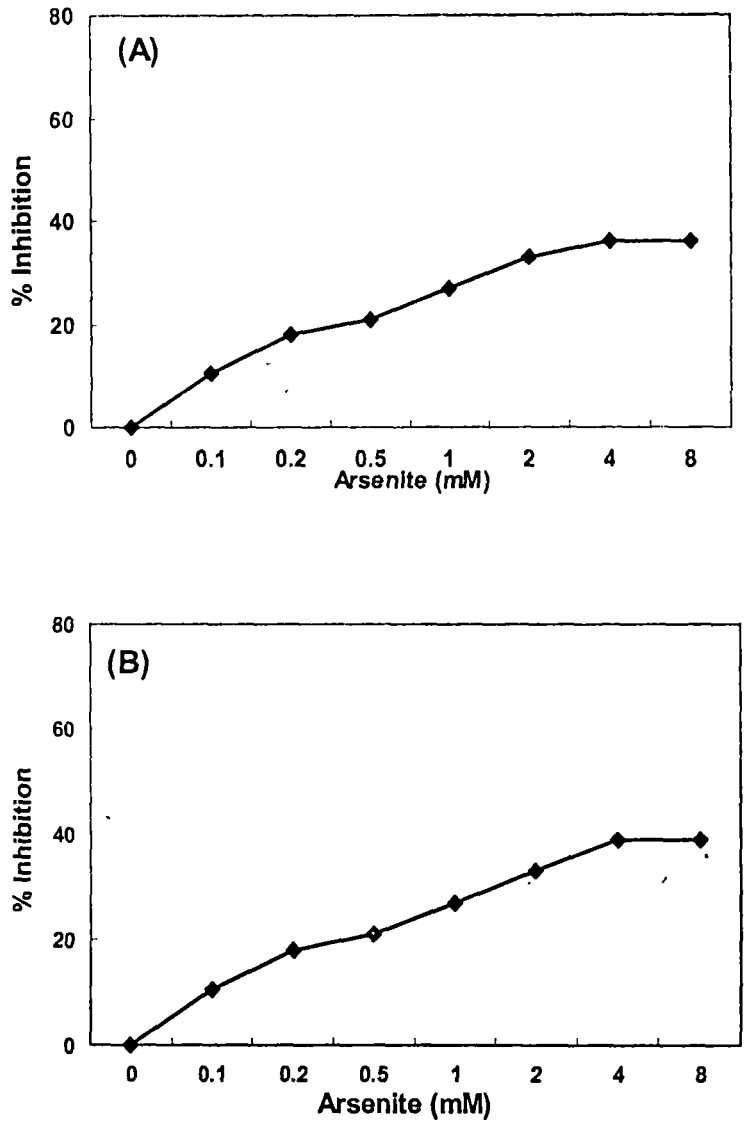


Figure 10. Effect of arsenite (AsO_2^-) on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes, as assessed by binding to DNA-cellulose **(A)** and purified nuclei **(B)**. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of arsenite. DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Heat activation in the absence (control) of arsenite is taken as 0% inhibition.

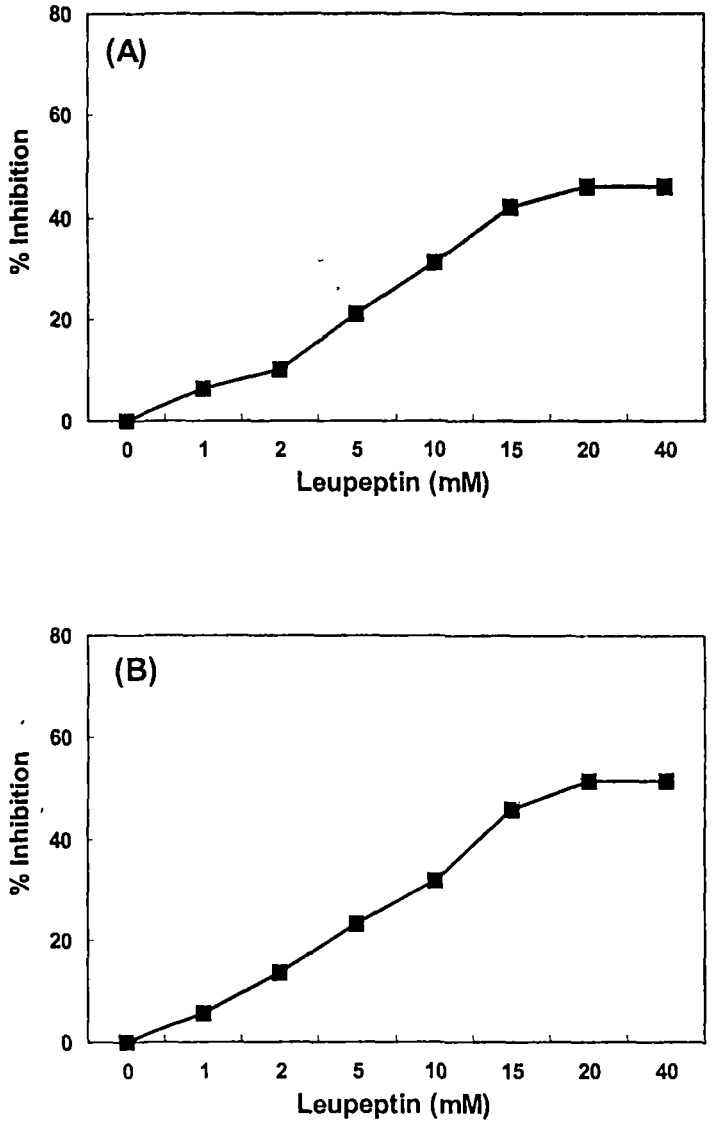


Figure 11. Effect of leupeptin on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes, as assessed by binding to DNA-cellulose **(A)** and purified nuclei **(B)**. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of leupeptin. DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Heat activation in the absence (control) of leupeptin is taken as 0% inhibition.

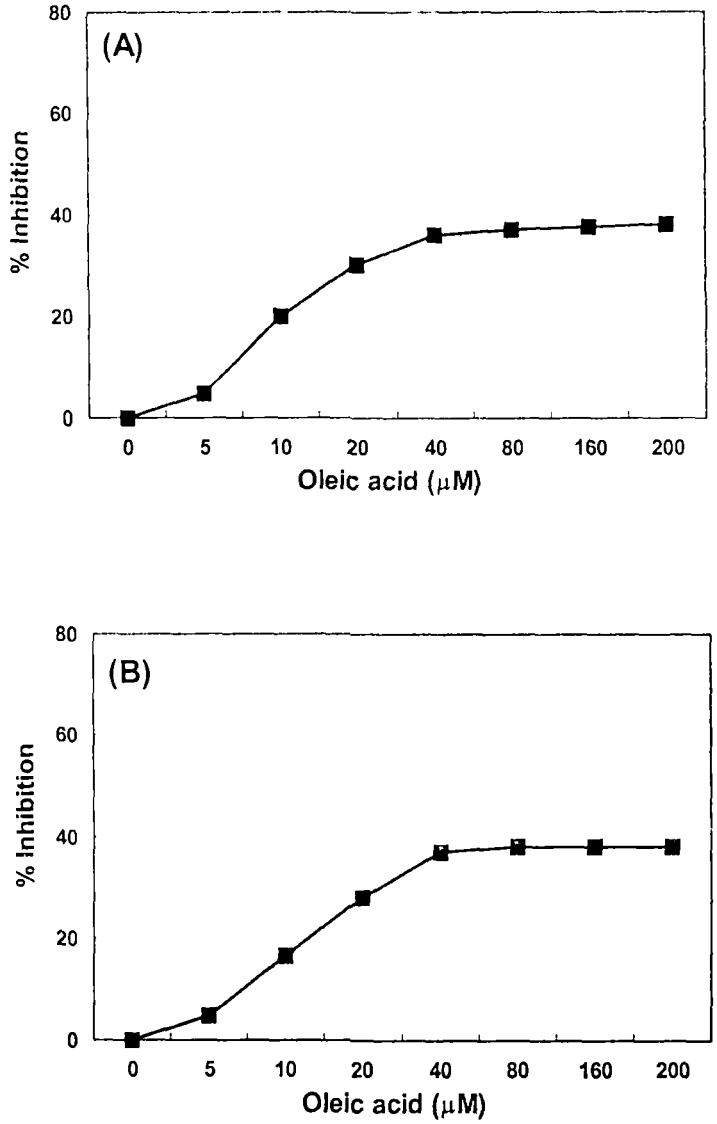


Figure 12. Effect of oleic acid (C18:1) on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes, as assessed by DNA-cellulose **(A)** and purified nuclear **(B)** binding assays. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of oleic acid (prepared as stock in dimethyl sulfoxide, DMSO). DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Control tubes received appropriate volume of DMSO instead of oleic acid. Heat activation in the absence (control) of oleic acid is taken as 0% inhibition.

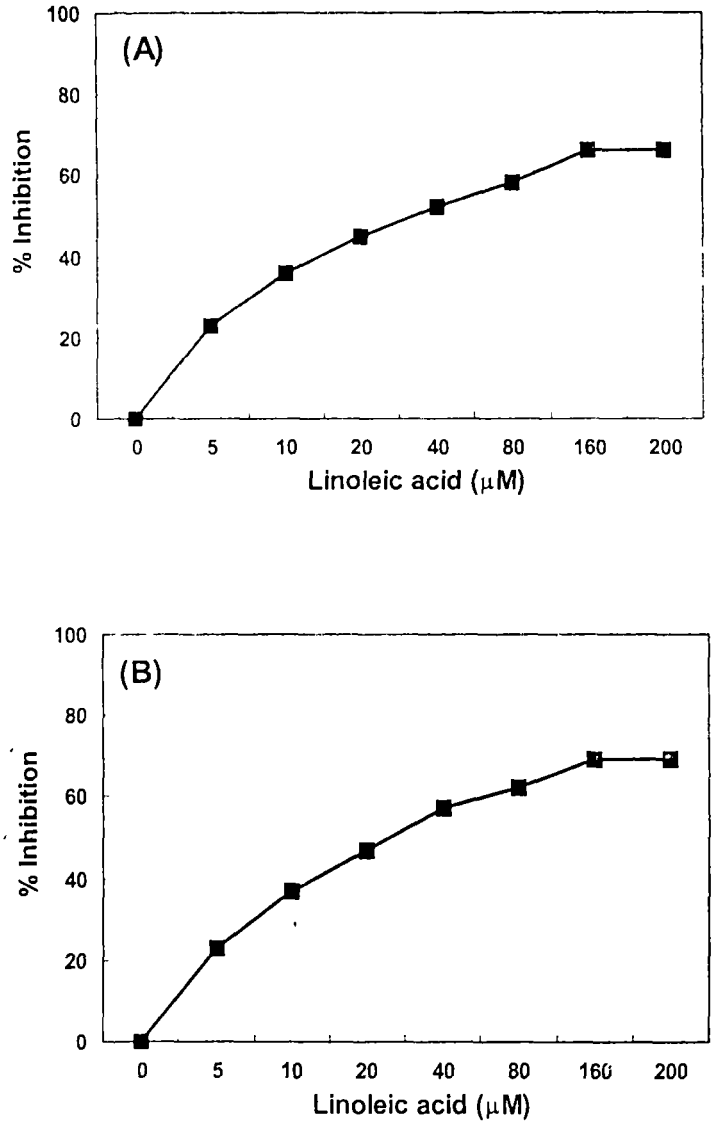


Figure 13. Effect of linoleic acid (C18:2) on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes, as assessed by DNA-cellulose **(A)** and purified nuclear **(B)** binding assays. Cytosol from liver was prepared in buffer B (I) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of linoleic acid (prepared as stock in dimethyl sulfoxide, DMSO). DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section Control tubes received appropriate volume of DMSO instead of linoleic acid. Heat activation in the absence (control) of linoleic acid is taken as 0% inhibition.

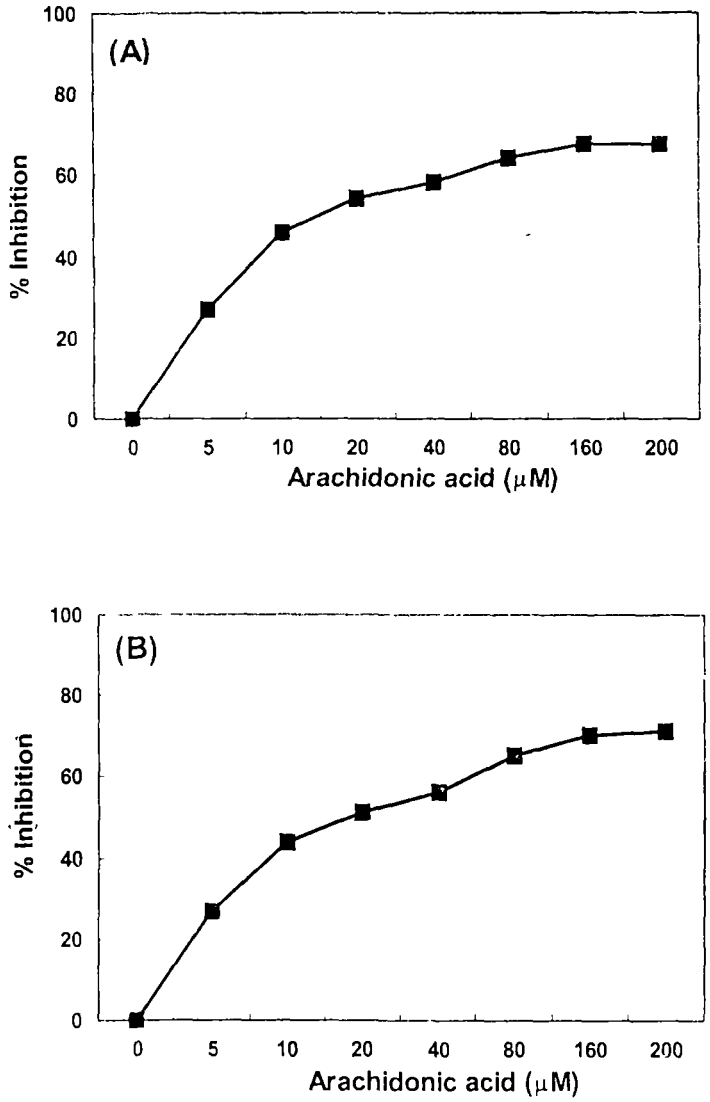


Figure 14. Effect of arachidonic acid (C20:4) on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes, as assessed by DNA-cellulose **(A)** and purified nuclear **(B)** binding assays. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of arachidonic acid (prepared as stock in dimethyl sulfoxide, DMSO). DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Control tubes received appropriate volume of DMSO instead of arachidonic acid. Heat activation in the absence (control) of arachidonic acid is taken as 0% inhibition.

Results indicate an increase in the magnitude of inhibition of hepatic GR activation concomitant to increase in chain length and unsaturation of PUFAs to a certain limit.

Pyrophosphates (PPi's) have been attributed to play important physiological and biochemical roles in animals. In this study, we have used tetrasodium pyrophosphate ($Na_4P_2O_7$) at a concentration range from 0-16 mM to see its effect on hepatic GR activation of 120-day old mice. $Na_4P_2O_7$ turned out to be an inducer of hepatic GR activation at 0°C, with maximum activation of ~65% at 8 mM, using both DNA-cellulose (Fig. 15A) and nuclear (Fig. 15B) binding assays.

All the above modulators of activation were also used to study the modulation of heat activation of liver and kidney GR from immature (15-) and mature (120- day) mice to see any tissue- and/or age-specific modulation. Cadmium was used at increasing concentration (0-4 mM) to see its inhibitory effect on GR heat activation in the liver and kidney of immature and mature mice. DNA-cellulose binding assay shows similar pattern of inhibition (~60% at 2 mM) of hepatic (Fig. 16A) and kidney (Fig. 16B) GR heat activation by cadmium in both the ages studied. Nuclear binding assay also revealed a similar pattern of inhibitory effect by cadmium in both the ages in the liver (Fig. 17A) and kidney (Fig. 17B) of mice. Selenite (0-8 mM), caused a maximal inhibition (~50% at 4 mM) of GR heat activation, but could not show any difference in % inhibition from the liver (Fig. 18A) and kidney (Fig. 18B) of immature and mature mice by DNA-cellulose binding assay, with similar result in the case of nuclear binding assay (Fig. 19A&B).

Arsenite (0-8 mM) also inhibited GR heat activation maximally 40% at 4 mM in both the tissues and ages albeit, to a similar extent as assessed by DNA-cellulose (Fig. 20 A&B) and nuclear (Fig. 21 A&B) binding assays. Leupeptin (0-40 mM) was also used to reveal any differences in GR heat activation inhibition in both the tissues and ages. However, both DNA-cellulose (Fig. 22 A&B) and nuclear (Fig. 23 A&B) binding assays exhibited similar extent of inhibition (~45-50% at 20 mM) in both the tissues and ages.

PUFAs (oleic, linoleic and arachidonic acid, 0-200 μM) were also employed to show any differences in tissue- and age- specific inhibition of heat activation. Oleic acid caused similar extent of inhibition (~38% at 40 μM) of heat activation of hepatic and renal GR in both the ages as observed by DNA-cellulose binding assay (Fig. 24 A&B), with a similar extent using in nuclear (Fig. 25 A&B) binding assay. Linoleic acid exhibited ~70% inhibition at 160 μM of hepatic and renal GR heat activation, albeit to a similar degree in both the ages studied using DNA-cellulose (Fig. 26 A&B) and nuclear (fig. 27 A&B) binding assays. Inhibition of heat activation of GR by arachidonic acid also showed a similar pattern (~70% at 160 μM) in both

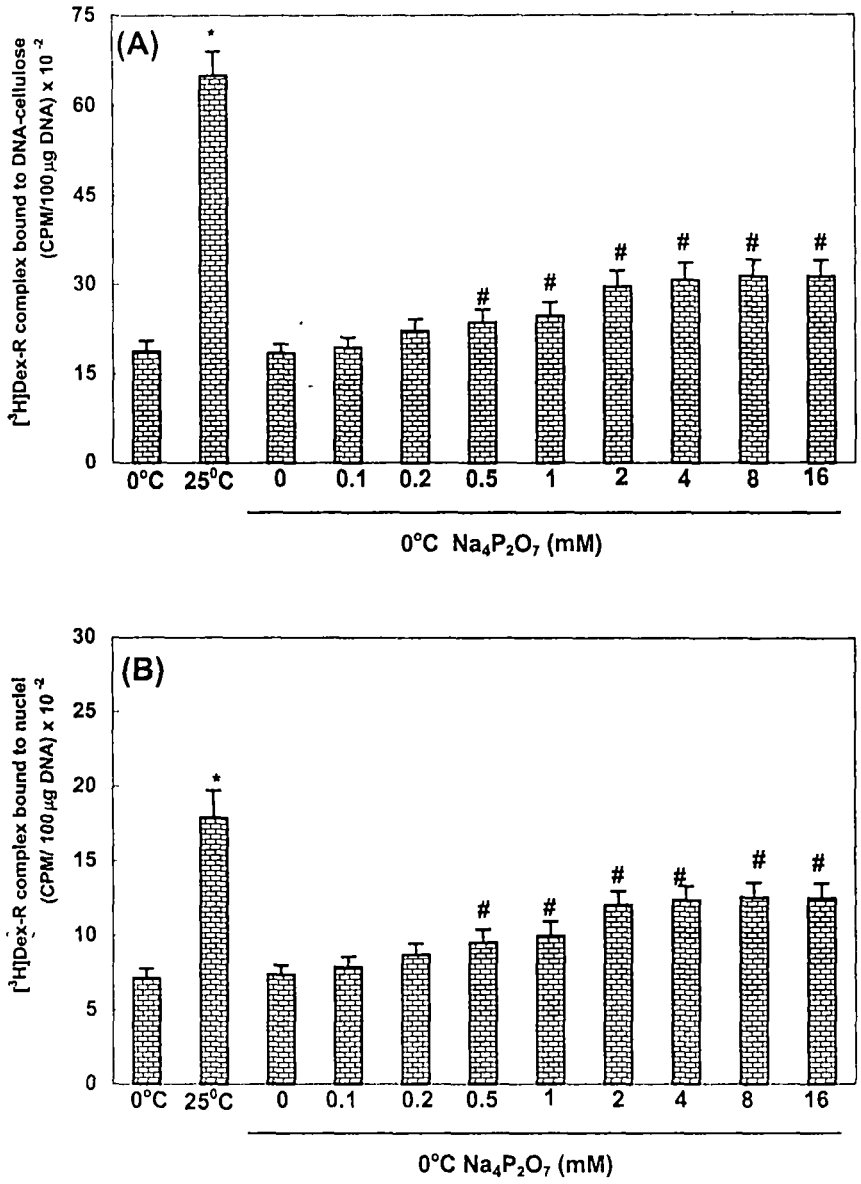


Figure 15. *Effect of tetrasodium pyrophoshate ($Na_4P_2O_7$) on the activation of hepatic* [^{3}H]*dexamethasone-receptor* complexes of mice using DNA-cellulose **(A)** and nuclear **(B)** binding assays. Cytosol from liver was prepared and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C. Hormone-receptor (H-R) complexes were then incubated with increasing concentrations of pyrophosphate [prepared as 0.1-1 . M stock in buffer B (i)] for 45 min at 0°C, control tubes received buffer B (i) only. Additionally, aliquots of H-R complexes were also incubated at 0°C and 25°C separately for 45 min, to yield unactivated and heat activated receptor complexes, respectively. The magnitude of activation was then assessed by allowing the H-R complexes to bind to DNA-cellulose and purified nuclear pellets for 60 min at 0°C. Further processing of the pellets was done as described in materials and methods in the text. Results are mean ± standard deviation of 4 separate experiments with 4-5 mice. *Significant (P<0.01) heat activation compared to 0°C. #Significant activation (P<0.05) compared to control.

