REGULATION OF GLUCOCORTICOID RECEPTORS DURING DIETARY RESTRICTION IN MICE

ABSTRACT

By

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ABSTRACT

Dietary restriction (DR), a reduction in calorie intake without malnutrition, influences several physiological processes namely, immunological, general protein and amino acid metabolism and also neuroendocrinological systems. DR has been reported to potentiate immune system, delay immunosenescence, reduce progression of diseases and extend life span in rodents and primates. It is an efficacious means of increasing longevity and reducing pathology in several groups of animals. DR inhibits the progression of carcinogenesis in animal model systems if allowed during the promotional phase. Glucocorticoid hormones regulate most of such effects. It is also known that the level of this hormone gets elevated during dietary restriction in animals. Glucocorticoids (GCs) produced by the adrenal cortices have multitude of effects within the body. They are involved in metabolism of lipid, carbohydrates and proteins, stress responses, fluid and electrolyte balance, as well as maintenance of immunological, renal and skeletal homeostasis. GCs exert their effects by binding to a single glucocorticoid receptor (GR), which is predominantly localized in the cytoplasm of the target cells. GR belongs to a phyllogenetically-conserved super family of largest known transcription factors. The knowledge of GR level, its activation process and interaction with chromatin, during dietary restriction might provide insight into the action mechanisms of glucocorticoids during such interventions, and will also help to understand the age-delaying role of DR in animals. Summarized below under various sections are the findings of the work done:

Changes in the body weight

Body weight graph of the mice after three months of alternate days of feeding showed a significant decrease (-34%; $p < 0.001$) in the body weight of the DR-fed mice (19.9 ± 1.1g) as compared to the AL-fed ones (30.4 ± 1.2 g). It has

assured that the animals were indeed subjected to dietary restriction during the experimentation.

Changes in the level of glucocorticoid receptors

Studies on glucocorticoid receptor (GR) level indicate a significant increase in the level of receptors in the dietary restricted (DR) mice as compared to the ad libitum (AL)-fed ones in both liver and kidney. Scatchard analyses confirm a higher concentration of receptor binding sites in the liver (41%) and kidney (37%) of DR as compared to that of AL-fed ones. However, the affinity (K_d) of GR for hormone remained the same in liver (~2.3 nM) and kidney (~2.1 nM) for both AL- and DR-fed mice. The slot blot analyses of receptor preparation corroborate the increased level of GR in the DR mice as compared the AL-fed ones in both the tissues. The increase in the level of GR may be a contributory factor in controlling the glucocorticoid-mediated responses during long-term dietary restriction in mice.

Activation studies of the glucocorticoid receptors

Higher activation of hepatic and renal GR complexes from both AL- and DR-fed mice was observed by temperature and salt. However, there was no significant change in the magnitude of activation (either by temperature or salt) of GR in both groups of animals in either of the tissues, which indicate no difference in the in vitro activation of hormone-receptor complexes from the liver and kidney of AL and DR mice under the conditions mentioned. It reflects that receptor activation may not be altered during such dietary interventions.

Physicochemical properties of the glucocorticoid receptors

Gel filtration analyses of unactivated and activated GR complexes from AL- and DR-fed mice did not reveal any change in terms of molecular weight for both groups of animals. Elution pattern from ion exchange chromatography also

showed a similar charge binding for both the groups of animals in unactivated and activated [^{3}H] dexamethasone-receptor preparations. From the above studies, it can be concluded that during dietary restriction the physicochemical properties of the GR remained unchanged.

DNase I extraction of bound GR from nuclei

DNase I digestion of liver and kidney nuclei from both AL- and DR-fed mice revealed a similar magnitude of extraction of bound GR complexes from both the groups of animals in either tissues. It shows that there may not be an appreciable change in the organization of chromatin in DR-fed animals as compared to the AL ones in both the tissues studied.

Studies on tryptophan 2,3 dioxygenase activity pattern

The activity pattern of tryptophan 2,3 dioxygenase (TO), a glucocorticoid inducible enzyme when measured in the liver showed a significantly higher activity (28%) in the DR animals as compared to the AL-fed ones. This increase in the activity of the enzyme may be due to increased level of GR in the DR animals. It reflects on the significance of increased GR level during DR in upkeeping the activity level of its inducible marker enzyme such as TO.

Aging studies

Our study indicates a significant decrease of GR level in the liver (25%) and kidney (28%) of old mice as compared to the adult ones in AL-fed animals. Whereas, the dietary restricted mice of both the age groups showed a marked increase in the receptor concentration in both liver and kidney as compared to the ad libitum-fed ones. However, the affinity of the GR for its ligand remained unchanged in both AL- and DR-fed animals from both the age groups in either of the tissues. Scatchard and the slot blot analyses of receptor preparation confirmed the higher level of GR in the liver and kidney of DR mice as compared

to the AL-fed ones in both the age groups. The high level of receptors in the adult animals compared to the old ones may be an essential factor for the role of this hormone during growth and development of animals. In older animals, an increase in GR level by DR might play a pivotal role in improving the metabolic activities and helping the animals to adapt better to the environmental stresses. The magnitude of activation of GR was lowered in both liver and kidney (15-20%) of old animals as compared to the adult ones. The decreased activation of GR in old animals 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 glucocorticoid receptors. In older group, the magnitude of activation was higher in both liver and kidney of dietary restricted animals as compared to the AL-fed ones, whereas, no such change was observed in the adult group of mice. The increase in the receptor activation in aged DR animals may help them to achieve better glucocorticoid action with lower level of receptors at that stage of life span. Our data also show a high degree of DNase I extraction of nuclear bound GR in the adult liver and kidney as compared to the old, whereas there was no marked effect of DR on such extractability at either age. This is indicative of more compact chromatin organization in old mice tissues. It also relates to the fact that there may not be an appreciable change in the organization of chromatin in DR animals as compared to the AL-fed ones.

To conclude, the findings summarized above indicate that –

- Dietary restriction does elevate the level of glucocorticoid receptor that may help animals to adapt to changing metabolic need during such interventions.
- DR could be used to elevate the level and the activation of GR in older animals, whose receptor levels get reduced because of age. This may be useful in better adaptation to stress in those animals to achieve greater survivility and longer life span.

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I, **Debipreeta Dutta**, hereby declare that the subject matter of thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University / Institute.

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INTRODUCTION

Dietary or calorie restriction is not starvation. It is a reduction of calorie intake without malnutrition. Dietary restricted animals receive a balanced reduction of the protein, carbohydrate, and fat content of the diet without a reduction in the micronutrients essential for normal growth and development. More than 70 years of research has supported dietary restriction as the only intervention that retards aging and lengthens life span in a wide variety of organisms. Clive M McCay and his colleagues were the first to discover that very low calorie diet can extend the life span in laboratory rodents (McCay et al. 1935). Since McCay's initial studies in rats, various laboratories have repeated, in various forms, dietary restriction regimens that extend life span, retard age-related diseases, and maintain the vitality of organism ranging from protozoa to mammals (Weindruch and Walford 1988). Such longevity extension results from the limitation of total calories derived from carbohydrates, fats or proteins to a extent of 25% - 60% below that of control animals fed ad libitum (Richardson 1985; Weindruch and Walford 1988). Recent studies showed that extension in life span can approach 50% in rodents kept on a defined dietary restriction (Sohal and Weindruch 1996). Virtually most informations regarding the effect of calorie restriction on aging and longevity come from research conducted with rodents and other short-lived species. However, emerging data suggest that long-lived non-human primates (NHP) also benefit similarly from dietary restriction (Mattison et al. 2003). The hypothesis that dietary restriction increases life span in non-human primates (NHP) received striking support from parallels between findings from studies in rhesus monkeys with those from rodents. These similarities include changes in body composition, maturation and reproduction, metabolism and the reduction of risk factors for diabetes and cardiovascular diseases (Lane et al. 1997).

Historical background of dietary restriction research

Research on dietary restriction (DR) was first initiated by McCay et al. in 1930s. First, they restricted all the dietary components to study the effect of

such restrictions. It was observed that the DR fed rats lived much longer than the AL fed ones (McCay et al. 1935). In their subsequent studies, McCay and his group used a diet where the amount of fat and carbohydrate was restricted but not proteins, minerals and vitamins. This study too revealed a longer life span in restricted animals as compared to their normally fed counterparts (McCay et al. 1937). From the above studies, McCay and his colleagues concluded that the longer life in dietary restricted rats was due to the decrease in the growth rate.

The next phase of DR research focused on the various beneficial effects of dietary restriction in different organisms. A number of studies performed during 1940s and 50s confirmed that dietary restriction inhibits carcinogenesis in both mice and rats, and markedly slowed the progression of age-associated diseases (Saxton and Kimball 1944). Reports were also available on life extending effect of food restriction in protozoa, rotifers and fishes (Rudzinska 1951; Fanestill and Burrows 1965; Comfort 1963). In 1960, a detailed study on the effects of long term dietary restriction on fertility, longevity and pathology in rats confirmed that DR retards reproductive aging, increases longevity and attenuates age-associated diseases in rodents and those beneficial actions of DR resulted from the reduction of body fat (Berg and Simms 1960). Ross and his collaborators studied dietary restriction mainly focusing on analysis of pathologic lesions and the manipulation of individual dietary components. Their data confirmed that reduced calorie intake is the nutritional factor responsible for life extension in rodents (Ross 1961).

During 1970s, research on dietary restriction got a new avenue with Roy Walford and his group who studied the effect of DR on DNA repair, mitochondrial function, metabolic characteristics, immune system and body temperature. Their findings indicated a significant increase in life span in both pre-and post-weaned mice by DR (Weindruch and Walford 1982; 1988). Later, Roy Walford himself was a member of the Biosphere 2 for 2 years. During that period, biochemical and physiological measurements were made of the crew members and was concluded that healthy non-obese humans on low calorie and nutrient dense diet show physiologic, hematological and

biochemical changes resembling those of rodents and monkeys on such diet (Walford et al. 2002). Contemporary to the Walford's group, another group of nutritionists headed by EJ Masaro initiated research on dietary restriction and aging in rodents, mainly focusing on the physiology and pathology. They used semisynthetic diet to determine the effects of restricting individual dietary components and concluded that the decrease in calorie intake is the major factor responsible for anti-aging action of food restriction. Their studies also revealed that DR results in a life long increase in the daily maximal level of plasma free corticosterone and a reduced level of plasma glucose and insulin throughout the lives of DR animals (Masaro et al. 1992).

In late 1970s, Brian J Merry along with his wife Ann Holehan initiated calorie restriction work on reproductive function and longevity of female rats. They concluded that female rats on restriction exhibit a reduced fertility at young ages, however, they remain fertile at much older ages than do fully fed rats. They also investigated the influence of food restriction on male reproductive function and found that in contrast to females, DR does not retard the age-associated decrease in male fertility (Merry et al. 1981). During 1980s, Merry and Holehan teamed up with Lewis and Goldspink to study the effect of dietary restriction on protein metabolism. They concluded that DR results in an increase in the whole body protein turnover and thereby prevents the accumulation of damaged proteins responsible for cellular aging (Merry et al. 1985). Influence of dietary restriction on autoimmune diseases and the mechanisms underlying the protective effects of DR were studied by Bob and his colleagues during 1970s. Their study revealed a protective role of DR in doubling the life span of the animals susceptible to autoimmune diseases (Good et al. 1976).

In late 1980s and early 1990s, calorie restriction and aging research on primates was initiated by George Roth and his collaborators at National Institute on Aging (NIA) and Joe Kemnitz and his collaborators at the University of Wisconsin (Roth et al. 1990; Kemnitz et al. 1993). From their initial studies, they arrived at a common conclusion that effects of calorie restriction also occur in the rhesus monkeys but it didn't give a conclusive idea due to lack of complete data. Now that the results of their pilot project on

rhesus monkeys indicate almost similar patterns of various effects as observed in rodent's studies (Roth 2003 and 04)

Scientists are utilizing the modern techniques for comparative study of protein synthesis, mRNA levels and nuclear transcription between dietary restricted and normally fed animals. It was shown that food restriction resulted in two-to three-fold increase of protein synthesis, mRNA levels and nuclear transcription in DR animals (Richardson et al. 1987). More recently, high-density gene expression array technology is used for the study of aging and dietary restriction (Weindruch et al. 2002). Transgenic mice models are used for the study of dietary restriction and aging with certain limitations to the use of these models.

Mechanisms underlying the effects of dietary restriction

The effects of dietary restriction are robust in every species tested (Weindruch and Walford 1988). DR delays a wide spectrum of diseases and reduces age-associated neurodegenerative disorders (Mattison et al. 2000). And most importantly, it is the only method known to extend mean and maximum life span in a variety of organisms (Weindruch and Walford 1988). In recent years, several theories have been proposed to explain the molecular mechanisms underlying the beneficial effects of dietary restriction but none of them could explain these effects conclusively. Its complexity lies in multiple effects including metabolic, neuroendocrine, and apoptotic changes, which vary in intensity and exhibit striking differences among specific organ system (Koubova and Guarente 2003).

Early theories suggested that restricted feeding acts through neuroendocrine mechanisms, which regulate development, metabolism, reproduction, adaptation to environment and senescence and can be modified by such interventions (Timiras 1983; Sonntag et al. 1999; Mobbs et al. 2001). Neuroendocrine system which coordinates effects on every tissue in the body (Nelson et al. 1995) plays an important role in senescence (Landfield 1978; Finch et al. 1984; Sapolsky et al. 1986; Weindruch and Walford 1988). This hypothesis was supported by the observations that hypophysectomy delays

many aspects of senescence and extends life span in rodents (Everitt et al. 1980).

Studies on free-living nematode, *C. elegans*, showed that loss of functions of several individual genes in the insulin-like signaling pathway extends maximum life span in that species (Guarente and Kenyon 2000). In fruit flies, *D. melanogastor*, also, loss of function of a hormone/ GTP-binding receptor like protein extends the life span of the flies (Lin et al. 1998). Many changes observed in the DR rodents trace directly or indirectly to changes in the neuroendocrine system. Animals on dietary restriction have lower levels of pituitary growth hormone (GH), thyroid stimulating hormone (TSH), insulin-like growth factor 1 (IGF-1) and gonadotropins (Mobbs et al. 2001). Conversely, DR increases glucocorticoids, catecholamines and glucagon in animals (Sabatino et al. 1991; Klebnov et al. 1995; Mobbs et al. 2001). Taken together, these studies suggest that actions of hormones may drive important processes in senescence including the limitations of life span. And it also supports the hypothesis that neuroendocrine responses to dietary restriction may mediate effects of DR to increase the life span in different organisms.

An alternate mechanism by which the neuroendocrine system may mediate effects of DR on senescence is obtained by regulating the glucose level (Mobbs et al. 2001). A wide range of data suggests that glucose metabolism in neuroendocrine cells of hypothalamus, cumulatively compromises the function of key neuroendocrine cells and leads to age-associated metabolic impairments (Mobbs 1990, 93). The hypothesis that glucose sensitive neuroendocrine cells regulate life span was supported by the study on nematode (*C. elegans)* and yeast (*S. cerevisiae*). Studies on *C. elegans* have demonstrated that life span of the worm is regulated by the action of an insulin-like pathway specifically in neurons (Mobbs et al. 2001). Studies involving *S. cerevisiae* revealed an essential metabolic step mediating effects of DR on the life span of yeast is the conversion of NAD^+ (nicotinamide adenine dinucleotide) to NADH (the reduced form of NAD^+) and not ATP. Several lines of evidences also indicate that hypothalamic neurons sense glucose by the conversion of NAD^+ to NADH, rather then production of ATP (Yang et al. 1999).

According to the evolutionary theories, dietary restriction could be viewed as part of a spectrum of neuroendocrine responses to environment with variable food levels that may have adaptive value during periods of short-term food storage in the wild (Navazio and Timiras 2003). According to the "female reproductive life span hypothesis" in nature, periods of low food availability (for example, drought) may outlast the reproductive life span of species such as mice and rats, and then there would be enormous selective advantage for those with genomes enabling them to respond by slowing reproductive aging (Harrison and Archer 1989). However, this hypothesis was rejected on the ground that reproductive senescence would be largely irrelevant to life in wild. The "energy apportionment hypothesis" states that there is clearly a selective advantage for individuals with genomes that respond to food shortages by directing energy expenditure to somatic maintenance rather than reproduction. Those individuals have an increased ability to survive the shortage and generate progeny when food again becomes plentiful. Moreover, when the reduction in food availability is sustained, as in dietary restriction laboratory protocols, this diversion of energy to somatic maintenance serves to slow senescent processes (Holliday 1989). Walford and Spindler proposed the "hibernation like hypothesis" of anti-aging action of dietary restriction. This hypothesis states that the functional characteristics of dietary caloric restriction and those of hibernation are two elements of a spectrum of evolutionary adaptations to food scarcity (Walford and Spindler 1997). Recently, the evolutionary hypothesis has been tested with the help of a computer modeling and the observations are in accord with the energy apportionment hypothesis of Holliday (Shanley and Kirkwood 2001; Kirk 2001).

Oxidative stress, free radicals and dietary restriction

According to the current theories, dietary restriction is thought to exert its effects by lowering the oxidative stress and protecting against free radicals accumulation, by upregulating apoptosis or by changing gene expressions and suppressing DNA damages. Oxidative stress is defined as the irreversible modification of cellular components resulting from the impact of pro-oxidant

generating processes (Yu 1996). In older animals, an elevation of oxidative stress can be observed as compared to the young ones and DR feeding retards the accural of accepted oxidative damages, such as the tissue concentration of peroxidized lipids, protein carbonyls and oxidative damage to bases in genomic and mitochondrial DNA (Merry 2000). The main cellular sites for the generation of free radicals and/or reactive oxygen species (ROS) are mitochondria, and are therefore susceptible to oxidative damages. Complexes I and III of the electron transport chain, located in the inner mitochondrial membrane, are considered to be the major sites of free radical generation (Bajra et al. 1998). Changes in membrane fluidity with age may compromise trans-membrane transport, the efficiency of oxidative phosphorylation and impair cell signaling. DR has been found to preserve mitochondrial and microsomal membrane order and fluidity with age in liver, brain and cardiac ventricular muscle (Yu et al. 1992; Choi and Yu 1995; Kim et al. 1996; Lee et al. 1999). Dietary restriction alters the membrane lipid composition making it less readily peroxidized (Pieri et al. 1990). DR animals sustain a higher unsaturation/ saturation index while lowering the content of the more readily peroxidized fatty acids (Laganiere and Yu 1987; Laganiere and Fernandes 1991). The preservation of membrane order and fluidity by restricted feeding is associated with the attenuation in age-related changes in membrane receptor number and binding affinity (Merry 2002). Both the oxidative lesions in DNA and the oxidatively damaged proteins have been shown to accumulate with aging. A significantly reduced accumulation of oxidatively damaged proteins was observed in animals subjected to restriction of proteins (5 to 10% of the diet as compared to the 20% of control) and dietary calorie (25 to 40% of control intake) (Merry 2002). The oxidative damage within a tissue represents the equilibrium between the rates of oxidant generation, and the rates of oxidant scavenging, repair and turnover processes (Beckman and Ames 1998). These four processes are all modified in a tissue-specific and time-dependent manner by dietary restrictions (Merry 2000). The precise mechanism by which DR results in lowered oxidative damage within a tissue is yet to be ascertained.

Apoptosis and dietary restriction

Apoptosis literally means programmed cell death. Several studies suggest role of apoptosis in limiting the life span of animals. Mice with a targeted disruption in the $p66^{shc}$ gene exhibit a longer life span than wild type animals (Migliaccio et al. 1999). $p66^{shc}$ is one of the down-stream targets of the p53, a key regulator of damage-induced apoptosis, (Trinei et al. 2002). Another finding, which implicates apoptosis in aging, is yeast Sir2 gene. The scope of Sir2 impact on life span has been extended recently to multicellular organisms. In the nematode, *C elegans*, elevated level of Sir2.1 extends life span by as much as 50% without affecting development (Tissenbaum and Guarente 2001). Recently, it has been shown that a cytoplasmic Sir2p homologue can promote survival in the protozoa (*Leishmania*) by preventing apoptosis (Vergues et al. 2002). The mammalian ortholog of SIR2 and SIRT1 represses the activity of p53 and therefore down regulates apoptosis (Luo et al. 2001; Vaziri et al. 2001). From the studies of survival function of Sir2, apoptosis thus appears to be important in limiting mammalian life span. It has been reported that presence of a hyperactive allele of p53 in transgenic mice leads to an early organ degeneration and promotes premature aging (Tyner et al. 2002). The above studies support the idea that mammalian life span is controlled by apoptosis, which enhances the aging process.

Gene expression and dietary restriction

Aging results in altered differential gene expression pattern indicative of a marked free response and lower expression of metabolic and biosynthetic genes. Most of these alterations can be completely or partially prevented by dietary restriction (Lee et al. 1999). DR has been shown to delay an age-associated change in hepatic gene expression and is reported to enhance the expression of mRNA level of the $\alpha 2\mu$- globulin gene in old animals which declines with age as compared to their fully fed counterpart (Richardson 1987). DR also delays the depression of androgen repressible senescence marker protein (SMP-2) in the liver of rats as compared to the control animals (Chatterjee et al. 1989). The effect of DR on age-dependent changes in gene expression correlated with the hepatic level of immunoreactive cytoplasmic

androgen-binding (CAB) protein. Androgen receptor mRNA is almost undetectable in prepubertal and senescent male rats. Only hepatocytes that express $\alpha 2\mu$- globulin gene contain androgen receptor mRNA, and the retardation of the age-dependant loss of androgen sensitivity by DR is due to a concomitant delay in decline of androgen receptor mRNA synthesis (Song et al. 1991).

Dietary restriction retards the age-related decline in DNA repair. Increased rate of DNA synthesis has been reported in the liver and kidney of DR-fed mice as compared to the AL- fed ones (Weraarchakul et al. 1989). Recently, the "hormesis hypothesis" was put forwarded by EJ Masaro to explain the mechanisms underlying the anti-aging and life-prolonging action of dietary restriction (Masaro 1998). Hormesis is defined as the biological action resulting from the response of an organism to low intensity stressors. Stresses that have been reported to delay aging and prolong life span in different systems (for example, yeast, fruit flies, nematodes, rodents and human cells) include temperature shock, irradiation, heavy metals, pro-oxidants, acetaldehyde alcohol, hypergravity, exercise and dietary restriction (Rattan 2004). DR in rats causes a moderate increase in the daily plasma corticosterone level, which indicate that it is a long term low intensity stressor. Dietary restriction may enhance the ability of old rodents to cope with a spectrum of acute intense damages by its hormetic action. In addition, DR also enhances the induction of stress proteins in response to damage (Heydari et al. 1993). Further support for the hormesis hypothesis is provided by the finding that single gene mutation that extends the life span also increases the ability of these organisms to cope with damaging agents (Martin et al. 1996).

Metabolic responses and dietary restriction

Although very little is known about the effects of long-term DR regimen on specific metabolic pathways. Indirect evidences suggest that DR alters the flux of intermediates through the glycolytic, gluconeogenic and nitrogen metabolizing pathways (Weindruch et al. 2001). During the adaptive phase of DR, animals breakdown carbohydrates and fats to compensate the loss of

blood glucose. As a result, loss of fat mass, a characteristic feature of dietary restriction is observed in the DR animals (Barziali and Gabriely 2001). And in the steady state, DR animals show a lower level and improved sensitivity of insulin, which may be due to the tissue-specific effects on cell insulin-receptor binding (Wang et al. 1997).

Another important metabolic alteration by DR is the reduction of core body temperature (Weindruch and Walford 1988; Duffy et al. 1990; Lane et al. 1996). This change is mainly due to an increase in the coupling of oxidative phosphorylation to ATP synthesis, perhaps by reduction of the level of uncoupling proteins. These proteins span the inner mitochondrial membrane and may allow proton leakage and thus hijack a fraction of the energy of electron transport chain (ETC) to generate heat rather than ATP (Weindruch et al. 2001). Other effects of DR that have been extensively reported are reduction of mitochondrial damage, free radical accumulation and lipid peroxidation. This reduction would be mediated, at least in part, by the decrease in energy expenditure brought about by the increased activity of these enzyme systems responsible for detoxification of reactive oxygen species (Ramsey et al. 2000).

Lipofuscin, a classical cellular biomarker of aging, accumulates with age in different tissues of many mammalian species. DR has been shown to lower the level of such fluorescence age pigments (Enesco and Kurk 1981). DR is reported to modify the membrane fatty acids composition in hepatocytes, which results in a membrane that is more resistant to peroxidation (Laganiere and Yu 1987).

Studies show that DR leads to an increase in the activity of key enzymes of hepatic gluconeogenesis, decreases the level of key enzymes of hepatic glycolysis and increases the activity of enzymes responsible for the disposal of nitrogen derived from muscle protein catabolism for energy production. The DR related changes in the activity and /or mRNA level of these enzymes suggest that DR enhances protein turnover in mice of all ages, resisting the well documented decline in peripheral tissue protein degradation with age (Dhahbi et al. 1999).

Cross-linking of a collagen, structural protein, is considered as another biomarker of aging. DR has been shown to retard the cross-linking and deposition of collagen in different tissues (Merry 2002). Numerous studies indicate that protein degradation declines with aging and is accompanied by an accumulation of damaged or aberrant proteins (Lavie et al. 1982; Gracy et al. 1985; Grune et al. 2001). DR has been shown to delay the age-related decline in protein degradation as well as retard the accumulation of oxidatively damaged or abnormal proteins. Some age-related pathologies, including those that result from the accumulation of damaged proteins, are also delayed by DR. The chymotrypsin like activity of SDS-stimulated proteasome changes with age and the process was delayed by DR (Scorfano et al. 1998). Age-related cataract is associated with the accumulation of cross-linked protein, called crystallin. DR has been shown to delay the age-related degeneration of eye lens (Li et al. 2003; Wang et al. 2004).