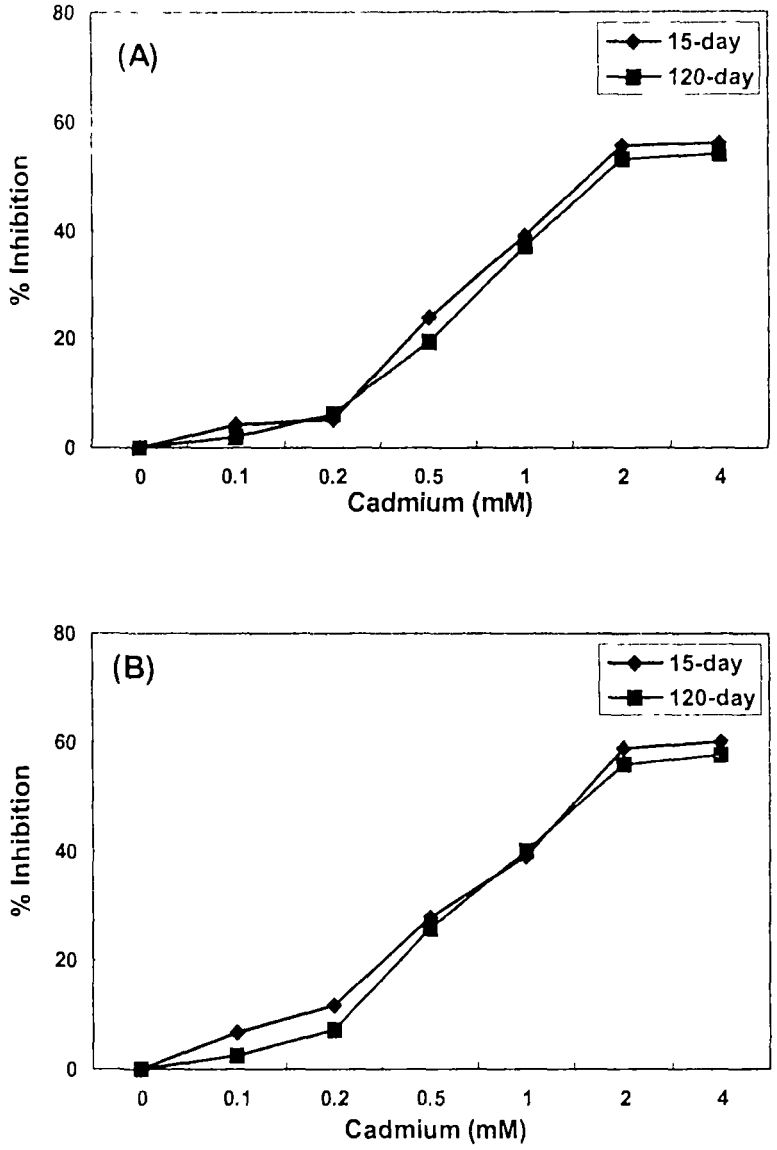


Figure 16. Effect of cadmium (Cd^{2+}) on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **DNA-cellulose binding assay**. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig.8.

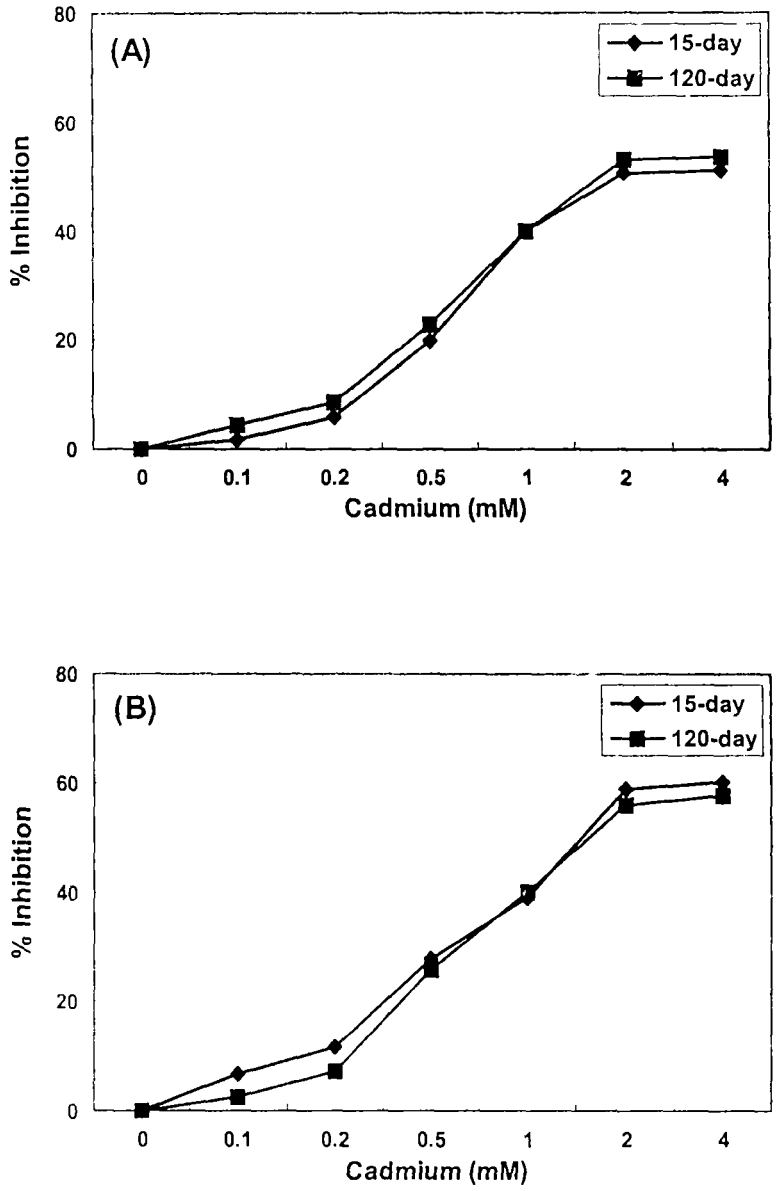


Figure 17. Effect of cadmium (Cd^{2+}) on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **nuclear binding assay**. Experimental procedures and assay conditions are similar as given in fig. 8.

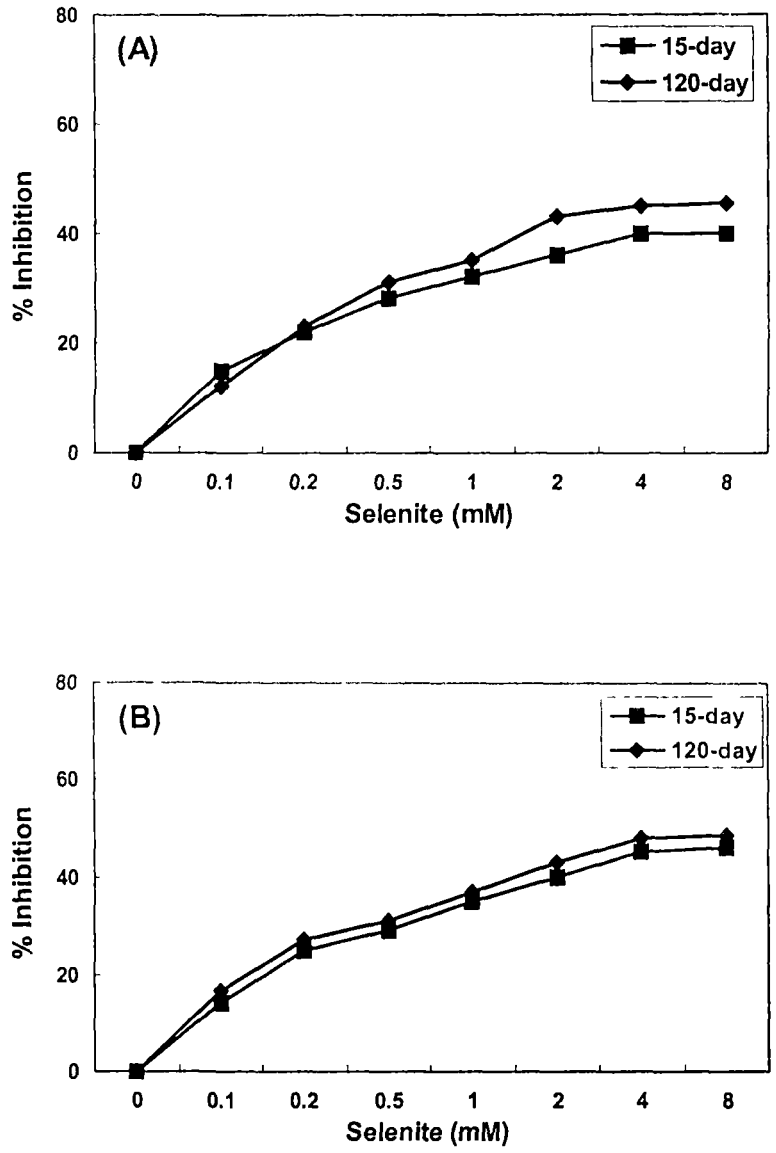


Figure 18. Effect of selenite on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **DNA-cellulose binding assay**. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 9

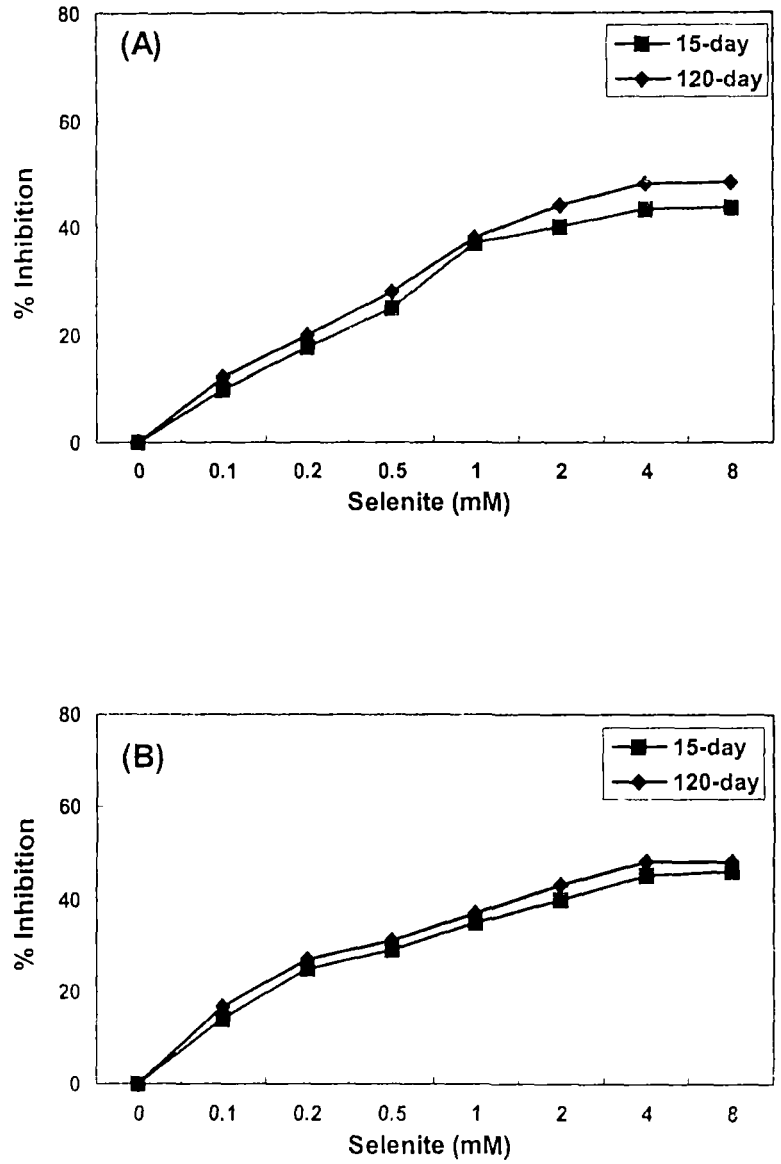
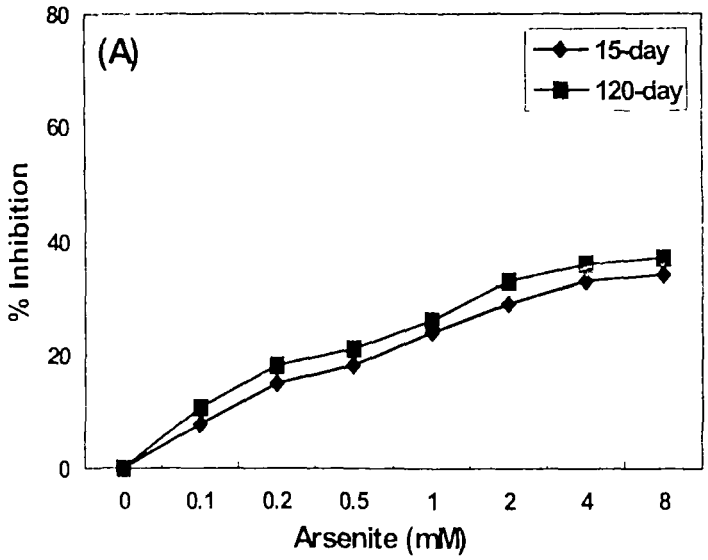


Figure 19. Effect of selenite on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **nuclear binding assay**. Details of experimental procedures and assay conditions are as given in fig. 9.



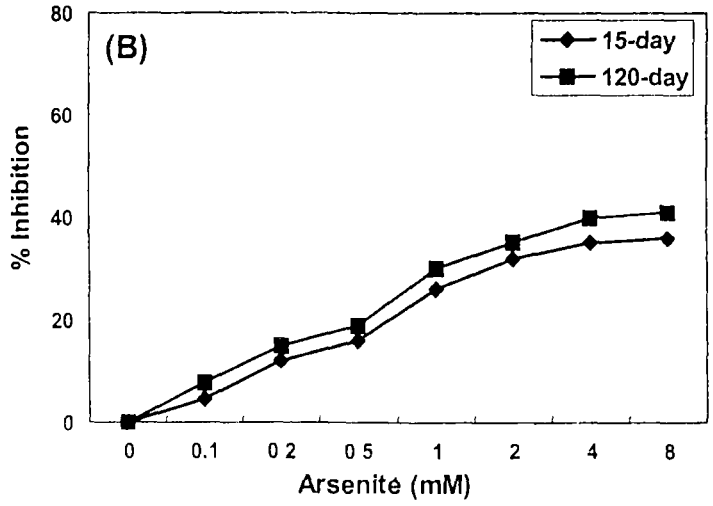


Figure 20 Effect of arsenite on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **DNA-cellulose binding assay**. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 10.

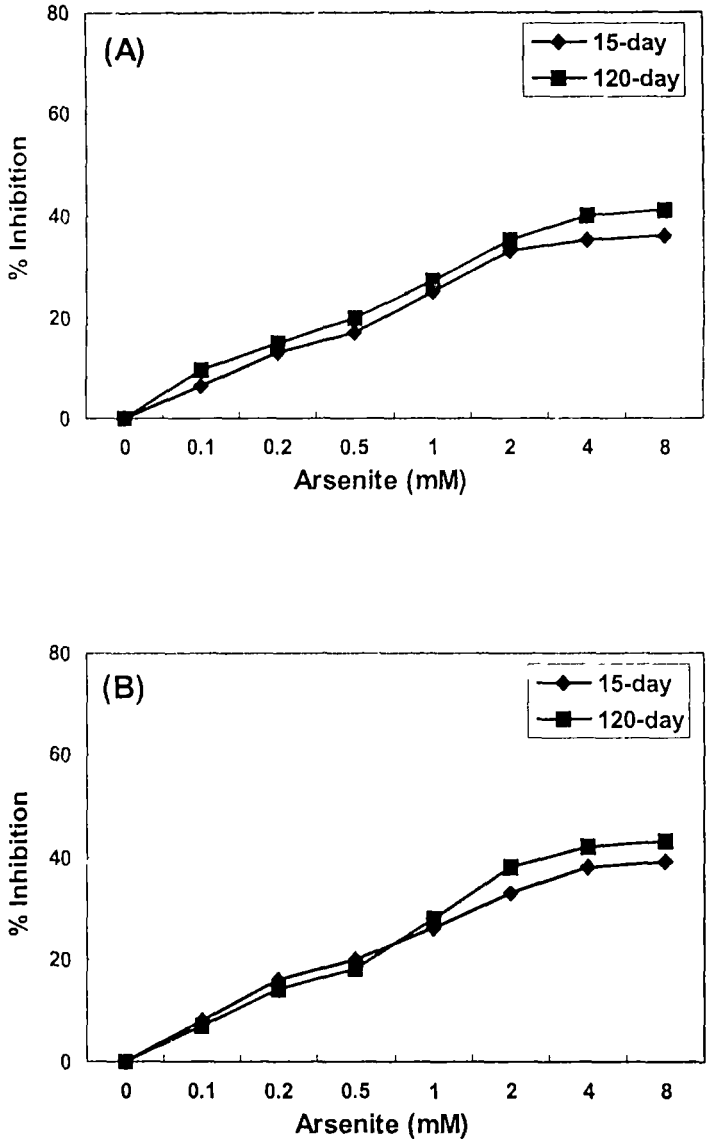
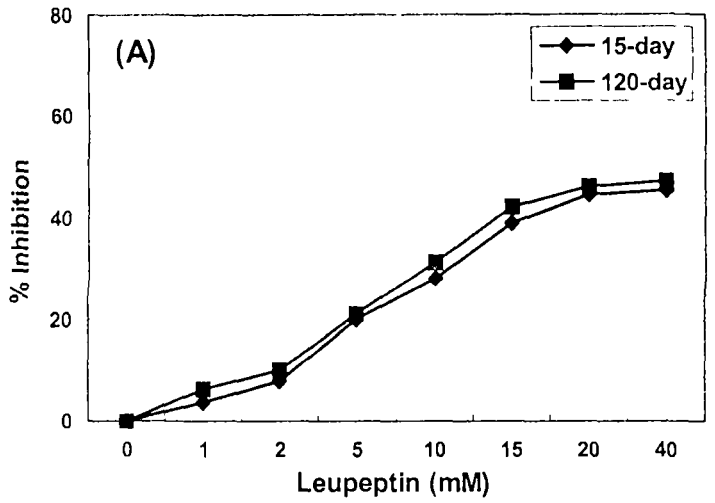


Figure 21. Effect of arsenite on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **nuclear binding assay**. Receptor preparation and activation procedures are same as described in fig. 10.



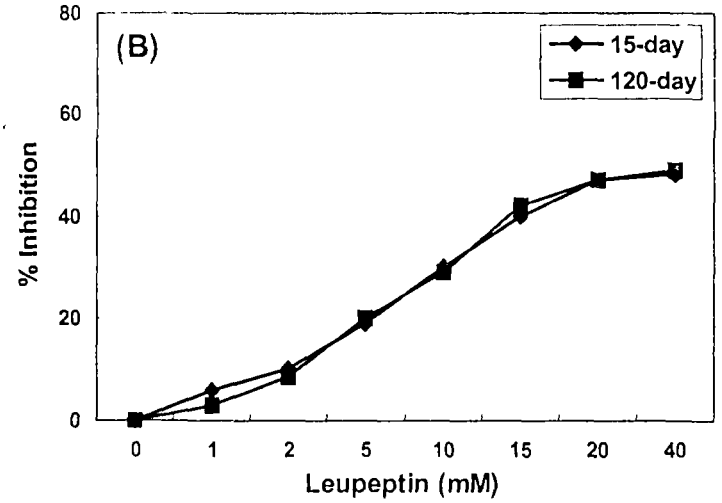


Figure 22. Effect of leupeptin on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor, complexes from 15- and 120-day old mice by **DNA-cellulose binding assay**. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 11.

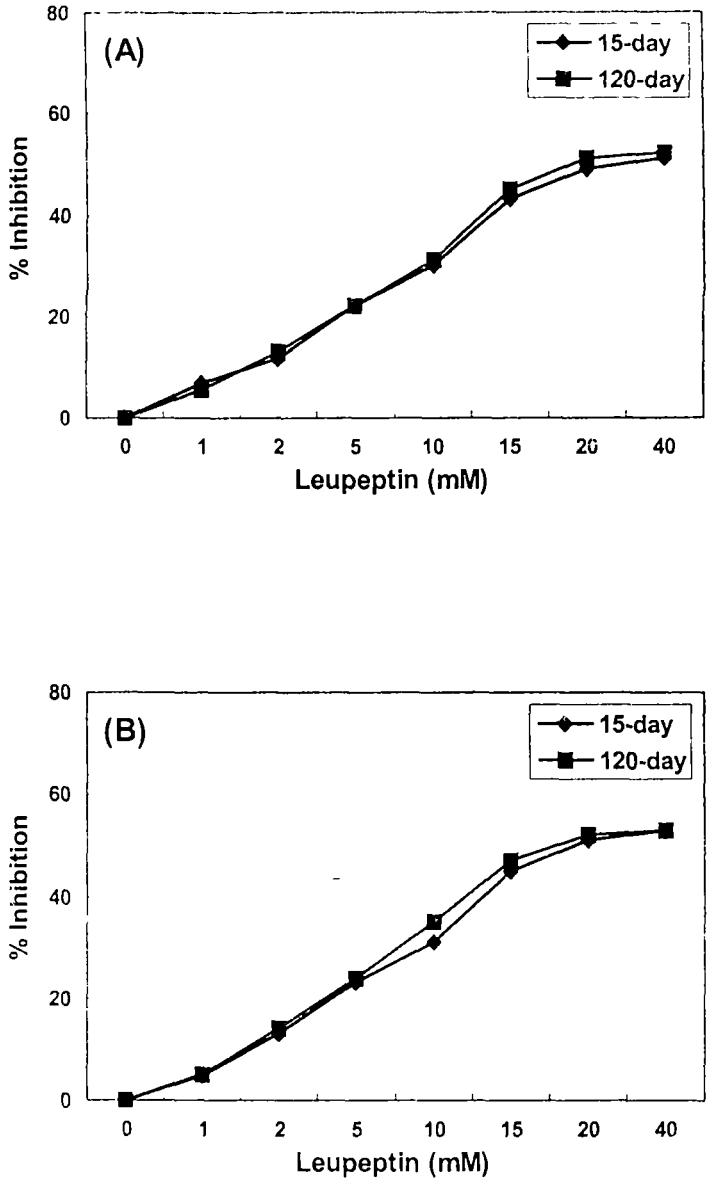
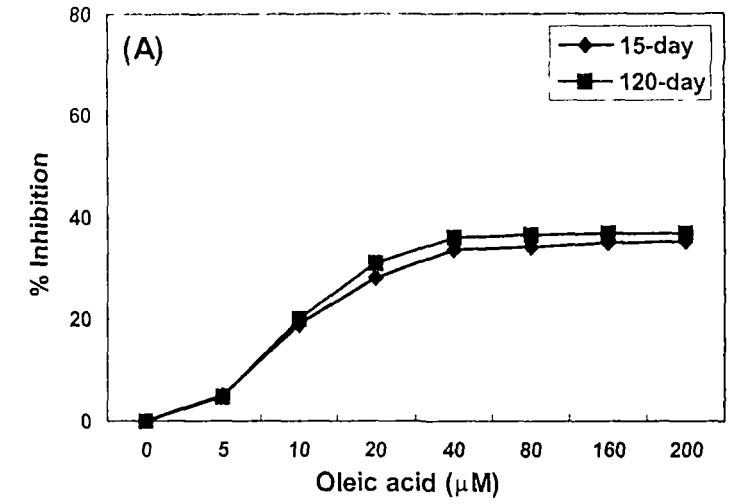


Figure 23. Effect of leupeptin on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **nuclear binding assay**. Receptor preparation and activation conditions are similar as given in fig. 11.



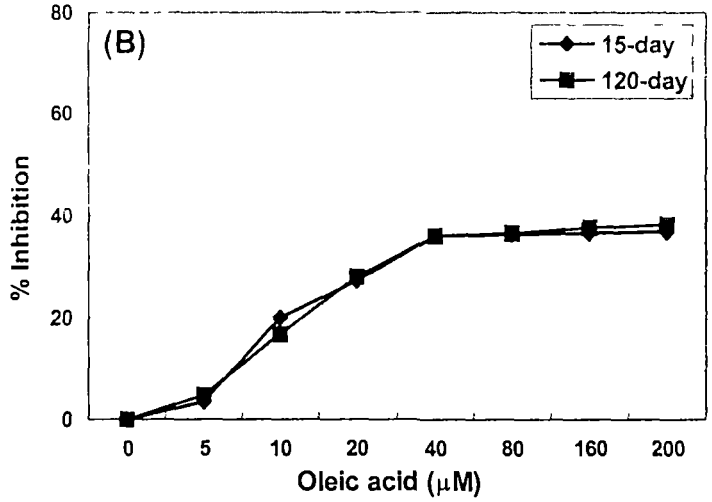


Figure 24 Effect of oleic acid (C18:1) on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **DNA-cellulose binding assay**. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig.12.

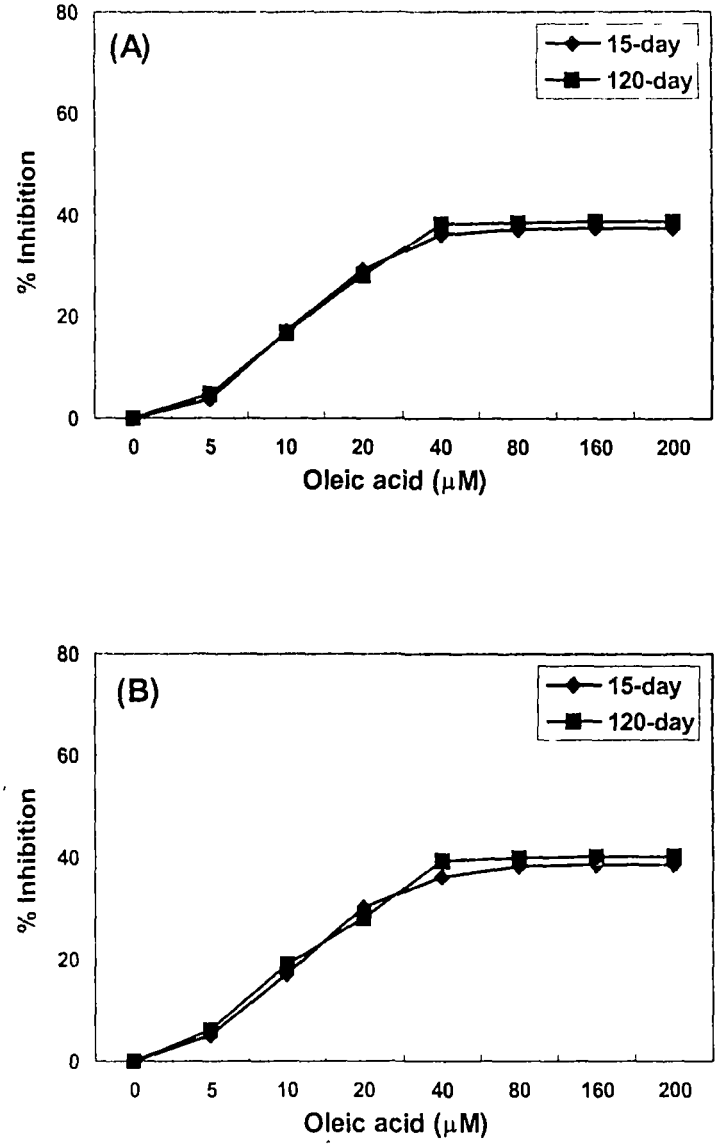


Figure 25. Effect of oleic acid (C18:1) on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **nuclear binding assay**. The details of procedures and experimental assay conditions are similar to fig. 12.

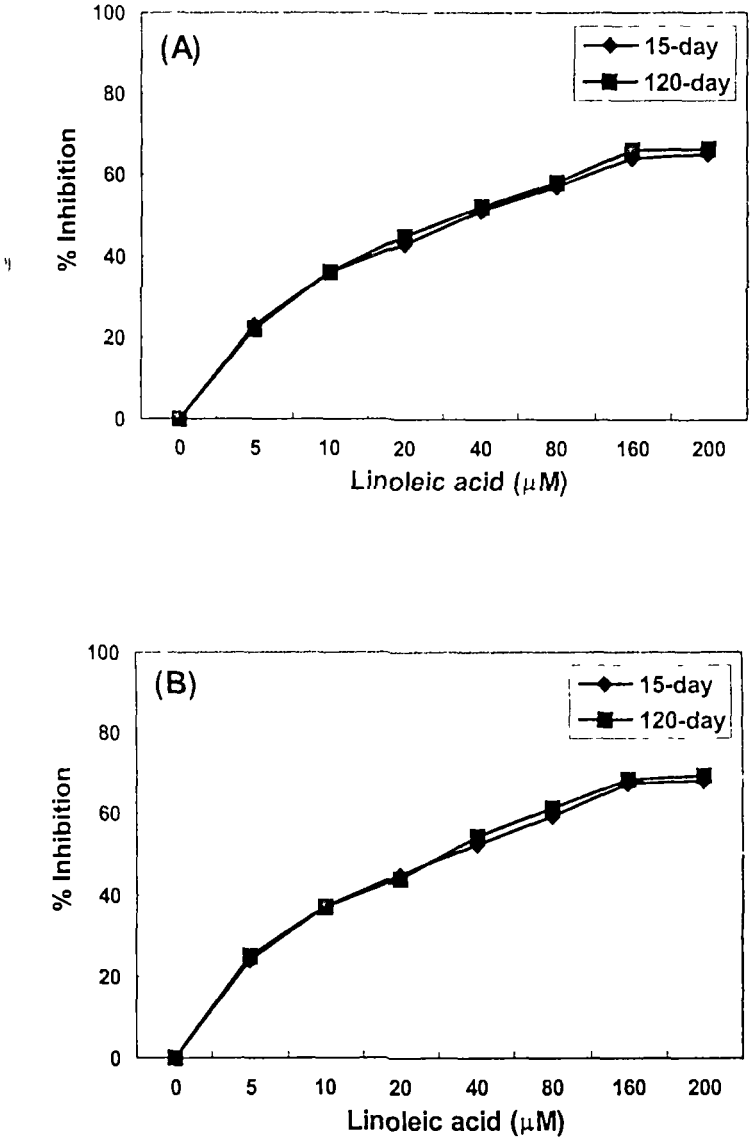


Figure 26. Effect of linoleic acid (C18:2) on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **DNA-cellulose binding assay**. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig 13

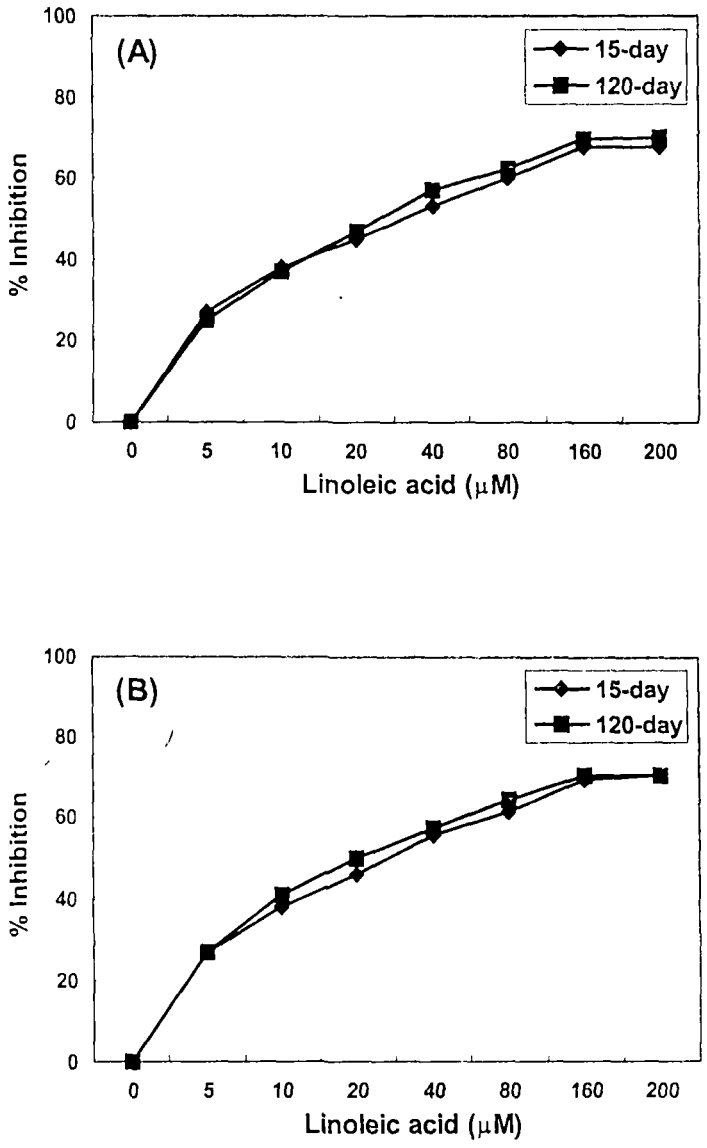
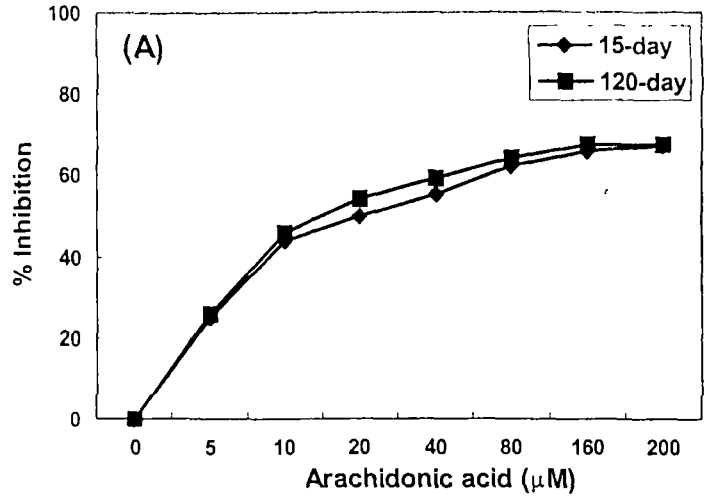


Figure 27. Effect of linoleic acid (C18:2) on the 'heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **nuclear binding assay**. Experimental procedures are same as detailed in fig. 13.



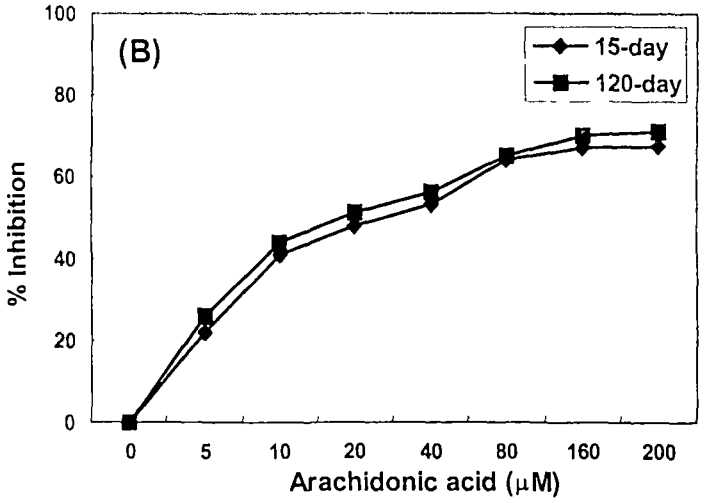
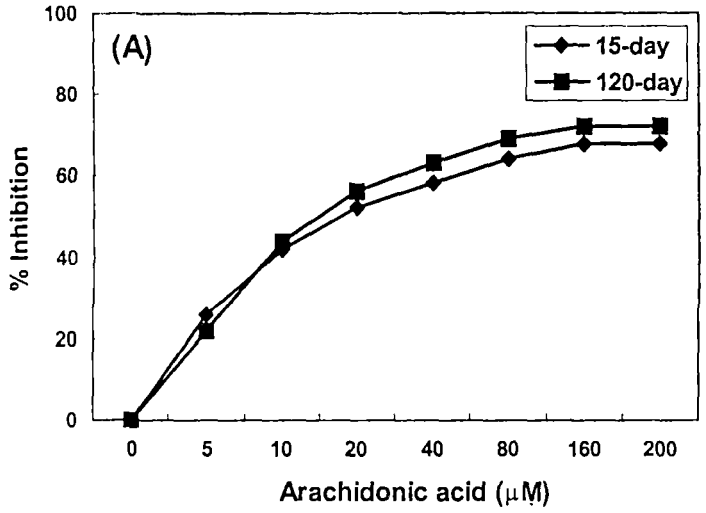


Figure 28. Effect of arachidonic acid (C20:4) on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **DNA-cellulose binding assay**. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 14.



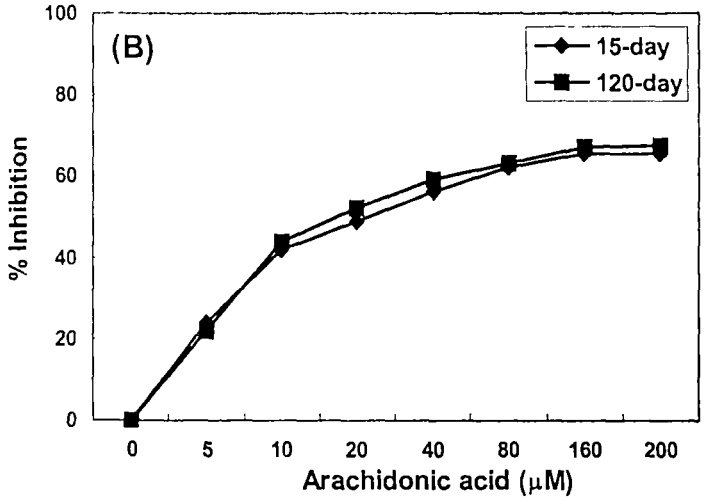


Figure 29. Effect of arachidonic acid (C20:4) on the heat activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes from 15- and 120-day old mice by **nuclear binding assay**. Details of experimental procedures are as given in fig. 14.

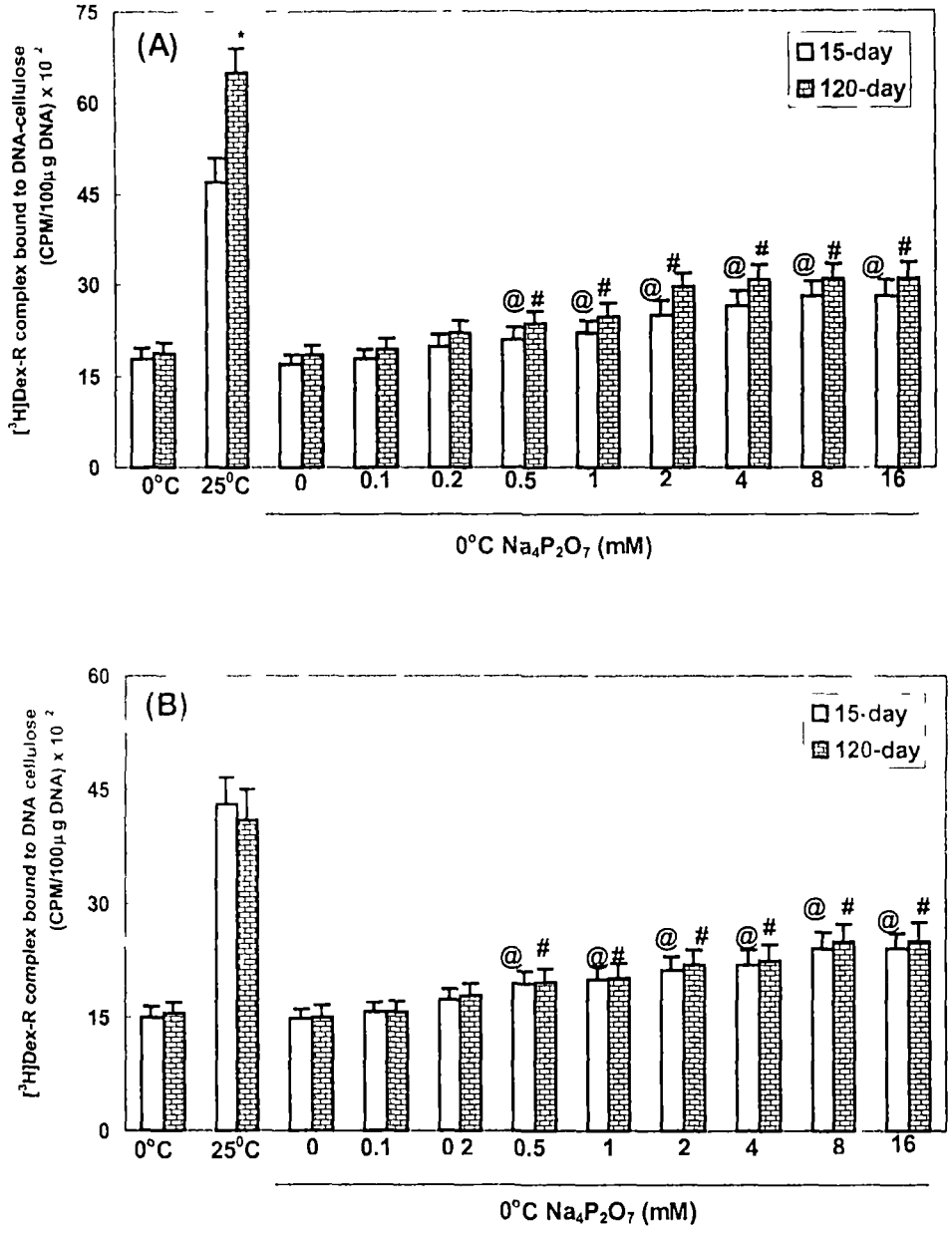


Figure 30 Effect of tetrasodium pyrophoshate ($Na_4P_2O_7$) on the activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes of 15- and 120-day old mice by using **DNA-cellulose** assay Cytosol preparation, activation conditions, binding to DNA-cellulose pellets and further processing of pellets are similar to those indicated in fig 15 Results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group *Significant (P<0 01) heat activation compared to 0°C #@Significant activation (P< 0 05) compared to respective control

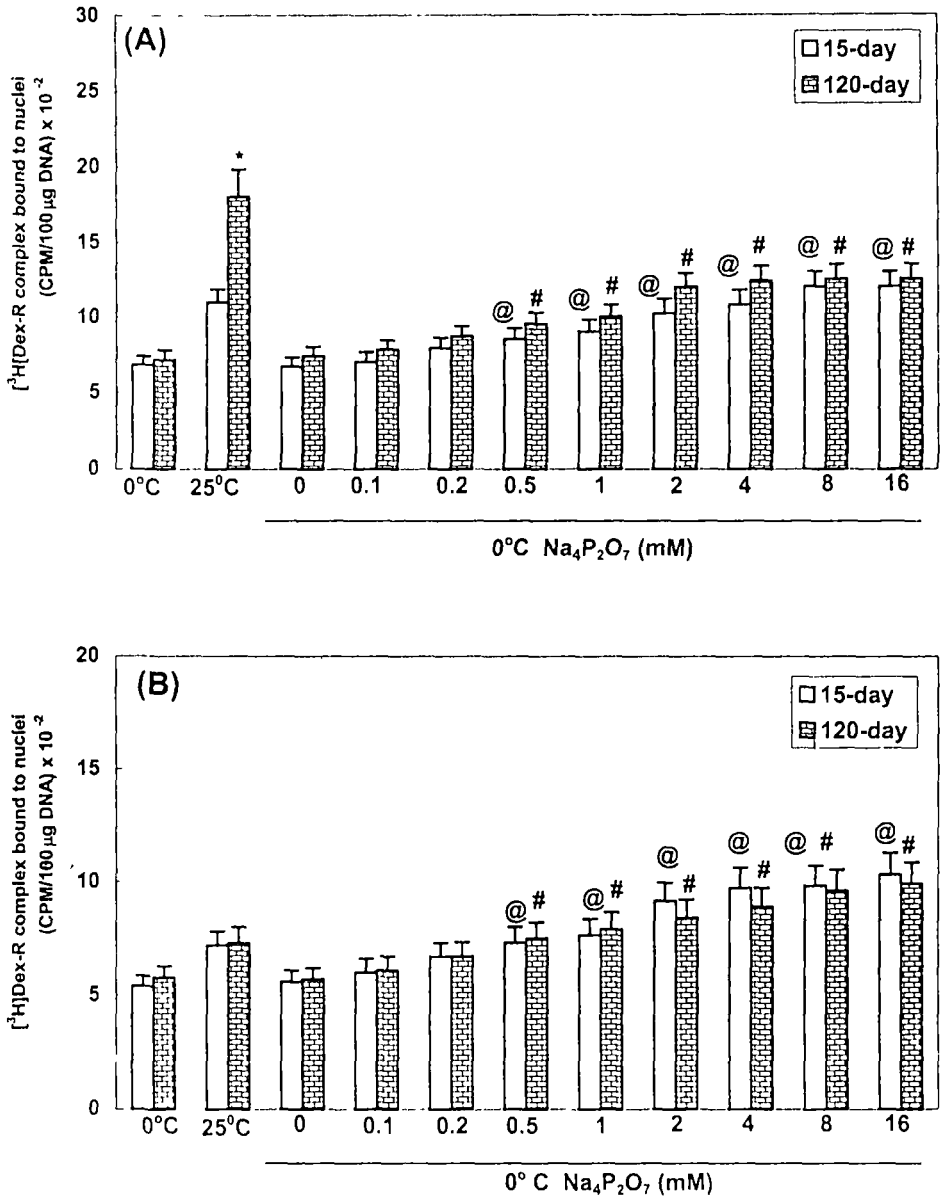


Figure 31. Effect of tetrasodium pyrophoshate ($Na_4P_2O_7$) on the activation of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes of 15- and 120-day old mice by using **purified nuclei** binding assay. Cytosol preparation, activation conditions, binding to nuclear pellets and further processing of pellets are similar to those indicated in fig. 15. Results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Significant (P<0.01) heat activation compared to 0°C. #,@Significant activation (P< 0.05) compared to respective control.

the ages and tissues of mice assessed by DNA-cellulose (Fig 28 A&B) and nuclear (Fig 29 A&B) binding assays

Tetrasodium pyrophosphate was turned out to be an inducer of GR activation (65% at 8 mM) at 0°C from both the tissues and ages of mice, but without any apparent change in per cent activation in terms of tissue- and age- specificity as assessed by binding to DNA-cellulose (Fig 30 A&B) and purified nuclei (Fig 31 A&B)

Taken together, results exhibit no significant change in per cent modulation of heat activation by these modulators in a tissue- and age- specific manner. This indicates that probably the mechanism(s) of activation modulation does not get altered in both the tissues at these ages of mice

Acceptor binding modulation of GR:

DNA binding of activated receptor can be modulated by a number of endogenous and exogenous agents In our study, acceptor (DNA) binding modulation of activated GR was carried out by using PLP, MMTS and ATA in the liver of 120- day old mice to observe their modulatory effect, if any Pyridoxal 5-phosphate (PLP) is an active coenzyme form of vitamin B_6 and a potent modifier of lysine residues in proteins It is known that the DNA binding domain (DBD) of GR contain many lysine residues that play an important role in DNA binding Hence, it was decided to use PLP for its modulatory effect on DNA binding by activated GR PLP when used at a concentration from 0-10 mM, maximally inhibited (98%) DNA binding of activated hepatic GR at 4 mM, showing an IC_{50} of 0 75 mM, by both DNA-cellulose (Fig 32A) and nuclear (Fig 32B) binding assays

Methyl methanethiosulfonate (MMTS) is a synthetic, strong modifier of thiol groups in proteins Hence, MMTS was used to study its modulatory effect on activated hepatic GR binding to acceptor at a concentration range from 0-8 mM MMTS was also found to be a potent inhibitor of activated hepatic GR binding to DNA, causing highest inhibition (85%) of receptor binding as assessed by DNA-cellulose (Fig 33A) and nuclear (Fig 33B) binding assays at a concentration of 6 mM, with an IC_{50} of 1 2 mM

Aurintricarboxylic acid (ATA), a synthetic triphenylmethane dye, was also a very potent inhibitor of acceptor binding ATA when used at 0-0 7 mM concentration caused a maximal inhibition (95%) of activated hepatic GR binding to both DNA-cellulose (Fig 34A) and nuclei (Fig 34B) at 0 2 mM, with an IC_{50} of 0 05 mM

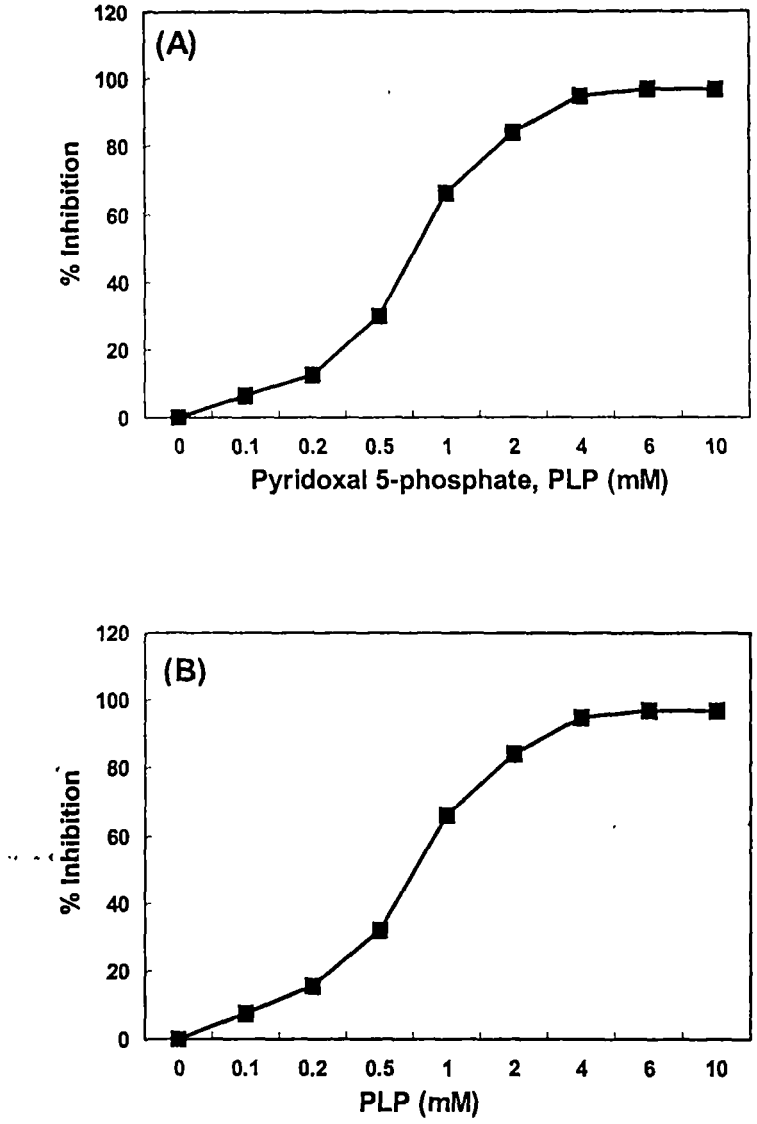


Figure 32. Effect of pyridoxal 5-phosphate (PLP) on the binding of heat (25°C)-activated hepatic [^{3}H]dexamethasone-receptor complexes to DNA-cellulose **(A)** and purified nuclei **(B)**. Cytosol from the liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min. Activated complexes were then incubated in the presence or absence (control) of varying concentrations of PLP for 30 min at 0°C. Control and PLP incubated [^{3}H]dexamethasone-receptor complexes were then allowed to bind to DNA-cellulose and purified nuclei at 0°C for 60 min. Further processing was done as described in the materials and methods section. Results are expressed as per cent (%) inhibition.

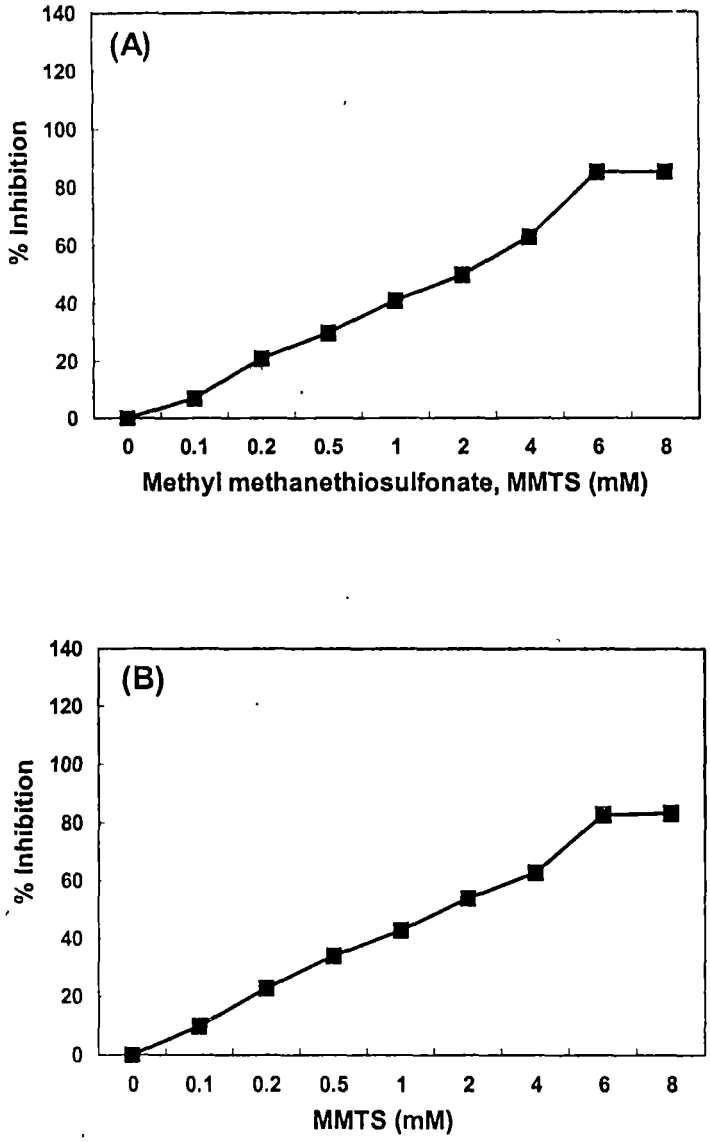


Figure 33. Effect of methyl methanethiosulfonate (MMTS) on the binding of heat (25°C)-activated hepatic [^{3}H]dexamethasone-receptor complexes to DNA-cellulose **(A)** and purified nuclei **(B)**. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min. Activated complexes were then incubated in the presence or absence (control) of varying concentrations of MMTS for 30 min at 0°C. Control and MMTS incubated [^{3}H]dexamethasone-receptor complexes were then allowed to bind to DNA-cellulose and purified nuclei at 0°C for 60 min. Further processing of pellets was done as described in the materials and methods section. Results are expressed as per cent (%) inhibition.

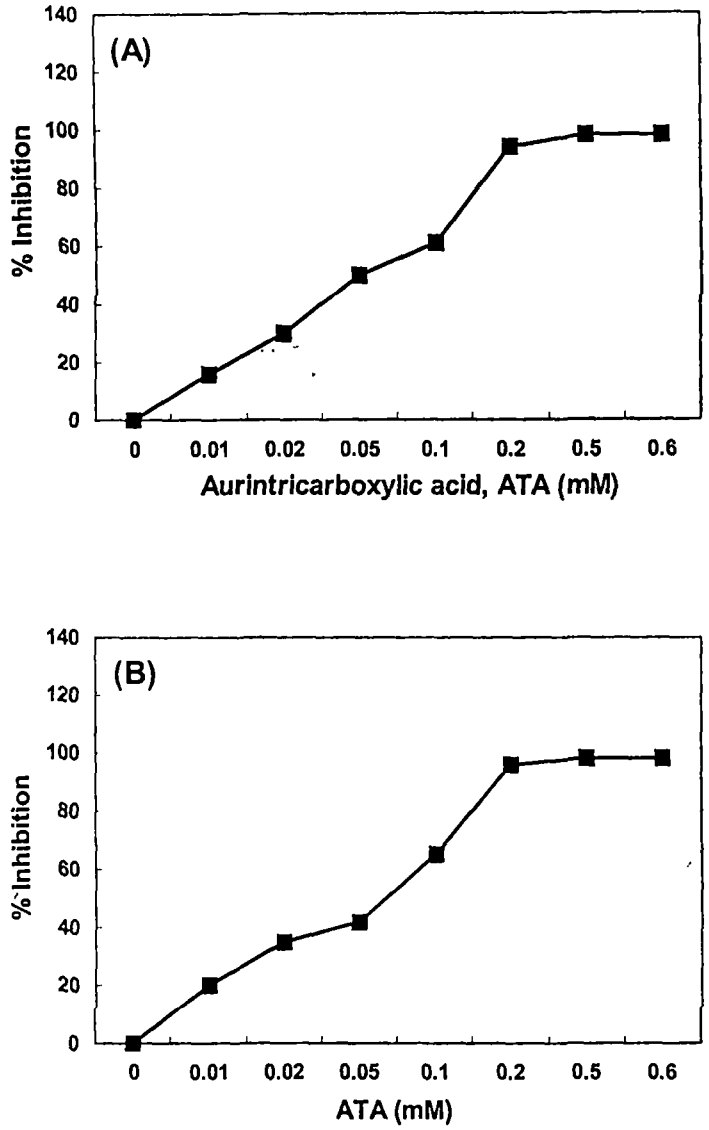


Figure 34. Effect of aurintricarboxylic acid (ATA) on the binding of heat (25°C)-activated hepatic [^{3}H]dexamethasone-receptor complexes to DNA-cellulose **(A)** and purified nuclei **(B)**. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min. Activated complexes were then incubated in the presence or absence (control) of varying concentrations of ATA for 30 min at 0°C. Control and ATA incubated [^{3}H]dexamethasone-receptor complexes were then allowed to bind to DNA-cellulose and purified nuclei at 0°C for 60 min. Further processing was done as described in the materials and methods section. Results are expressed as per cent (%) inhibition.

Acceptor binding modulation study was also performed in the liver and kidney of immature (15-) and mature (120-day old) mice to reveal any tissue- and/or age- specific modulation. PLP was employed (0-10 mM) as an inhibitor of acceptor binding of heat-activated GR from both the tissues and ages. There was a significant inhibition (98% at 4 mM) of acceptor binding of GR in the liver and kidney in the presence of PLP, however, it could not exhibit any significant difference in acceptor binding inhibition by activated GR in terms of tissue- and age-specificity by DNA-cellulose (Fig. 35A&B) and nuclear (Fig. 36 A&B) binding assays. MMTS (0-8 mM) was also a significant inhibitor (85% at 6 mM) of acceptor binding by GR in both the tissues, but result indicates no apparent differences in per cent inhibition at either of the age and tissue by DNA-cellulose (Fig. 37 A&B) and nuclear (Fig. 38 A&B) binding assays. ATA (0-0.7 mM) also turned out to be a potent inhibitor (95% at 0.2 mM) of acceptor binding by GR in the liver and kidney. It also does not reveal any significant change in per cent inhibition at such ages and tissues studied, as assessed by binding to DNA-cellulose (Fig. 39 A&B) and purified nuclei (40 A&B).

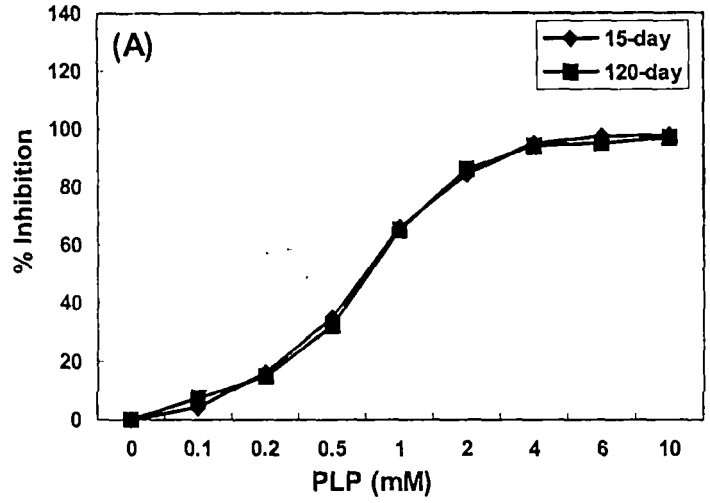
It seems, none of these agents showed any age- and tissue- specific response in modulating the acceptor binding of heat-activated GR complexes, thereby indicating that the mechanism(s) of inhibition modulation of activated receptor binding to DNA do(es) not get altered during these phases of animal's life span.

Diabetes and GR modulation:

Diabetes was experimentally induced in mice (15- and 120- day old) by injecting intraperitoneally a single dose (20 mg/100 g body weight) of streptozotocin (STZ). Blood glucose level was determined at regular intervals, and on day 7 of STZ treatment, animals were sacrificed. Thereafter, STZ-induced diabetic effects were analyzed for GR level, affinity, and for *in vitro* activation (by heat and salt) in the liver and kidney of immature and mature animals.

In this study, blood glucose levels (mg/dl) of STZ-treated animals were elevated by approximately 3.5 fold to that of controls and hence, ensured that animals had responded to STZ and were diabetic (Fig. 41). The diabetogenic effect of STZ in inducing blood glucose level was similar at immature and mature ages of mice.

Scatchard plot analyses of the data obtained indicates a reduced (22-33%) level of GR in the liver (Fig. 42A) and kidney (Fig. 42B) of mature (120-day) mice as compared to immature (15-day) in control groups (Table I). However, slopes of the plots exhibit no alteration in the affinity (K_d) of GR for its cognate hormone at these two ages. STZ-induced diabetes (Fig. 43A&B) had no effects on the level and affinity of GR in either of the tissues and ages of mice studied (Table I). Studies on the activation process of the GR complexes at two ages in the liver and kidney of control and diabetic mice using DNA-cellulose (Fig. 44A&B) and nuclear (Fig.



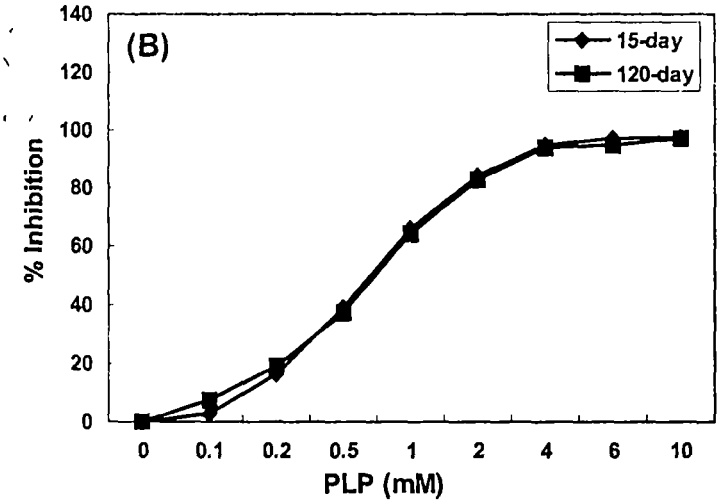


Figure.35 Effect of PLP on the binding of heat-activated [^{3}H]dexamethasone-receptor complexes from liver **(A)** and kidney **(B)** of 15- and 120- day old mice to **DNA-cellulose.** Cytosol from liver and kidney of respective ages were prepared in buffer B (i) and incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min. Activated complexes were then incubated in the presence or absence (control) of varying concentrations of PLP for 30 min at 0°C. Control and PLP incubated [^{3}H]dexamethasone-receptor complexes were then allowed to bind to DNA-cellulose at 0°C for 60 min. Further processing was done as described in the materials and methods section. Results are expressed as per cent (%) inhibition for each age group.

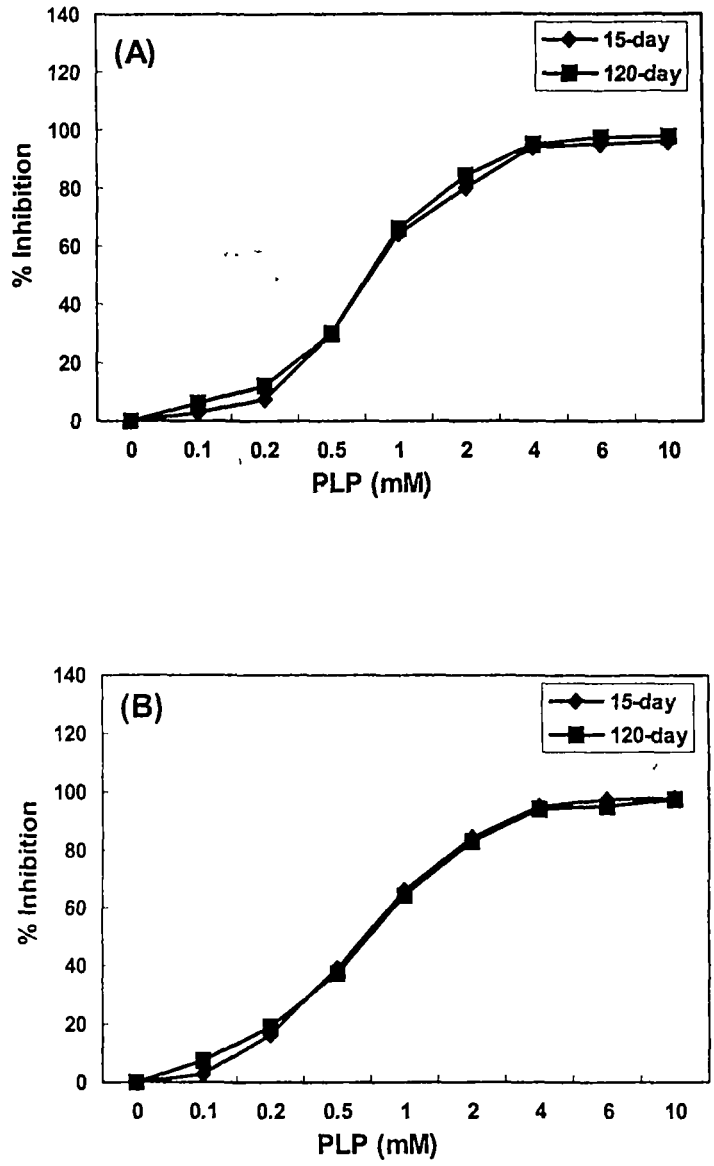


Figure 36. Effect of PLP on the binding of heat-activated [^{3}H]dexamethasone-receptor complexes from liver **(A)** and kidney **(B)** of 15- and 120- day old mice to **purified nuclei**. Cytosol preparation, heat activation, treatment with PLP, binding to purified nuclei and further processing were performed as described in fig. 32. Results are expressed as per cent (%) inhibition.

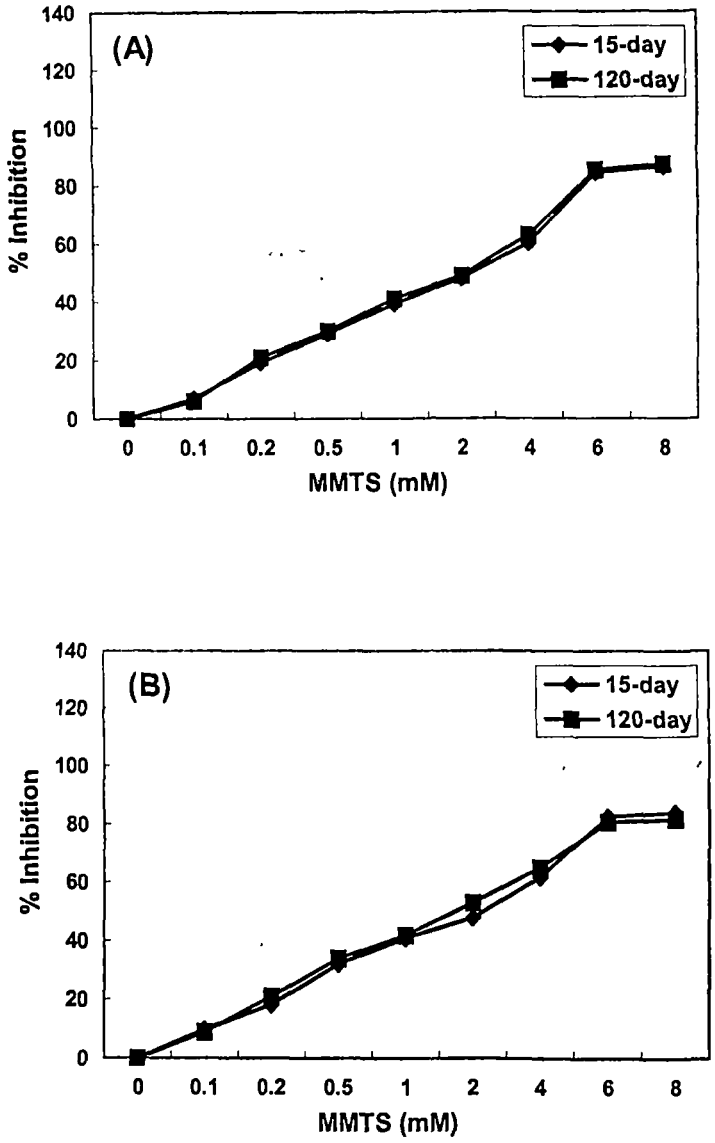


Figure 37. Effect of methyl methanethiosulfonate (MMTS) on the binding of heat-activated [^{3}H]dexamethasone-receptor complexes from liver **(A)** and kidney **(B)** of 15- and 120- day old mice to **DNA-cellulose**. Cytosol preparation, heat activation, treatment with MMTS, binding to DNA-cellulose pellet and further processing were performed as described in fig. 33. Results are expressed as per cent (%) inhibition for each age group.