Hormonal changes and dietary restriction

Dietary restriction influences various physiological functions of an organism. It has been observed that secretion of pituitary hormone is reduced during food restriction in rats resulting in hypophysectomized state in the animal. Studies also revealed that complete removal of pituitary gland results in a greater anti-aging action. From these studies, it was concluded that DR by reducing the secretion of pituitary hormones, such as GH, diminishes the oxidative damage and delays the development of age-related diseases and extends life span in rodents (Everitt 2003). DR is reported to retard the puberty by reducing the circulating levels of LH, FSH, testosterone and its peripheral metabolite 5α-dihydrotestosterone in male mice, whereas in female it does the same by reducing the level of FSH and significantly elevating the circulating level of 17β-estradiol (Merry and Holehan 1981, 85).

DR is reported to delay the reproductive aging in mice as judged by the rate of follicular depletion and estrous cycle. With increasing age, the estrous cycles of *ad libitum* (AL) fed females are associated with characteristic irregularities. Chronic dietary restriction abolishes the age-related increase in cycle length and results in much later appearances of irregularities in the

cycle (Lu 1983). A number of studies suggest that dietary restriction delays reproductive aging in rodents (Holehan and Merry 1999). DR is said to attenuate the age-related hyperparathyroidism in old rodents. The detailed mechanism by which DR prevents hyperparathyroidism is not known, but a concomitant decrease in the incidence of renal lesions has been reported (Kalu et al. 1984). During DR in the rat, total body thyroxine (T_4) to triiodothyronine (T_3) conversion is significantly reduced (Van Doorn et al. 1984). Thus, moderate restricted feeding that is sufficient to prolong life span has a greater effect on the peripheral conversion of the thyroid hormones than on their central neuroendocrine control through the release of thyroid stimulating hormone (Herlihy et al. 1990).

In contrast to thyroid hormones, the level of glucocorticoids gets elevated during dietary restriction due to the stimulation of hypothalamo-pituitary-adrenal (HPA) axis (Everitt 1976). It has been reported that three months of alternate days feeding increases the level of glucocorticoid receptors (GR) in both young and old male mice, which might help the animal in better adaptation during more stressful condition (Dutta and Sharma 2003, 04). DR is also reported to decrease the expression of GR in the hippocampus and cerebral cortex of rats (Lee et al. 2000). Dietary restriction in Lobund-Wistar rats results in modulating the age-associated changes in adrenal catecholamines, dopamine, norepinephrine, epinephrine and dihydroxymendelic acid (Kingsley et al. 1991). The age-related loss in glucagon-promoted lipolysis in adipocytes is prevented by dietary restriction. Underfeeding preserved the response to insulin and completely prevented the age-related decline in the lipolytic response (Bertrand et al. 1980; Volicer et al. 1983).

Immune responses and dietary restriction

Dietary restriction is known to be an effective method for prolonging the life span, delaying immunosenescence, and reducing pathology in various strains of rodents. The decrease of IL-2 expression with aging was attenuated by dietary restriction and was shown to be altered at the level of transcription. The increase in expression of IL-2 expression by T-cells from DR rats in

response to antigens would result in a more robust immune response, which could provide an organism better protection against foreign antigens (Pahlavani 1998). DR strongly inhibits the blood levels of IL-6 and tumor necrosis factor-α (TNF- α), commonly increased during aging in rodents and humans. DR has anti-inflammatory roles in brain, which normally show prominent activation of microglia-monocytes during aging and neurodegenerative diseases (Lee et al. 2000; Patel and Finch 2002). DR attenuates cellular immune aging changes in rodent genotypes for example, lectin-induced proliferation of spleenic lymphocytes, which decreases sharply during aging in AL-fed animals, is increased two folds by DR in all ages (Weindruch et al. 1982).

Neuroprotective effects of dietary restriction

DR can have profound effects on brain function and vulnerability to injury and diseases. It protects the brain against various neuorodegenerative diseases and also stimulates the production of new neurons from stem cells. DR regimen has been shown to retard the age-related increase in the level of glial fibrillary acidic protein and oxidative damage to proteins and DNA (Dubey et al. 1996; Major et al. 1997). It is reported to have beneficial effects on synapses also. The synaptosomes from the DR rats exhibited improved glucose transport and mitochondrial function following exposure to oxidative and metabolic stresses (Guo et al. 2000). Dietary restriction also helps in increasing the neurogenesis in the brains of adult rats and mice (Lee et al 2000). DR was shown to attenuate the age-related loss of spiral ganglion neurons in C57BL/6 mice (Park et al. 1990). Rats maintained on DR exhibit increased resistance of hippocampal neurons to excitotoxic degeneration (Bruce-Keller et al. 1999). DR may be beneficial for epilepsy patients by reducing seizure incidences and severity (Mahoney et al. 1983). DR can also counteracts adverse effects of deficiencies of certain nutrients. Thiamine deficiency impairs oxidative metabolism and can cause degeneration of neurons in certain susceptible brain regions such as the thalamus. Rats maintained on a DR regimen are able to tolerate thiamine deficiency such that damage to thalamic neurons was greatly reduced (Calingasan and Gibson

2000). A major contribution of the beneficial effects of DR on neurons comes from a cellular stress response in which levels of protein chaperons and neurotrophic factors are increased. The cellular stress response may be induced by a mild metabolic stress associated with DR resulting from hunger. Finally, the hormesis hypothesis for the beneficial effects of DR in the brain also provides a satisfactory explanation for the increased neurogenesis observed in mice and rats maintained on DR (Lee et al. 2000).

Human studies and dietary restriction

Dietary restriction studies in human are still in their infant stage. Very little is known about the effect of diet on the rate of human aging. Although, many studies of DR on human are conducted but none of them could forward a definite conclusion about the impact of DR on aging in human. A recent study conducted among Islamic people during 'Ramadan' period revealed an elevated level of plasma HDL by 25 to 30% (Maislos et al. 1998). This result may be analogous to the HDL increase observed in DR monkeys (Verdery et al. 1997). HDL is infact inversely related to atherosclerosis and other cardiovascular diseases.

Using a unique set of experimental design, Rao et al. (1996) performed a study of naturally occurring south Indian population of three age groups (Young, 8-14 years; adult, 20-35 years; old, above 55 years) who were having normal and undernourished diets as judged on the basis of body mass index and the history of their dietary intake. The DNA-repair capacity was found to be higher in the lymphoytes of age-matched undernourished individuals. They also observed a slower reduction in DNA repair capacity as a function of age in undernourished individuals as compared to normal fed ones. These studies provide a potential link between DNA-repair mechanisms and nutritional state in humans.

In another study conducted on the Japanese of Okinawa island, it was reported that Okinawans have a longer life span (81 years) with six times as many centenarians per 100,000 people than those living in the United States, and 97% of their lives were spent free from disabilities. Their longevity has been correlated with a diet relatively low in calories and high in fruits and

vegetables (Weindruch and Walford 1988). Two recent studies of Biosphere 2 in Arizona and the TNO Toxicology and Nutrition Institute study in Netherlands (Walford et al. 1992; Velthuis et al. 1994) exemplified a better-planned DR study in human. Biosphere 2 is a close structure containing an approximately 3-acre ecosystem in the desert near Tuscon, Arizona. Several biomedical studies were conducted on the four male and female occupants. Level of consumption approximated an average degree of restriction of about 30%. However, the diet was both nutrient dense and low in fat. Biosphere 2 crew showed a marked decrease in the body weight and reductions in the level of fasting glucose, blood pressure, serum cholesterol and serum triglycerides (Walford et al. 1992). TNO Toxicology and Nutrition Institute Organisms subjects also lost weight and lowered blood pressure (Velthuis et al. 1994).

Aging studies and dietary restriction

Aging is usually defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality with advancing age (Kirkwood and Austad 2000). It is a result of accumulation of random damages to the building blocks of life especially DNA, certain proteins, carbohydrates and lipids. This damage gradually impairs the functioning of cells, tissues, organs and organ systems, thereby increasing vulnerability to disease and give rise to the characteristic manifestations of aging (Hayflick et al. 2002). Aging is viewed as an extremely complex multitactorial process (Kowald and Kirkwood 1996). Thus, keeping in mind the multiplicity of mechanisms regulating aging, various theories have been broadly divided into four main categories viz., evolutionary, molecular, cellular and systemic (Weinert and Timiras 2003). The theories of aging may overlap at various levels of organization, alterations with aging of molecular events may lead to cellular alterations, and these, in turn, contribute to organ and systemic failure with evolutionary implications for reproduction and survival.

Evolutionary theory was first proposed in 1940s based on the observation that, a dominant lethal mutation, remained in the population even though it should be strongly selected against (Haldane 1941). The

evolutionary theories include mutation accumulation theory (Medawar 1952), disposable soma theory (Kirkwood 1977) and antagonistic pleiotropy (Williams 1957). Mutation accumulation theory states those detrimental, late-acting mutations may accumulate in the population and ultimately lead to pathology and senescence (Medawar 1952). Disposable soma theory is based on optimal allocation of metabolic resources between somatic maintenance and reproduction. It means that longevity has a cost; the balance of resources invested in longevity vs. reproductive fitness determines the life span of an organism (Kirkwood 1996). Whereas, the theory of antagonistic pleiotropy suggests that some genes may be selected for beneficial effects early in life and yet have unselected deleterious effects with age, thereby contributing directly to the aging.

According to the molecular theories, mainly the genes regulate the life span of an organism by interacting with different environmental factors. Molecular theories include, codon restriction theory, somatic mutation theory, error catastrophe theory and gene regulation theory. Codon restriction theory is based on the assumption that fidelity/ accuracy of mRNA translation is impaired due to inability to decode codon in mRNA (Strehler at al. 1971). The somatic mutation theory suggests mutations occurring randomly in the somatic tissues accumulate with age and leads to structural disabilities and finally to the death of an organism (Szilard 1959). Error catastrophe theory of aging proposed that decline in fidelity of gene expression results in increased fraction of abnormal proteins, which ultimately lead to the senescence (Orgel 1963). Gene regulation theory proposes that aging results from the differential gene expression regulating both development and aging of an organism (Kanungo 1994).

Cellular theories relate to the changes that occur in structural and functional elements of cells with the passage of time. These theories include cellular senescence-telomere theory, free radical theory, wear and tear theory and age pigments theory. Hayflick and Moorhead (1966) proposed the cellular senescence theory, according to which phenotypes of aging are caused by an increase in frequency of senescent cells. Aging might be a result of telomere loss (replicative senescence) or cell stress (cellular senescence) (Sitte et al. 1998). Free radical theory of aging proposed that damages of DNA, proteins

and lipids by highly reactive free radicals, produced as a result of oxidative metabolism, are critical in determining the life span of an organism (Harman et al. 1956). Since not only free radicals lead to damage of biomolecules but also other cellular reactive by-products of the normal metabolism, the theory has been extended to the 'oxidative damage theory' which suggests that reactive oxygen species or reactive oxygen intermediates are responsible for the accumulation of age-related cellular damages, and that these damages represent an important contributor to aging (Osiewacz et al. 1997). According to the wear and tear theory, accumulation of normal cellular injuries results in the aging of an organism (Sacher 1977). Lipofuscin is a potential biomarker of aging, which shows an age-dependent increase in different tissues of mammals and flies (Sohal 1973; Shedal and Tappel 1974). According to this theory, aging is the result of various side reactions essential to biological processes that lead to the formation of age-pigments, and contribute to the senescence (Hammer and Braum 1988; Yin 1996; Grune et al. 2001).

Systemic theories of aging include neuroendocrine and immunologic theories. Neuroendocrine theory suggests that aging results due to changes in neural and endocrine functions crucial for coordinating communications and programming physiological responses to environmental stimuli and maintaining an optimal functional state in response to the environmental demands. The neuroendocrine system is thus responsible for preservation and maintenance of the internal homeostasis despite the continuing changes in the environment. Alterations in these control points of homeostasis result in age-related physiological changes (Weinert and Timiras 2003). The immune system is also essential for controlling and eliminating the foreign agents from the body while at the same time recognizing self and sparing from destruction the molecules (cells and tissues) from oneself. The efficiency of immune system decreases with the increase of age characterized by decreased protection against infection in old ages (Franceschi et al. 2000). The immunological theory suggests a decline of immune function with aging results in decreased incidence of infectious diseases and increased incidence of autoimmunity.

The ability of dietary restriction to modulate the life span and retard pathogenesis provides an opportunity to test the aging hypotheses and to

prolong the mean and maximum life span (Masaro et al. 1988). DR has been shown to retard the decline of severe physiological functions and delay the onset of many age-related pathologies (Aksenova 1998). The decrease in the rate of aging by DR is often accompanied by a decrease or loss in the ability to reproduce which has been interpreted as a part of the fundamental trade off between reproduction and maintenance (Grune et al. 2001). Although the basic mechanisms by which DR retards aging and reduces susceptibility to diseases are not yet understood, several hypotheses have been put forth for that. Factors that may contribute are decreased body temperature, altered metabolism, increased metabolic efficiency, differential gene expression and decreased DNA damage or increased DNA repair (Duffy et al. 1989 and 90; Feurers 1989). More recently, it has been shown that DR stimulates the production of growth factors and stress proteins that may increase the resistance of cells to age-related diseases (Mattson et al. 2001).

Glucocorticoids and their physiological effects

Glucocorticoids (GCs) are synthesized and released into the circulation by the adrenal gland (Miller et al. 1995). They have multitude of effects within the body. The endogenous members of this family are involved in a breadth of endocrine functions, and are necessary for maintenance of many important biological activities. They are important for metabolism of lipids, carbohydrates, and proteins. As the name suggests they are mainly gluconeogenic in nature and are responsible for regulation of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), serine dehydrogenase (SDH) and tyrosine aminotransferase (TAT) (Kellondonk et al. 1999). Glucocorticoids directly or indirectly maintain fluid and electrolyte balance and are crucial for the regulation of basal and stress related homeostasis (Coghlan et al 2003). Highly important is the role of GCs in the dynamic modulation of inflammatory and immune responses (De Bosscher et al. 2003). In brain, glucocorticoids influence emotions and cognitive processes like learning and memory (Mattison et al. 2003). Glucocorticoids are also involved in the developmental processes, for example, they play an important role in lung maturation,

chromaffin cells differentiation and erythroblast proliferation (Kellendonk et al. 1999). Secretion of GCs is under the tight regulation of corticotropin releasing hormone (CRH), which is secreted from neuronal hypothalamus on sensory input. CRH then stimulates anterior pituitary to secrete adrenocorticotropin (ACTH); ACTH released into the blood then stimulates adrenal cortical cells to secrete glucocorticoids. These three regions are collectively called Hypothalamus-Pituitary-Adrenal (HPA) axis, an integrated system that maintains the appropriate level of glucocorticoids in the body (Fink et al. 1997).

Glucocorticoid receptor (GR)

The most known cellular effects of glucocorticoids are mediated by a ubiquitous intracellular receptor protein, the glucocorticoid receptor (GR), predominantly localized in the cytoplasm of target cells (Kino et al. 2003). GRs are expressed in almost all cell types and their density varies from 2,000-30,000 binding sites per cell (Adcock et al. 1996). The magnitude of a cell's response to glucocorticoids depends both on the hormone level it is exposed to and on its glucocorticoid sensitivity i.e., efficiency of GR mediated signal transduction (Bamberger et al. 1996). The GR is expressed in cell- and tissue-specific manner and the level of GR is negatively regulated by glucocorticoids contributing to the fact that long-term treatment with glucocorticoids results in a decrease of the physiological response (De Bosscher et al. 2003). Glucocorticoid receptor belongs to a largest known superfamily of ligand-activated eukaryotic transcription factors and its members mediate signaling pathways in a wide variety of physiological systems. The superfamily is broadly divisible into three subclasses: the type I receptors for steroid hormones, including progesterone receptor (PR), estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR); the type II receptors for thyroid hormone (THR), vitamin D receptor (VDR), 9 cis retinoic acid (RXRs) and all trans retinoic acid (RARs); and type III for those whose cognate ligands have not yet been characterized,

the orphan receptor subclass (O'Malley et al. 1999). The GR can function either by direct protein-protein interaction i.e., independent of DNA binding or by modulating the chromatin structure to allow the assembly of other gene regulatory proteins at transcription machinery on DNA (McEwan et al. 1997; Biola and Pallardy 2000).

Studies on GR-deficient mice have demonstrated that functions of GR are essential for survival, although, the molecular mechanism is yet to be elucidated (Cole et al. 1995). Reichardt et al. (1998) showed that DNA-binding of GR is not essential for survival by generating a mice with a point mutation known to impair dimerization and DNA binding. Despite the impairment of several important physiological functions of the GR, the mutation does not reduce viability, indicating its non-genomic role in varied physiological functions.

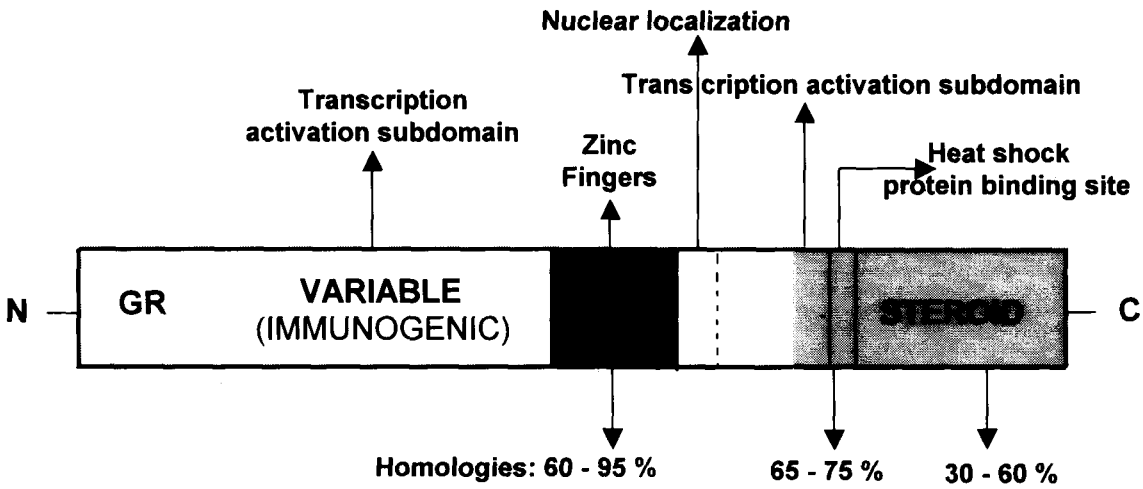
Isoforms of GR

The human GR gene consists of nine exons and is located on chromosome 5 (Kino and Chorosus 2001). Exon 1 consists solely of 5′-untranslated sequence, and exon 2 encodes for amino terminal portion of the GR. Two exons separately encode the Zn fingers. A total of five exons combine to form the cortisol-binding domain. Alternating splicing of the terminal exons 9α and β results in two variants of GR, i.e. GRα and GRβ. The 94 kDa hormone binding GRα is made of a 777 amino acid protein, while the 91 kDa non-hormone binding GRβ contains 742 amino acids. The first 727 amino acids from the N-terminus are identical in both the isoforms. GRα possesses additional 50 amino acids, while the GRβ contains an additional 15 non-homologous amino acids in their C-terminus. This variation renders GRβ unable to bind glucocorticoids (Bamberger et al. 1995; Yudt MR and Cidlowski JA 2001). However, GRβ is capable of binding to glucocorticoid response elements (GRE) and forms homodimers as well as heterodimers with GRα. GRβ can also associate with heatshock protein (Hsp 90), although with lower affinity than GRα. Transactivation and transrepression activities of glucocorticoids are mediated by GRα (Bamberger et al. 1995). Formation of

GRα/GRβ heterodimer is an important component of the mechanism responsible for the inhibitory effects of glucocorticoids (Bamberger et al. 1996). A recent report suggests that over-expression of the GRβ in the inflammatory cells might contribute to steroid insensitivity in diseases such as asthma (Hamilos et al. 2001).

Modular structure of GR

The modular structure of GR has been elucidated using the cDNA analysis and site directed mutagenesis, which have revealed distinct domains in the receptor protein (Muller and Renkawitz 1991). As with other members of the nuclear receptor family, glucocorticoid receptor contains a modular structure consisting of three major domains, C-terminal ligand binding domain followed by a central DNA binding domain and the N-terminal variable or immunologic domain (Kino and Chorosus 2001).



MODULAR STRUCTURE OF GLUCOCORTICOID RECEPTOR

The ligand binding domain: The C-terminal ligand binding domain (LBD) which extends approximately 250 amino acids serves as the binding site for the hormone (Chen et al. 1994; Warrier et al. 1994). The function of this domain is very complicated. It contains sequence critical for binding of heat shock protein, nuclear translocation, dimerization, transactivation and sequence for silencing the receptor in the absence of hormone. In addition to the above, LBD is important determinant in receptor mobility (Bamberger et al. 1996).

The DNA binding domain: The three-dimensional structure of the DNA binding domain (DBD) of GR in solution and bound to DNA has been resolved by 2D-NMR (Hard et al. 1990; Luisi et al. 1991). The central DBD is composed of two highly conserved zinc finger regions and is most conserved region among nuclear receptors. The first zinc finger is primarily thought to be responsible for target site recognition. Three residues in the carboxyl half of the first zinc finger termed as "P box", are responsible for response element discrimination (Luisi et al. 1991). The second zinc finger stabilizes protein-DNA interactions and contains the "D box" region critical for receptor dimerization (Luisi et al. 1991). The DNA binding domain is also required for the repression of other transcription factors such as NFkB and AP-1.

The variable or immunologic domain: The N-terminal variable domain (tau1) is involved in transcriptional trans-activation of genes and is also involved in binding to other transcription factors (Dahlman-Wright et al. 1994). This is the least conserved part of receptor protein between individuals and between species. Deletion analysis has demonstrated a 41 amino acid core at the C-terminal end of tau1, which is critical for transactivation. In human GR, there is another trans-activating domain (tau2) adjacent to the glucocorticoid binding domain and this region is also important for the translocation of the receptor (Barnes 1998). Other functional constituents of this region are nuclear localization signal and immunogenic epitopes. Most of the known antibodies generated against GR are from the antigenic epitopes localized in this region (Dahlman-Wright 1994).

GR and molecular chaperones

The cycling of GR and chaperone is responsible for maintaining hormone binding form of the GR. Though heat shock protein 90 (Hsp 90) and heat shock protein 70 (Hsp 70) are sufficient for interaction and activation of GR but presence of Hsp organizer protein (Hop), heat shock protein 40 (Hsp 40), phosphoprotein (p23) and immunophillins (IPs) acts as non-essential co-chaperones which optimize the assembly of Hsp 90, Hsp 70 and GR as a hetero-complex (Prodromou et al. 2000). First, Hsp 70 in association with Hsp 40 and Hsp 70 interacting protein (Hip) binds to GR (Meacham et al. 1999). Then, a dimer of Hsp 90 binds to Hsp 70-bound GR. Simultaneously Hop, a

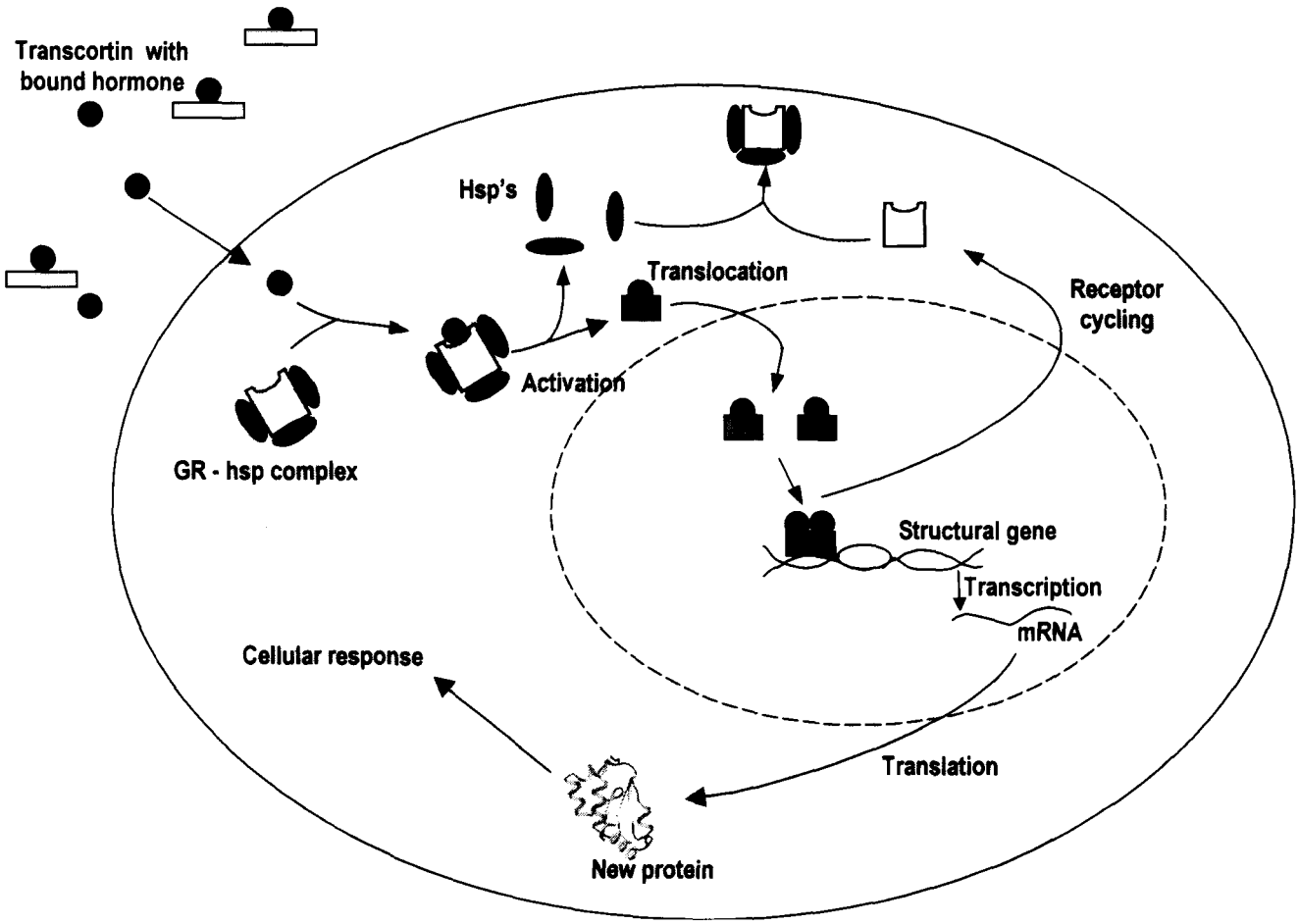
60 kDa co-chaperone acts as a docking protein to bind both Hsp 90 and Hsp 70 and completes the formation of intermediary complex (Morishima et al. 2000). Another co-chaperone called, Bcl-2 associated gene product 1 (BAG-1), promotes dissociation of Hop bound Hsp 70 complex from the intermediate complex, and simultaneous association of IP and p23 to GR-Hsp 90 complex leading to the formation of mature complex. Then, p23 facilitates dissociation of IP. Finally, an interaction partner of Hsp 70 called carboxy terminal of Hsp 70 interacting protein (CHIP) stimulates the dissociation of p23 from the Hsp 90 dimer by direct interaction with a tetracopeptide repeat acceptor (TPR) site of Hsp 90 (Ballinger et al. 1999). The Hsp 90 heteromeric complex undergoes constant cycle of dissociation and ATP- and Hsp-dependent association in absence of corticosteroids. The main function of the GR-Hsp complex is to keep the receptor in a transcriptionally inactive, yet in the high affinity glucocorticoid binding state (Prima et al. 2000). The functional GR either binds a glucocorticoid, otherwise it re-enters the chaperone cycle in order to maintain its three dimensional functional structure. Further, the steroid binding activities and transactivation potential of the receptor are abolished by CHIP, which is also responsible for induction of ubiquitylation of GR and its degradation by proteasomal enzymes (Meacham et al. 2001).