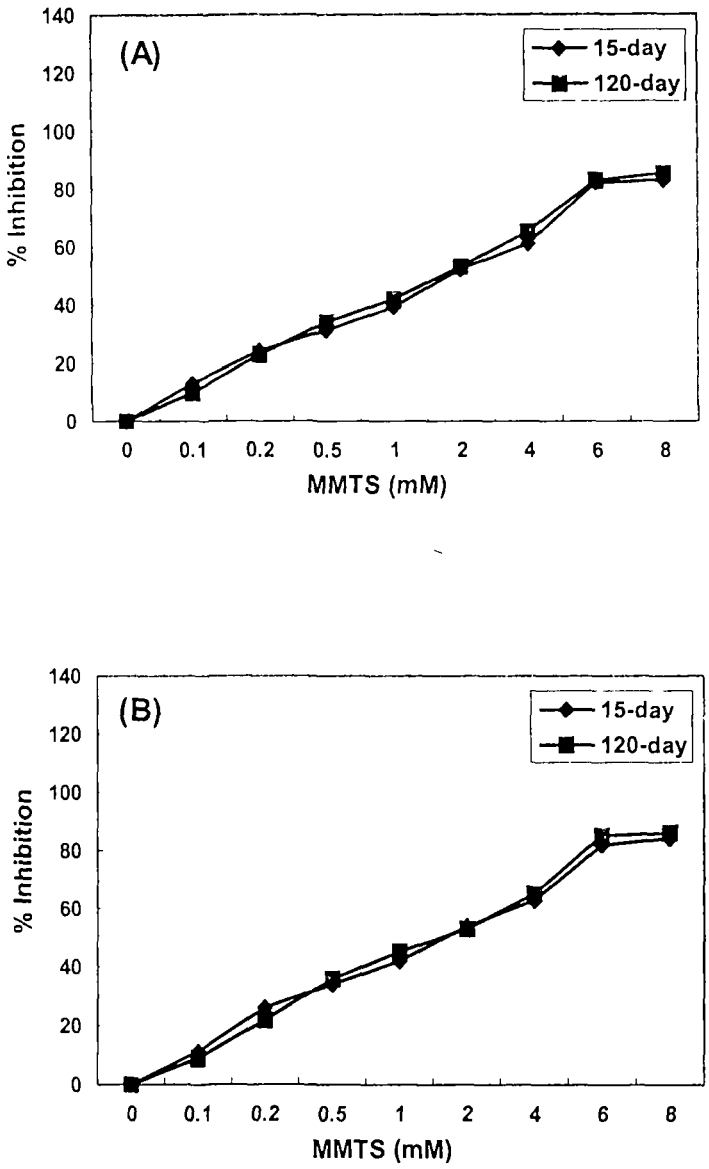


Figure 38. Effect of methyl methanethiosulfonate (MMTS) on the binding of heat-activated [^{3}H]dexamethasone-receptor complexes from liver **(A)** and kidney **(B)** of 15- and 120- day old mice to **purified nuclei** Cytosol preparation, heat activation, treatment with MMTS, binding to purified nuclear pellet and further processing were performed as described in fig 33. Results are expressed as per cent (%) inhibition for each age group.

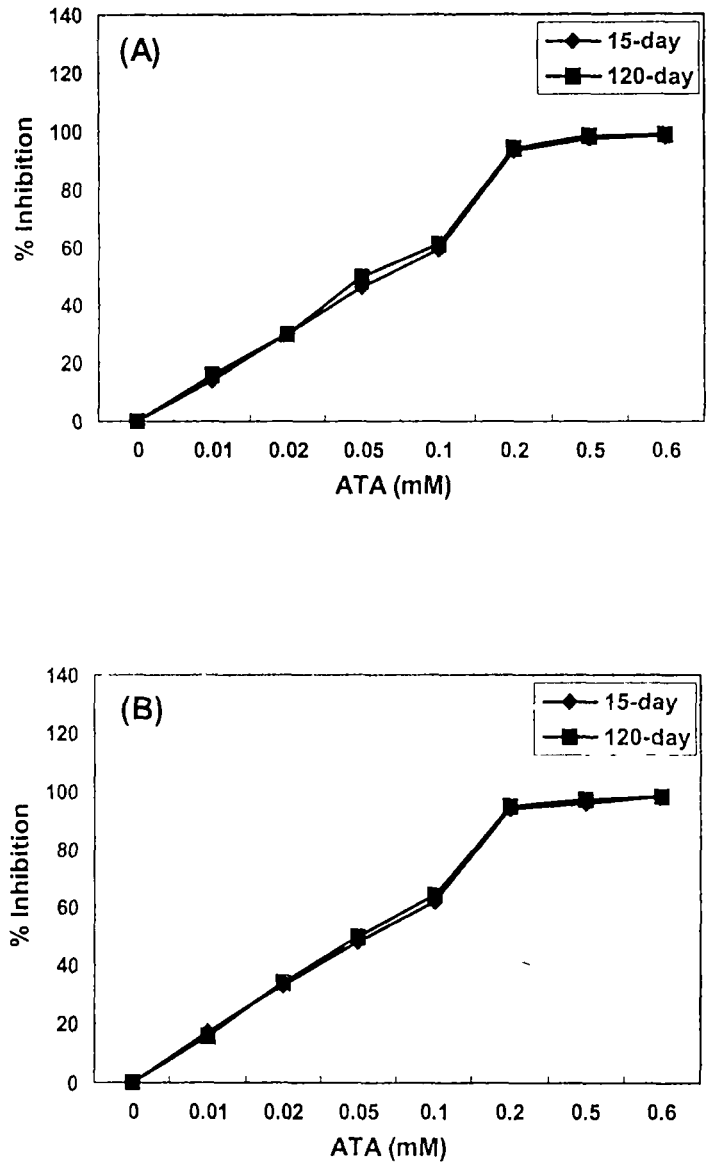


Figure 39. Effect of aurintricarboxylic acid (ATA) on the binding of heat-activated [^{3}H]dexamethasone-receptor complexes from liver **(A)** and kidney **(B)** of 15- and 120- day old mice to **DNA-cellulose**. Cytosol preparation, heat activation, treatment with ATA, binding to DNA-cellulose pellet and further processing were performed as described in fig. 34. Results are expressed as per cent (%) inhibition for each age group.

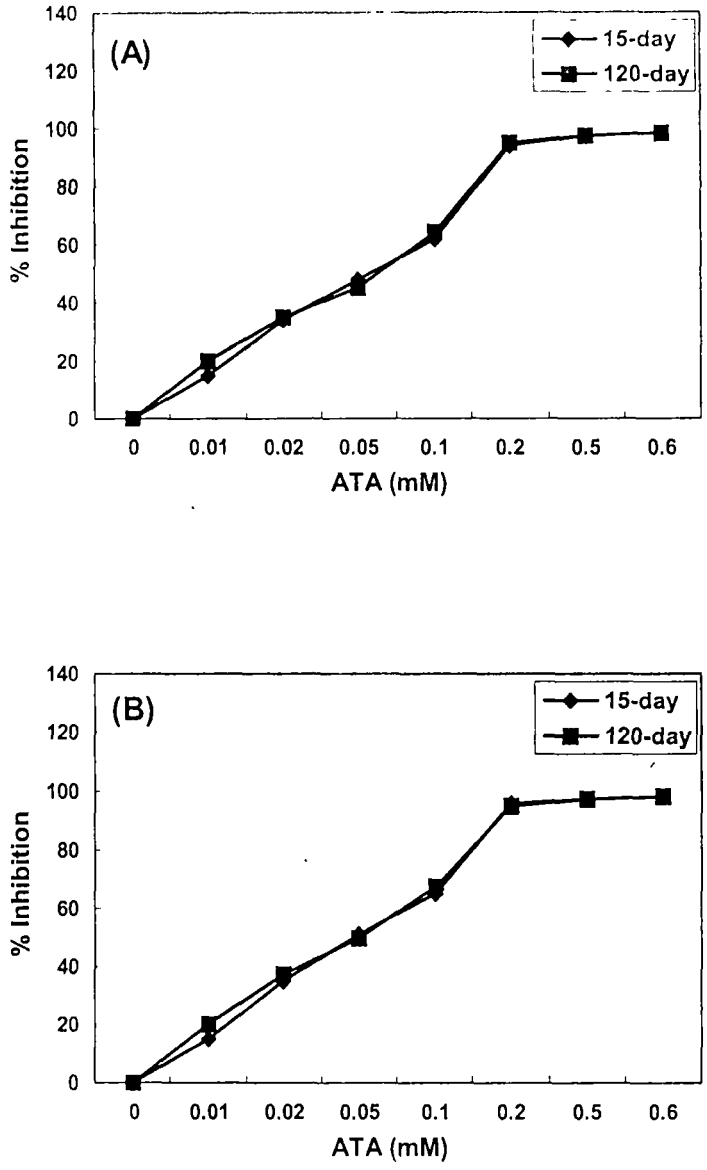


Figure 40. Effect of aurintricarboxylic acid (ATA) on the binding of heat-activated [^{3}H]dexamethasone-receptor complexes from liver **(A)** and kidney **(B)** of 15- and 120- day old mice to **purified nuclei**. Cytosol preparation, heat activation, treatment with ATA, binding to nuclear pellet and further processing were performed as described in fig. 34. Results are expressed as per cent (%) inhibition for each age group.

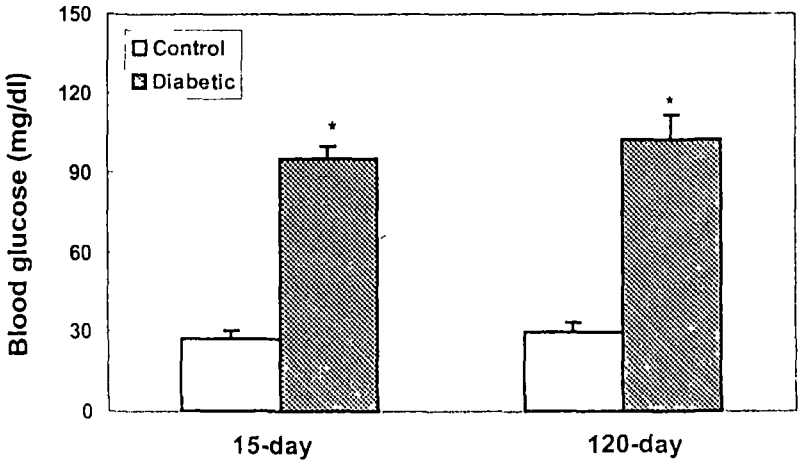


Figure 41. Blood glucose level in streptozotocin-treated diabetic and control mice of 15- and 120-day old. Values are mean of 4-5 mice of each age group. Bars represent standard deviation. *Statistically significant (P<0.001) as compared to control.

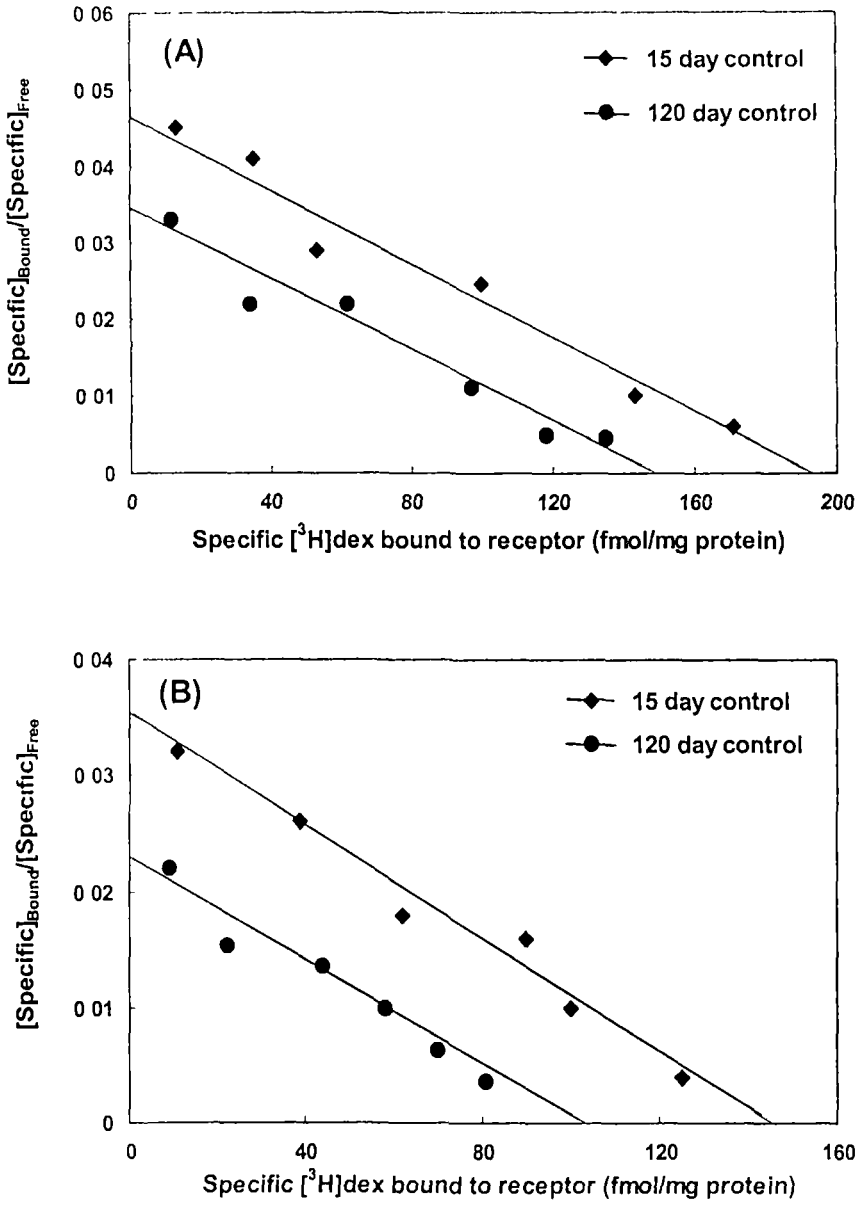


Figure 42 Scatchard plot of the [^{3}H[dexamethasone binding studies from the liver **(A)** and kidney **(B)** of 15- and 120- day old **control** mice Cytosols from these tissues were incubated with 1-120 nM [^{3}H]dexamethasone ± 500-fold excess of nonradioactive dexamethasone for 4 hr at 0°C. The specific binding at each concentration was obtained by subtracting nonspecific binding from total binding and the data obtained was analyzed according to the method of Scatchard The slope of the curve gave the dissociation constant (K_d), while the intercept on the X-axis gave the maximal number of specific binding sites Each data point represents the mean of 4 separate experiments with 4-5 mice of each age group

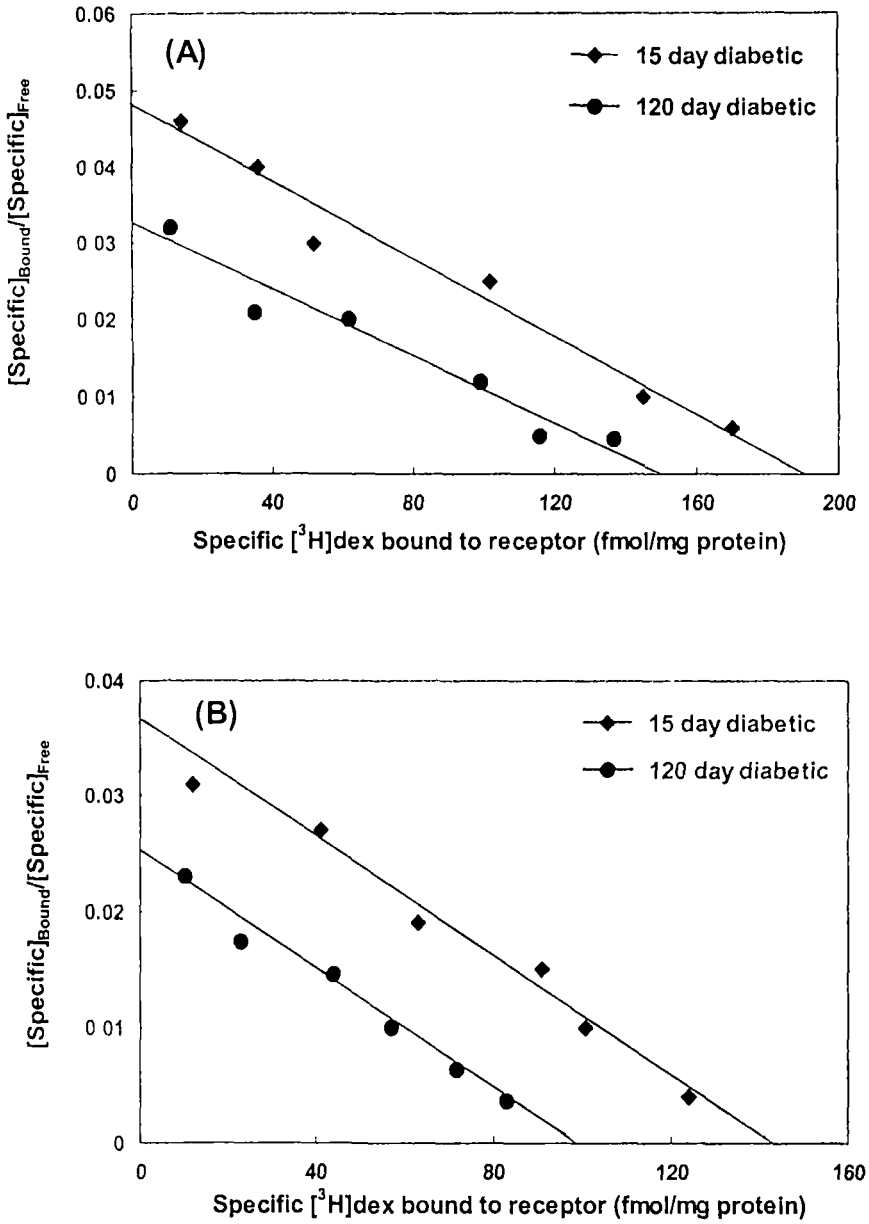


Figure 43. Scatchard plot of the [^{3}H[dexamethasone binding studies from the liver **(A)** and kidney **(B)** of 15- and 120- day old **diabetic** mice. Experimental procedure, Scatchard analysis of the data were carried out as indicated for fig. 42. Each data point represents the mean of 4 separate experiments with 4-5 mice of each age group.

Table I.

Concentration and affinity of [^{3}H]dexamethasone-receptors in the liver and kidney of immature (15-) and mature (120-day) control and diabetic mice [a]

Conditions	Tissues	Age (Days)	B_{max} (fmol/mg protein)	K_d (nM)
Control	Liver	15	192.00±7.10	3.10±0.25
		120	148.80±6.63 [b]	2.78±0.30
	Kidney	15	144.00±5.93	3.80±0.36
		120	97.00±4.89 [b]	3.70±0.33
Diabetic [NS]	Liver	15	189.70±6.79	3.20±0.28
		120	147.30±5.69	2.78±0.30
	Kidney	15	141.00±6.62	3.70±0.35
		120	95 10±5.08	3.80±0.35

[a] The data were collected from 4-5 mice of each age group and analyzed using **Scatchard plot** as given under materials and methods. The results are mean ± standard deviation of four separate experiments for each age group.

[b] Statistically significant (P<0.05) with respect to day 15; NS, not significant as compared to control.

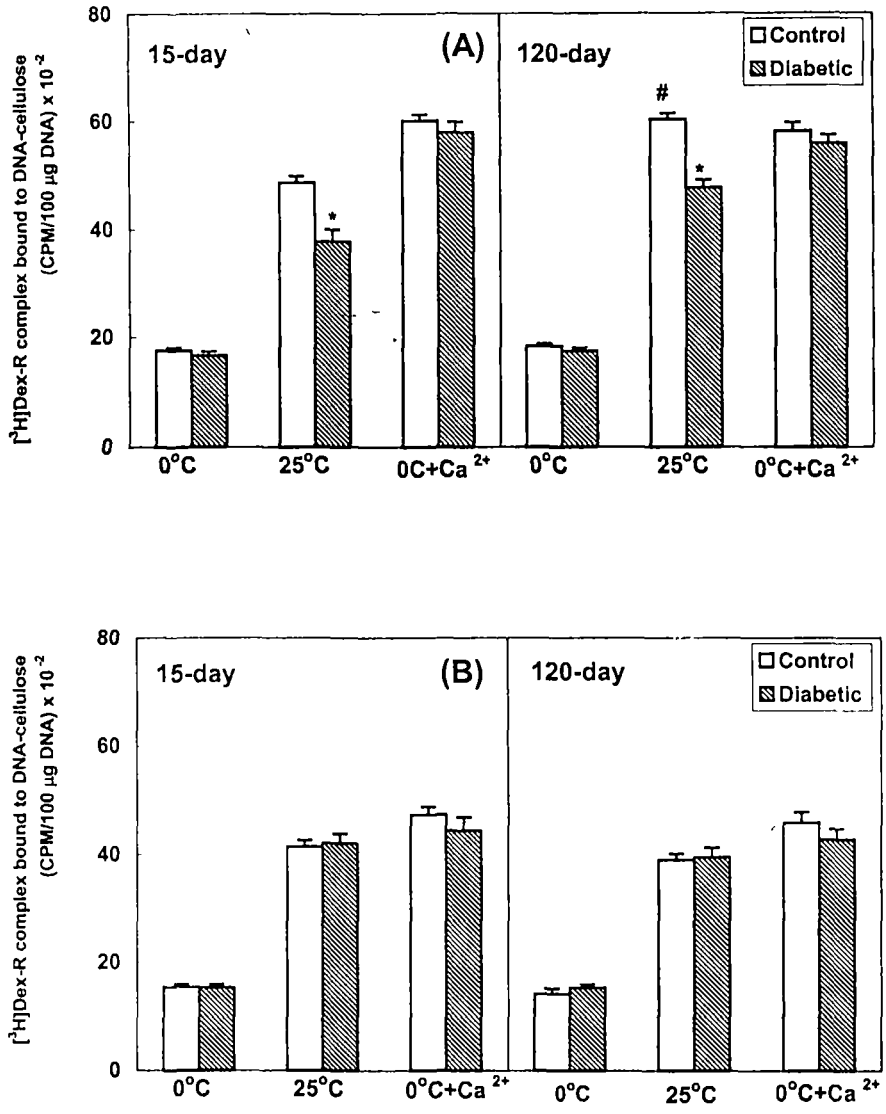


Figure 44. Binding of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes to **DNA-cellulose** in 15- and 120-day old control and diabetic mice. Cytosols from these tissues were prepared in buffer B (i) and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C. Activation procedure, DNA-cellulose binding and further processing of the pellets were performed as described in materials and methods section in the text. The results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Statistically significant (P<0.05) compared to control. #Significantly (P<0.05) higher heat activation as compared to 15-day control.

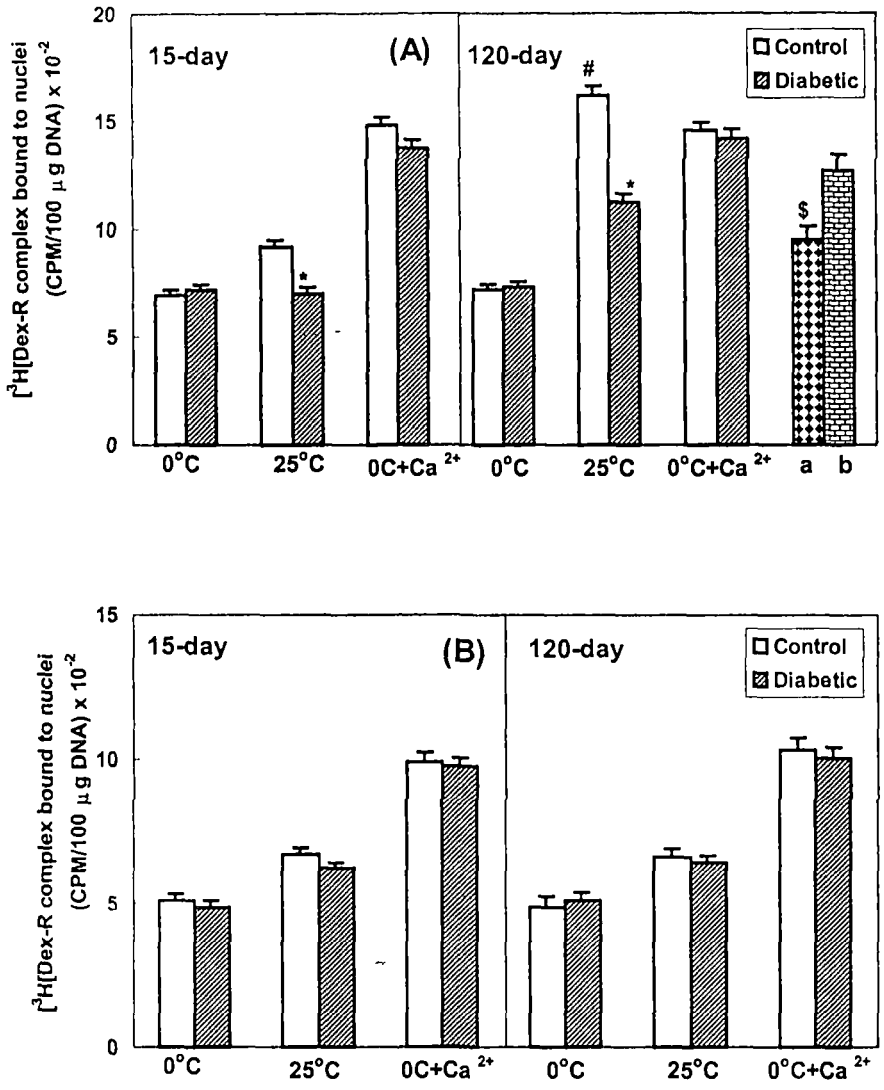


Figure 45. Binding of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes to **purified nuclear pellets** in 15- and 120-day old control and diabetic mice. Purified nuclei of these tissues were used instead of DNA-cellulose for activation studies. Other experimental procedures are same as for fig.44. The **(a)** and **(b)** barograms in **(A)**, represent cross-mixing experiments in which heat-activated GR complexes from 120-day old diabetic mice were incubated with the nuclei of 120-day old control **(a)** and heat-activated GR complexes from 120-day old control mice with the nuclei of 120-day diabetic mice **(b)**. The results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Statistically significant ($P< 0.05$) compared to control.. #Significantly ($P< 0.05$) higher heat activation as compared to 15-day control. $Statistically significant ($P< 0.05$) compared to (b).

45A&B) binding assays indicate that both temperature and salt significantly increase the DNA-cellulose binding of the H-R complexes in both the ages and tissues in diabetic and control animals. The GR from both the tissues underwent heat activation, albeit the extent of activation was more pronounced in mature liver compared to immature (Fig. 44A), with no such difference of activation in the kidney (Fig. 44B). In diabetic mice, the heat activation of hepatic GR exhibits reduced (~20-23%) DNA-cellulose binding (Fig. 44A) compared to control. In contrast, thermal activation of kidney GR does not show marked difference in diabetic mice at either of the ages studied (Fig. 44B). Salt-dependent activation of GR complexes does not exhibit any change in either of tissues and ages of mice (Fig. 44A&B). Nuclear binding of heat-activated hepatic GR complexes is also significantly reduced (24-39%) in diabetic mice compared to control at both the ages (Fig 45A). However, the nuclear binding of heat-activated H-R complexes from kidney (fig. 45B) does not show marked difference in diabetic mice at either of the ages. Data from cross-mixing experiments (heat-activated GR of diabetic and nuclei of control and vice-versa) performed only on mature mice's liver showed significantly decreased (25%) nuclear binding (Fig. 45A) by diabetic GR, thus indicating receptor specificity.

Aging and GR modulation:

The regulation of GRs in young (4-) and old (120-week) mice was studied to investigate possible changes in GR level, affinity, activation (by heat), activation modulation by PUFAs and nuclear-bound GR extraction by DNase I in the liver and kidney.

Scatchard plot (Fig. 46A&B) analyses of the data indicates a decreased level of GR in the liver (25%) and kidney (33%) of old mice compared to young ones. However, slopes of the plots exhibit no alteration in the affinity (K_d) of GR for its ligand at these two different ages (Table II).

Pilot experiments performed reveal that heat (25°C for 45 min) significantly enhanced the activation of H-R complexes from liver (2-2.5 fold) (Fig. 47A & 48A) and kidney (1.5-2.5 fold) (47B & 48B) in both the ages, albeit the magnitude of activation was higher (24-29%) in young hepatic and renal GR with respect to old. Our earlier experiments reveal that PUFAs (linoleic and arachidonic acid) inhibit the heat activation of H-R complexes in a dose-dependent manner. Both were most effective at a concentration of 160 μM, exhibiting 40-75% maximal inhibition of receptor activation. Linoleic acid caused significant magnitude of inhibition in the liver (64%) (Fig. 47A) and kidney (68%) (Fig. 47B) of young mice as compared to old (41% and 43%, respectively). Arachidonic acid also showed a similar extent of activation inhibition of hepatic GR (Fig. 47A) in young (74%) compared to old (50%) animals. In kidney (Fig. 47B), however, arachidonic acid inhibited the heat activation of GR, albeit to a similar magnitude (~57%) in both the ages. Thus, arachidonic acid showed its age-specific inhibitory effect only in

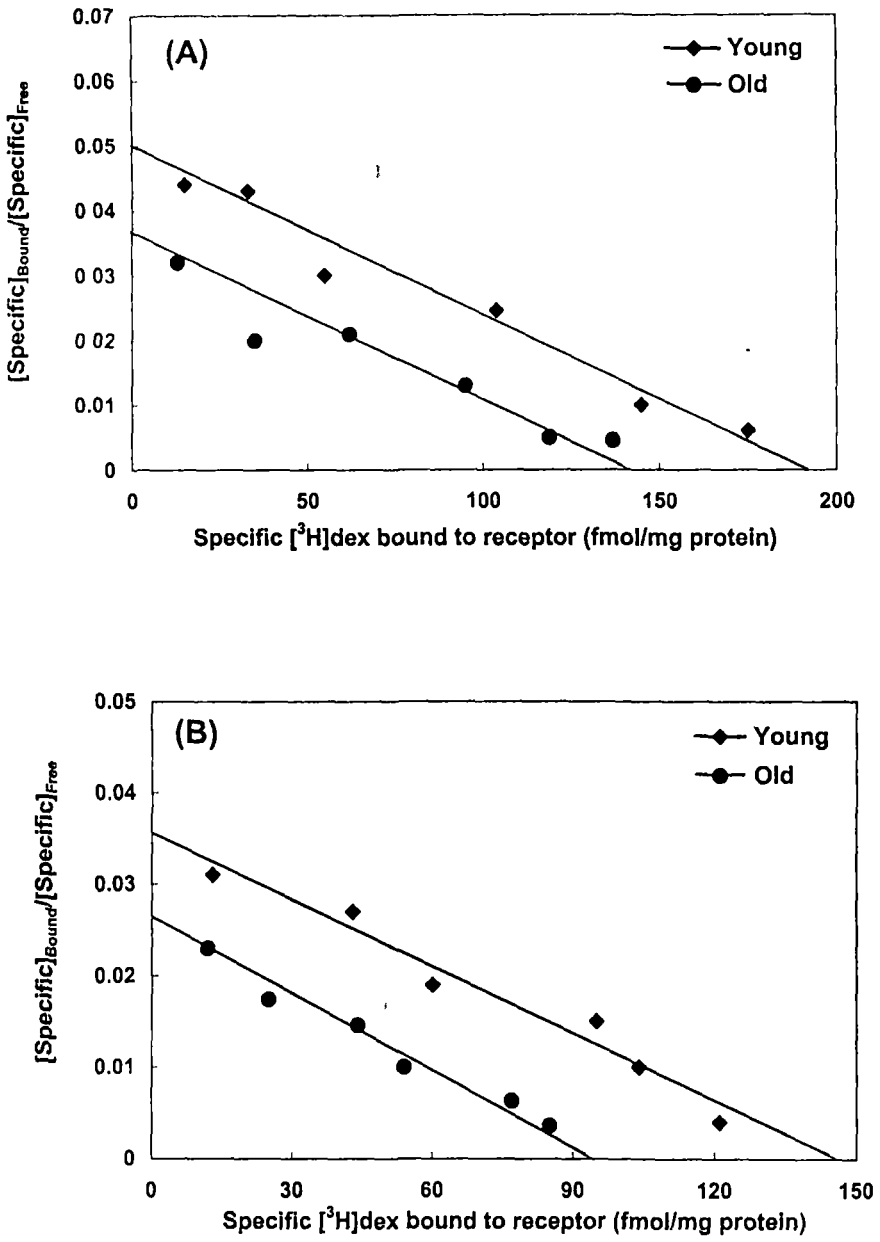


Figure 46. Scatchard plot of the [^{3}H[dexamethasone binding studies from the liver **(A)** and kidney **(B)** of young (4-) and old (120-weeks) mice. Cytosols from these tissues were incubated with 1-120 nM [^{3}H]dexamethasone ± 500-fold excess of nonradioactive dexamethasone for 4 hr at 0°C. The specific binding at each concentration was obtained by subtracting nonspecific binding from total binding and the data obtained was analyzed according to the method of Scatchard. The slope of the curve gave the dissociation constant (K_d), while the intercept on the X-axis gave the maximal number of specific binding sites. Each data point represents the mean of 4 separate experiments with 4-5 mice of each age group.

Table II.

Specific binding sites (B_{max}) and dissociation constant (K_d) of glucocorticoid receptors in the liver and kidney of young (4-) and old (120- week) mice [a]

Tissues	Age (Weeks)	B_{max} (fmol/mg protein)	K_d (nM)
Liver	4	195±20.00	3.40±0.27
	120	146±13.70*	3.68±0.24
Kidney	4	143±15.10	3.17±0.39
	120	96±8.73*	3.38±0.33

[a] Data were obtained from 4-5 mice of each age group and analyzed using **Scatchard plot** as given in materials and methods section. The results are mean ± standard deviation of 4 separate experiments for each age group. *Statistically significant (P< 0 001) with respect to 4-week (young) mice.

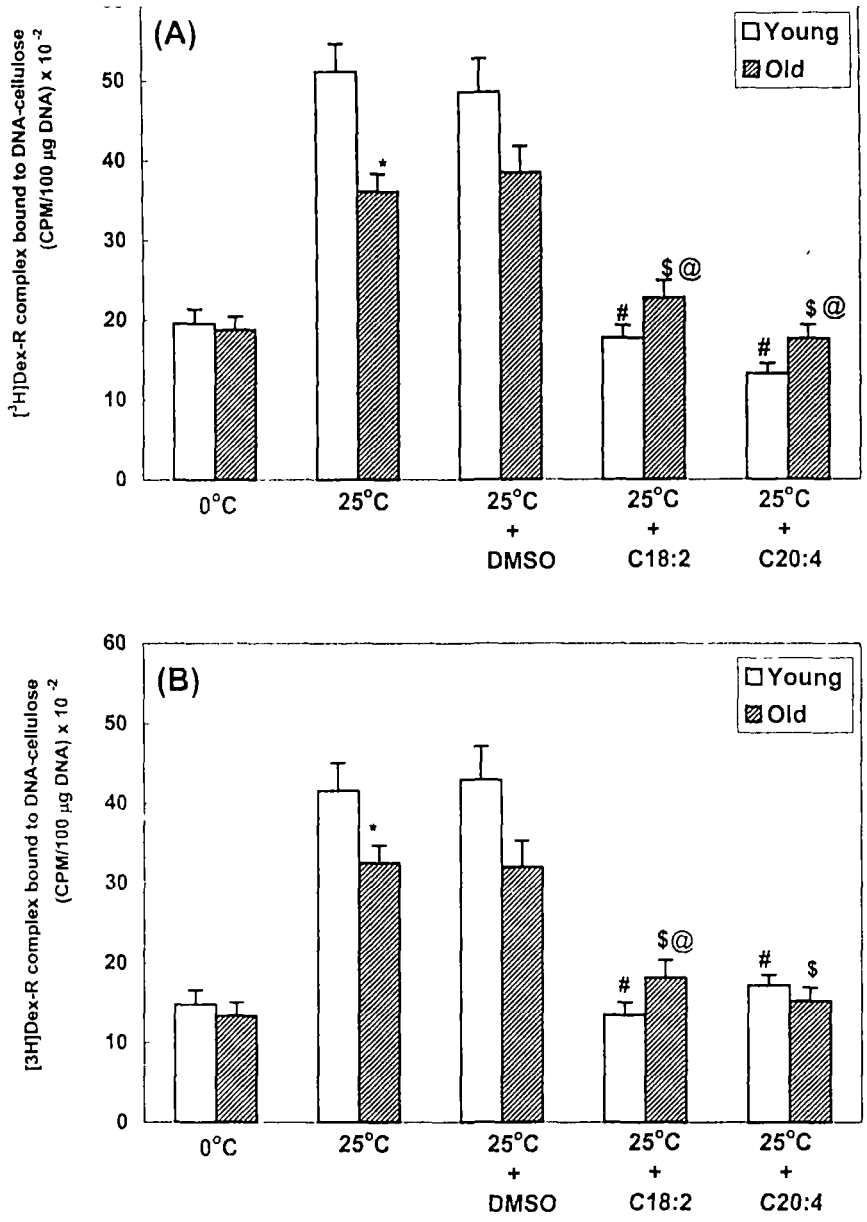


Figure 47. Binding of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes to **DNA-cellulose** in young (4-) and old (120-weeks) mice. Cytosols from these tissues were prepared in buffer B (i) and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H]dexamethasone for 4 hr at 0°C. The hormone-receptor complexes were then subjected to heat (25°C) activation for 45 min alone or in presence of dimethyl sulfoxide (DMSO) as control, linoleic (C18:2) and arachidonic (C20:4) acids, which were added to a final concentration of 160 µM. DNA-cellulose binding and further processing of the pellets were performed as described in materials and methods. The results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Statistically significant (P<0.01) as compared to young mice. #, $ Statistically significant (P<0.01) to control for their respective ages. @Significantly higher binding compared to young mice.

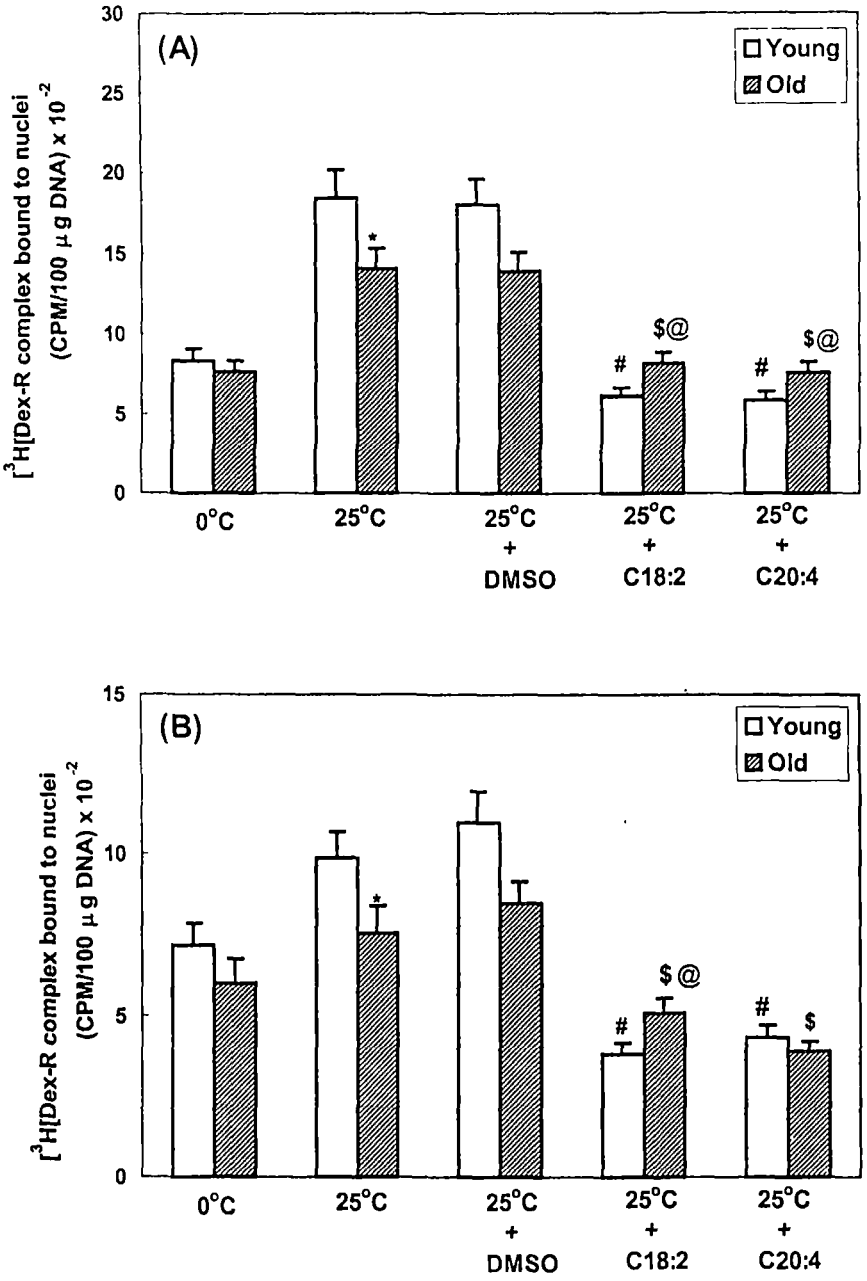


Figure 48. Binding of hepatic **(A)** and kidney **(B)** [^{3}H]dexamethasone-receptor complexes to **purified nuclei** in young (4-) and old (120-weeks) mice. Purified nuclei from respective tissues were utilized instead of DNA-cellulose for activation-inhibition studies. Other experimental procedures are same as mentioned for fig. 47. Results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Statistically significant (P<0.01) compared to young mice. #, $ Statistically significant (P<0.01) to control for their respective ages. @Significantly (P< 0.05) higher binding as compared to young mice.

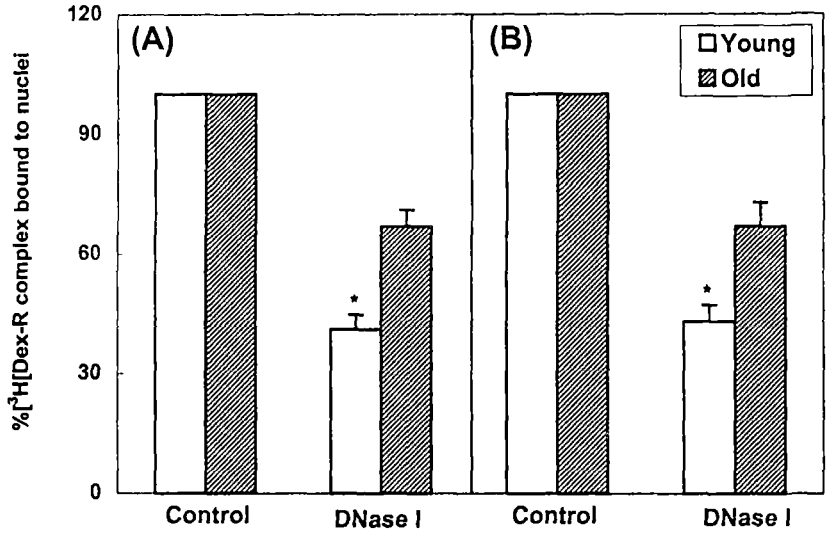


Figure 49. DNase I digestion-extraction of hepatic **(A)** and kidney **(B)** nuclear-bound [^{3}H]dexamethasone-receptor complexes of young (4-) and old (120-weeks) mice. Heat-activated, nuclear-bound hormone-receptor complexes from both the tissues were extracted using DNase I (prepared in buffer C) at a concentration of 100U/100 μg DNA for 45 min at 2-4°C. Experimental protocols are explained under materials and methods. The results are mean ± standard deviation of 4 separate experiments performed with 4-5 mice of each age group. *Statistically significant (P<0.001) compared to old mice.

the liver of mice. Since DNA-cellulose being a non-specific assay system, could not unequivocally implicate differences in the inhibitory effects of PUFAs on acceptor binding by activated H-R complexes. Hence, the purified nuclei from both the tissues of respective ages were used to provide a more relevant physiological assay system. Nuclear binding assay results also show linoleic acid as being equally effective in causing inhibition of GR heat activation, with greater magnitude of inhibition in young liver (66%) (Fig. 48A) and kidney (65%) (Fig. 48B) as compared to old age tissues (42% and 40%, respectively). Again, arachidonic acid showed tissue specificity in causing greater inhibition of hepatic GR activation (Fig. 48A) in young (68%) as compared to old (45%). The age-specific difference in arachidonic acid-mediated inhibition of activation was not significant in case of kidney (Fig. 48B), wherein the inhibition was ~57% at both the ages of mice. Hence, both DNA-cellulose and nuclear binding assays revealed a similar pattern of inhibition of heat activation of GR in the liver and kidney by these two PUFAs.

DNase I digestion (Fig. 49) of hepatic and renal nuclei bound with activated GR complexes from young and old mice revealed significant higher extraction of nuclear-bound, heat-activated [^{3}H]dexamethasone-receptor complexes from young liver and kidney with respect to old age tissues. The degree of extraction of the nuclear-bound receptors in the young mice's liver (Fig. 49A) was higher (59%) as compared to old (33%) ones, when compared to their respective controls, taken as 100%. In kidney too (Fig. 49B), the per cent extraction was higher in young mice (57%) as compared to aged (33%) ones.