The unliganded GR

Based on cell activation studies, the unliganded receptors were originally thought to be localized in the cytoplasm and to translocate to the cell nucleus upon binding of the ligand (Truss and Beato 1993). Careful analyses have shown that the GR is loosely associated with the cell nucleus in the absence of hormone, and that binding of ligand leads to a tighter nuclear binding (Welshons et al. 1985). Moreover, heat shock treatments of cells seem to induce nuclear translocation of unliganded GR (Sanchez 1992). Furthermore, there is an energy-dependent continuous shuffling of receptors from the cytoplasm into the cell nucleus (Hache et al. 1999). Nuclear retention of GR is prevented by inhibition of protein phosphatase 2A and 1 trapping GR in the cytoplasmic compartment. Receptor phosphorylation may influence the interactions of GR with other transcription factors required for transactivation. Phosphorylation of the unliganded GR on a tyrosine residue in the glucocorticoid-binding domain may block subsequent hormone binding and nuclear translocation (Kido et al. 1987).

Mechanism of action of glucocorticoids

Glucocorticoids exert their cellular and molecular actions through a cascade of regulatory events initiated by high affinity binding to the ~ 94 kDa intracellular protein, the GR. The GR in its unliganded but ligand-friendly state is located primarily in the cytoplasm. Being lipophilic in nature, free glucocorticoids enter the cell by simple diffusion through the plasma membrane and binds to GR noncovalently to form the hormone-receptor complex. After binding to its agonist ligand, the GR undergoes conformational changes, dissociates from the heat shock proteins, homodimerizes, and translocates into the nucleus through the nuclear pore, a process called activation/transformation. In the nucleus, the ligand-activated GR directly interacts with the specific DNA sequences, the glucocorticoid response elements (GREs), in the promoter region of the target genes or with other transcription factors via protein-protein interactions, to modulate the gene expression.



GLUCOCORTICOID ACTION MECHANISM

Activation of GR: Lipophilic glucocorticoids enter the cell by free diffusion into the cytoplasm to interact with the intracellular GR. Once inside the cell, the glucocorticoid binds the GR and induces a process known as receptor activation that converts non-DNA binding form of receptor to a DNA binding form. The activation of receptor is crucial in the glucocorticoid action as it is a rate limiting step for nuclear chromatin binding (Bamberger et al. 1996). In vitro activation of GR can be achieved by exposing the cytosol containing hormone-receptor complexes to a temperature i.e., 25°C or to a selective salt concentration in the cold, i.e. 0°C. Dialysis, gel filtration and elevated pH also transform the hetero-oligomeric receptor to a monomeric nuclear binding form (Milgrom et al. 1981; Meshinchi et al. 1990; Sharma 1991). Although the exact mechanism of receptor activation is still not clear, it is thought to be controlled by subunit dissociation (Picard and Yamamato 1987), endogenous heat stable cytoplasmic/ nuclear factors (Bailley et al. 1977; Litwack 1988), phosphorylation/ dephosphorylation and presence of small RNA molecules. The partially phosphorylated receptor becomes hyperphosphorylated , mostly at serine residues (Orti et al. 1992). Nuclear localization signal-1 (NLS1) adjacent to DNA binding domain (DBD) and Nuclear localization signal-2 (NLS2) that overlaps with the ligand binding domain (LBD) of the receptor are unmasked, resulting in the movement of the GR to nucleus (Tsai and O'Malley 1994).

The relevance of GR phosphorylation is not well understood. There is a possibility that the phosphorylation status of the GR co-determines its subcellular localization rather than its transcriptional activity (Borror et al. 1995). Phosphorylation does not act as on-off switch but has more subtle effects regulating the activity by 0-90% depending on mutated site, cell type or reporter gene (Bodwell et al. 1998). Receptor phosphorylation may influence the interactions of GR with other transcription factors required for transactivation (Kido et al 1987).

Translocation of GR: The mechanism of nuclear export of GR is not well established. It has been demonstrated that receptors that accumulate within nuclei upon ligand binding are not statically confined to that compartment, but

rather have the capacity to reversibly traverse the nuclear envelope. Further, the ability of various GR mutants to shuttle between nuclei of heterokaryons excludes transcriptional activation and DNA binding as prerequisite for nucleocytoplasmic shuttling of GR. Hsp 90 plays multiple roles to facilitate chromatin recycling of GR. In addition to Hsp complex, protein phosphatase 5 (PP5) is also involved in the regulation of GR nucleocytoplasmic shuttling, and its inhibition lead to accumulation of GR in the nucleus (De Franco et al. 1991). It seems that DNA binding domain of GR, which is unrelated to known nuclear export sequence (NES), is necessary and sufficient for nuclear export of GR. There are indications that NLS can influence nuclear processing pathways that culminate in efficient nuclear export. It seems that NLS mediated import and DBD-mediated export jointly define a shuttling cycle that integrates the compartmentalization and activity of these receptors.

Glucocorticoid response elements (GREs)

The GREs have been reported to contain two defined stretches of nucleotides separated by undefined nucleotide creating two-half sites. The consensus sequence of GRE is 5′-AGAACAnnnTGTTCT-3′, where 'n' could be any nucleotide (Luisi et al. 1991; Del Monaco et al. 1997). Members of the nuclear receptor superfamily often recognize response elements with similar or even identical half-site sequences, but with different orientations or spacing (Kasper et al. 1999). The GREs exhibiting the highest affinity for GR are imperfect palindromes separated by 3 bp. However, natural regulatory elements lack one half of the palindrome. The affinities of GR for these half-palindromes depend on the dimerization status and on the GR interactions with neighboring sequences. Optimal binding is seen within the homodimers. Within imperfect palindrome one monomer interacts strongly with the good half of the palindrome, whereas the other monomer can accommodate great deviations from the consensus sequences due to protein-protein interactions within the dimer (Truss and Beato 1993). The number of GREs and their positions relative to the transcriptional start site may be an important determinant of the magnitude of the transcriptional response to glucocorticoids. Thus, an increased number of GREs and proximity to the

TATA box increases the glucocorticoid inducibility of a gene (Jantzen et al. 1987; Wieland et al. 1990).

In addition to its function as a ligand-dependent activation of transcription, in some promoters, binding of the activated GR to negative glucocorticoid response elements (nGREs) causes inhibition of transcription. The nGREs are less well-defined than positive GREs and have a more variable sequence ATYACnnTnTGATC (Truss and Beato 1993). The mechanism of action of GR on nGREs seems to involve displacement of a positive regulatory protein from the promoter. The prototype of the nGRE is located on the POMC promoter that slightly resembles the classic GRE. Instead of binding to GR homodimer, the nGRE reportedly accommodates three molecules of GR (Drouin et al. 1993).

GR coactivators

Some proteins function as transcription co-activators of the GR, and enhance and/ or enable the transcriptional activity of ligand-activated GR (Jenkins et al. 2001). In addition to serving as "bridging" factors, coactivators contain intrinsic histone acetyltransferase (HAT) activity (Mc Kennan et al. 1999). Coactivators include molecules, consisting of homologue p300 and cAMP-responsive element binding protein (CREB)-binding protein (CBP) and the family of p160 nuclear receptor coactivators. P300/CBP also serves as molecular docking platforms for transcription factors from several signal transduction cascades, including, in addition to nuclear receptors, CREB, activator protein-1 (AP-1), nuclear factor kB (NFkB), p53, Ras-dependent growth factor, and signal transducers and activators of transcription (STATs) (Goodman and Smolik 2000).

Steroid receptors potentially interact with p160 family of coactivators: steroid receptor coactivator-1 (SRC-1); transcriptional intermediate factor-II (TIF-II) or glucocorticoid receptor-interacting protein-1 (GRIP-1), also called SRC-2; and the p300/CBP/co-integrator -associated protein (p/CIP), activator of thyroid receptor (ACTR) or receptor-associated coactivator-3 (RAC-3), also called SRC-3 (Mc Kennan et al. 1999; Leo et al. 2000). P300 / CBP and p160 family coactivators contain one or more copies of the coactivator signature

motif sequence LXXLL where L is leucine and X is any amino acid through which they interact with GR (Heery et al. 1997; Lee et al. 2000).

Transactivation by GR

The interaction of GR to GRE changes the rate of transcription, resulting either in induction or repression of glucocorticoid responsive genes. GR-DNA interactions change DNase I sensitivity indicating that there may be a local change in the DNA or chromatin configuration, which may expose previously masked areas, resulting in increased binding of other transcription factors and the formation of a more stable transcription initiation complex (Truss and Beato 1993). Glucocorticoid increases the synthesis of lipocortin-1, a 37 kDa protein that has an inhibitory effect on phospholipase A_2 (PLA_2), and therefore may inhibit the production of leukotrines, and prostaglandins in leukocytes (Adcock 2000). Glucocorticoid is reported to increase the expression of β_2 -receptors by increasing the rate of transcription via three potential GREs. Glucocorticoids increase β-receptors gene transcription in human lung in vitro (Mak et al. 1995). Survival of certain inflammatory cells as eosinophils that is dependent on certain cytokines is reduced by the glucocorticoids. Exposure to glucocorticoids blocks the effects of these cytokines and leads to programmed cell death or apoptosis involving increased expression of specific endonucleases (Wallen et al. 1991). Osteocalacin is an example of nGRE, which is repressed by GR acting through GRE overlapping the TATA box thus blocking the binding of the basal transcription complex and preventing mRNA expression (Meyer et al. 1997). There is compelling evidence that increased gene transcription is associated with an increase in histone acetylation, whereas deacetylation is correlated with reduced transcription or gene silencing (Imhof et al. 1998).

Transrepression by GR

The major anti-inflammatory effects of glucocorticoids are through repression of inflammatory and immune genes. The inhibitory effect of glucocorticoids appears to be largely due to a protein-protein interaction between activated GR and transcription factors such as AP-1/ and NFkB,

which mediate the expression of these inflammatory genes (Barnes 1998). Direct protein-protein interaction have been demonstrated between GR and AP-1 (Jonat et al. 1990; Pfahl 1993), between the p65 component of NFkB (Ray et al. 1994; Caldenhoven et al. 1995) and some STAT proteins such as STAT3, STAT5 and STAT6 (Stocklin et al. 1996; Zhang et al. 1997; Moriggl et al. 1997) suggesting that glucocorticoids modulate either the binding or activation of these factors and thus modify the expression of inflammatory genes. There has been increasing evidence that GCs may have effects on the chromatin structure and function. CBP and p300 have an intrinsic histone acetylation (HAT) activity that is activated by binding of AP-1 and NFkB (Ogryzko et al. 1996). Repression of gene expression may occur due to inhibition of histone acetylation or by increasing deacetylation. The interplay between the acetylation and deacetylation in the control of chromatin structure is markedly dependent upon the exact acetylase (HAT) or deacetylase (HDAC) activated by particular signals (Gray et al. 1995). The repressive action of GCs may be due to competition between GR and the binding sites on CBP for other transcription factors, including AP-1, NFkB, Sp1, Ets, NF-AT and STAT (Adcock 2000). Alternatively, activated GR may bind to one of several transcription corepressor molecule, such as receptor interacting protein receptor interacting protein 140 (RIP140) and glucocorticoid receptor interacting protein 1 (GRIP1), that associates with proteins that have differing histone deacetylase activity (Ding et al. 1998). GR may also inhibit protein synthesis by reducing mRNA half-life through the enhanced transcription of specific ribonucleases that target AU-rich regions in the 3′-untranslated region of some genes such as granulocyte macrophage-colony stimulating factor (GM-CSF) and cycloxygenase-2 (COX-2) (Bickel et al. 1990; Ristimaki et al. 1996; Newton et al. 1998).

Glucocorticoids in selective therapeutics

Many of the anti-inflammatory effects of GCs are mediated by transrepression (binding with transcription factors), whereas the endocrine and metabolic effects of glucocorticoids are mediated via transactivation (binding with GRE). This has lead to a search for novel glucocorticoids that

selectively transrepress, thus reducing the risk of systemic side effects. A separation of transrepression and transactivation has been demonstrated using reporter gene constructs in transfected cell using selective mutations of GR (Heck et al. 1994). Furthermore, some glucocorticoids such as RU24858, RU486 and ZK98299, have a greater transrepression than transactivation effect (Heck et al. 1994). Recently, a novel class of glucocorticoids has been described in which there is potent transrepression with relatively little transactivation. These specifically designed glucocorticoids including RU24858 and RU40066, have potent anti-inflammatory effects in vivo (Vayssiere et al. 1997; Schacke and Rehwinkel 2004).

Study of tryptophan 2,3-dioxygenase

The hepatic hemoprotein, tryptophan 2,3-dioxygenase (TO) is a key regulatory enzyme that through irreversible degradation, controls the flux of tryptophan through physiologically relevant pathways. This enzyme is composed of four identical subunits and in its fully assembled tetrameric form requires two moles of heme-protoporphyrin IX for functional competence (Ren et al. 1996). Tryptophan 2,3-dioxygenase (EC 1.13.11.11; TO) is a liver specific enzyme, found only in the mature parenchymal liver cells, and is induced by various hormones, such as glucocorticoids, glucagon and catecholamines (Nakamura et al. 1987; Sharma et al. 1990). It appears in rat liver after 2 weeks of birth and reaches the peak level after 4-5 weeks of birth (Nakamura et al. 1987; Kaltschmidt et al. 1994). It has been reported that the TO gene is switched on as early as 1 day after birth in a few differentiated hepatocytes that respond fully to various hormones, and that the number of these differentiated cells then increase during early neonatal development (Nagao et al. 1986). Therefore, TO is a very useful marker in studies on hormonal regulation of gene expression and the molecular mechanism of cellular differentiation.

The degradation of tryptophan by TO results in the formation of kynurenine. Though TO is known to be a liver specific enzyme, many studies suggest the presence of TO in other tissues, such as brain and lung (Sadler et al. 1983; Keith and Brownfield 1985; Haber et al. 1993). Glucocorticoids seem

to be the primary stimulator of expression of the TO gene and glucagon potentiates its effect. TO gene contains a consensus sequence of the binding sites for the GR complex between nucleotide -101 and -119 at the 5′-end (Danesch et al. 1987; Chikhirzhina et al. 1999; Ren and Correia 2000). Reports suggest that dietary restriction elevates the level of tryptophan 2,3-dioxygenase in the liver of rats (Shanker et al. 1982). Hence, TO could be a good model enzyme to assess the impact of DR where in glucocorticoid and its receptor play a pivotal role.

Glucocorticoid mediated effects of dietary restriction

Dietary restriction not only evokes anti-inflammatory and antineoplastic effects consistant with chronic hypercortisolism but also protects aging rodents against insulin-resistant diabetes (Kalant et al. 1988; Harris et al. 1995; Ohneda et al. 1995; Bodkin et al. 1995), impaired tissue growth and regeneration (Lewis et al. 1985; Wolf et al. 1995), certain neurologic impairments (Forster et al. 1996; Lal et al. 1995), and reproductive senescence (Kennan et al 1995). Although few of these effects appear initially to be consistent with elevated level of glucocorticoids, on further analysis they appear to be the natural consequences of the nutrient stress produced by DR under experimental condition (Leaky et al. 1998). The antiinflammatory effects of dietary restriction are also generally consistent with effects resulting from elevated level of glucocorticoids. For example, DR repeatedly induces lipocortin 1 immunoreactive proteins in rat liver (Leaky et al. 1994), inhibits carrageenan-induced inflammation in mice (Klebnov et al. 1995), decreases lipooxygenase activity in rat liver and testes, delays the onset of autoimmunity in autoimmune-prone mice (Engelman et al. 1993), and inhibits promotion of mouse skin papillomas by phorbol esters (Schwartz et al 1994). Dietary restriction decreases the incidence of both spontaneous and chemically induced carcinogenesis and also reportedly decreases the acute toxicity of several chemicals (Kennan et al. 1994 and 97). Such effects are consistent with the anti-inflammatory and antineoplastic effects of elevated glucocorticoids (Leaky et al. 1998).

The observations indicate an important role of glucocorticoids during dietary restriction. The exact mechanism of glucocorticoid-dependent mediation of the above processes during DR is not elucidated as yet. Since the action of glucocorticoid depends primarily on the level of its receptors and steps following hormone binding to receptor (post-receptor events), the knowledge of its receptor and interaction with chromatin might provide insight into the mechanism of the glucocorticoid action in DR. Hence, keeping in view that these studies may provide a basic knowledge on glucocorticoid action, mediated by GR during DR, which might also help in understanding the age-delaying role of DR, we have studied the regulation of glucocorticoid receptors during dietary restriction in two different age groups of mice.

The work embodied in this thesis was conceived with the following objectives.

- To determine the endogenous level and the affinity of glucocorticoid receptors in different tissues of mice in control and dietary restricted animals.
- To determine the receptor activation and also physicochemical properties in these two conditions.
- To determine the receptor binding and chromatin organization in control as well as dietary restricted animals.
- To determine the regulation of marker glucocorticoid - inducible enzyme (tryptophan 2,3 dioxygenase) in control and dietary restricted mice.
- To study the effects of DR on GR level, affinity, activation and DNase I digestion of nuclear bound GR from young and old mice.

EXPERIMENTAL PROCEDURES

MATERIALS

Animals and diet

Swiss albino (Balb/c strain) male mice maintained under standard laboratory conditions (24 ± 2°C; 12 h light/dark cycle) were used for experimentation. The animals were caged in polycarbonate cage and fed with standard pellet diet (Amrut Laboratory, Pune) and tap water ad libitum as per the experimental schedule. Male mice of two different age groups (5- and 20- months old) were used for aging studies.

Chemicals and reagents

All chemicals and reagents used were of highest analytical grade. They were obtained from following companies:

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

Sigma Chemical Company, St. Louis, USA: Nonradioactive dexamethasone, Tris, sodium molybdate, EDTA, dithiothreitol, bovine serum albumin (BSA), activated charcoal, dextran T-70, Triton X-100, DNase I, diphenylamine, DNA-cellulose, deoxyribonucleic acid, Sephadex G-100, G-200, anion exchanger DE-52, and methemoglobin.

Roche Molecular Biochemicals, Germany: Nitrocellulose membrane (0.45 μm pore size).

Boehringer Mannheim, Germany: Calibration proteins II for chromatography (MW 18,000-30,000).

Merck, Germany: Metaphosphoric acid.

Wako Pure Chemicals, Japan: L-Tryptophan.

Whatman, England: Qualitative filter papers.

Qualigens, India: Sucrose, glycerol, potassium chloride, sodium hydroxide, hydrochloric acid, acetic acid, orthophosphoric acid.

Merck, India; Calcium chloride, magnesium chloride, sodium chloride, acetaldehyde.

Himedia, India: Potassium di-hydrogen phosphate and di-potassium hydrogen phosphate.

Bangalore Genei, India: Goat-antirabbit-IgG-HRP, Tween-20, TMB/H_2O_2.

Bengal Chemicals and Pharmaceuticals, India: Ethanol.

Sisco Research Laboratories, India: Scintillation cocktail-W

Instruments and apparatus

Tissues were homogenized in Remi tissue homogenizer (model RQ-127A). All the centrifugations were done using Hitachi (himac CR20B2) and Beckman Coulter (Allegra™ 64R) high-speed refrigerated centrifuges. Absorbance measurements were taken in Hitachi U-2000 double beam UV/visible spectrophotometer. Tissues were stored at -80°C in Heto ultra freezer until use. pH meter was from Control Dynamics, India. All radioactive countings were performed in Wallac 1409 liquid scintillation counter having an efficiency of 68% for tritium. The slot blotting was performed on a Bio-Rad Bio-Dot® SF Micro filtration apparatus following the instructions given in the user's manual.

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. The following buffers were used:

A. 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

B. 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6

C. 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6/ 0.5% (v/v) Triton X-100

D. 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6/ 4.2 mM $MgCl_2$

E. (I) 100 mM potassium phosphate buffer, pH 7.5/ 1 mM EDTA/ 1 mM β-mercaptoethanol/ 20 mM sodium molybdate

(II) 100 mM potassium phosphate buffer, pH 7.5/ 1 mM EDTA/ 1 mM β-mercaptoethanol/ 20 mM sodium molybdate/ 300 mM KCl

F. 10 mM potassium phosphate buffer, pH 7.5/ 1 mM β-mercaptoethanol/ 20 mM sodium molybdate. For eluting the bound proteins, 0-400 mM KCl in the same buffer was used.

G. 0.02 M Potassium phosphate buffer, pH 7.0/ 2.5 mM L-Tryptophan/ 2 μM *methemoglobin*

METHODS

Dietary restriction schedules

Mice were subjected to dietary restriction (DR) regimen of alternate days of feeding for a period of one to three months (Merry 1999; Dutta and Sharma 2003, 04). However, they had free access to water on all days. Animals were sacrificed both on the day of feeding as well as on the fasting day.

Tissue Preparation

Both the ad libitum (AL) and dietary restricted (DR) fed mice were sacrificed by cervical dislocation at a fixed time of the day (13:00 h). Their liver and kidney tissues were quickly excised, freed of fat and connective tissues and washed in chilled normal saline (0.9% NaCl). They were then blotted dry and stored in deep freezer (-80°C) until use.

Receptor preparation and radio receptor assay (RRA)

A 20% (w/v) homogenate of the minced tissue was prepared in chilled buffer A using motor driven Potter-Elvejham homogenizer in ice bucket for 1-1.5 min at 800-1000 rpms. The homogenates were then subjected to centrifugation at 2,000 x g for 10 min at 2°C and the nuclear and other cellular debris were discarded. The supernatants were further centrifuged at 40,000 x g for 45 min at 2°C to obtain a clear fat free cytosol. Radio receptor assay was performed using the method of Kalimi et al. 1983; Sharma and Timiras 1987; Borbhuiya and Sharma 1995; Ranhotra and Sharma 2001. Aliquots (100 μl) of cytosol were

incubated with 40 nM of [^{3}H] dexamethasone alone or with 500- fold excess of non-radioactive dexamethasone for 4 hr at 0°C to get maximum saturable binding. For Scatchard analyses (Scatchard 1949), aliquots of cytosol (100 µl) were incubated at 0°C for 4 h in duplicate with 20 µl [^{3}H] dexamethasone of increasing concentrations (5-120 nM) and 20 µl buffer A, and were gently vortexed at regular intervals to ensure proper binding of hormone to the receptors; this gave the total binding. For non-specific binding, 100 µl of cytosol was simultaneously incubated under identical conditions with 20 µl [^{3}H] dexamethasone (5-120 nM) and 500-fold excess of non-radioactive dexamethasone to that of respective [^{3}H] dexamethasone concentrations, 50 µl of dextran-coated charcoal (4% activated charcoal + 0.4% dextran T-70 in buffer A) prepared in buffer A was added and incubated for 10 min at 0°C to remove any unbound steroid (Beato and Fiegelson 1972). The charcoal was then pelleted by centrifugation at 2000 x g for 10 min at 2°C. 100 µl of the charcoal free cytosol was carefully pipetted into scintillation vials and 4 ml of cocktail-W added. The contents were thoroughly mixed and the bound radioactivity (CPM) measured in the liquid scintillation counter. The protein content in the final reaction mixture for each set of assay was determined according to Bradford's dye-binding method (Bradford 1976) (Appendix I). Specific binding of [^{3}H] dexamethasone was calculated 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) and expressed as fmol / mg protein. Details of CPM conversion to concentration are given in appendix II. 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).

GR slot blot analysis

Polyclonal rabbit anti-GR-ab, raised against amino acid (407-423) sequence (SVFSNGYSSPGMRPDVS) from the central region of the rat-GR was a gift from Profs. N. Katunama and H. Kido, Japan. The blotting was performed on Bio-Rad Bio-Dot® SF Micro filtration apparatus following the instructions given in the user's manual. Clear fat free cytosol (obtained by the process mentioned above) was used for the slot blotting experiment. A nitrocellulose (NC) membrane was soaked overnight in ddH_2O for activation and proper binding. After placing the NC membrane in the slot blot apparatus, the slots used were rehydrated with ddH_2O for uniform binding. To each slot, 100 μl of (x 10) cytosol adjusted to equal protein (50 μg) from both AL and DR and from both age groups were applied in the center and was allowed to filter through the membrane by gentle vacuum. The NC membrane was then placed in blocking solution (5% non-fatty milk in TBS) for an hour. It was washed in TTBS (20 mM Tris-HCl, pH 7.5/ 500 mM NaCl/ 0.05% Tween-20) twice with gentle agitation and was kept for overnight incubation with the anti-GR-ab solution (1: 1500). After washing the membrane twice in TTBS, it was transferred to goat anti-rabbit IgG-HRP (1:3000) conjugate solution and kept for 3 h. The membrane was later washed twice in TTBS and finally in TBS (20 mM Tris-HCl, pH 7.5/ 500 mM NaCl) to remove the detergent. The substrate (TMB/H_2O_2) was added in TBS solution (1:1500) to the membrane and after the development of color, the reaction was stopped by washing the membrane in ddH_2O. It was then photographed using digital hp Photosmart 315 camera and/ or scanned using hp Scannerjet 7400 c.

Activation studies

Glucocorticoid receptor activation studies were performed in liver and kidney of AL- and DR-fed mice and also at two different age groups (5- and 20-months). The magnitude of receptor activation was judged using DNA-cellulose as well as purified nuclei binding assays.

Preparation of Activated Glucocorticoid Receptor Complexes

A 20% (w/v) homogenate of the tissues were prepared in buffer B. It was centrifuged at 2,000 x g for 10 min at 2°C to sediment the nuclei. The supernatant was then centrifuged at 40,000 x g for 45 min at 2°C and to the clear cytosol was added [^{3}H] dexamethasone to a final concentration of 40 nM; bound hormone-receptor (H-R) complexes were separated by dextran-coated charcoal (in buffer B) treatment. Aliquots of these complexes were then subjected to heat (25°C) and salt (20 mM Ca^{2+}) activation for 45 min to obtain activated hormone-receptor complexes (Sharma and Timiras 1987). Aliquots of the cytosols were also kept at 0°C for 45 min to provide the unactivated receptor complexes as controls.

DNA-cellulose Binding Assay

Commercially available DNA-cellulose was suspended overnight in buffer B at 2°C. The mixture was then gently vortexed to give a homogenious slurry. From the slurry, aliquots containing 100-150 μg DNA was transferred to microfuge tubes and 1 ml of chilled buffer B was added to each tube. The cellulose suspension was pelleted by centrifuging at 2,000 x g for 10 min at 2°C and the supernatant was discarded. Equal aliquots of unactivated and activated H-R complexes were added to the cellulose pellets separately in duplicates and mixed well. The mixture was incubated for an hour at 0°C with regular vortexing to keep the cellulose in suspension. The reaction was stopped by adding 1 ml of ice-cold buffer B followed by centrifugation at 2,000 x g for 10 min at 2°C (Kalimi et al. 1975; Sharma and Timiras 1987; Ranhotra and Sharma 2001; Dutta and Sharma 2003). The cellulose pellets thus obtained were washed twice with the same buffer. To the final pellet was added 1 ml of cocktail-W and transferred to scintillation vials, to which 3 ml cocktail was again added and the content was thoroughly mixed. The radioactivity bound in pellet was counted and expressed as [^{3}H] dexamethasone-receptor complex bound to DNA-cellulose (CPM/100 μg DNA).

Nuclear Binding Assay

The crude nuclear pellets obtained as earlier were further processed (Eberhardt et al. 1978; Ranhotra and Sharma 2001; Dutta and Sharma 2003) for the nuclear binding assay. To the pellet was added chilled buffer C and the contents were gently homogenized and centrifuged at 2,000 x g for 10 min at 2°C. The pellet was then washed thrice with buffer B followed by centrifugation at 2,000 x g for 10 min at 2°C and the final pellet thus obtained was suspended in buffer B to give a homogeneous slurry. Aliquots of which containing 100-150 μg of DNA were pipetted in microfuge tubes and washed in buffer B. Tubes were then subjected to centrifugation at 2,000 x g for 10 min to give the final purified nuclear pellets and the supernatants were decanted. Aliquots of the activated H-R complexes were added to it in duplicate and the content is gently vortexed to keep the pellet in suspension for better interaction. 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] dexamethasone-receptor complex bound to nuclei (CPM/ 100 μg DNA).

DNase I digestion studies

DNase I digestion studies were performed on purified nuclei obtained from both liver and kidney of AL- and DR-fed mice and also in both the age groups according to a modified method of Chaturvedi and Kanungo (1983). Heat activated H-R complexes 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 in a total volume of 100 μl at 2°C for 45 min. The DNase I was dissolved in buffer D and used at a concentration of 150 units/100 μg DNA. The control tubes received 100 μl of buffer only (Ranhotra and Sharma 2001). The nuclear pellets were properly mixed and the reaction was stopped by

adding 1 ml buffer B followed by centrifugation at 2,000 x g for 10 min at 2°C. The pellets were processed and the bound radioactivity determined as described above. The results were expressed as % [^{3}H] dexamethasone-receptor complexes bound to nuclei. Controls were taken as 100% bound.

Physicochemical characterization of glucocorticoid receptor

Physicochemical properties of glucocorticoid receptors were studied during dietary restriction in the liver of both AL- and DR-fed mice to see if there is any change in various physicochemical parameters (molecular weight, stokes radius, elution profiles) of the receptor due to dietary restriction as compared to the AL-fed ones. To determine the molecular weight and the stokes radii of the unactivated as well as activated [^{3}H] dexamethasone –receptor complexes, gel filtration analyses were carried out using sephadex G-200 and G-100, respectively. To study the charge content, anion exchange chromatography of the unactivated and activated receptors were done. All the gel filtration and ion exchange procedures were carried out in the cold at 2-4°C, unless otherwise mentioned.

Gel filtration analyses of the unactivated receptor

The dry sephadex G-200 was suspended in distilled water and allowed to swell overnight at 25°C. The gels were washed repeatedly to remove the gel fines and the supernatant was decanted. Thick gel slurry was prepared and degassed for an hour; this was then poured into a glass column (1.7 x 40 cm) kept in the cold. The gel, after being allowed to stand for some time, was equilibrated with buffer E (I), using a peristaltic pump attached to the lower end of the column. The buffer was pumped into the column at a flow rate of 20 ml/h and equilibration was continued till the pH of the eluent was same as that of the buffer used. After proper equilibration, the column was calibrated using [^{3}H] dexamethasone, blue dextran 2000 and standard molecular weight markers at a flow rate of 12ml/h. [^{3}H] dexamethasone-receptor complexes from the liver of AL- and DR-fed mice were prepared in buffer A as mentioned and the unbound

hormone removed using dextran-coated charcoal. Aliquots (2 ml) from the two groups containing approximately the same radioactivity were loaded onto the column separately and eluted with buffer E (I) at 12ml/h flow rate. 2 ml fractions were collected and 100 µl aliquots from each fraction transferred to the scintillation vials, 4 ml cocktail-W added and the radioactivity counted for each fraction.

The elution volume of [^{3}H] dexamethasone provided the total gel volume (V_p) and that of blue dextran, the void volume (V_o). The distribution coefficient (K_d) *and the available distribution coefficient* (K_{av}) *of the marker proteins and the* unactivated receptors were calculated with the help of the following equations

$$K_d = (V_e - V_o) / (V_i)$$

$$K_{av} = (V_e - V_o)/ (V_t - V_o)$$

Where, $V_i = V_p - V_o$; V_e = elution volume of the marker proteins and the sample and $V_t = \pi r^2 l$; r = radius of the column and l = length of the gel bed. The apparent molecular weights and stokes radii of unactivated receptors, from both AL- and DR-fed animals were calculated (Andrews 1970; Porath 1963; Laurant and Killander 1964) and plotted

For molecular weight	**For stokes radii**
V_e/ V_o Vs. log M	$-(\log K_{av})^{½}$ Vs. R_s
$M^{1/3}$ Vs. $K_d^{1/3}$	$K_d^{1/3}$ Vs. R_s

Gel filtration analyses of the activated receptor

The molecular weights and stokes radii of heat activated glucocorticoid receptors were analyzed on a sephadex G-100 column (1.7 x 35 cm). The column was prepared with buffer E (II) at a flow rate of 20 ml/h. After equilibration, the column was calibrated using [^{3}H] dexamethasone, blue dextran 2000 and standard low molecular weight markers at a flow rate of 12 ml/h. The [^{3}H] dexamethasone-receptor complexes from the liver of AL- and DR-fed mice were prepared in buffer B and activated at 25°C for 45 min. Aliquots (2 ml) from the two groups containing approximately the same radioactivity were loaded onto

the column separately and eluted with buffer E (II). 2 ml fractions were collected and radioactivity in 100 µl, from each fraction counted. The apparent molecular weights and the stokes radii were determined as that for the unactivated receptors.

Ion exchange chromatography of unactivated and activated receptors

To determine the difference in the net charge content of unactivated and activated glucocorticoid receptors from AL- and DR-fed mice, anion exchange chromatography on DEAE-cellulose (DE-52) was done according to Grandics et al. (1984); Borbhuiya and Sharma (1995). The ion exchange resin was washed thoroughly with 0.5 M NaOH followed by extensive washing with distilled water to neutral pH. A glass syringe of 5 ml capacity was used as a column. A thin film of dextran-coated charcoal (≈ 2 mm) was layered at the bottom of the column. The washed resin was then gently poured over the charcoal layer and allowed to settle under pressure to give the gel bed a height of 3 cm. The resin was then washed extensively with buffer F, in the cold at a flow rate of 25 ml/h till the pH of the eluent equals to that of the buffer. Unactivated [^{3}H] dexamethasone-receptor complexes from the liver of both AL- and DR-fed mice were prepared in buffer C and 2 ml of it was loaded onto the column. Upon sample application, the column was washed with 30 ml of buffer F to remove all the unbound proteins. The bound receptors were subsequently eluted with a 50 ml linear gradient of KCl (0-400 mM in buffer F), applied with the help of a gradient mixer at 25 ml/h flow rate. 1 ml fractions were collected and 100 µl from each fraction transferred to scintillation vials and 4 ml cocktail-W added. The radioactivity in each fraction was counted and from the elution plot, concentration of salt at which the receptor peak eluted was determined. A similar set of experiments provided the data for the heat activated receptors from both groups of animals.

Assay of tryptophan oxygenase (TO)

Tryptophan oxygenase (TO) is a known glucocorticoid inducible marker enzyme. Induction patterns of this enzyme by dietary restriction in the liver of

both AL- and DR-fed mice were studied. A 10% homogenate of the liver was prepared in 0.02 M potassium phosphate buffer (pH 7.0) containing 2.5 mM tryptophan and 2 μM methemoglobin and centrifuged at 800 x g for 10 min. The supernatant was collected and centrifuged at 14,000 x g for 30 min. The supernatant was incubated at 37°C in shaking water bath for 10 and 20 min. The reactions were stopped by the addition of 0.25 ml of 15% metaphosphoric acid and centrifuged at 3,000 x g for 15 min. To the supernatant 0.4 ml of 1N NaOH was added for neutralization and was read at 365 nm. The activity of TO is expressed as – unit (μmol kynurenine formed per hour) / mg protein (Seglen and Jervell 1969).

RESULTS

Dietary restriction regimen

Various modes of dietary restriction regimen that extend life span, retard age-related diseases and maintain vitality in a wide range of organisms are being followed by different laboratories. In our laboratory, we have studied the effect of one and three months of dietary restriction (alternate days of feeding) in Swiss albino (Balb/ c strain) male mice. The animals were divided into two groups. The ad libitum (AL) group had continuous supply of food whereas the animals subjected to dietary restriction (DR) were provided with food on alternate days for a period of one and three months. Water was supplied regularly to both the groups. After our careful evaluation, we have followed the three months DR regimen that gave the best results in terms of adaptive response of dietary restriction in experimental animals.

For the aging studies we have also followed the similar dietary regimen for both adult and old mice for a period of three months.

Body weight

The record of body weight was maintained regularly for both AL and DR animals for the whole of restriction period to ascertain the impact of such restriction in mice. It has been observed that, after three months of dietary restriction, the adult DR mice showed a significant decrease (-34%; $p<0.001$) in body weight (19.9 ± 1.1g) as compared to the AL-fed (30.4 ± 1.2 g) mice (Fig 1). Our observation of body weight reduction confirmed that the animals must have had a reduced food intake during the restriction period.

Changes in the glucocorticoid receptor level

The GR level was studied in both liver and kidney of AL- and DR-fed male mice. In liver, the DR-fed animals showed a significant increase (41%) in the

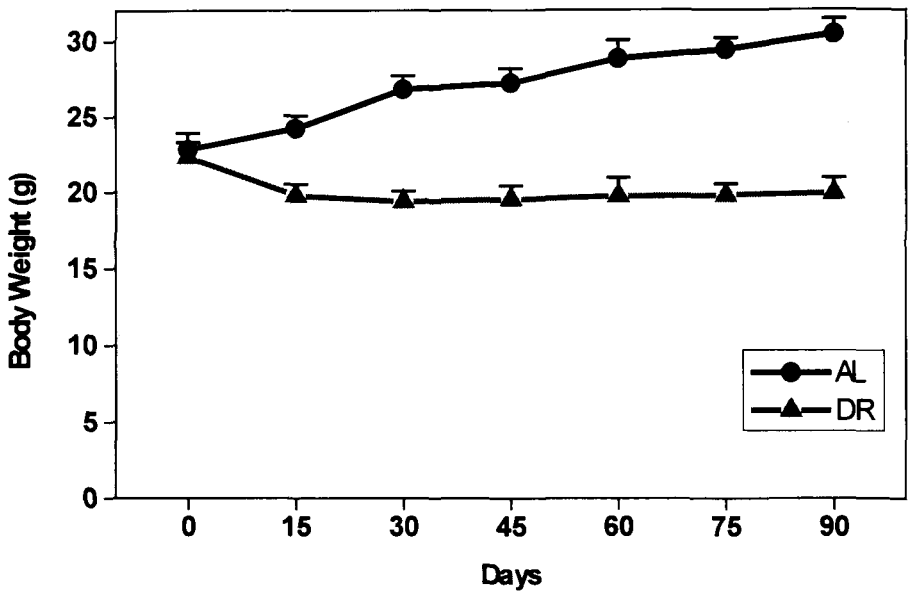


Fig 1 : Body weight changes during dietary restriction of adult (5- months) male mice. Animals were divided into two groups. One group was subjected to ad libitum (AL) dietary feeding while the other group was fed on alternate days for a period of three months. Body weight of individual mouse was monitored during this period. Each point represents mean body weight for 5-6 animals and bar represent standard deviation.

level of GR over the AL-fed ones (Table I). Scatchard analyses of the data confirmed a higher concentration of receptor binding sites in DR animals (173.0 fmol / mg protein) as compared to the AL-fed (122.2 fmol / mg protein) ones. However, slopes of the plot did not reveal any change in the affinity (K_d) of GR for its ligand in both the groups of animals. The affinity remained same (~ 2.3 nM) in both AL and DR mice (Fig 2). In kidney too, the level of GR was significantly higher (37%) in dietary restricted mice compared to the ad libitum fed ones (Table I). The increase in the receptor level was confirmed by the Scatchard analyses, which showed a higher binding site in DR animals (146.2 fmol / mg protein) than that of the AL- (106.0 fmol / mg protein) fed ones. The affinity remained same (~2.1 nM) in both AL- and DR-fed mice (Fig 3).

Slot blot analyses of the GR in both liver (Fig 4A) and kidney (Fig 4B) confirmed the increase in the concentration of the receptors in DR animals as compared to the AL-fed ones.

Activation studies of the GR

Activation studies of the GR were performed in the liver and kidney of both AL- and DR-fed mice. The magnitude of temperature (25°C) and salt (Ca^{2+}) dependent activation was studied using DNA-cellulose and purified nuclear binding assays.

DNA-cellulose binding assay

In liver, the magnitude of both temperature (25°C for 45 min) and salt (20 mM Ca^{2+} at 0°C for 45 min) dependent activation of [^{3}H] dexamethasone-receptor complexes was significantly high (~2.1-2.6 fold) as compared to the unactivated receptor complexes (O°C for 45 min). However, both the temperature and Ca^{2+}-mediated activation of GR did not reveal any significant change in DR-fed animals as compared to the AL-fed ones (Fig 5A). Kidney too showed a significantly higher degree (~ 2.0 – 2.5 fold) of temperature and salt dependent activation of the [^{3}H] dexamethasone-receptor complexes as compared to the unactivated hormone receptor complexes incubated at 0°C for 45 min. No significant changes were observed in the temperature and salt activation of GR in either groups (Fig 5B).

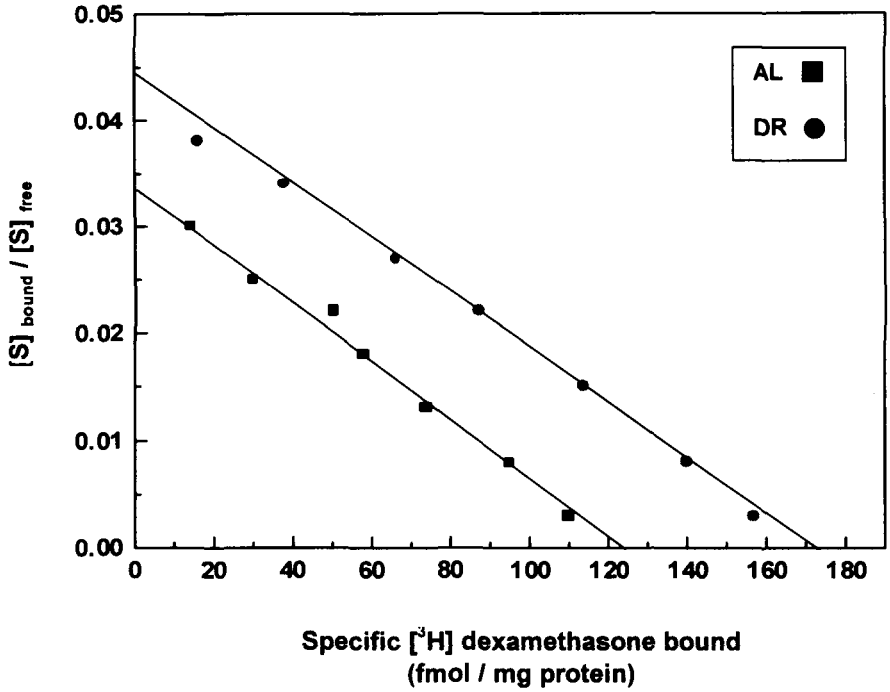


Fig 2: Scatchard plot of [^{3}H] dexamethasone binding in the liver of adult AL- and DR-fed male mice. Cytosols were incubated with 5 - 120 nM [^{3}H] dexamethasone ± 500 fold excess cold dexamethasone for 4 h at 0°C. Specific binding at each concentration was calculated by subtracting non-specific binding from total binding and the data obtained were analyzed by Scatchard method. The slope of the curve gave the dissociation constant (K_d) and the intercept on X-axis gave the maximum receptor binding sites. Each point is the mean of four separate experiments with 5-6 mice of each group.

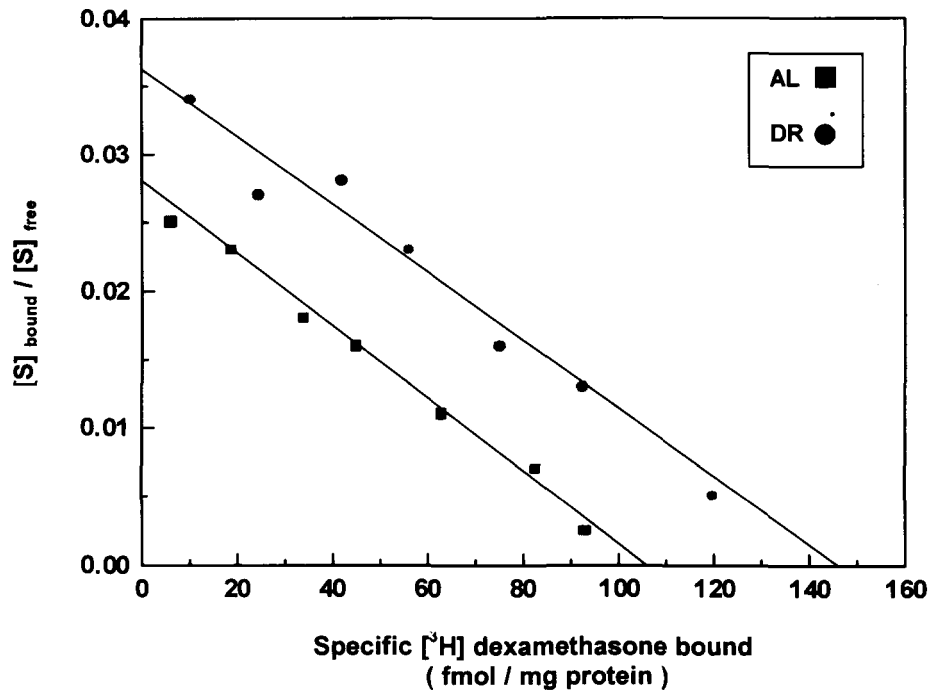


Fig 3: Scatchard plot of [^{3}H] dexamethasone binding in the kidney of adult AL- and DR-fed male mice. Cytosols were incubated with 5-120 nM [^{3}H] dexamethasone ± 500 fold excess cold dexamethasone for 4 h at 0°C. Specific binding at each concentration was calculated by subtracting non-specific binding from total binding and the data obtained were analyzed by Scatchard method. The slope of the curve gave the dissociation constant (K_d) and the intercept on X-axis gave the maximum receptor binding sites. Each point is the mean of four separate experiments with 5-6 mice of each group.

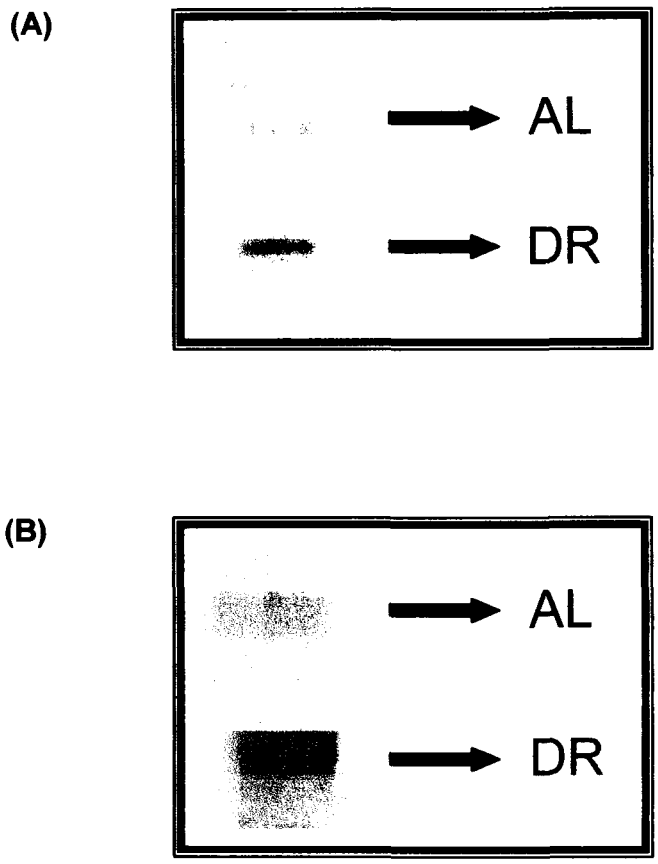


Fig 4: Slot blot analysis of adult liver (A) and kidney (B) glucocorticoid receptor from ad libitum (AL) and dietary restricted (DR) fed male mice. The details of experimental conditions are described in 'materials and methods' section. Equal amount of liver and kidney cytosols containing GR from AL and DR mice was applied onto each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-HRP conjugate. Arrows indicate the position and content of GR from AL- and DR-fed adult mice.

Table I: Concentration (fmol/mg protein) and affinity (nM) of [^{3}H] dexamethasone-receptor in the liver and kidney of *ad libitum* (AL) and dietary restricted (DR) fed male mice.

	AL		DR	
Tissue	B_{max}	K_d	B_{max}	K_d
Liver	122.2 ± 7.3	2.3 ± 0.3	173.0 ± 5.7*	2.3 ± 0.2
Kidney	106.0 ± 3.3	2.0 ± 0.2	146.2 ± 3.5*	2.1 ± 0.2

* Statistically significant ($p<0.001$) with respect to AL-fed mice

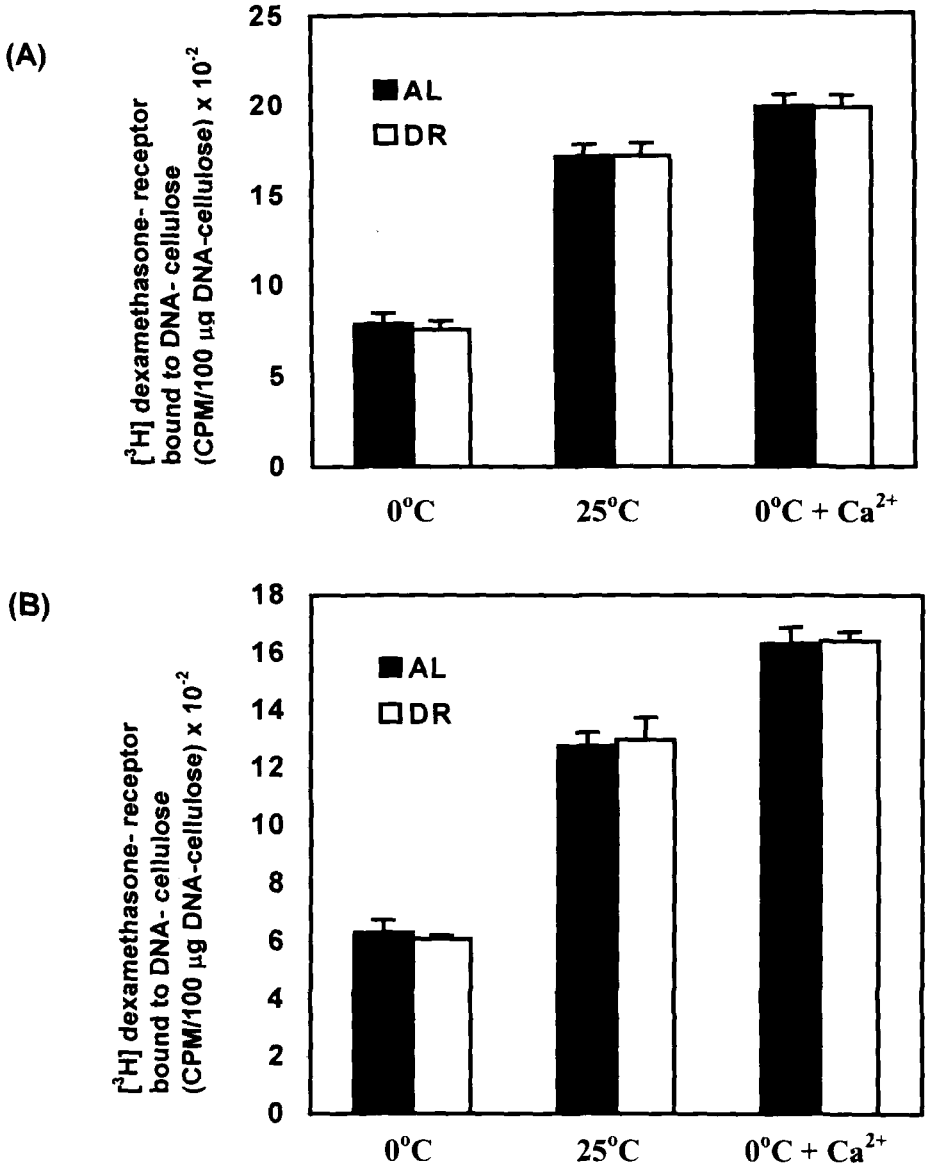


Fig 5: Binding of [^{3}H] dexamethasone-receptor complexes to DNA-cellulose in the liver (A) and kidney (B) of AL- and DR-fed male mice. Cytosols were prepared in buffer B and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H] dexamethasone for 4 h at 0°C. The hormone-receptor complexes were then subjected to heat (25°C) and Ca^{2+} (20 mM at 0°C) activation for 45 min as against 0°C control. Details of DNA-cellulose binding and further processing conditions are described in 'materials and methods' section. The results are mean ± standard deviation of four separate experiments performed with 5-6 mice of each group.