Glucocorticoids (GCs) play a major role in orchestrating homeostasis in vertebrates (Carlstedt-Duke 1999). The name 'glucocorticoid' derives from early observations that these hormones were involved in glucose metabolism (Norman and Litwack 1997). Now it is well known that they have a wide range of functions including regulation of glucose, fat and protein metabolism, anti-inflammatory and immunosupressive actions, cellular differentiation and development (Borski 2000; Jenkins 2001), along with regulation of mood and cognitive functions in the brain (Welberg and Seckl 2001). Most of the above functions of GCs are mediated by binding to its high-affinity intracellular cytoplasmic receptor, the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily. Binding of hormone to GR leads to generation of activated hormone-receptor complex, which then undergoes translocation into the nucleus. In the nucleus this complex binds to specific DNA sequence, termed as glucocorticoid response element (GRE) in the chromatin and modulates transcription of a gene, leading to changes in cellular physiology (Chen et al 2000). The GR is a multifaceted transcription factor, present in almost all the tissues. Modulation of GR by a number of factors is important in the control of signal transduction as it is now widely believed that GC action on target cells is regulated not only by the level of receptors, but also by endogenous modulators. Each of the steps in the steroid-receptor action mechanism, that is, steroid binding, activation, DNA binding and transcriptional regulations of specific genes, may be controlled by the receptor interactions with other proteins and modulators (Pratt and Toft 1997). In view of the multiple activities of GR, it was decided to investigate its modulation by various endogenous and exogenous agents and also by diabetic state and senescence in mice. Results obtained are discussed below under different sections to explain the modulation of GRs.

Steroid binding and stabilization studies on GR:

Sulfhydryl groups play an important role in determining the functional state of the GR. The hormone-binding domain (HBD) of GR contains many cysteine residues, which play crucial role in hormone binding (Rees and Bell 1975). Hence, any agent that stabilizes the thiol groups in the HBD, may influence steroid binding activity of GR and the stability of hormone-bound receptors (Simons et al 1990). Dithiothreitol (DTT), 2-mercaptoethanol (ME), and glutathione (GSH) are known to stabilize the thiol groups in proteins and enzymes (Kalimi and Gupta 1982). Hence, the choice of using these reducing agents to study the steroid binding to GR and the stability of hormone-bound GR, as a function of time was a reasonable one.

Adrenocorticoid receptors are extremely labile in steroid binding activity *in vitro*, as it was observed that the hormone binding ability of the unliganded GR decayed rapidly, whereas the

steroid-bound receptor was quite stable (Munck et al 1972; Kirkpatrick et al 1972; Pratt and Ishii 1972; Rafestin-Oblin et al 1977). Steroid binding to GR is a crucial step in glucocorticoid mediated signal transduction in target cells. Interaction of hormone with the receptor must fulfill two conditions: the redox conditions must be such that the thiols in the HBD are not oxidized to form intramolecular disulfide bonds, and the receptor must be bound to Hsp 90 (Pratt and Toft 1997). A series of reports (Miller and Simons 1988; Simons et al 1990; Chakraborti et al 1992) have demonstrated that steroid-binding activity of GR is inactivated by the formation of disulfide bonds between cysteine SH groups that are vicinally spaced in the HBD when bound to Hsp 90. The protective effect of milli molar concentration of DTT in cytosolic GR binding was reported earlier (Kalimi and Gupta 1982). Experiments performed to study hormone binding to GR and the stability of hormone-receptor (H-R) complexes in the liver of 120- day old mice show increased steroid binding to GR by DTT, which was maximal at 4-8 hrs of incubation at 0°C compared to control. This observation corroborates with the findings of Granberg and Ballad (1977) and Kalimi and Gupta (1982), who reported increased binding of steroid to cytosolic GR in the presence of DTT in rat liver. However, DTT could not stabilize the H-R complexes after attaining maximal specific binding. Greater steroid binding to hepatic GR in the presence of DTT is most likely due to protection of thiol groups in the HBD and by maintaining them in a reduced state. The inability of DTT to stabilize the H-R complexes after maximal specific binding reflects that DTT has no effect on steroid-prebound GR.

Mercaptoethanol and glutathione had no effect on steroid binding to hepatic GR and stabilization of H-R complexes. The increased potency of DTT on steroid binding as compared to ME and GSH may be due to the fact that DTT is bidentate.

Interestingly, the hepatic GRs of immature (preweaned) mice showed higher specific binding compared to mature ones in control groups, with similar observation in case of kidneys, thereby showing age-specificity. One possible explanation for this observation could be the presence of modulator(s) in an age-specific manner, that modify receptor property or perhaps due to developmentally related changes in the nature of GRs (Kalimi and Gupta 1982). The findings could not reveal any alteration in the stability of hepatic and renal GRs from these ages in control groups. Furthermore, none of the three reducing agents were able to reveal any differences in steroid binding to GR and its stability in an age- and tissue- specific manner. These findings indicate that the mechanism(s) of steroid binding to receptor and the stabilization of steroid-bound receptor by these agents is not altered in an age- and tissue-specific manner.

Activation modulation of GR:

Activation of GR is a well-defined intricate process. It has been reported to occur *in vivo* also and limits the nuclear binding of GR (Meshinchi et al 1990). However, the process of receptor activation *in vivo* is still unclear and seems to be little doubtful that receptor activation based on purely cytosolic observations will be simplistic (Pratt and Toft 1997). *In vitro* studies have shown that the nuclear or DNA-cellulose binding capacity can be achieved by incubating the H-R complexes at 25°C, and also by salt, elevated pH etc (Denis et al 1988; Pratt and Toft 1997), which dissociate the bound Hsp's and probably result in the exposure of positively charged amino acid residues (lysine and arginine) on the surface of the receptor (Meshinchi et al 1990). The requirement of hormone for the heat activation (DNA binding form) of GR is an absolute necessity (Denis et al 1988). The activated receptor exhibits increased binding affinity towards isolated nuclei, DNA-cellulose, phosphocellulose etc. Many exogenous agents influence this activation process, like molybdate, tungstate, N-ethylmaleimide (NEM) etc, which upon incubation with preformed H-R complexes block the activation at 25°C.

Activation studies reported in this thesis revealed that GR from the liver and kidney of immature and mature mice underwent heat and salt activation, as assessed by binding to DNA-cellulose and purified nuclei. In both the assays, the magnitude of heat activation was more pronounced in case of mature mice's liver compared to immature, without any such differences in kidney, thereby exhibiting age- and tissue- specificity in heat activation of GR. This result corroborates with the findings of Sharma and Timiras (1987), who reported greater thermal activation of hepatic GR in adult male Long-Evans rats compared to young ones. Greater heat activation of GR in mature animals may compensate for the low receptor level and support for the role of this hormone at such phase of life span. The observed difference in heat activation of hepatic GR from mature animal may be due to differences in the endogenous receptor modifying factor(s)/enzymes. Nuclear exchange studies were also performed to confirm that whether the increased nuclear binding of heat activated GR from liver of mature mice is due to nuclear or receptor specificity. Our data indicate that this alteration is due to change in receptor property and not nuclear property with respect to binding of heat-activated GR complexes. This observation corroborates with the findings of Sharma and Timiras (1987), who reported receptor specificity as the reason for greater heat activation of hepatic GR from adult Long-Evans rats compared to young ones.

A large number of exogenous and endogenous agents have been described that modulate the activation of GR *in vitro*. We have utilized a number of such agents, most of them turned out to be inhibitors of GR activation, except PPi, which stimulated the activation of GR at 0°C. Study was also extended to find out tissue- and age- specific modulation of activation process of GR by these modulators. Thiols have been reported to play a significant role in steroid

receptor activation (Tienrungroj et al 1987). Divalent cadmium (Cd^{2+}) has been shown to be an efficient inhibitor of enzymes containing essential dithiols (Joshi and Hughes 1981). Recent report suggests that Cd^{2+} reduce the steroid binding to rat hepatic GR (Dundjerski et al 2000). Hence, in our search for modulators of GR activation, we were attracted to Cd^{2+}. Cadmium specifically reacts with vicinal dithiols in proteins. Findings revealed that Cd^{2+} inhibits the *in vitro* heat activation of hepatic H-R complexes in a dose-dependent manner, attaining a maximal inhibition (60%) at 2 mM. However, the precise influence of Cd^{2+} on inhibition of heat activation of GR is not known. Cadmium probably interacts with dithiols present in a particular domain responsible for activation, or it may discourage the dissociation of bound Hsp's, so as to inhibit activation process. Also, cadmium might interact with some other unidentified group(s) in the H-R complex, thereby inhibiting the heat activation.

Selenite (SeO_3^{2-}) is a potent inhibitor of steroid binding to receptors (Surks et al 1989). We wanted to extend this role of selenite on heat activation of hepatic H-R complexes as assessed by binding to DNA-cellulose and purified nuclei, to reveal any modulatory effect on such activation. Results indicate that selenite is quite effective in blocking heat activation of hepatic H-R complexes, attaining ~50% maximal inhibition at a concentration of 4 mM. Selenite is a strong oxidant and reacts rapidly with vicinal dithiols to yield disulfides (Simons et al 1990). To our knowledge, there are no reports on the role of selenite on *in vitro* activation of glucocorticoid receptors. Our result indicates that the mechanism of inhibition by selenite of heat activation of hepatic GR is probably similar to the one suggested for cadmium.

Arsenite (AsO_2^-), a thiol-specific reagent, is particularly attractive because thiols are the most reactive nucleophiles found in biological systems (Simons et al 1990). As stated earlier, thiols have been reported to play important role in steroid receptor activation. Our results show that arsenite is also capable of blocking the *in vitro* heat activation of hepatic GR complexes, though to a lesser extent as compared to cadmium and selenite, causing a maximal inhibition (~40%) at 4 mM. Arsenite has earlier been shown to react with vicinal dithiols in GR (Simons et al 1990). The effects of arsenite on receptor activation probably involve interactions with vicinal dithiols or some other associated components of GR complexes, such as Hsp's. A recent report (Kaltreider et al 2001) reveals that non-toxic doses of arsenite directly interacts with the GR complexes and interferes with the GR-mediated gene expression in rat hepatoma cells, rather than hormone-induced GR activation.

Activation, *in vivo*, is a well-regulated intricate process that involves several factors like hyperphosphorylation, endogenous proteases, and modulators such as Litwack's modulator (Hubbard et al 1984; Litwack 1988). Leupeptin, a bacterial peptide, is an inhibitor of ser/cys proteases (Umezawa 1976). It is also known that leupeptin is an stabilizer of untransformed (non-DNA binding form) GR, similar to molybdate (Kasayama et al 1987). Hence, we thought of employing leupeptin to see the modulatory role on *in vitro* activation of hepatic GR. The

observation reveals a dose-dependent inhibition of activation, which was maximally 45-50% at 20 mM. The mechanism of this inhibition of activation probably involves leupeptin's inhibition/inactivation of proteases in cytosol, which may be essential for activation. However, the targets of these proteases, whether it is the steroid binding receptor subunit or Hsp's are unknown. Leupeptin might inhibit endogenous protease(s), which may be involved in cleavage of receptor-bound Hsp's so as to dissociate them from the receptor complex and facilitate activation. Earlier studies reported serine protease inhibitors such as TPCK and PMSF also inhibited the activation of rat liver GR (Hubbard et al 1984).

Glucocorticoids (GCs) are known to regulate fatty acid metabolism in adipocytes as well as in liver and kidney (Garrett and Grisham 1999). GCs acting through GRs facilitate lipolysis in adipocytes and the released fatty acids are metabolized in many tissues including liver and kidney. Earlier reports have shown that free fatty acids modulate receptor function of many steroidal hormones including GCs (Bresnick et al 1990). Polyunsaturated fatty acids (PUFAs) have been attributed to play important roles in growth and development in mammals, where GCs also act as a regulator of such processes (Simopoulous 1991). PUFAs have been earlier shown to reduce hormone binding to GR (Sumida et al 1993; Haourigui et al 1994). However, the role of PUFAs on GR activation has not been ascertained. Keeping in mind the importance of both these regulators on target tissues, the modulatory role of PUFAs (oleic, linoleic and arachidonic acids) on *in vitro* GR activation was studied. All the three PUFAs turned out to be inhibitors of heat activation of hepatic GR complexes in a dose-dependent manner. Linoleic and arachidonic acids were found to be more potent inhibitors of GR activation than oleic acid. These studies also indicated a possible involvement of degree of unsaturation and fatty acid chain length in inhibiting the activation of GR. Earlier experiments performed in this laboratory revealed that saturated fatty acids [palmitic (C16:0) and stearic (C18:0) acid] were unable to inhibit the heat activation of GR complexes, indicating the involvement of unsaturated moiety. However, the exact mechanism(s) of these inhibitory effects are unclear as yet. The particular domain in the GR responsible for the inhibition of activation has not been delineated, nor the group(s) in the PUFAs that interact with such domain is known. Probably, PUFAs induce a conformational change in the receptor molecule through the involvement of their unsaturated moieties, thereby inhibiting the release of bound Hsp's, that keeps the receptor in an unactivated (non-DNA binding) form.

It is known that nucleoside triphosphates such as ATP, GTP, CTP etc induce activation of GR complexes at 0°C, along with estrogen receptor (ER) and progesterone receptor (PR) and the interaction is direct rather than involving cytoplasmic mediator(s) (Diehl and Schmidt 1987). Previous studies suggest that the triphosphate moiety in these nucleotides may be important in inducing activation, as the di- and mono- phosphate form of these nucleotides appeared to be less potent in inducing activation, indicating the involvement of phosphate moiety in these

nucleotides (Kalimi 1986). Earlier reports (Moudgil et al 1986) indicate pyrophosphate (PPi) as being equally potent compared to ATP in inducing activation at 0°C. Inorganic pyrophosphate (PPi) play crucial physiological and biochemical roles in various animal tissues (Syiem and Sharma 1997). They are generated in a number of metabolic pathways, such as biosynthesis of nucleotides, amino acid, fatty acids and coenzymes and regulate oxidative metabolism in mitochondria (Syiem and Sharma 1997). They are potential donors of phosphate groups in various metabolic reactions, as well as in regulating protein and enzymatic activities (Ganong 2000). These functions of PPi turned our attention to study their role in *in vitro* hepatic GR activation, as very scanty reports are available on modulation of GR activation by PPi. Results indicate that PPi induces activation at 0°C, giving a maximal activation (~65%) at 8 mM. The exact mechanism of stimulation of this activation is not known. PPi's might induce the dissociation of bound Hsp's in H-R complexes, so as to stimulate activation. Also, PPi may alternatively be binding to a site in the receptor and exposing region(s) of receptor that are necessary for activation. Moudgil et al (1986) showed that PPi stimulated the conversion of 9S form (unactivated) of the receptor to the 4S (activated) form in 5-20% sucrose density gradients. PPi seems to play pivotal role on GC action in responsive tissues.

Furthermore, experiments were also conducted to reveal differences in modulation in the liver and kidney of immature (15-) and mature (120-day) mice. However, no alterations in modulation were observed in an age- and tissue- specific manner. Our data corroborate with the findings of Kalimi et al (1983), who reported no alterations in the sensitivity of liver GR from adult and aged rats to inhibitors of heat activation.

Acceptor binding modulation of GR:

Under physiological conditions, hormone binding to GR causes *activation,* and the activated H-R complexes translocate to the nucleus where they bind to the glucocorticoid responsive elements (GREs) of target genes to modulate gene expression. The interaction of GR with chromatin is modulated by a number of parameters, such as phosphorylation of the receptor, and other factors that vary in response to activation of other signal transduction pathways (Orti et al 1992). It has been shown by *in vitro* experiments that, nuclear extracts from different rat and human cell lines can increase the binding of activated GR complexes to DNA/or chromatin (Cavanaugh and Simons 1994; De Vos et al 1994). One such factor, a protein of low molecular mass (700-3000 Da) has been characterized, which facilitates ~40% of the activated GR complexes to bind to DNA from calf thymus *in vitro* (Cavanaugh and Simons 1994). A second factor termed as ASTP (ATP-stimulated glucocorticoid-receptor translocation promoter), a 93 kDa histone binding protein has also been isolated from rat liver, that facilitates increase GR binding to nuclei/chromatin (Okamoto et al 1993). *In vivo*, pyridoxal 5-phosphate (PLP) has

been demonstrated to influence the translocation and binding of activated GR complexes to chromatin in the nucleus (Allgood et al 1990; Tully et al 1994), thus modulating cognate gene expression (Allgood and Cidlowski 1992). *In vitro* studies have also shown that there are known exogenous agents that do not influence receptor activation (9S to 4S conversion), but rather modulate the binding of activated GR complexes to acceptors such as DNA-cellulose, ATP-Sepharose, and purified nuclei etc (Moudgil et al 1984). In the present study, pyridoxal 5-phosphate (PLP), methyl methanethiosulfonate (MMTS) and aurintricarboxylic acid (ATA) were utilized as modulators of acceptor binding sites (DNA-cellulose and purified nuclei) of activated GR in order to find out extent of modulatory responses, if any.

The biological manifestation of glucocorticoid action through the mediation of GR may be affected by a number of endogenous modulators such as PLP and biotin (Hapgood and Holt 1987). PLP is an active coenzyme of vitamin B_6 (pyridoxine), an essential, water-soluble vitamin that is required for normal growth and development (Allgood et al 1990). A number of studies have reported that PLP influences several biochemical properties of steroid receptors, such as molecular structure, surface charge and polyanion binding (Allgood and Cidlowski 1992). PLP is also a coenzyme for many aminotransferases, such as tyrosine aminotransferase (TAT) that are induced several folds by glucocorticoids (Allgood and Cidlowski 1992). It has been reported that PLP forms Schiff's base with lysine residues of many other proteins (Litwack et al 1985). The DNA binding domain of GR contains many lysine residues that play an important role in DNA binding along with cysteine residues (Moudgil et al 1984). Hence, PLP was employed to see any modulatory effect on DNA binding of heat activated hepatic GR complexes from mice. Results show that PLP is very effective in blocking DNA binding of activated GR complexes in a dose dependent manner, exhibiting ~95% inhibition at 4 mM. Our observations corroborate with the findings of Disorbo et al (1980) and Moudgil et al (1984) where PLP was shown to inhibit the binding of activated rat hepatic GR to DNA. It is now established that *in vivo* translocation of GRs from the cytoplasmic to the nuclear compartment in HeLa S_3 cell lines is increased under conditions of PLP deficiency and declines under elevated PLP concentrations (Allgood et al 1990; Allgood and Cidlowski 1992). The mechanism of action of PLP appears to involve the formation of Schiff's base with lysine residue(s) in the DBD of the receptor (Litwack et al 1985).

Thiol groups are required for DNA binding of activated hormone-receptor complexes (Tienrungroj et al 1987). In our search for modifiers of sulfhydryl groups, a well-known modifier of thiol groups in proteins attracted us, methyl methanethiosulfonate (MMTS). Hence, MMTS provided another means of studying the DNA binding of activated hepatic GRs. MMTS significantly inhibited the activated hepatic GR complexes binding to both DNA-cellulose and nuclei. These inhibition studies support the observation of Tienrungroj et al (1984) that, MMTS inactivates the DNA binding capacity of heat-activated GRs in rat thymocyte. The mechanism of action of MMTS on activated GR binding to DNA possibly involves derivatization of thiol

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moieties in the DNA binding domain of GR by adding a thiomethyl group through the formation of a mixed disulfide (Tienrungroj et al 1987).

Aurintricarboxylic acid (ATA) is a synthetic triphenylmethane dye, which was earlier shown to inhibit the acceptor (DNA) binding of progesterone and estrogen receptors (Moudgil and Eessalu 1980a,b). Both DNA-cellulose and nuclear binding assays reveal a similar pattern of inhibition of activated hepatic GR binding to DNA maximally (94%) at 0.2 mM. These observations support the results of Moudgil et al (1984) and Moudgil and Caradona (1985), where they have revealed that the inhibitory effects of ATA on activated GR binding to DNA-cellulose were most likely on the receptor rather than on the acceptor sites in DNA. ATA does not inhibit the process of receptor activation and that the effect could be exerted through an interaction with the DNA binding domain of the GR (Moudgil and Caradona 1985). This interaction could probably alter the conformation of the activated GR rendering it incapable for optimal DNA binding.

Furthermore, modulation of acceptor (DNA) binding by GR was carried out in the liver and kidney of immature (15-) and mature (120-day) mice to observe any age- and tissue- specific differences. Data, however, could not reveal any age- and tissue- specific differences in the inhibition of acceptor binding of GR in these ages. These findings indicate that probably the mechanism of acceptor binding modulation does not get influenced at these ages studied.

Diabetes and GR modulation:

Glucocorticoids exert a range of metabolic effects on a variety of animal tissues including liver and kidney. These hormone-mediated responses are attributed by binding to specific high affinity intracellular receptors, by activation of H-R complexes and subsequent nuclear translocation of these complexes where they interact with acceptor sites in the promoter regions of responsive genes. GCs are diabetogenic hormones since they decrease glucose uptake and increase hepatic glucose production under normal conditions (Delaunay et al 1997). In liver, GCs are primarily gluconeogenic as they enhance the expression of enzymes involved in gluconeogenesis and is probably the best known metabolic function of GCs (Scott et al 1998; Tronche et al 1998). In kidney, they influence renal Na^+ reabsorption and K^+ and H^+ elimination and other metabolic functions (Tronche et al 1998). A variety of factors have been reported to modulate GR function including dietary restriction, nutritional state, age, disease etc. We decided to look into GR modulation, if any, in streptozotocin (STZ)-induced diabetic mice. STZ has earlier been reported to induce diabetes in experimental animals (Yourick and Beuving 1985). A single large dose of STZ is sufficient to induce hyperglycemia resulting from a loss of pancreatic β-cells, thereby providing a model of type 1 diabetes. This alkylating agent induces a high level of DNA strand breaks in β-cells and finally leads to cell death (Pieper et al 1999).

It has been reported that glucocorticoid sensitivity of target cells is impaired during some pathophysiological conditions, such as arthritis, visceral obesity etc (Bamberger et al 1996). This alteration in sensitivity towards glucocorticoids could be due to out of many reasons such as, changes in the level of GR and/or its affinity towards its cognate ligand and receptor activation (Chrousous et al 1993; Harrison et al 1995). Diabetes is known to alter the glucose homeostasis in animals including humans. Glucocorticoids acting through GRs are also known to regulate glucose metabolism, and this property of GR perhaps, might be altered in diabetic animals. Our finding of higher level of GR in the liver and kidney of immature mice may be a contributory factor for the role of this hormone in early growth and development of mice (Munck et al 1984). It has been reported earlier that the level of GR is higher in the liver and kidney of young rats compared to adult ones (Sharma and Timiras 1987; Kalinyak et al 1989). Also, our finding, indicate that while the level of GR is higher in immature animal tissues, the affinity (K_d) for the hormone remains unaltered. These findings agree with earlier reports (Kalimi 1984; Kalimi et al 1988) that there is no apparent age-related alteration in the binding affinity of GR in both these tissues. STZ-induced diabetes did not alter the level of GR in either of the tissues and ages studied. These observations reveal that the level of GR exhibits tissue- and age-specific correlation and is not influenced under diabetic conditions (Ranhotra and Sharma 2000). Our findings of no change in the level of GR contrast with the report of reduced GR-specific binding sites by Yourick and Beuving (1985). However, it corroborates with the finding of Svec (1985) who reported no such changes in the level of GR during diabetes in experimental animals.