Nuclear binding assay

As observed in DNA-cellulose binding studies in the liver, the degree of temperature (25°C for 45 min) and salt (20 mM Ca^{2+} at 0°C for 45 min) dependent activation of [^{3}H] dexamethasone-receptor complexes was higher (~ 2.0 – 2.5 fold) as compared to the unactivated receptor complexes (0°C for 45 min), using purified nuclear binding assays. No difference in the magnitude of activation of GR was observed between AL- and DR-fed mice (Fig 6A).

Kidney revealed the same pattern of nuclear binding in both AL and DR mice by temperature and salt activation of receptor complexes as observed in the liver. Both temperature and Ca^{2+}-dependent activation of GR was significantly high (~ 2.0 – 2.5 fold) as compared to the unactivated receptor complexes. However, no changes in the rate of activation were observed in either group (Fig 6B).

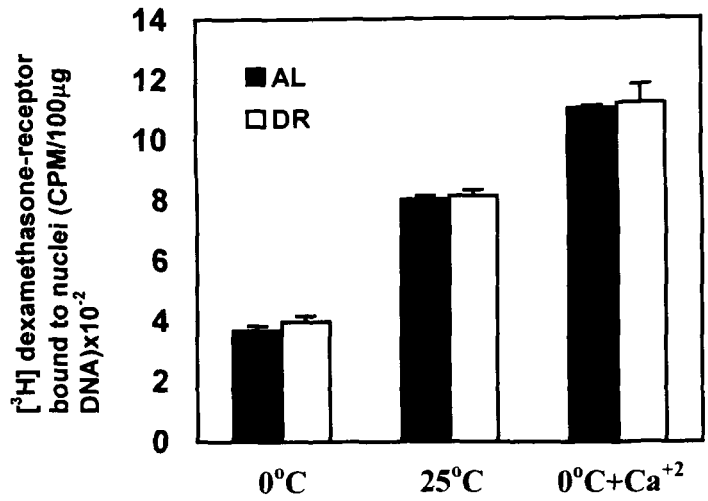
DNase I digestion studies

DNase I digestion of nuclear bound GR was performed in both liver and kidney of AL- and DR-fed mice to ascertain the chromatin organization and its possible role in nuclear binding of activated GR complexes during dietary restriction. In liver, DNase I digestion of hepatic nuclei bound to activated GR-complexes revealed similar magnitude of extraction (60 – 61.5%) in both AL- and DR-fed animals (Fig 7A) as compared to their respective controls taken as 100%. In Kidney also, the magnitude of extraction observed (59 – 60.3%) for both AL and DR animals was same. No significant change was marked in DR mice as compared to that of the AL-fed ones (Fig 7B).

Study of physicochemical properties of GR

To study some of the physicochemical properties such as molecular weight, stokes radius and the charge content of the GR during dietary restriction, gel filtration and ion-exchange chromatographic analyses were performed. The studies were carried out for both unactivated as well as activated GR obtained from the liver of both AL- and DR-fed mice.

(A)



(B)

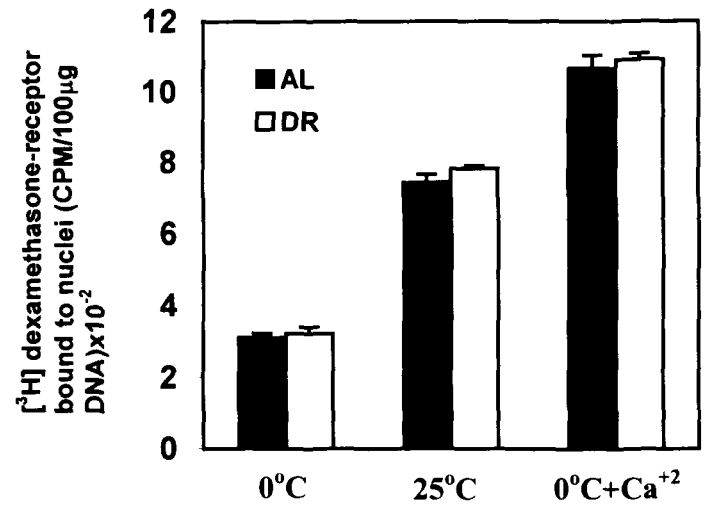
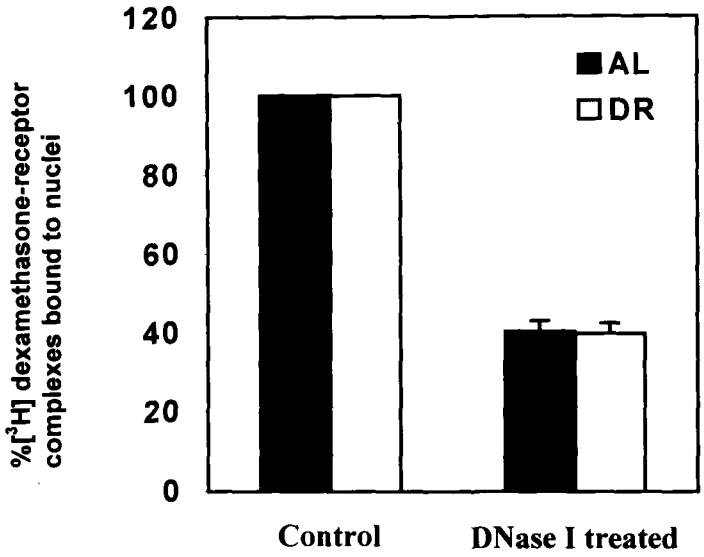


Fig 6: Binding of [^{3}H] dexamethasone-receptor complexes to purified nuclei in the liver (A) and kidney (B) of AL- and DR- fed male mice. Cytosols were prepared in buffer B and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H] dexamethasone for 4 h at 0°C. The hormone-receptor complexes were then subjected to heat (25°C) and Ca^{2+} (20 mM at 0°C) activation for 45 min as against 0°C control. Details of purified nuclear binding and further processing procedures are described in 'materials and methods' section. The results are mean ± standard deviation of four separate experiments performed with 5-6 mice of each group.

(A)



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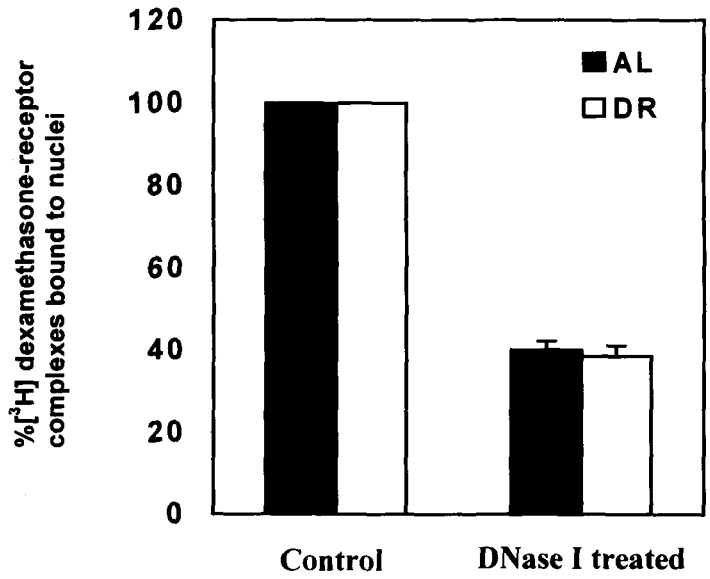


Fig 7: DNase I digestion of bound [^{3}H] dexamethasone-receptor complexes from the liver (A) and kidney (B) of AL- and DR-fed male mice. Heat-activated, nuclear-bound hormone-receptor complexes were extracted using DNase I as per experimental protocols given in 'materials and methods' section. The results are mean $\pm$ standard deviation of four separate experiments performed with 5-6 mice of each group.

Gel filtration analyses

The unactivated [^{3}H] dexamethasone-receptor complexes from both AL and DR animals were analyzed on sephadex G-200 column. The hepatic receptors from both groups of animals eluted at same elution volume and as a single peak between the standard molecular weight markers ferritin (450 kDa) and β-amylase (200 kDa) (Fig 8). Linear regressed curves were obtained by plotting the above experimental data and from them molecular weight and stokes radii (R_s) were calculated. The plot of log M vs. V_e / V_o gave a molecular weight of 292 kDA for AL and 296 kDa for DR-fed mice (Fig 9). Porath plot of $M^{1/3}$ vs. $K_d^{½}$ gave the values 287 kDa and 300 kDa for the receptors from AL- and DR-fed animals, respectively (Fig 10). Stokes radii of GR for both the groups were calculated using the plot of $(-\log K_{av})^{1/2}$ vs. R_s and were found to be 5.8 nm and 6.0 nm for AL- and DR-fed mice, respectively (Fig 11). Another plot of $K_d^{1/3}$ vs. R_s gave the similar values of 5.9 nm for AL and 6.0 nm for DR animals (Fig 12).

The activated glucocorticoid receptor complexes from the liver of both AL and DR animals were analyzed using a column of sephadex G-100. The elution profile of the receptors from both the groups showed that they eluted as a single peak at the same elution volume between the standard molecular weight markers aldolase (158 kDa) and BSA (67 kDa) (Fig 13). A small radioactive peak was also eluted in early fractions, which may be due to the presence of a tiny fraction of receptors that remained unactivated. Molecular weight of 98 kDa and 95 kDa were calculated using the plot of log M vs. V_e / V_o (Fig 14) for activated hepatic GR from AL- and DR-fed mice, respectively. Porath plot of $M^{1/3}$ vs. $K_d^{1/3}$ gave the molecular weight values of activated GR as 97 kDa for AL and 96 kDa for DR-fed animals (Fig 15). Stokes radii of activated GR were determined using the plot of $(-\log K_{av})$ vs. R_s, which gave the values 3.6 nm and 3.5 nm for both AL- and DR-fed animals, respectively (Fig 16). Plot of $K_d^{1/3}$ vs. R_s also gave the stokes radii as 3.5 nm and 3.3 nm for AL- and DR-fed mice (Fig 17).

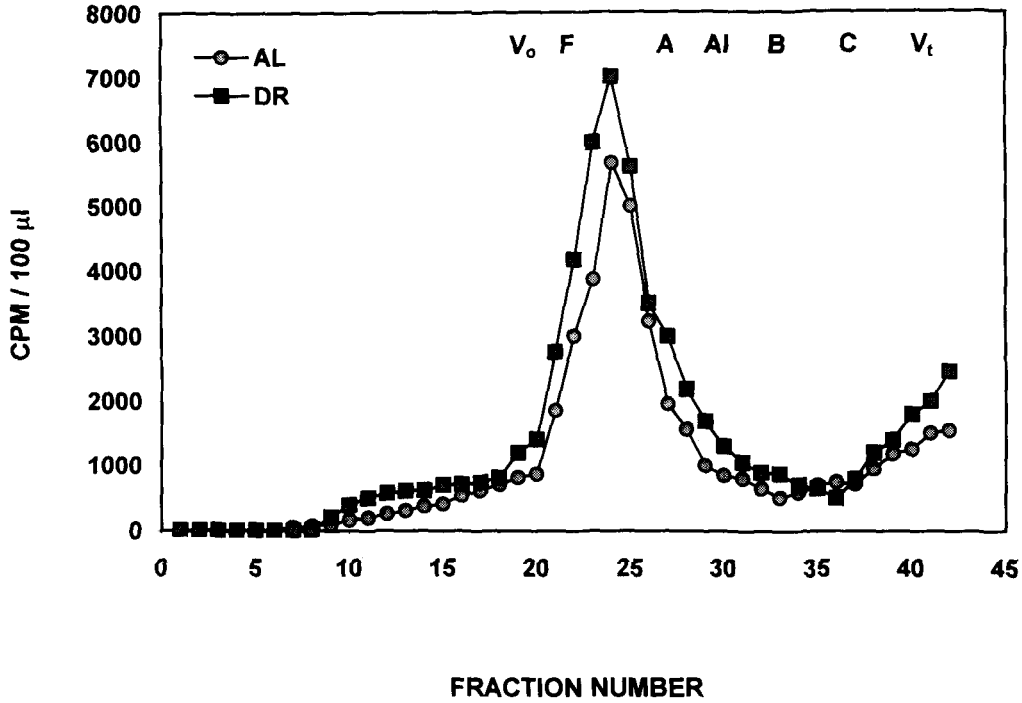


Fig 8: Gel filtration chromatography of unactivated glucocorticoid receptors from the liver of AL- and DR-fed male mice. Sephadex G-200 column (1.7 x 40 cm) equilibrated with buffer E (I) at 4°C was used. Cytosols were prepared in buffer A and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H] dexamethasone for 4 h at 0°C. After DCC treatment, 2 ml of the cytosol was loaded onto the column and eluted.with the buffer E (I). Each point in the elution profile represents the mean value of four separate experiments. Molecular weight markers used were ferritin (F), β-amylase (A), aldolase (Al), bovine serum albumin (B) and cytochrome C (C). V_o and V_t represent the elution volume of blue dextran and [^{3}H] dexamethasone, respectively.

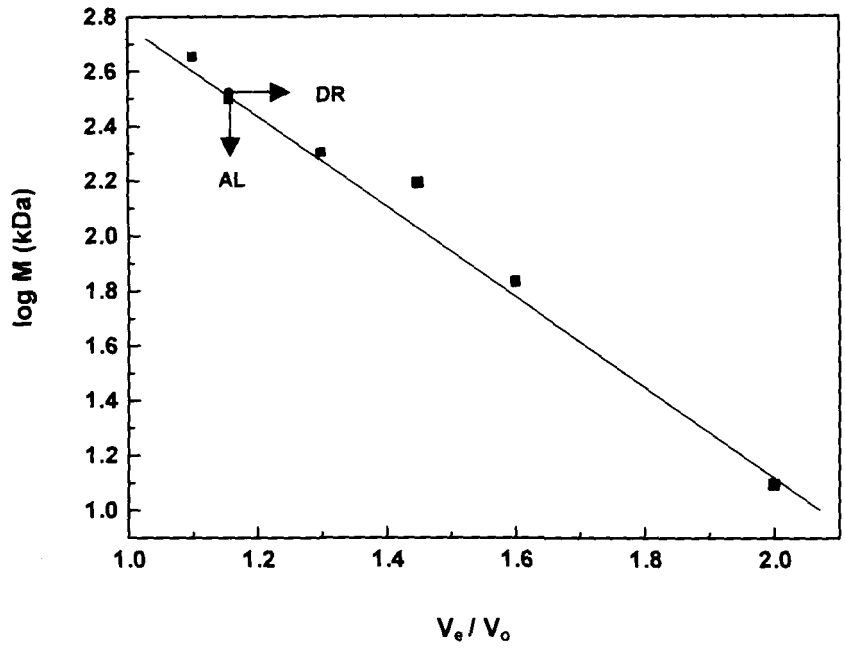


Fig 9: Plot of log M vs. V_e / V_o for the determination of molecular weight of unactivated receptor in AL- and DR-fed male mice. Data obtained from sephadex G-200 column were plotted for a linear regressed curve. The standard protein molecular weight marker used were ferritin (450 kDa), β-amylase (200 kDa), aldolsae (156 kDa), BSA (67 kDa) and cytochrome C (12.5 kDa).

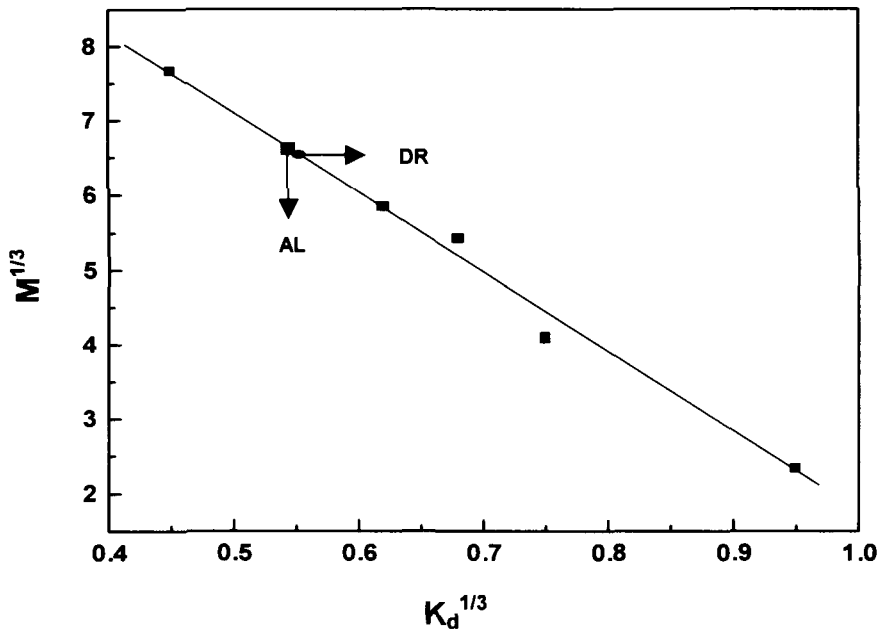


Fig 10: Porath plot of $M^{1/3}$ vs. $K_d^{1/3}$ for the determination of molecular weight of unactivated receptors from AL- and DR-fed male mice. Data from sephadex G-200 chromatography were used to generate the linear-regressed plot. Standard marker proteins were used same as mentioned in the legend to Fig 9.

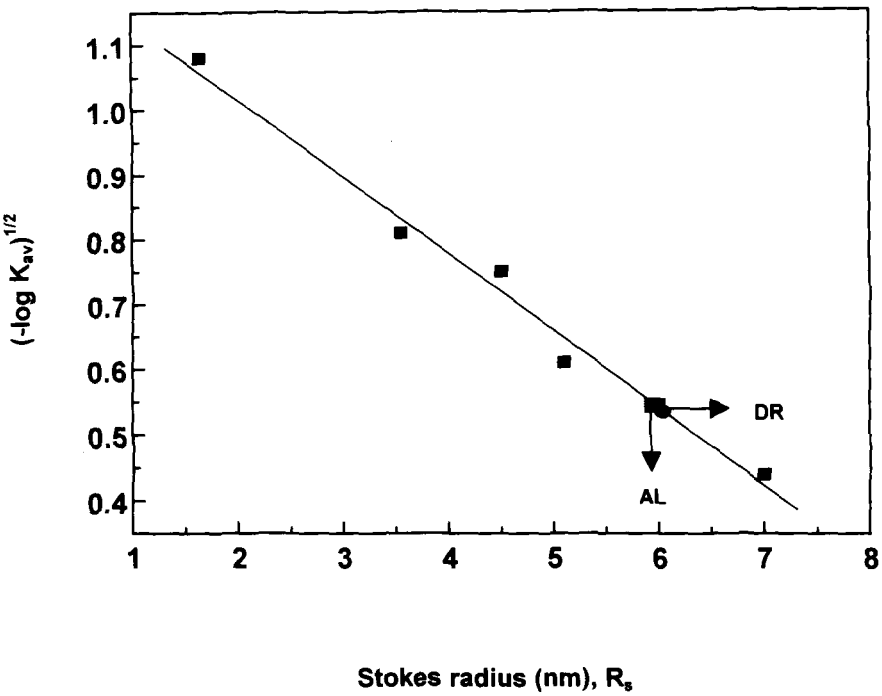


Fig 11: Plot of (-log K_{av})$^{1/2}$ vs. R_s for the determination of stokes radii of unactivated receptors in AL- and DR-fed male mice. The plot was generated using data from sephadex G-200 chromatography. The standard markers used were ferritin (7.0 nm), β-amylase (5.1 nm), aldolase (4.5 nm), BSA (3.55 nm) and cytochrome C (1.64 nm).

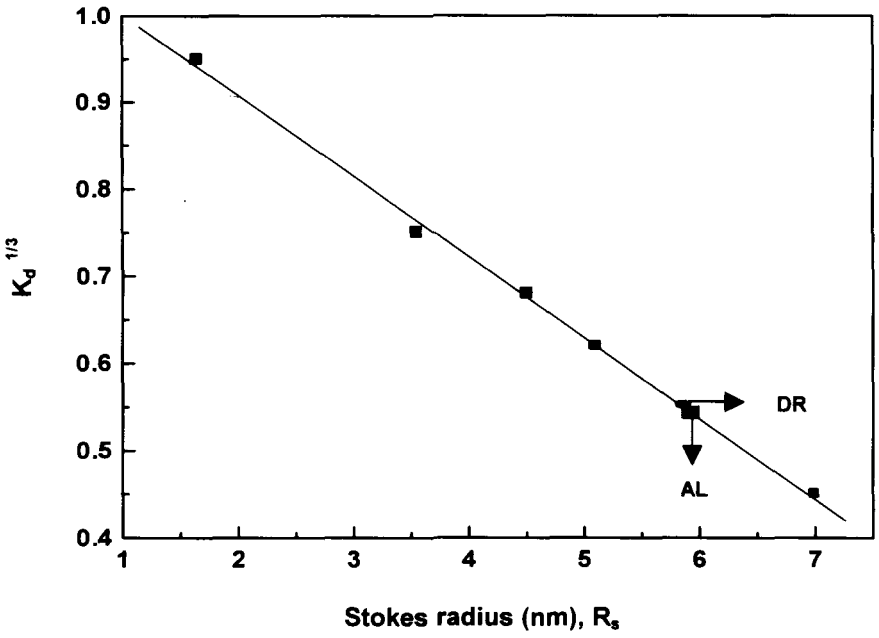


Fig 12: Plot of $K_d^{1/3}$ vs. R_s for the determination of stokes radii of unactivated receptors in AL and DR fed male mice. Sephadex G-200 chromatography data were used to obtain the plot. Standard markers and other details are the same as given in the legend to Fig 11.

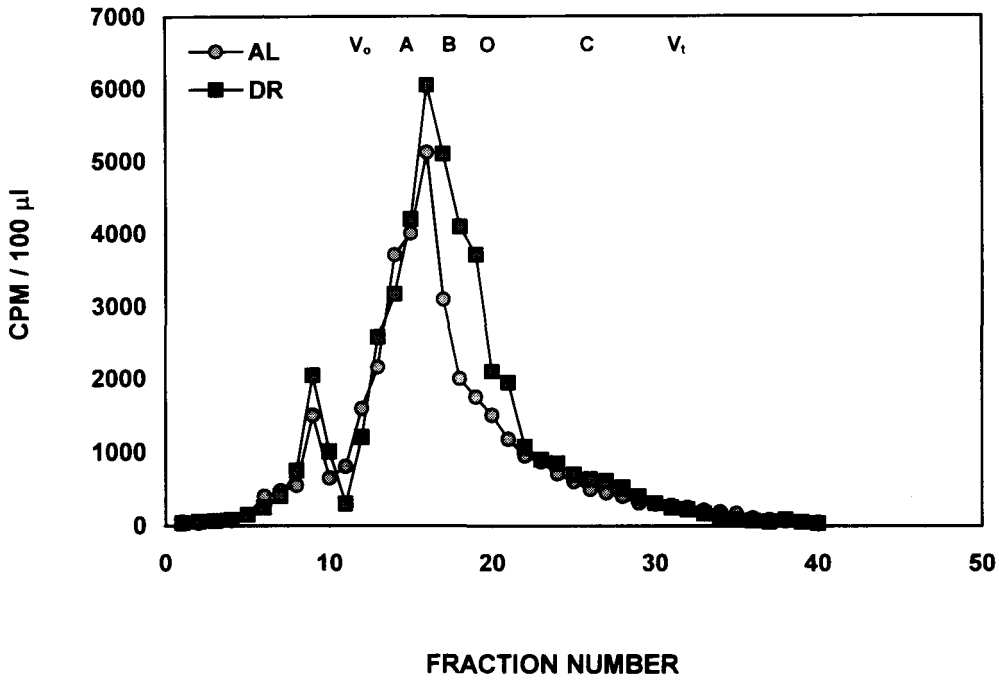


Fig 13: Gel filtration column chromatography of hepatic activated glucocorticoid receptors from AL- and DR-fed male mice. Sephadex G-100 column (1.7 x 35 cm) equilibrated with buffer E (II) at 2°C - 4°C was used. Cytosols were prepared in buffer B and the hormone-receptor complexes are obtained by incubating with 40 nM [^{3}H] dexamethasone for 4 h at 0°C. After DCC treatment, the hormone-receptor complexes were activated at 25°C for 45 min and 2 ml of the cytosol was loaded onto the column and eluted.with the buffer E (II). Each point in the elution profile represents the mean value of four separate experiments. Standard protein markers used were aldolase (A), bovine serum albumin (B), ovalbumin (O) and cytochrome C (C). V_o and V_t represent the elution volume of blue dextran and [^{3}H] dexamethasone, respectively.

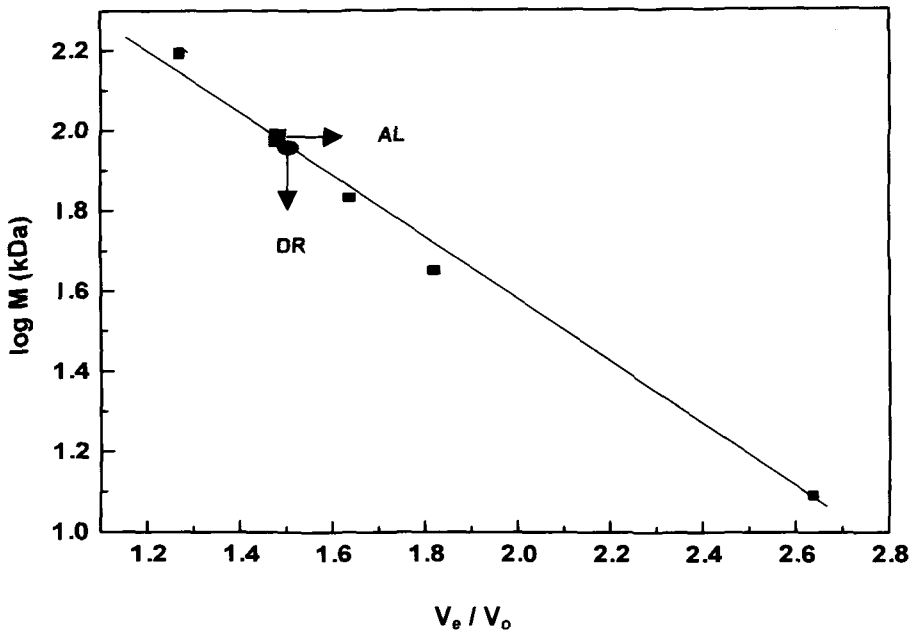


Fig 14: Plot of log M vs. V_e/V_o for the determination of molecular weight of activated receptors in AL- and DR-fed male mice. The data from sephadex G-100 gel chromatography were plotted to obtain a linear-regressed curve. The standard protein markers used were aldolase (156 kDa), BSA (67 kDA), ovalbumin (45 kDa) and cytochrome C (12.5 kDa).

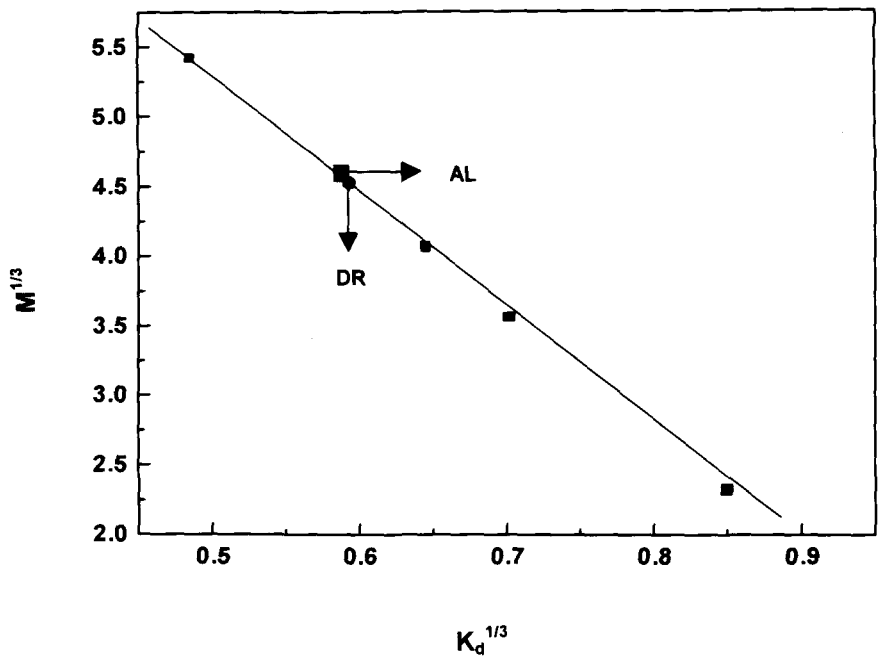


Fig 15: Porath plot of $M^{1/3}$ vs. $K_d^{1/3}$ for the determination of molecular weight of activated receptors from the liver of AL- and DR-fed male mice. Data from sephadex G-100 chromatography were used to generate the linear-regressed plot. Standard marker proteins used were as mentioned in the legend to Fig 14.

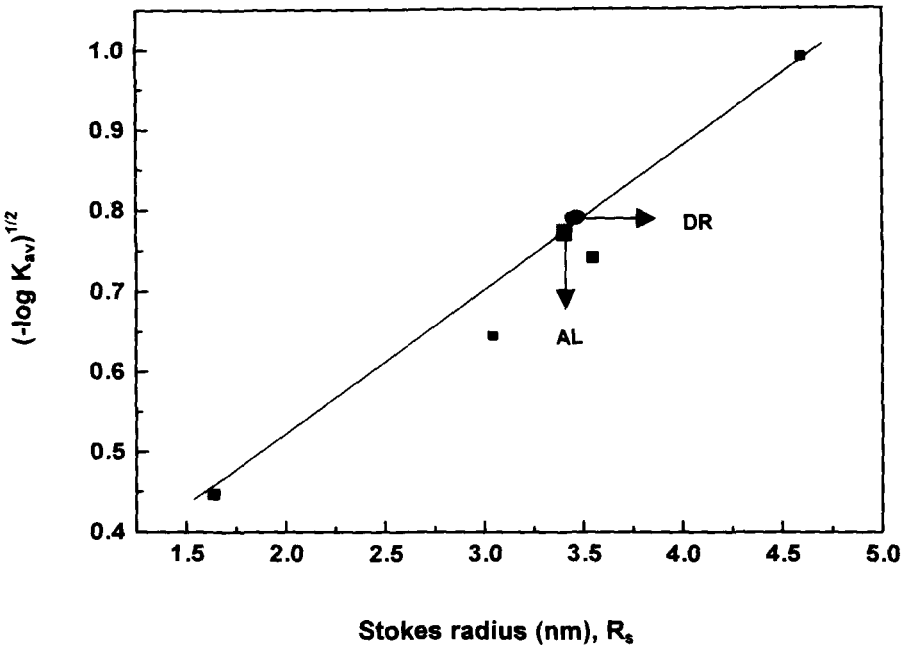


Fig 16: Plot of $(-\log K_{av})^{1/2}$ vs. R_s for the determination of stokes radii of activated receptors from the liver of AL- and DR-fed male mice. The plot was generated using data from sephadex G-100 chromatography. The molecular markers used were aldolase (4.6 nm), BSA (3.55 nm), ovalbumin (3.05 nm) and cytochrome C (1.64 nm).

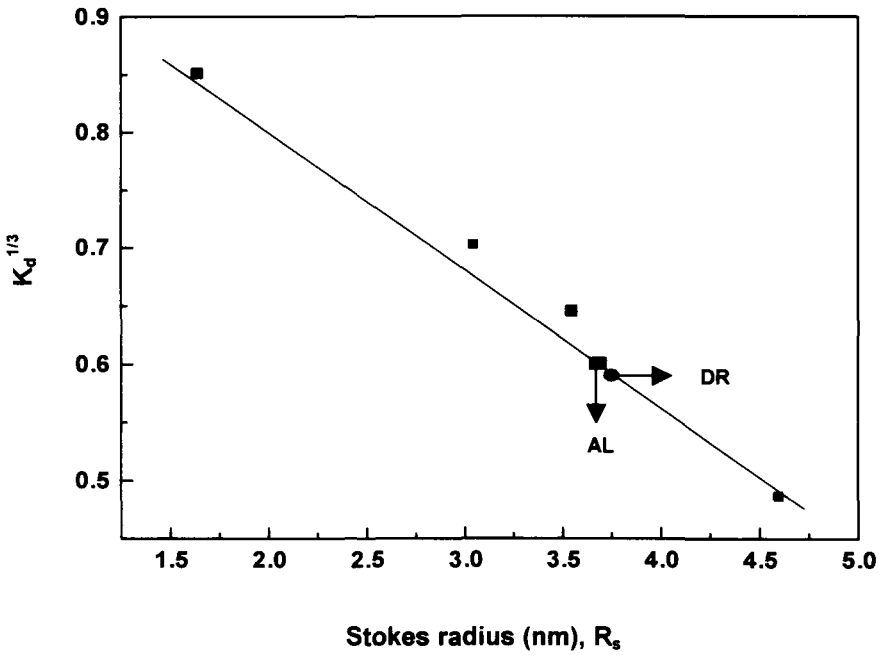


Fig 17: Plot of $K_d^{1/3}$ vs. R_s for the determination of stokes radii of activated receptors from the liver of AL- and DR-fed male mice. Sephadex G-100 gel chromatography data were used to obtain the plot. The molecular markers used were aldolase (4.6 nm), BSA (3.55 nm), ovalbumin (3.05 nm) and cytochrome C (1.64 nm).

Ion exchange chromatographic analyses of GR

Ion exchange chromatographic analyses of the [^{3}H] dexamethasone-receptor complexes from the liver of both AL and DR animals were performed to study the charge difference, if any, of the hepatic glucocorticoid receptors in both unactivated and activated state. The hormone receptor complexes from both AL and DR fed mice eluted as single peak at ~ 250 mM KCl (Fig 18). The elution profile, therefore, did not reveal any change in the salt concentration used for eluting unactivated receptors from both AL- and DR-fed animals.

Elution profile of the temperature activated receptors from both AL- and DR-fed animals revealed two radioactive peaks, one at ~ 250 mM of KCl (Fig 19). The peak eluted at ~120 mM KCl represents the activated receptors, whereas the other one at ~250 mM of KCl is due to the fraction of receptors that remained as unactivated. However, no difference was observed in the charge contents of both unactivated and activated receptors in either groups. Physicochemical characterization data of GR from AL- and DR-fed animals are tabulated and presented in Table II.

Studies on TO activity patterns

The induction pattern of tryptophan oxygenase (TO) was studied in the liver of AL- and DR-fed mice. There was a significantly higher (38 %) level of the TO activity in the liver of DR animals as compared to the AL -fed ones (Fig 20).

Aging studies

Regulation of glucocorticoid receptors during dietary restriction was studied in two different age groups (adult, 5- months and old, 20- months) of male mice. The receptor level, its activation (by temperature and salt) and DNase I digestion patterns were assessed in the liver and kidney of both age groups of mice to find out, if there is any age-related change in the GR due to dietary restriction.

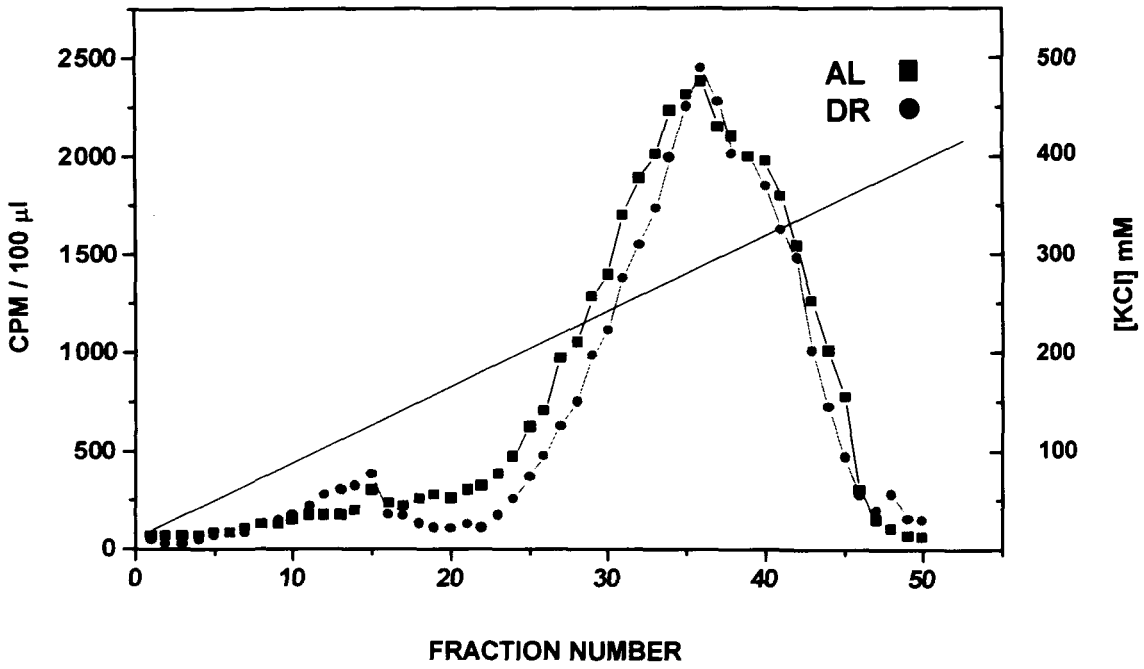


Fig 18: Elution profile of the unactivated glucocorticoid receptors from anion-exchanger DE-52. A 5 ml glass syringe containing 3 cm high gel-bed layered over a 2 mm thick DCC layer was used. The gel was equilibrated at 2-4°C with buffer (10 mM potassium phosphate, pH 7.5/ 1 mM β-mercaptoethanol/ 5 mM sodium molybdate). Unactivated [^{3}H] dexamethasone receptor complexes from liver were prepared as given in the Materials and Methods section and 500 µl loaded onto the column. After washing off the unbound proteins, the bound proteins were eluted with a salt gradient (0-400 mM KCl in the above buffer). 1 ml fractions were collected and 100 µl from each fraction counted for radioactivity. Each point in the profile represents the mean of 4 experiments.

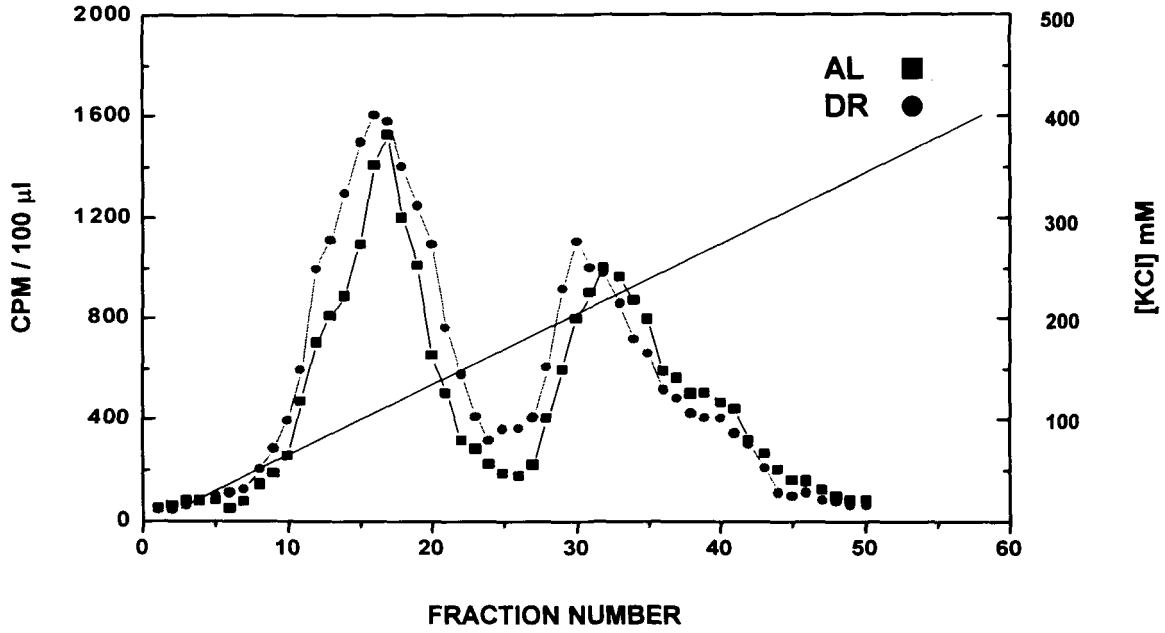


Fig 19: Elution profile of the activated glucocorticoid receptors from anion-exchanger DE-52. A 5 ml glass syringe containing 3 cm high gel-bed layered over a 2 mm thick DCC layer was used. The gel was equilibrated at 2-4°C with buffer (10 mM potassium phosphate, pH 7.5/ 1 mM β-mercaptoethanol/ 5 mM sodium molybdate). Heat activated [^{3}H] dexamethasone receptor complexes from liver were prepared as given in the Materials and Methods section and 500 µl loaded onto the column. After washing off the unbound proteins, the bound proteins were eluted with a salt gradient (0-400 mM KCl in the above buffer). 1 ml fractions were collected and 100 µl from each fraction counted for radioactivity. Each point in the profile represents the mean of 4 experiments.

Table II: Physicochemical properties of GR from the liver of AL- and DR-fed adult male mice.

Parameters	AL	DR
<u>Unactivated receptors</u>		
Molecular weight (kDa)	289.5 ± 3.5	298.0 ± 2.8
Stokes radius (nm)	5.90 ± 0.07	6.00 ± 0.14
Elution from DE 52 by KCl (mM)	250 ± 4.0	248 ± 5.0
<u>Activated receptors</u>		
Molecular weight (kDa)	97.6 ± 4.0	95.3 ± 7.0
Stokes radius (nm)	3.55 ± 0.07	3.40 ± 0.07
Elution from DE 52 by KCl (mM)	120 ± 2.0	115 ± 5.0

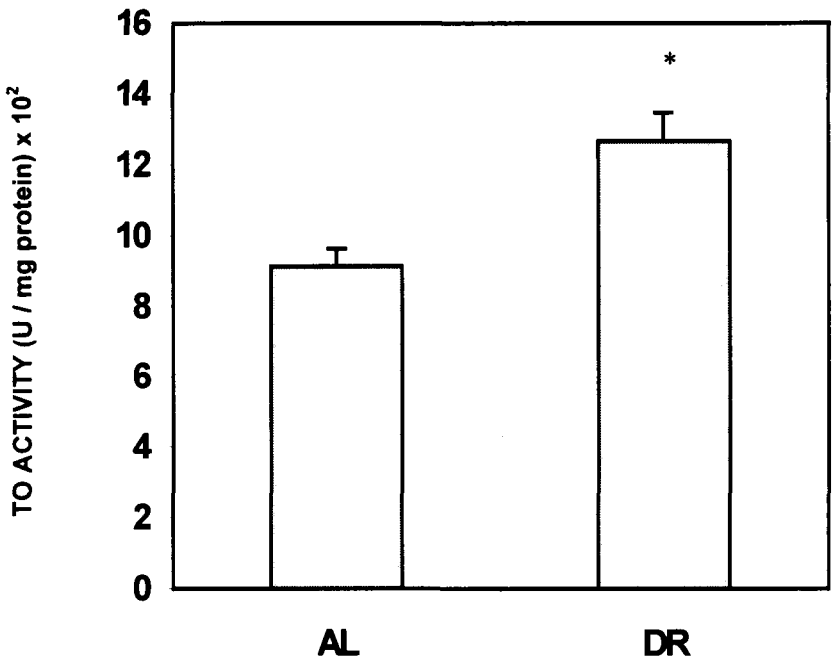


Fig 20: Activity of tryptophan oxygenase in the liver of AL- and DR-fed male mice. *Statistically significant ($p<0.001$) compared to the AL-fed mice. The values are the mean of four separate experiments performed with 5-6 mice of each group.

Body Weight

In old animals too, a significant decrease (-23%; $p < 0.001$) was observed in the body weight of DR (26.3 ± 2.1 g) animals as compared to the AL- (34.1± 3.2 g) fed ones (Fig 21). The decrease was relatively more pronounced in adult animals as compared to the older ones

Changes in the GR level

Studies on the level of GR revealed a decreased receptor level in both liver (25%) and kidney (30%) of AL-fed old mice as compared to the adult ones, whereas the DR animals of both the age groups showed a significant increase in the receptor concentration in both liver (37% in adult; 31% in old) and kidney (31% in adult; 28% in old) as compared to the AL-fed animals (Table III). Scatchard analyses of the data from both adult and old animals confirmed, both, the age specific decrease of receptors in AL-fed mice in both the tissues and also increase of receptors in DR animals in both liver and kidney as compared to the AL-fed ones. However, slopes of the plot did not exhibit any alteration in the affinity (K_d) of GR for its ligand at the two different ages in both liver (Fig 22A and B) and kidney (Fig 23 A and B) of AL- and DR-fed mice. Slot blot analyses of the receptor preparation from both liver (Fig 24A) and kidney (Fig 24B) at two different age groups also confirmed the increased level of GR in DR animals as compared to the AL-fed ones.

Activation studies of the GR

Temperature (25°C for 45 min) and salt (20 mM Ca^{2+} at 0°C for 45 min) dependent activation of the GR was studied in both liver and kidney of AL- and DR-fed mice at both adult and old ages using DNA-cellulose and purified nuclear binding assays. Results indicated a lower activation of receptors in both liver and kidney (15 – 20%) of older animals compared to the AL-fed adult ones. Although, the adult AL- and DR-fed mice did not show any change in the magnitude of receptor activation in both the tissues, it was significantly higher in both liver and

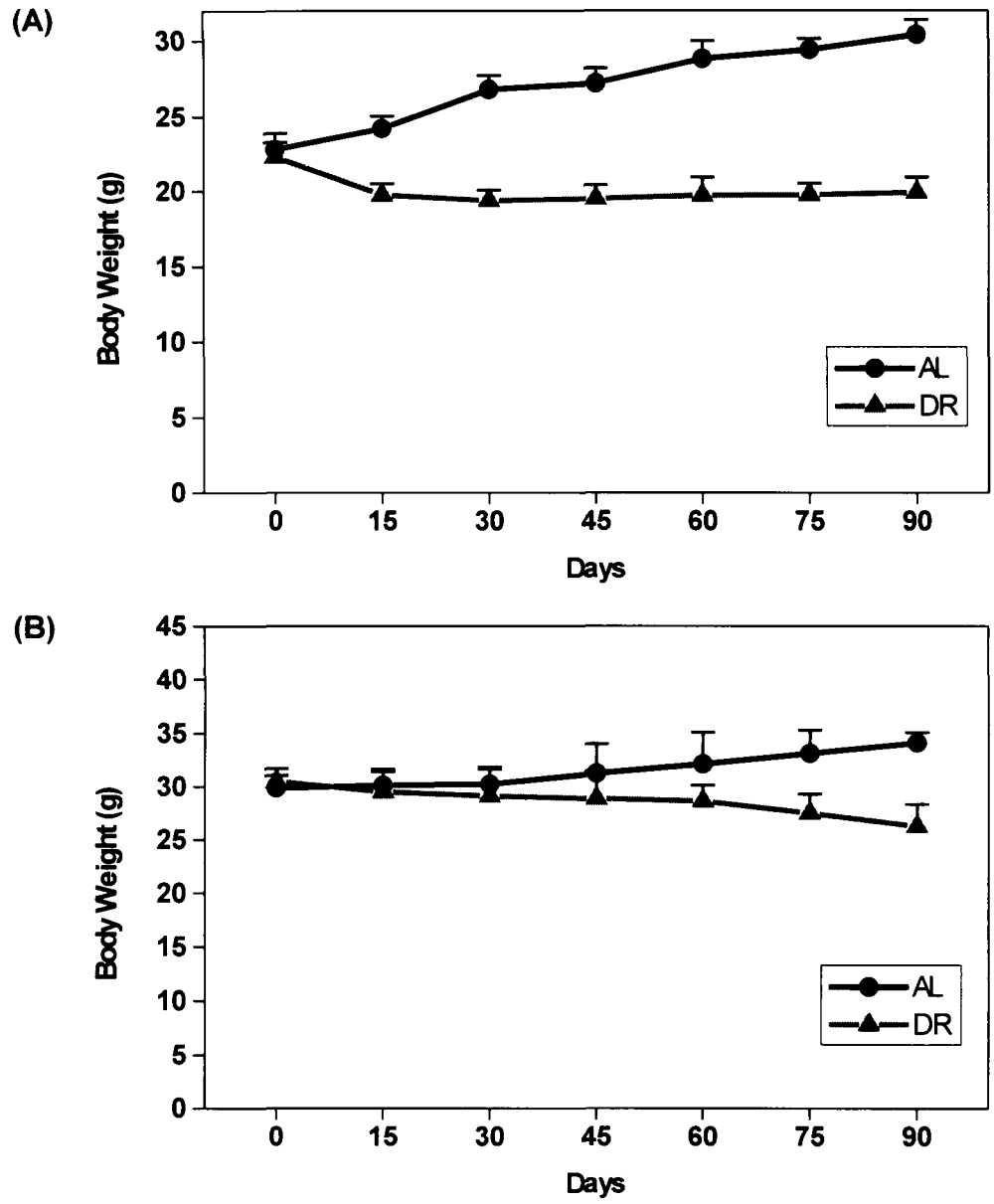


Fig 21: Body weight changes during dietary restriction of (A) adult (5-months) and (B) old (20-months) male mice. Animals were divided into two groups. One group was subjected to ad libitum (AL) dietary feeding while the other group was fed on alternate days for a period of three months. Body weight of individual mouse from each group were monitored during this period. Each point represents mean body weight for 5-6 animals of either group and bar represents standard deviation.