The activation of GR can be subjected to modulatory influences that may alter the glucocorticoid responsiveness of tissues, as activation *in vivo*, is a rate-limiting step for chromatin binding of GR. Experiments were carried out to study the effect(s) of diabetic state, if any, on GR activation in mice. The GR from both the tissues underwent heat activation, albeit the magnitude of heat activation was more pronounced in mature liver compared to immature, with no such alteration of activation in the kidney. A higher level of heat activation of hepatic GR has been earlier reported in male Long-Evans rats (Sharma and Timiras 1987). Greater heat activation of hepatic GR in mature animals may compensate for the low receptor level and support for the role of this hormone at such phases of life span. The observed difference in heat activation of GR may be due to the differences in the endogenous modulators of GR functions (Borbhuiya and Sharma 1995 a,b). The magnitude of heat activation of hepatic GR from diabetic animals was significantly less as compared to control at both the ages studied, however, without any such difference in the kidneys. Cross-mixing experiments conducted to see whether the decrease in activation of hepatic GR in diabetic animal is due to alteration in the receptor or nuclear property indicate receptor specificity (Ranhotra and Sharma 2000).

During diabetes several metabolic adjustments take place to tailor high circulating blood glucose level (Singh and Sharma 1998). GCs and GR may play a critical role in such metabolic conditions. Our findings that STZ-induced diabetes reduce activation of hepatic GR that may play a pivotal role in controlling glucose homeostasis in diabetic animals. The precise mechanism of GR activation regulation during STZ-induced diabetes is, however, unclear. Perhaps, diabetic condition might inhibit stimulators of activation (such as kinases, required for hyperphosphorylation) that may result in inhibition of GR activation. Additionally, diabetes might have elevated the level of ketone bodies (acetone, acetoacetate and β-hydroxybutyrate) which probably act as inhibitors of receptor activation. Diabetes is also known to cause dehydration (change in osmolarity) in cells, which may be another factor to inhibit receptor activation, as dilution *in vitro* activates GR. Moreover, it will be of much clinical significance to unravel the correlation between STZ-induced diabetes and the regulation of GR function.

Aging and GR modulation:

Glucocorticoids, acting through their cognate receptors, exert several physiological and biochemical effects in a wide range of target tissues and organs such as liver, kidney, lungs, heart, skin, brain and immune cells (Carlstedt-Duke 1999). The responsiveness of tissues and cells to certain hormonal modulators has been shown to be altered during development and aging (Roth 1988; Kalimi 1988; Kanungo 1994; Singh and Sharma 1995). Most of the known actions of GCs involve high-affinity interaction with the specific cytoplasmic receptors, subsequent activation and translocation of receptors to the nucleus, where they interact with cognate acceptors' sites in the promoter region of responsive genes and modulate their expression. GRs are attributed to regulate the organismal response to stress (Li et al 2000). It has been reported that aging is accompanied with a decline in maintaining homeostasis in animals (Kanungo 1994). GCs acting through their receptors have pivotal role in maintaining homeostatic balance in quite large group of animals (Sapolsky et al 2000) by regulating glucose, fat, protein and hydro-mineral metabolism (Norman and Litwack 1997). Keeping in mind the critical role of GCs, it was planned to investigate the possible changes in GR property, if any, during aging of mice.

Tissue's responsiveness towards glucocorticoids depends on many factors, including the concentration of receptor molecules and its affinity for the hormone. There is a significant decrease in GR level in the liver and kidney of aged mice, however, without any change in the affinity for the hormone. The higher level of GR in the liver and kidney of young mice may be a contributory factor for the role of this hormone in early growth and developmental phase of animal's life span. This phase involves appearance of several new proteins and enzymes performing specialized functions, some of which are under the genetic control of

glucocorticoids. The level of these proteins exhibits alterations with advancing age (Kanungo 1994). Significant decrease in the level of GR in old mice might impair metabolic functions, which could be one of the reasons for reduced ability to maintain homeostatic balance during this phase of life span (Ranhotra and Sharma 2001, in press). There have been contradictory reports on the level of GR in different tissues of various groups of aged animals. It has been reported earlier that GR level in liver is increased in senescent rats as compared to young/adult animals (Kalimi et al 1988), with similar observation in rat brain (Martin et al 1999), albeit, without any change in the affinity of hormone for the receptor. However, previous reports have also suggested a decrease in receptor level with aging in rat liver (Djordjevic-Markovic et al 1999) and brain (Ozawa, personal communication), while in rat adipocytes there have been no such alterations (Kalimi and Banerji 1981). Our findings also reveal that the level of GR exhibits tissue- and age- specific correlation, which may modulate GC responsiveness of various tissues and thus may play an important role in adaptive responses as a function of age (Ranhotra and Sharma 2001, in press).

GR activation *in vivo* is a critical step in glucocorticoid action, as it is the rate limiting step for nuclear or chromatin binding. Hence, alterations in the activation process could also determine the responsiveness of cells towards glucocorticoids. Activation studies were carried out in the liver and kidney of young and senescent mice to reveal any differences, which might, in part explain the altered responsiveness of senescent animals towards glucocorticoids. Our findings show a significant decreased heat activation of GR in both the tissues of aged animals compared to young ones. The decreased magnitude of activation in both the tissues of aged mice compared to young may be due to differences in the endogenous modulators of GR functions at these two phases of life span and/or alterations in the physicochemical properties of GR (Bodine and Litwack 1988). One such endogenous modulator has been isolated by Bodine and Litwack (1988) and was found to be a novel ether aminophosphoglyceride that inhibited rat liver GR activation. This endogenous modulator was suggested to be mimicking the effect of exogenous molybdate on receptor activation.

Free fatty acids have been shown to modulate receptor function of many steroid hormones including glucocorticoid (Bresnick et al 1990). In mammals, PUFAs have been attributed to play important roles in growth and development (Simopoulous 1991). PUFAs have been shown to reduce the binding of glucocorticoid to its receptor (Sumida et al 1993; Haourigui et al 1994). However, the modulatory role of PUFAs on receptor activation in aged animal's has not been ascertained. Hence, it was interesting to carry out studies on the role of PUFAs, such as linoleic (C18:2) and arachidonic (C20:4) acids on *in vitro* heat activation of hepatic and renal GR to reveal any modulatory effect. Using DNA-cellulose binding assays, our study clearly indicated a reduced magnitude of GR activation inhibition by both C18:2 and C20:4 in aged mice compared to young ones (Ranhotra and Sharma 2001, in press). Since DNA-cellulose being a non-

specific assay system, it could not unequivocally implicate differences in the inhibitory effects of PUFAs on DNA binding by activated H-R complexes. Hence, purified nuclei from both the tissues of respective ages were used to provide a more relevant physiological assay system. Both DNA-cellulose and nuclear binding assays revealed a similar pattern of inhibition in terms of age- and tissue- specificity on the activation of GR by PUFAs. However, as observed in the case of DNA-cellulose binding assay, the effect of arachidonic acid on GR activation inhibition using purified nuclei in the kidneys was age-independent. The exact mechanism of these inhibitory effects is unclear as yet. The particular domain in the GR responsible for the inhibition of activation has not been delineated, nor the group(s) in the PUFAs that interact with such domain is known. Probably, PUFAs induce a conformational change in the receptor molecule through the involvement of their unsaturated moieties, thereby discouraging the dissociation of bound Hsp's that keep the receptor in an unactivated (non-DNA binding) form. The age-specific differences in the inhibitory property of PUFAs onto GR activation may be attributed to such alterations in the receptor site(s), that may be modulated by these PUFAs. Greater inhibition of GR activation in young animal tissues may indicate such physicochemical status of GRs and other associated factors those modulate the receptor activity. However, the probable explanation for the similar magnitude of GR activation inhibition by arachidonic acid in young and aged mice kidney may be due to no such age-dependent changes in the domain/region that interacts with arachidonic acid. This also justifies the multiplicity of GR in the liver and kidney of mice. Previous studies showing the inhibitory effects of PUFAs on the binding of [^{3}H]dexamethasone to hepatic GR in fishes (Lee and Struve 1992) suggested the unsaturated moiety(ies) as the likely candidate for such inhibitory roles, but this remains to be elucidated.

It has been reported that alterations in chromatin organization influence the accessibility of genes to transcription factors necessary for their expression (Tsai and O'Malley 1994; Bamberger et al 1996). There have been observations that chromatin becomes more compact with increasing age and its digestibility by DNase I is decreased in aged animals (Kanungo 1994). The digestion and extraction of nuclear bound GR by DNase I was studied in young and aged mice in order to reveal differences in extraction, which might give an idea about the extent of compactness of chromatin and its accessibility to DNase I and transcription factors like GR. DNase I, a ~31 kDa endonuclease that makes single-strand cuts in double-stranded DNA at 10-bp intervals and its multiples, is widely used to study chromatin organization in animals (Chaurasia and Thakur 1997). DNase I digestion of chromatin DNA also depends on the degree of its condensation, as has been reported that DNase I preferentially cuts the DNA where it is maximally exposed (Kanungo 1994). The findings on DNase I digestion extraction of nuclear-bound H-R complexes from young and old mice corroborate with the observation of Chaturvedi and Kanungo (1983) and Chaurasia and Thakur (1997), who reported reduced digestibility of chromatin by DNase I of old rat brain compared to young and adult. Lesser

extraction of DNA bound GRs from aged animal tissues revealed a more compact chromatin organization in old mice as compared to the chromatin from young, thereby limiting the access to DNase I and also to transcription factors like GRs (Ranhotra and Sharma 2001, in press). These studies also entail that DNase I is probably unable to detect the sites for digestion of chromatin from aged mice as efficiently as it could in case of chromatin from young animals. These differences in chromatin compactness may play an important role in determining tissue- and age- specific responsiveness to glucocorticoids by the animals (Borbhuiya and Sharma 1995a,b). Also, there are earlier reports on age-dependent decline in nuclear binding efficiency of estradiol-receptor complexes in rat liver (Konoplya et al 1986) and uteri (Chuknyiska et al 1985; Belisle et al 1986). These studies on changes in the GR level, activation, activation modulation by PUFAs and chromatin organization during aging of mice may contribute towards functional changes in glucocorticoid responsiveness in aged animals.

SUMMARY

Glucocorticoids play a key role in regulating a wide variety of biological responses. Virtually all tissues in the body are target organs for glucocorticoids and can respond in one way or another (Borski 2000). In spite of the broad spectrum of biological effects induced by glucocorticoids, there is a set mechanism whereby target cells can respond to the hormonal signal, through the mediation of a receptor protein called glucocortocoid receptor (GR) in most target cells (Carlstedt-Duke 1999). Now it is known that there is a single gene for GR in all vertebrates. Thus, there must be other mechanisms that modulate the action of GR to enable the myriad roles of glucocorticoid actions in animals, including humans. A number of reports indicate the presence of tissue-specific factors/proteins/enzymes that may specify and/or modulate the cellular role of GCs through GR (Bamberger et al 1996). These factors may act at a particular or distinct step of steroid action. A number of such factors such as pyridoxal 5-phosphate, free fatty acids, biotin, melatonin, parathymosin etc have been attributed under physiological conditions to regulate GR action. Apart from these, a number of signal molecules and tissue-specific transcriptional co-activators/co-repressors have also been demonstrated to regulate the GR mediated gene expression in target cells (Tronche et al 1998). Hence, GR action can be modulated by a number of factors, which may alter its tissue responsiveness. In view of the diverse role of GR and the susceptibility of its regulation by various agents, it was decided to study the modulatory effects of some endogenous and exogenous agents on hormone binding to receptor and its stabilization, activation, DNA binding and also the effects of diabetic state and senescence onto GR.

Findings of work done are summarized below under various sections:

Steroid binding to GR and stabilization of steroid-bound receptor

Dithiothreitol (DTT) significantly increased (maximally at 4-8 hr) hormone binding to hepatic GR compared to control. However, DTT did not show any significant increase in stabilization of hepatic hormone-bound GR. Mercaptoethanol and glutathione were ineffective in enhancing the steroid binding to hepatic GR compared to control, with no influence on hormone-bound receptor stabilization. Furthermore, none of these reducing agents could show any differences in steroid binding to GR and the stabilization of hormone-bound receptor in an age- and tissue-specific manner.

Activation modulation of GR

Both heat (25°C) as well as salt (20 mM Ca^{2+}) were able to activate the GR from the liver and kidney of mice. Interestingly, the activation of GR by heat is more pronounced in mature (120-day) animal's liver as compared to immature (15-day), without any such changes in the case of kidneys. This difference in heat activation of immature and mature hepatic GR was attributed to alterations in the receptor property as evident from cross-mixing experiments. Various exogenous and endogenous agents used to modulate the activation of receptor did modulate the activation process. Cadmium, selenite and arsenite were found to be inhibitors of heat activation of GR in liver and kidney. The potency of these modulators was cadmium > selenite > arsenite. Additionally, leupeptin and PUFAs were also found to be potent inhibitors of heat activation of GR. Among the PUFAs, both linoleic and arachidonic acid had greater potency (~70% at 160 μM) in inhibiting GR heat activation compared to oleic acid (38% at 40 μM). Interestingly, pyrophosphate (PPi), unlike other modulators, was found to significantly induce (~65%) activation of GR at 0°C from both liver and kidney. However, the magnitude of activation modulation by these modulators remains the same at the two ages studied, indicating that the mechanism(s) of activation modulation does not get altered during these ages of mice.

Acceptor binding modulation of GR

Various modulators of activated receptor binding to DNA inhibited the binding of GR to DNA. Pyridoxal phosphate (PLP) was found to be a potent inhibitor of activated receptor binding to DNA. Besides PLP as a physiological inhibitor of activated receptor binding to DNA, aurintricarboxylic acid (ATA), a synthetic triphenylmethane dye, and methyl methanethiosulfonate (MMTS) were also found to be effective inhibitors of GR binding to DNA. Among these, ATA exhibits strong inhibition followed by PLP and MMTS as evident from their IC_{50} values. These modulators do not yield any age- and tissue- specificity in inhibiting the activated receptor binding to DNA-cellulose and nuclear DNA.

Diabetes and GR modulation

STZ-induced diabetic mice exhibited a similar level of GR in the liver and kidney of immature (15-) and mature (120-day) animal as compared to control, without any change in the affinity (K_d) for the hormone. This shows that STZ-induced diabetes have no effect on modulating the level of GR and the affinity for the hormone in either liver or kidney at these two ages studied. However, STZ-induced diabetes decreased the heat activation of hepatic GR from diabetic animals in both the ages studied, with no such decrease in the kidney, thereby indicating tissue- specificity. Such decrease in activation of hepatic GR in diabetic mice is attributed to receptor specificity as judged by cross-mixing experiments. These observations indicate that

the reduced hepatic GR activation during STZ-induced diabetes might play an important role in controlling glucose homeostasis in diabetic animals.

Aging and GR modulation

Aging and GR modulation studies have indicated changes in GR concentration, heat activation, activation modulation by PUFAs and chromatin organization during old age of mice. The level of GR is significantly reduced in the liver and kidney of older (120-week) mice as compared to young (4-week) ones, however, with no change in the affinity (K_d) for the hormone. Also, the magnitude of heat activation of GR was more pronounced in the liver and kidney of young mice than those from older ones. Polyunsaturated fatty acids (PUFAs), linoleic and arachidonic acid showed variable impact on activation inhibition of GR in an age-specific manner. Linoleic acid caused greater inhibition of GR heat activation in the liver and kidney of young mice as compared to old ones. Whereas, arachidonic acid exhibited greater inhibition of GR activation only in the liver of young mice as compared to old. In contrast, the inhibition of renal GR heat activation by arachidonic acid was age-independent. DNase I digestion of hepatic and renal nuclei from young and aged mice revealed significant higher digestion extraction of bound GR complexes from young animal tissues compared to old ones. These findings indicate more compact nuclear chromatin organization in old mice's tissues. Such alterations may contribute towards functional changes in glucocorticoid action and responsiveness in target tissues of senescent animals.

In conclusion, the findings summarized in this thesis indicate glucocorticoid receptor modulation by various endogenous/exogenous modulators and also by diabetic state and old age in mice. Such modulation of GR may in turn be responsible for tissue's responsiveness towards glucocorticoids during animal's health and diseases.

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APPENDIX I

Total protein concentration determination by the dye-binding method of Bradford

The dye-binding method of **Bradford** (1976) was used to determine the protein concentrations of samples.

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml of absolute ethanol, and to this further 100 ml of orthophosphoric acid (85% w/v) was added. This stock solution was mixed and stored under refrigeration.

Just before use, 15 ml of the stock solution was made up to 100 ml by adding water and filtered through Whatman No.1 qualitative filter paper to give the **working solution**.

Bovine serum albumin (BSA) was used as a standard protein. Stock solution of BSA was dissolved in distilled water (1 mg/ml). Then, aliquots containing 0.01-0.1 mg protein were pipetted out into the test tubes in duplicate, and further distilled water was added to make the final volume to 100 µl. To this, 5 ml of working solution was added and mixed. The test tubes were then incubated for 10 min at room temperature for color development. Finally, absorbance of each test solutions was determined at a visible wavelength of 595 nm, against a reagent blank. Next, a plot of absorbance against protein concentrations used was obtained.

20-30 µl of the cytosol, from which total protein concentration to be estimated was pipetted out in duplicate, into the test tubes and the final volume was made to 100 µl with distilled water. Furthermore, 5 ml of working solution was added and the absorbance determined as indicated above. Total cytosolic protein concentration was then determined from the standard plot.

APPENDIX II

Use of conversion factor to convert CPM into concentration (mmol)

2 µl of [^{3}H]dexamethasone (specific activity, 89 and 91 Ci/mmol) was diluted to 20 µl with the homogenization buffer. Then, 5 µl of this was pipetted into scintillation vials in triplicate and further 4 ml of Scintillation cocktail-T was added, and mixed throughly. The radioactivity (CPM) was obtained using liquid scintillation counter. The average CPM (X) was converted to concentration unit (mmol), using

$$\frac{\text{Average CPM (X)}}{\text{Vol. Of [}^3\text{H]dex (ml)}} \times \frac{1}{\text{Radioactive concentration (Ci/ml)}} \times \text{Specific activity of [}^3\text{H]dex (Ci/mmol)} \times \text{dilution factor}$$

Using the above equation, a value of X CPM/mmol was obtained. It was inverted to give1/X mmol/CPM and then used as a factor to convert CPM into concentration of receptor in mmol. This was further converted to fmol by multiplying with 10^{12}.

APPENDIX III

DNA concentration determination by diphenylamine method.

The method of Burton (1968) which is a modified version of Burton (1956) procedure was used to determine DNA content in purified nuclear suspension.

Preparation of diphenylamine reagent

1.5 g of diphenylamine was dissolved in 100 ml of glacial acetic acid. To this, 1.5 ml of concentrated sulfuric acid was added and the solution stored in amber bottle at room temperature. Prior to use, 0 5 ml of an aqueous solution of acetaldehyde from a stock (1.6 %) was added.

Preparation of standard DNA solution

2 mg of calf thymus DNA was added to 5 ml of 5 mM sodium hydroxide solution and incubated overnight at room temperature to allow the DNA to dissolve. Furthermore, to this, 5 ml of 1 N perchloric acid was added and the solution incubated at 70°C, in a water bath for 15 min. The solution was then brought to room temperature and the final volume was made to 10 ml by adding 1 N perchloric acid. This resulted in a standard DNA solution of concentration 200 µg/ml.

For standard plot, 0.1-1 ml (20-200 µg DNA) of standard DNA solution was pipetted out in duplicate into the test tubes and the volume made to 1 ml by adding 0.5 N perchloric acid. Next, to each tube 2 ml of diphenylamine reagent was added and the solution mixed properly by vortex machine. The tubes were then incubated at 30°C for 17 hr in a water bath. The tubes were then brought to room temperature and the absorbance obtained at a wavelength of 600 nm against a reagent blank.

DNA estimation in nuclear suspension

50 µl of purified nuclear suspension was pipetted out into the test tubes and the volume made to 1 ml with 0.5 N perchloric acid and the solution incubated at 70°C for 15 min. The solution was then brought to room temperature and further 2 ml of diphenylamine reagent was added, followed by incubation for 17 hr at 30°C in a water bath. The absorbance was determined as mentioned above. DNA concentration in the samples was then obtained from the standard plot.

BIO-DATA

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Date of birth 2nd December 1971

Educational qualifications

M.Sc$_{NEHU}$ (Biochemistry) (1994). 1st Class. 66% (Rank 3rd)

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Research experience	Five years
Teaching experience	3 years, as Lecturer in Biochemistry, St. Edmund's College, Shillong-3
Research field	Steriod receptor biology, Signal Transduction, Aging.
Fellowship awarded	CSIR Junior Research Fellowship, 1998.

Experience in Biochemical techniques and methods

Radioreceptor assay, liquid scintillation counting, centrifugation, spectrophotometry, urea-PAGE, gel filtration chromatography, agarose gel electrophoresis, SDS-PAGE, radioimmuno assay (RIA), ELISA, genomic DNA isolation and purification, working knowledge of HPLC, PCR, gas chromatography, western blotting, Pharmacia Phast system.

Experience in computer

Windows 98, Microsoft Word 97, MS Excel 98, MS paint, MS photoeditor, Internet explorer, Netscape Communicator, Macintosh.

Research publications

Ranhotra HS and Sharma R (1999). Glucocorticoid receptor regulation during streptozotocin-induced type 1 diabetes in mice. In: **Geriatrics Update** Ed.: O.P.Sharma. Proceedings of International Conference on Geriatrics and Gerontology. Geriatric Society of India, New Delhi, 132-139.

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Ranhotra HS and Sharma R (2001). Modulation of hepatic and renal glucocorticoid receptors during aging of mice. ***Gerontology*** **(Karger Publisher, Basel, Switzerland)** 47 (suppl 1): 503 (Abstract).

Conferences/symposia/seminars

1. Attended the International Conference on Radiation Biology: DNA Damage, Repair and Carcinogenesis and Indo-German Satellite Symposium on Molecular Biology of Radiation Damage and Repair, 7-10 April 1998, Department of Biochemistry, NEHU, Shillong, India.
2. Attended and presented a paper titled "Glucocorticoid Receptor Regulation During Streptozotocin-Induced Type 1 Diabetes in Mice", During International Conference on Geriatrics and Gerontology, Organized by Geriatric Society of India, 12-14 Nov, 1999, New Delhi, India.
3. Presented an abstract titled "Effects of Modulators on Glucocorticopid Receptor of Mice", presented during the National Symposium on Cellular and Molecular Biology of Aging, 20-21 Oct, 2000, Jawaharlal Nehru University, New Delhi, India.
4. Paper titled "Modulation of Hepatic and Renal Glucocorticoid Receptor During Aging of Mice", presented during the World Congress on Gerontology, Organized by the International Association of gerontology, 1-6 July, 2001, Vancouver, Canada.

National tests cleared GATE'96 and National Eligibility Test (NET), 1997.

Scholarship/fellowship NEHU Post Graduate Scholarship (1992-94), CSIR Junior Research Fellowship (1998).

Refresher course attended One, in UGC-Sponsored "Recent trends in Zoology", 15 Nov-7 Dec 2000. Department of Zoology, NEHU, Shillong.

Member of Scientific bodies

1. Geriatric Society of India (GSI), New Delhi, India.
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