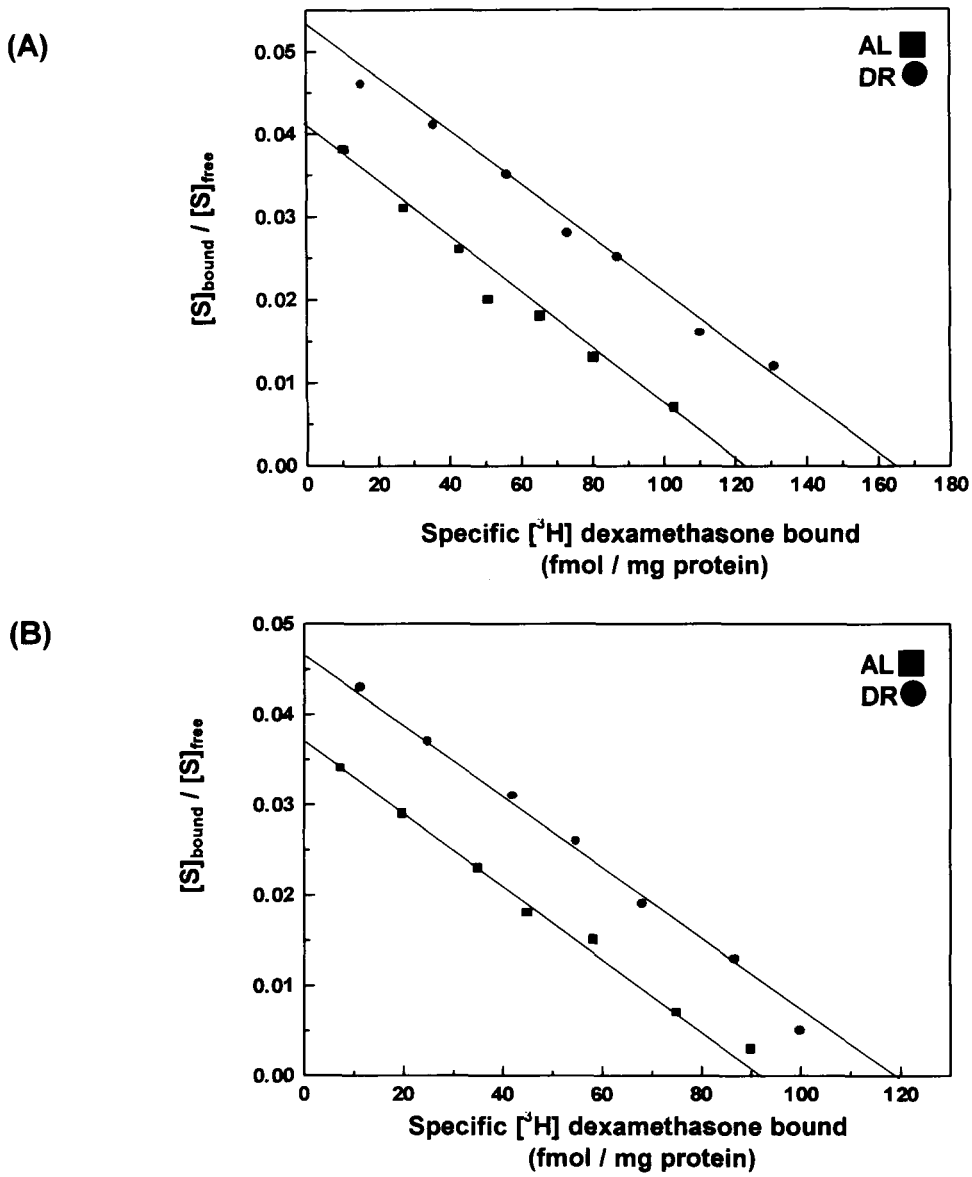


Fig 22: Scatchard plot of the [^{3}H] dexamethasone binding in the liver of (A) adult (5- months) and (B) old (20- months) AL- and DR-fed male mice. Cytosols were incubated with 5-120 nM [^{3}H] dexamethasone ± 500 fold excess cold dexamethasone for 4 h at 0°C. Specific binding at each concentration was calculated by subtracting non-specific binding from total binding and the data obtained were analyzed by Scatchard method. The slope of the curve gave the dissociation constant (K_d) and the intercept on X-axis gave the maximum receptor binding sites. Each point is the mean of four separate experiments with 5-6 mice of each group.

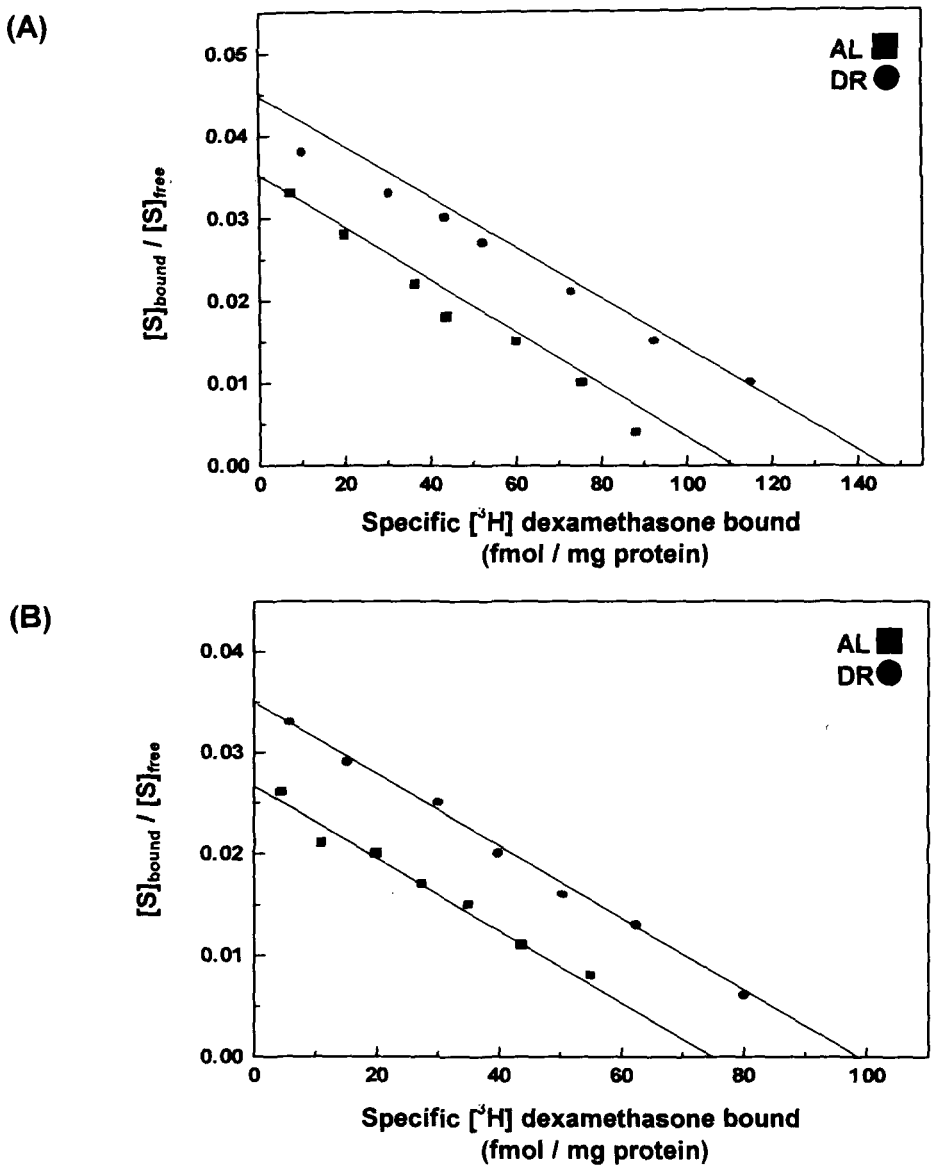
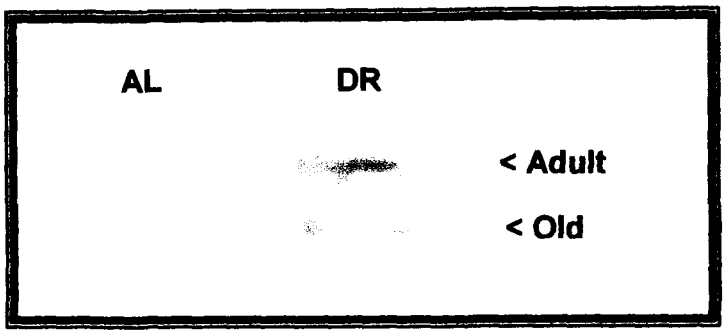


Fig 23: Scatchard plot of the [^{3}H] dexamethasone binding in the kidney of (A) adult (5- months) and (B) old (20- months) AL- and DR-fed male mice. Cytosols were incubated with 5-120 nM [^{3}H] dexamethasone ± 500 fold excess cold dexamethasone for 4 h at 0°C. Specific binding at each concentration was calculated by subtracting non-specific binding from total binding and the data obtained were analyzed by Scatchard method. The slope of the curve gave the dissociation constant (K_d) and the intercept on X-axis gave the maximum receptor binding sites. Each point is the mean of four separate experiments with 5-6 mice of each group.

(A)



(B)

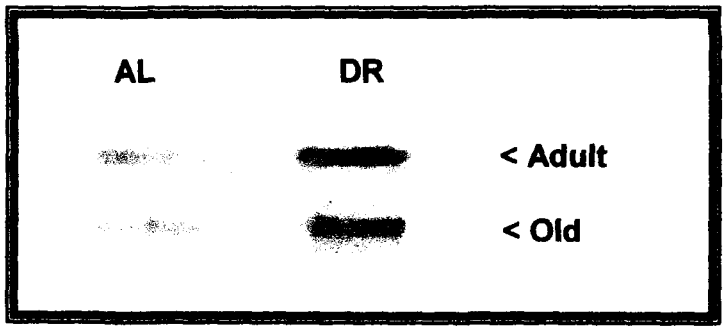


Fig 24: Slot blot analysis of adult and old liver (A) and kidney (B) glucocorticoid receptors from ad libitum (AL) and dietary restricted (DR) fed male mice. The details of experimental conditions are described in 'materials and methods' section. Equal amount of liver and kidney cytosol containing GR from AL and DR mice was applied onto each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-HRP conjugate. Arrows indicate the position and content of GR from AL- and DR-fed adult and old animals.

Table III: Concentration (fmol/mg protein) and affinity (nM) of [^{3}H] dexamethasone-receptors in the liver and kidney of adult (5 - months) and old (20 - months) *ad libitum* (AL) and dietary restricted (DR) fed male mice.

		AL		DR	
Tissue	**Age (months)**	**B_{max}**	**K_d**	**B_{max}**	**K_d**
Liver	**5**	122.0 ± 1.21	2.61 ± 0.07	167.21 ± 5.97*	2.65 ± 0.14
	20	91.18 ± 4.34	2.62 ± 0.02	119.93 ± 5.03*	2.55 ± 0.08
Kidney	**5**	111.2 ± 6.91	2.41 ± 0.03	146.21 ± 6.55*	2.44 ± 0.04
	20	77.37 ± 6.27	2.36 ± 0.11	99.50 ± 7.08*	2.46 ± 0.08

*Statistically significant ($p < 0.001$) with respect to AL-fed mice

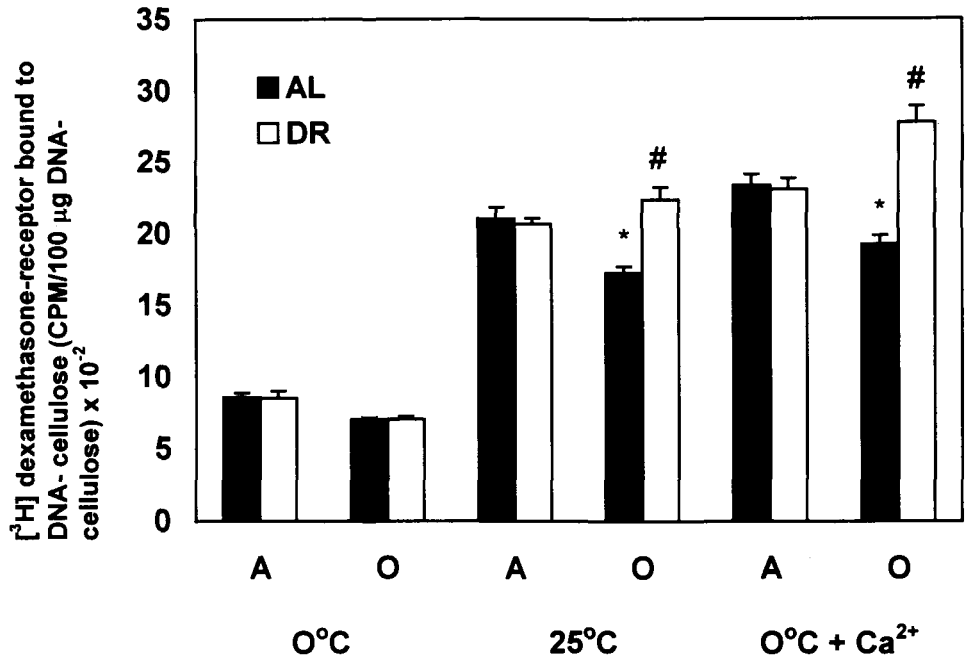


Fig 25 (A): Specific binding of liver [^{3}H] dexamethasone-receptor complexes to DNA-cellulose from the AL- and DR-fed mice of adult (A) and old (O) ages. Liver cytosols were prepared in buffer B and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H] dexamethasone for 4 h at 0°C. The hormone-receptor complexes were then subjected to heat (25°C) and Ca^{2+} (20 mM at 0°C) activation for 45 min as against 0°C control. Details of DNA-cellulose binding and further processing are described in 'materials and methods' section. The results are mean ± standard deviation of four separate experiments performed with 5-6 mice of each group*. Statistically significant compared to the adult. # Statistically significant compared to the AL-fed mice of the respective age group.

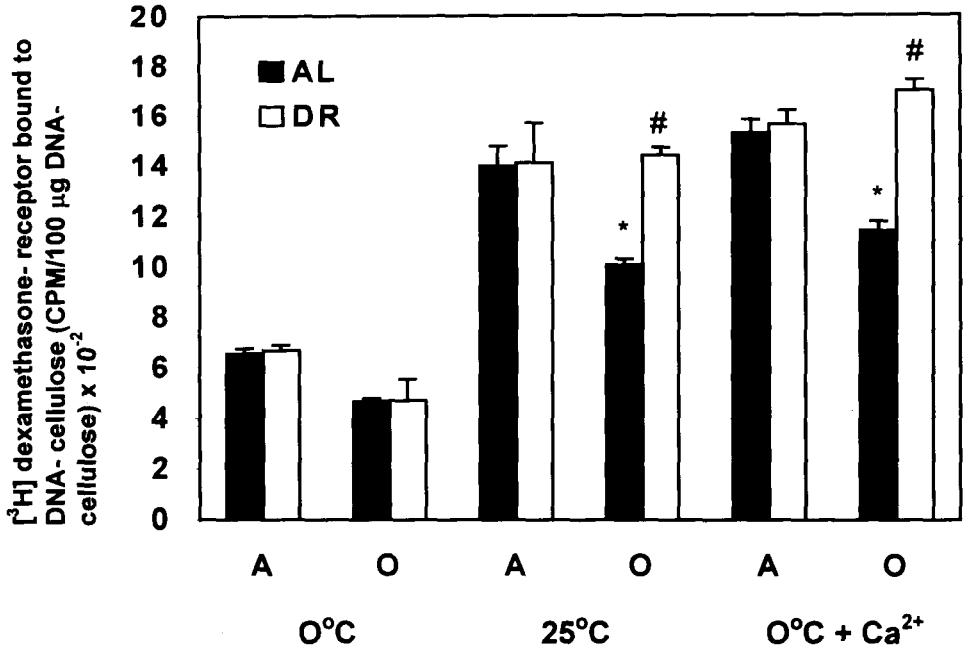


Fig 25 (B): Specific binding of kidney [^{3}H] dexamethasone-receptor complexes to DNA-cellulose from the AL- and DR-fed mice of adult (A) and old (O) ages. Kidney cytosols were prepared in buffer B and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H] dexamethasone for 4 h at 0°C. The hormone-receptor complexes were then subjected to heat (25°C) and Ca^{2+} (20 mM at 0°C) activation for 45 min as against 0°C control. Details of DNA-cellulose binding and further processing are described in 'materials and methods' section. The results are mean $\pm$ standard deviation of four separate experiments performed with 5-6 mice of each group*. Statistically significant compared to the adult. # Statistically significant compared to the AL-fed mice of the respective age group.

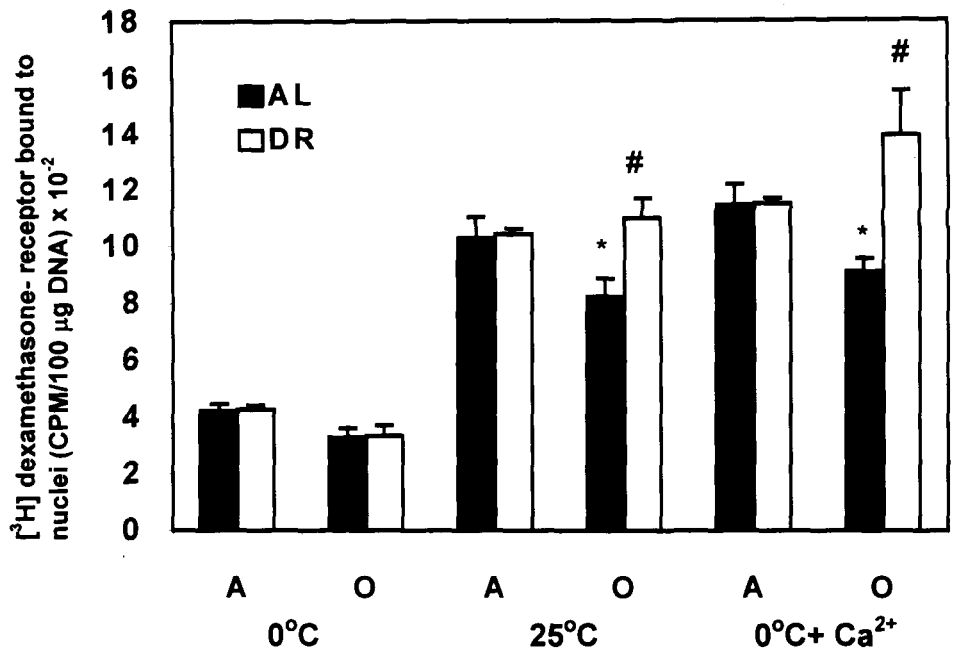


Fig 26 (A): Specific binding of liver [^{3}H] dexamethasone-receptor complexes to purified nuclei from the AL- and DR-fed mice of adult (A) and old (O) ages. The hormone-receptor complex preparation and activation conditions are same as given in Fig 25. Activated hormone-receptor complexes were incubated with purified nuclei instead of DNA-cellulose as mentioned in 'materials and methods' section. The results are mean ± standard deviation of four separate experiments performed with 5-6 mice of each group.* Statistically significant compared to the adult. # Statistically significant compared to the AL-fed mice of the respective age group.

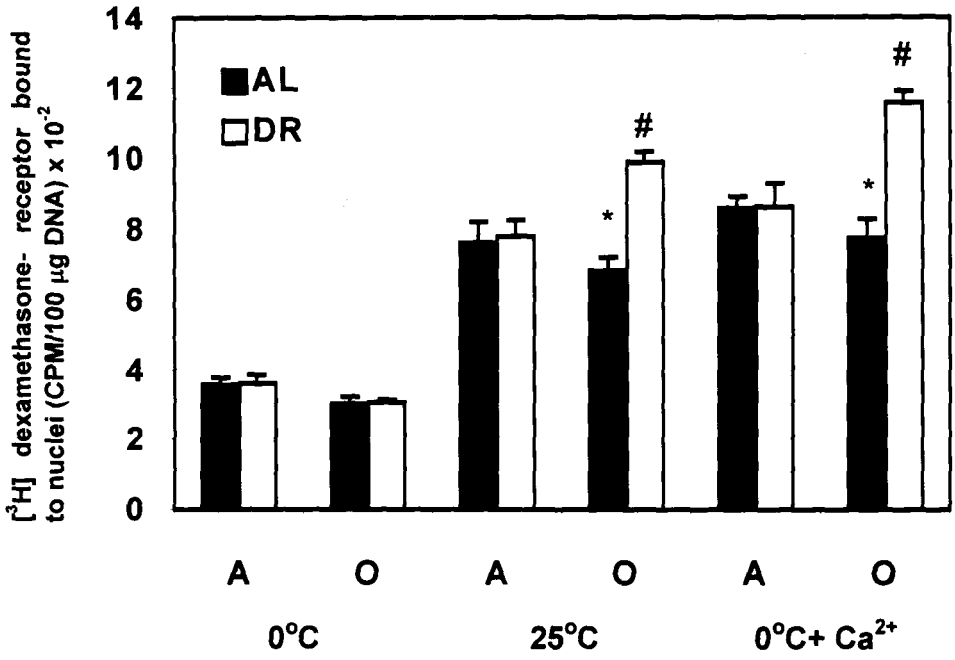


Fig 26 (B): Specific binding of kidney [^{3}H] dexamethasone-receptor complexes to purified nuclei from the AL- and DR-fed mice of adult (A) and old (O) ages. The hormone-receptor complex preparation and activation conditions are same as given in Fig 25. Activated hormone-receptor complexes were incubated with purified nuclei instead of DNA-cellulose as mentioned in 'materials and methods' section. The results are mean $\pm$ standard deviation of four separate experiments performed with 5-6 mice of each group. * Statistically significant compared to the adult. # Statistically significant compared to the AL-fed mice of the respective age group.

kidney of older DR animals compared to the AL-fed ones as judged by both the DNA-cellulose (Fig 25A and B) and nuclear binding (Fig 26A and B) assays.

DNase I digestion of GR

DNase I digestion of bound GR from the liver and kidney revealed a significantly higher extraction of nuclear bound heat activated [^{3}H] dexamethasone-receptor complexes from adult as compared to the old animals. However, no significant change in the magnitude of extraction was observed between AL and DR animals of both the age groups. In liver, the magnitude of extraction of the receptors from adult mice was significantly higher (57% - 59%) than that of the older (32% - 33%) mice (Fig 27A). In kidney too, similar pattern of higher extraction was observed in case of adult animals (56% - 57%) when compared to the aged (30% - 31%) ones (Fig 27B).

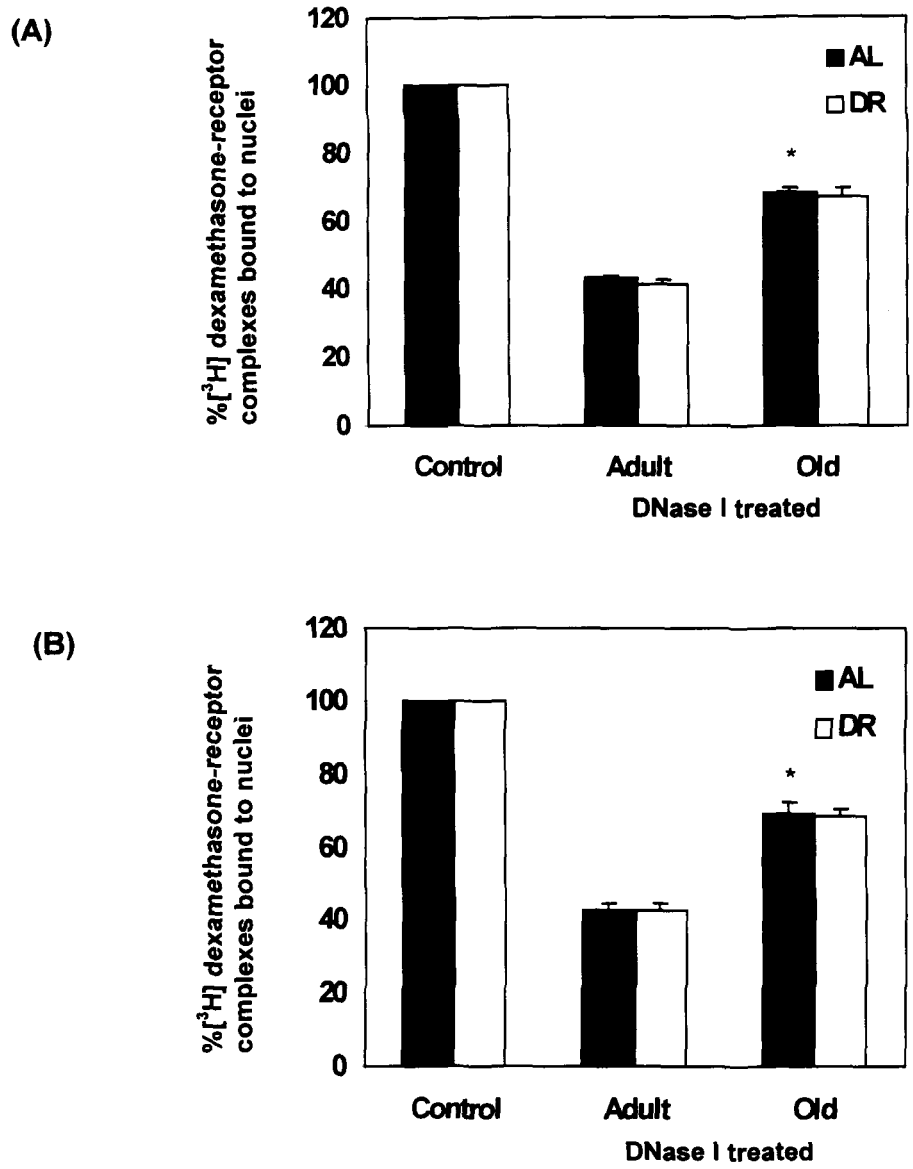


Fig 27: DNase I digestion of bound [^{3}H] dexamethasone-receptor complexes from the liver (A) and kidney (B) nuclei of adult and old AL- and DR-fed mice. Heat-activated, nuclear-bound hormone-receptor complexes were extracted using DNase I as per experimental protocols given in 'materials and methods' section. The results are mean $\pm$ standard deviation of four separate experiments performed for each group. *Statistically significant compared to the adult mice

DISCUSSION

Dietary restriction (DR) refers to a dietary regimen low in calories without malnutrition (Yu et al. 1994). Animals on a standard DR regimen enjoy meals that are nutritionally balanced, containing adequate proteins, fats, carbohydrates, vitamins and minerals. The results of such dietary moderation are largely positive. McCay and his collaborators were the first to show that DR dramatically increased the life span of rats (McCay et al. 1935). Since then, numerous laboratories with a variety of strains of rats and mice have confirmed this initial observation and shown that of reducing the calorie intake significantly increases both the mean and maximum survival of different group of organisms (Weindruch and Sohal 1997; Lin et al. 2000; Mattson et al. 2003; Sharma 2004). In rodents, primates and humans, dietary restriction reduces blood glucose and insulin concentrations (Dhahbi et al. 1999). DR evokes anti-inflammatory, anti-neoplastic effects and also protects the rodents against diabetes, impaired tissue growth and reproductive senescence (Leaky et al. 1998; Jolly 2004). Dietary caloric restriction is the most robust means of slowing aging and extending life span in short lived mammals and also in many lower organisms. Recent studies also suggest a similar role of DR in non-human primates (Mattison et al. 2003; Roth 2003 and 04).

Aging is the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to diseases and death. Over the past two decades, gerontological and nutritional researchers have made remarkable advances toward a better understanding of the mechanistic interactions between nutrition and aging (Yu and Chung 2001). Although the precise molecular basis of the life prolonging effects of dietary restriction has not been fully elucidated but recent work on DR suggests it's diverse and beneficial anti-oxidative role on aging. Experiments have repeatedly shown that DR provides a variety of protective mechanisms against oxidative damage while upholding antioxidant defense systems during aging (Yu 1996).

DR has been shown to reduce the age-associated cellular accumulation of oxidatively damaged molecules such as lipid peroxidative products (Matsu et al. 1993), oxidized proteins (Dubey et al.1996), and oxidatively damaged DNA (Sohal et al. 1994). These remarkable actions to resist cellular oxidative damage and to maintain a cellular redox balance are proposed to be the hormetic response of DR's anti-aging actions (Yu and Chung 2001).

Alterations in the adaptive responses to hormones and other biochemical stimuli are characteristics of aged animals and include a decreased ability to respond to stress (Kalimi et al. 1983). And the mechanisms controlling the adaptive response to reduced calorie intake may involve a dynamic interplay between the hormones that control energy balance, appetite, cell proliferation and apoptosis, stress responses, inflammation and repair systems (Leaky et al. 1998). All the above effects in a way or the other are regulated by glucocorticoids (GCs). They play an essential role in maintaining basal and stress related homeostasis (Bamberger et al. 1996). GCs are produced by the adrenal cortical cells and are regulated by CRH (from hypothalamus) and ACTH (from anterior pituitary). They exert their cellular and molecular actions by modulating the expression of genes through a cascade of regulatory events initiated by high affinity interaction with glucocorticoid receptors (GR) (Biola and Pallardy 2000; Schaff and Cidlowski 2002; Kino et al. 2003). GR belongs to a largest known superfamily of eukaryotic transcription factors that alter the expression of target gene either by transrepression or by transactivation (Schaff and Cidlowski 2003). As with the other members of the nuclear receptor family, the glucocorticoid receptor contains a modular structure consisting of three major domains: an amino terminal transactivation domain, a central zinc finger DNA-binding domain, and a carboxy terminal ligand-binding domain (Tsai and O'Malley 1993).

In the absence of hormone, GR resides in the cytoplasm as a multiprotein complex with the chaperone proteins (hsp 90, hsp 70, the immunophilin p59 and the phosphoprotein p23) (Pratt and Toft 1997). Upon hormone binding, GR dissociates from this complex and undergoes a conformational change that unmasks nuclear localization signals (NLS) found within the receptor (Hache et

al. 1999). The glucocorticoid receptor then translocates to the nucleus and binds to glucocorticoid response elements (GREs) as a dimer in the promoter region of the target genes (Barnes 1998). The association of the GR dimer with the GRE results in an allosteric induced conformational change within the receptor and subsequent recruitment of coactivator complexes critical for chromatin remodeling and transcription (Jenkins et al. 2001). The functional results of the GR-GRE interaction can be largely cell-type, promoter and ligand specific. In some instances, the glucocorticoid receptors bind negative GREs in promoter of genes and inhibit transcription (Truss and Beato 1993). Alternatively, GR represses gene transcription by physically interacting with other transcription factor such as AP-1 and NF-kB (Barnes 1998).

Glucocorticoids are of major importance for protection of the body against stress by regulating glucose metabolism and blood pressure. Besides the metabolic actions, GC effects have also been described with respect to behavior and brain function (De Bosscher et al. 2003). Furthermore, GCs affect organ development, tissue maturation, wound healing, and calcium reabsorption (Inazu et al. 1990; Gaynon and Lustlg 1995). Highly important is the role of GCs in the dynamic modulation of inflammatory and immune responses (De Bosscher et al. 2003). It is now known that the level of this hormone gets elevated during dietary restriction. Several lines of evidences also suggest that glucocorticoid elevation in dietary restriction mediates the prevention of cancer (Birt et al. 1999).

Since dietary interventions have attained a significant place in the extension of life span and in the inhibition of carcinogenesis and potentiation of the immune system, our data would provide better understanding of the link, controlling the role of glucocorticoids during dietary restriction. We have studied the long-term effect of DR on the endogenous level of GR in the liver and kidney of two different age groups of AL and DR fed mice. We have studied the effect of one- and three-months of dietary restriction on Swiss albino (Balb/c strain) male mice. And after our careful evaluation, we followed the three-month dietary restriction regimen that gave the maximum stable change in the GR expression. In our experimental schedule, it has been observed that DR for three months

significantly reduced the body weight of mice. 34% reduction in body weight was observed in the DR mice as compared to the AL-fed ones. This observation of body weight reduction confirmed that the animals were indeed subjected to dietary restriction and that has been consistent with the data of an earlier report (Lee et al. 2000).

Changes in the GR level

GR is known to have tissue-specific expression which is altered during development and aging. GCs are involved not only in the metabolic functions of various animal tissues but also in cellular growth and differentiation. Development and aging of animals may partly be characterized by changes in the responsiveness of tissues and of quantitative changes in the receptor molecules (Cake and Litwack 1976; Sharma and Timiras 1988).

We have studied the level of GR in the liver and kidney of AL- and DR-fed male mice. Our data on GR level suggest an increase in the concentration of GR in both the tissues of DR animals as compared to the AL-fed ones. In liver, the GR level increased significantly in DR animals as compared to the AL-fed ones. The increased level of receptors in the liver may help to maintain the anabolic role of this hormone during dietary restriction. It has earlier been reported that the dietary calorie restriction in mice leads to an increase in the mRNA and/or activity of key enzymes (phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) of hepatic gluconeogenesis (Dhahbi et al. 1999). The up-regulation of glucocorticoid receptors in the liver of mice might have functional role in inducing such enzymes for better metabolic regulation during dietary restriction.

In kidney too, we have found a statistically significant increased level of receptor in DR animals as compared to the AL-fed ones. Higher level of GR in kidney of mice may be a contributory factor for the role of this hormone in glomerular filtration rate and ion transport during such restriction (Borbhuiya and Sharma 1995). Glucocorticoids are known to influence glomerular filtration rate, ion-transport and other metabolic functions in kidney (Fanestill and Park 1981; Sharma and Timiras 1988). There are reports that DR rodents are more resistant

to a variety of stresses such as trauma, heat shock and drug toxicity (Weindruch et al. 2001). The increased resistance in DR rodents may be a result of the glucocorticoid's involvement in cellular protection possibly by modulating GR functions. There are few reports on the differential tissue-specific expression of GR during dietary interventions in varied groups of animals. Recently, maternal under-nutrition during early to mid-gestation has been attributed to increase in the expression of GR mRNA in the liver of neonatal sheep (Whorwood et al. 2001). DR has also been reported to selectively decrease the expression of GR in the hippocampus and cerebral cortex of rats (Lee et al. 2000).

To confirm the observed increase in the GR concentration of liver and kidney of DR-fed mice as compared to the AL-fed ones, we performed the Scatchard analyses of the binding data. The data of the Scatchard analyses confirmed the increased level of GR concentration in both liver and kidney of DR animals as compared to the AL-fed ones. However, no alteration in the affinity of the receptors for it ligand was found in both the groups of animals in either of the tissues. It clearly indicates that the level of GR is increased in both the tissues during dietary restriction without altering the affinity for the hormone (Dutta and Sharma 2003).

Again, the slot blot analyses were performed to reaffirm the increased receptor level in DR animals. The presence of more intense bands on the DR-fed slots confirmed the increased concentration of receptor in the DR-fed mice as compared to the AL-fed ones (Dutta and Sharma 2003). The higher level of GR in both the tissues studied during DR may help respective tissues to suitably adapt their metabolic and physiological functions when mice were subjected to dietary restriction.

Activation studies of the GR

A very important aspect of the steroid-induced alterations in gene transcription is the mechanism(s) by which the steroid–receptor complex interacts with the nuclear acceptor sites. The activation of steroid-receptor complexes has been described as a not well-defined, conformational change that

enables the complexes to interact with specific acceptor sites on chromatin and modulate gene expression (Yamamoto 1985). This nuclear binding capacity can be achieved in vitro by incubating the hormone receptor complexes at 25°C and under high ionic condition (Sharma and Timiras 1987). Activation of the GR by both temperature (25°C for 45 min) and salt (20 mM Ca^{2+} at 0°C for 45 min) was performed to detect the change, if any, in the physicochemical properties of the receptors during dietary restriction in the liver and kidney of mice. To judge the magnitude of activation, DNA-cellulose and nuclear binding assays were performed. As DNA-cellulose is a non-specific binding media so to have a more physiological in vitro binding medium, purified nuclear pellet was used.

Results indicate similar pattern of activation by both temperature (25°C for 45 min) and salt (20 mM Ca^{2+} at 0°C for 45 min) as compared to the unactivated (0°C for 45 min) receptors in the liver and kidney of both AL- and DR-fed animals as judged by the DNA-cellulose and purified nuclear binding assays. It is indicative of the fact that receptor activation may not be altered during such dietary interventions.

DNase I digestion studies

Chromatin structure and organization play modulatory role in the in vivo gene expression and any change in these may affect the interaction of transcription factors with cis-acting elements (Tsai and O'Malley 1994, Bamberger et al. 1996). In order to study the GR- chromatin interactions, we studied the extraction of bound GR complexes from the nuclei of both groups of animals (AL and DR) by digestion with DNase I. The super coiling of DNA around histone core confers the specificity of digestion of DNA in chromatin by pancreatic DNase I (Chaturvedi and Kanungo 1983). DNase I makes single stranded cuts in the double stranded DNA at 10 bp- intervals and its multiples which is widely used to study chromatin organization in animal tissues (Chaurasia and Thakur 1997). Our data, on digestion of hepatic and renal chromatin in both AL- and DR-fed mice revealed no change in the extent of digestion of nuclear bound GR complexes. It seems that there may not be an

appreciable change in the organization of chromatin in DR-fed animals as compared to the AL ones.

Studies on the physicochemical parameters of glucocorticoid receptors

Studies of the physicochemical properties of the hepatic glucocorticoid receptors did not reveal any change in terms of molecular weight and stokes radii. Gel filtration analyses of both unactivated as well as thermally activated liver glucocorticoid receptors showed similar values for both AL- and DR-fed animals. However, the lower molecular weight of the activated receptors from both the group is indicative of monomeric form upon activation as compared to the oligomeric form of the unactivated receptors where the molecular weight is higher because of presence of other associated proteins. A similar phenomenon was observed while looking at stokes radii for both unactivated as well as activated receptors in AL- and DR-fed animals.

The elution pattern of DE-52 also did not reveal any difference in the charge binding of the receptors from both the groups of animals. The concentration of salt required to elute both unactivated as well as activated receptors was same for both AL- and DR-fed animals. From the above studies, it can be concluded that DR might not have any regulatory role in altering the physicochemical properties of the GR.

Considering all the studies performed above, it has been observed that the level of receptors is elevated during dietary restriction while physicochemical properties of the receptors remained unaltered. In order to correlate its physiological significance, the induction pattern of tryptophan 2,3-dioxygenase (TO), a known glucocorticoid inducible enzyme (Nakamura et al. 1987; Chikhirzhina et al. 1999; Ren and Correia 2000) was studied. TO converts tryptophan to kynurenine. The activity of this enzyme was measured in the liver of both AL- and DR-fed mice. A higher level of TO was present in the liver of DR-fed mice as compared to the AL-fed ones. The presence of the enhanced level of TO in DR animals is may be due to the higher GR concentrations in the DR

animals that may help upkeeping of TO level during dietary restriction (Shanker et al. 1982).

Aging studies

The long-term effect of DR on the endogenous level, activation properties and nuclear binding of the GR was studied in the liver and kidney of two different age groups of AL- and DR-fed mice. In such experimental schedule, it has been observed that DR for three months significantly lowered the body weight in both adult and old mice. However, the decrease was more pronounced in adult animals (34%) as compared to older ones (23%). It assured that the mice were indeed dietary restricted (Dutta and Sharma 2004).

Data on GR level suggest an overall decrease in the receptor level in the liver (25%) and kidney (30%) of old mice as compared to the adult ones. The high level of receptors in both the tissues of the adult animals may be a contributory factor for the role of this hormone during early growth and development of animals (Kanungo 1994; Ranhotra and Sharma 2001). Decrease in the GR level of old mice may impair metabolic functions, which may be one of the reasons for reduced ability to maintain homeostasis during old age (Ranhotra and Sharma 2001). Other reports also suggest a decrease in the receptor level with aging in rat liver and kidney (Sharma and Timiras 1988; Djordjovic - Markovic et al. 1999), whereas there was no such alteration in rat adipocytes (Kalimi and Benarjee 1981). Scatchard and slot blot analyses of the binding data confirmed the decreased level of receptor concentration with increasing age. However, slopes of the Scatchard plots did not reveal any change in the affinity of receptor for its ligand at the two different age groups in both the tissues. Experimental findings also indicate a significant increase in the receptor level in the liver and kidney during dietary restriction in both age groups as compared to the AL-fed ones. Though the increase was almost similar in magnitude at both the ages, such increase may help the animal in controlling the glucocorticoid mediated responses of metabolic need during dietary restriction. DR rodents are more resistant to a variety of stresses due to increased production of stress

proteins that may increase the resistance of cells (Weindruch et al. 2001). In old animals, an elevated level of GR by dietary restriction might help improving the metabolic activities and quite likely provide better adaptability to the environmental changes (Dutta and Sharma 2004).

Activation studies of GR were carried out from the liver and kidney of both adult and old mice during dietary restriction to see any change in the magnitude of temperature (25°C for 45 min) and salt (Ca^{2+} at 0°C for 45 min) dependent activation. Results indicate a significant decrease in the magnitude of activation of hepatic and renal (15 - 20%) GR in old animals compared to the adult ones in the AL-fed mice. The decreased activation of GR in old mice 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 glucocorticoid receptors (Bodine and Litwack 1988). Low temperature Ca^{2+}-dependent activation of the H-R complexes was more pronounced than temperature-mediated activation in the liver and kidney of older animals as judged by the DNA-cellulose and nuclear binding assays. The exact mechanisms of low temperature Ca^{2+}-mediated activation of the glucocorticoid-receptor complexes are not well understood. However, Ca^{2+}enhancement of nuclear and DNA-cellulose binding of GR may be due to direct interaction of Ca^{2+} with the receptor molecule and/or receptor transforming factor(s). This interaction could cause a conformational change capable of exposing the DNA- and/of chromatin-binding domain (Groday et al. 1982; Sharma and Timiras 1988). Low temperature Ca^{2+}-dependent activation was more pronounced using DNA-cellulose than purified nuclei. This may be due to the open DNA binding sites in DNA-cellulose compared with intact nuclei (Sharma and Timiras 1988). Surprisingly, we have also observed a greater activation (30 - 40%) of receptors in both the tissues in old mice during dietary restriction as compared to the AL-fed ones, whereas, the adult group showed no such changes. This increase in the receptor activation in aged DR animals may help them to achieve better glucocorticoid action with lower level of receptors at that stage of life span.

The digestion and extraction of nuclear bound GR by DNase I was studied in the liver and kidney of adult and old mice during dietary restriction in order to reveal differences, if any, in extraction of nuclear bound receptors. There are reports suggesting increased compactness of chromatin with aging and its reduced digestibility by DNase I in aged animals (Kanungo 1994). DNase I cut the DNA where it is maximally exposed and thus depends on the degree of chromatin condensation. Our data showed a higher degree of extraction of nuclear bound hormone-receptor complexes in the adult liver and kidney as compared to the old, whereas there was no marked effect of DR on such extractability at either ages. It relates to the fact that there may not be an appreciable change in the organization of chromatin in DR animals as compared to the AL-fed ones (Dutta and Sharma 2004). Our findings of decreased extractability in aged animals corroborate with the observations of others (Chaturvedi and Kanungo 1983; Chaurasia and Thakur 1997) who have also reported reduced digestibility of chromatin by DNase I in old rat brain compared to the young and adult.

In conclusion, our findings indicate that long-term dietary restriction results in an increase of glucocorticoid receptor level in the liver and kidney of both adult and old mice. Whereas the activation properties of the GR complexes remained unaltered during such interventions in the adult animals, they do exhibit greater values in older mice. Hence, dietary restriction could be used to elevate the GR concentration in older animals whose receptor level gets already reduced during that period of life span. Furthermore, the higher magnitude of receptor activation in older DR mice could be an advantage to such animals for attaining better glucocorticoid-mediated responses and for adapting to stress in old age (Dutta and Sharma 2004).

SUMMARY

Dietary restriction (DR), a reduction in calorie intake without malnutrition, influences several physiological processes namely, immunological, general protein and amino acid metabolism and also neuroendocrinological systems. DR has been reported to potentiate immune system, delay immunosenescence, reduce progression of diseases and extend life span in rodents and primates. It is an efficacious means of increasing longevity and reducing pathology in several groups of animals. DR inhibits the progression of carcinogenesis in animal model systems if allowed during the promotional phase. Glucocorticoid hormones regulate most of such effects. It is also known that the level of this hormone gets elevated during dietary restriction in animals. Glucocorticoids (GCs) produced by the adrenal cortices have multitude of effects within the body. They are involved in metabolism of lipid, carbohydrates and proteins, stress responses, fluid and electrolyte balance, as well as maintenance of immunological, renal and skeletal homeostasis. GCs exert their effects by binding to a single glucocorticoid receptor (GR), which is predominantly localized in the cytoplasm of the target cells. GR belongs to a phyllogenetically-conserved super family of largest known transcription factors. The knowledge of GR level, its activation process and interaction with chromatin, during dietary restriction might provide insight into the action mechanisms of glucocorticoids during such interventions, and will also help to understand the age-delaying role of DR in animals. Summarized below under various sections are the findings of the work done:

Changes in the body weight

Body weight graph of the mice after three months of alternate days of feeding showed a significant decrease (-34%; $p < 0.001$) in the body weight of the DR-fed mice (19.9 ± 1.1g) as compared to the AL-fed ones (30.4 ± 1.2 g). It has assured that the animals were indeed subjected to dietary restriction during the experimentation.

Changes in the level of glucocorticoid receptors

Studies on glucocorticoid receptor (GR) level indicate a significant increase in the level of receptors in the dietary restricted (DR) mice as compared to the ad libitum (AL)-fed ones in both liver and kidney. Scatchard analyses confirm a higher concentration of receptor binding sites in the liver (41%) and kidney (37%) of DR as compared to that of AL-fed ones. However, the affinity (K_d) of GR for hormone remained the same in liver (~2.3 nM) and kidney (~2.1 nM) for both AL- and DR-fed mice. The slot blot analyses of receptor preparation corroborate the increased level of GR in the DR mice as compared the AL-fed ones in both the tissues. The increase in the level of GR may be a contributory factor in controlling the glucocorticoid-mediated responses during long-term dietary restriction in mice.

Activation studies of the glucocorticoid receptors

Higher activation of hepatic and renal GR complexes from both AL- and DR-fed mice was observed by temperature and salt. However, there was no significant change in the magnitude of activation (either by temperature or salt) of GR in both groups of animals in either of the tissues, which indicate no difference in the in vitro activation of hormone-receptor complexes from the liver and kidney of AL and DR mice under the conditions mentioned. It reflects that receptor activation may not be altered during such dietary interventions.

Physicochemical properties of the glucocorticoid receptors

Gel filtration analyses of unactivated and activated GR complexes from AL- and DR-fed mice did not reveal any change in terms of molecular weight for both groups of animals. Elution pattern from ion exchange chromatography also showed a similar charge binding for both the groups of animals in unactivated and activated [^{3}H] dexamethasone-receptor preparations. From the above studies, it can be concluded that during dietary restriction the physicochemical properties of the GR remained unchanged.

DNase I extraction of bound GR from nuclei

DNase I digestion of liver and kidney nuclei from both AL- and DR-fed mice revealed a similar magnitude of extraction of bound GR complexes from both the groups of animals in either tissues. It shows that there may not be an appreciable change in the organization of chromatin in DR-fed animals as compared to the AL ones in both the tissues studied.

Studies on tryptophan 2,3 dioxygenase activity pattern

The activity pattern of tryptophan 2,3 dioxygenase (TO), a glucocorticoid inducible enzyme when measured in the liver showed a significantly higher activity (28%) in the DR animals as compared to the AL-fed ones. This increase in the activity of the enzyme may be due to increased level of GR in the DR animals. It reflects on the significance of increased GR level during DR in upkeeping the activity level of its inducible marker enzyme such as TO.

Aging studies

Our study indicates a significant decrease of GR level in the liver (25%) and kidney (28%) of old mice as compared to the adult ones in AL-fed animals. Whereas, the dietary restricted mice of both the age groups showed a marked increase in the receptor concentration in both liver and kidney as compared to the ad libitum-fed ones. However, the affinity of the GR for its ligand remained unchanged in both AL- and DR-fed animals from both the age groups in either of the tissues. Scatchard and the slot blot analyses of receptor preparation confirmed the higher level of GR in the liver and kidney of DR mice as compared to the AL-fed ones in both the age groups. The high level of receptors in the adult animals compared to the old ones may be an essential factor for the role of this hormone during growth and development of animals. In older animals, an increase in GR level by DR might play a pivotal role in improving the metabolic activities and helping the animals to adapt better to the environmental stresses. The magnitude of activation of GR was lowered in both liver and kidney (15-20%) of old animals as compared to the adult ones. The decreased activation of GR in

old animals 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 glucocorticoid receptors. In older group, the magnitude of activation was higher in both liver and kidney of dietary restricted animals as compared to the AL-fed ones, whereas, no such change was observed in the adult group of mice. The increase in the receptor activation in aged DR animals may help them to achieve better glucocorticoid action with lower level of receptors at that stage of life span. Our data also show a high degree of DNase I extraction of nuclear bound GR in the adult liver and kidney as compared to the old, whereas there was no marked effect of DR on such extractability at either age. This is indicative of more compact chromatin organization in old mice tissues. It also relates to the fact that there may not be an appreciable change in the organization of chromatin in DR animals as compared to the AL-fed ones.

To conclude, the findings summarized above indicate that –

- Dietary restriction does elevate the level of glucocorticoid receptor that may help animals to adapt to changing metabolic need during such interventions.
- DR could be used to elevate the level and the activation of GR in older animals, whose receptor levels get reduced because of age. This may be useful in better adaptation to stress in those animals to achieve greater survivility and longer life span.

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APPENDIX I

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). 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, 3 ml of working solution was added and mixed. The test tubes were then incubated for 10 min. at room temperature for colour development. Finally, absorbance of each test solutions was determined at a wavelength of 595 nm, against a reagent blank. A plot of absorbance against protein concentrations used was obtained and employed for deducing the protein concentrations of experimental samples.

20 – 30 μl of the cytosol, from which 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, 3 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

Determination of conversion factor to convert CPM into concentration (fmol)

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

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

Using the above equation, a value of X CPM/mmol was obtained. It was inverted to give 1/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

Determination of DNA content by diphenylamine method

The method of Burton (1968), a modified version of Burton (1956), was used to determine DNA content in purified nuclear suspensions.

Diphenylamine reagent preparation

Diphenylamine (1.5 g) was dissolved in 100 ml of acetic acid. To this, 1.5 ml of concentrated sulfuric acid was added and then the solution was 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

Calf thymus DNA (2 mg) 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 1N perchloric acid was added and then 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 1N perchloric acid. This resulted in a standard DNA solution of concentration 200 μg/ml.

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. To each tube, 2 ml of diphenylamine reagent was added and the solutions were mixed properly by vortexing. 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 measured at a wavelength of 600 nm against a reagent blank.

Estimation of DNA in nuclear suspension

Purified nuclear suspension (50 μl) was pipetted out into the test tubes and the volume made to 1 ml with 0.5 N perchloric acid and the solution was 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 measured as mentioned above. DNA concentrations in the samples were determined from the standard plot.

BIO-DATA

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Paper published in journal

- Dutta D and Sharma R (2003) Regulation of hepatic glucocorticoid receptors in mice during dietary restriction. Horm. Metab. Res 35: 415-420
- Dutta D and Sharma R (2004) Age-dependent dietary regulation of glucocorticoid receptors in the liver of mice . Biogerontology 5: 177 - 184

Papers presented in conferences

- D Dutta and R Sharma (2001) Regulation of glucocorticoid receptors during dietary restriction in mice. 70th Annual SBC (I) meeting, Dec 27-29, Osmania University , Hyderabad.
- D Dutta and R Sharma (2002) Dietary restriction regulates hepatic glucocorticoid receptors in mice. 71st Annual SBC (I) meeting, Nov 14-16, Punjab Agricultural University, Ludhiana.
- R Sharma and D Dutta (2003) Dietary modulation of glucocorticoid receptors in the liver of mice at adult and old ages. 7th Asia / Oceanic Regional Congress of Gerontology, Nov 24-28, Tokyo, Japan.