



Research article

## Age-dependent dietary regulation of glucocorticoid receptors in the liver of mice

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### Abstract

Dietary restriction (DR) increases the resistance to different stresses, retards various age-related diseases and extends life span in a variety of animals. Here we have investigated the effect of DR (alternate days of feeding for 3 months) on glucocorticoid receptors (GRs) in the liver of adult (5 months) and old (20 months) male mice. A significant decrease was observed in the level of receptors in old mice (25%) as compared to the adult ones. DR subjected mice of both age groups showed a marked increase in the GR concentration (37% in adult and 31% in old mice) as compared to the *ad libitum* (AL) fed mice, whereas the affinity remained the same in both groups of animals at both ages. Scatchard analyses and the protein slot blot experiment confirmed the increase in the receptor level in AL and DR fed animals for both age groups. The magnitude of heat and salt activation of GR was higher in the adult mice as compared to the old mice who were fed AL. DR, however, significantly increased (40%) the magnitude of activation of GR in the older mice as compared to the AL fed animals, whereas no such change was observed in the adult animals. Further, DNase I digestion and extraction of nuclear bound GR-complexes showed a higher degree of extraction in adult animals (57%–59%) as compared to the old (31%–33%) animals. Mice subjected to DR revealed no significant change at either age. These findings indicate that DR regulates GR in an age-dependent manner and that it may allow animals to better adapt to metabolic regulation in older ages.

### Introduction

Dietary restriction (DR) without malnutrition, has been known to influence various physiological processes and is the only means to delay aging and extend the mean and maximum life spans in various groups of animals. Calorie deprivation without deficiency in essential nutrients retards the pathophysiological changes associated with aging (Weindruch and Walford 1988; Masoro 1993; Lane et al. 1996; Sohal and Weindruch 1996; Lee et al. 1999). In rodents, primates and humans, caloric restriction (CR) reduces the 24-h-fasting blood glucose and insulin concentration (Dhahbi et al. 1999). In addition, DR increases the resistance of neurons to age-related neurodegenerative disorders (Lee et al. 2000). It evokes anti-inflammatory, anti-nepotistic effects and also protects

aging rodents against diabetes, impaired tissue growth and reproductive senescence (Lynn et al. 1998). Recent studies have suggested DR as a mild stress which mobilizes cellular defenses against later exposure to more severe (age-, disease-, or injury-associated) stresses (Leaky et al. 1994).

The mechanism controlling the adaptive response to reduced caloric intake may involve the complex dynamic interplay between the hormones that control energy balance, appetite, cell proliferation and apoptosis, stress response, metabolic rate, inflammation and repair system (Leaky et al. 1994). All the above effects in one way or the other are regulated by glucocorticoid hormone (GCs). The synthesis and secretion of glucocorticoid hormone from adrenal cortical cells is under the tight regulation of corticotropin release hormone (CRH) from the hypothalamus and adrenocorti-

cotrophic hormone (ACTH) from the anterior pituitary. It is of major importance for the protection of the body against stress by regulating glucose metabolism and blood pressure. Besides the metabolic action, glucocorticoid effects have also been ascribed to behavioral and brain functions. Most important is the role of GC in the dynamic modulation of inflammatory and immune responses (Bosscher et al. 2003). At the cellular level, the most known effects of GCs are mediated by high affinity binding with ~94 kDa intracellular protein, the glucocorticoid receptor (GR). GR belongs to a phylogenetically conserved superfamily of ligand inducible transcription factors (McEwan et al. 1997). All the members of this family share a characteristic three-domain structure, an amino terminal transactivation domain, a central Zn-finger DNA binding domain and a carboxy terminal ligand-binding domain. The unliganded GR resides in the cytoplasm as a hetero-oligomeric complex, comprising of two Hsp 90, one each of Hsp 70 and Hsp 56 and a p23 acidic protein which allow the receptor to bind steroid ligands with high affinity (Biola and Pallardy 1999; Prima et al. 2000). When a hormone binds to the receptor the complex dissociates by a process called 'activation', and the activated hormone-receptor (H-R) complex translocates to the nucleus and binds to specific glucocorticoid response elements (GREs), ultimately modulating responsive gene expression (Biola and Pallardy 1999; McNally et al. 2000). Keeping in view the diverse but interrelated roles of DR and GCs and to know whether there is any age-specific change in the GRs during DR, we studied the level of GR, its activation and interaction with nuclear chromatin in the liver of both adult and old mice during DR.

## Materials and methods

### *Animals and diet*

Swiss albino (Balb/c strain) male mice of two different age groups (5- and 20-months old) maintained under normal laboratory conditions were used. They were fed with the standard pellet diet (Amrut Laboratory, Pune) and water *ad libitum* (AL) as per the experimental schedule. Mice subjected to DR were fed on alternate days for a

period of 3 months (Merry 1999). Animals were sacrificed at the end of a feeding day. Previous studies have shown that mice maintained on such an alternate day feeding schedule will consume 30% less food over a period of time and live up to 30% longer, compared to AL fed animals (Lee et al. 2000).

### *Chemicals*

[1,2,4,6,7-<sup>3</sup>H] dexamethasone, a synthetic glucocorticoid (specific activity 91 Ci/mmol) was from Amersham, England. Non-radioactive dexamethasone was obtained from Sigma Chemical Co., USA. All the other chemicals used were of analytical grade. The radioactive counting (CPM) was carried out using a Wallac 1409 liquid scintillation counter having a 68% efficiency for tritium.

### *Buffers*

We used the following buffers: (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<sub>2</sub>.

### *Receptor preparation and radio receptor assay (RRA)*

Animals were sacrificed by cervical dislocation at a fixed time of the day (13.00 h). Their livers were dissected out, washed in normal saline (0.9% NaCl) and blotted dry. A 20% (w/v) homogenate of the tissue was prepared in chilled buffer A. The homogenate was centrifuged at 40,000 × *g* for 45 min at 2 °C to obtain clear fat free cytosol used for RRA. Aliquots (100 μl) of cytosol were incubated with 40 nM of [<sup>3</sup>H] dexamethasone with or without 500-fold excess of non-radioactive dexamethasone for 4 h at 0 °C to get maximum saturable binding. For Scatchard analyses, 5–120 nM [<sup>3</sup>H] dexamethasone was used in a similar manner. Fifty microliters of DCC (4% activated charcoal + 0.4% dextran T-70 in buffer A) was added to remove any unbound steroid (Beato and Fiegelson 1972). Specific saturable binding was ob-

tained by subtracting the radioactivity (CPM) bound in the presence of unlabeled hormone from that bound in the presence of labeled dexamethasone. The number of specific binding sites (fmol/mg protein) and the dissociation constants ( $K_d$ ) were calculated according to the method of Scatchard (1949).

#### *GR slot blot analysis*

Polyclonal rabbit anti-GR Ab, raised against the amino acid (407–423) sequence (SVFSNGYSS-PGMRPDVS) from the central region of the rat-GR was a gift from Profs. N. Katunama and H. Kido, Japan. Goat-antirabbit-IgG-HRP conjugate was obtained from Bangalore Genei, India. The blotting was performed on a 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<sub>2</sub>O for activation and proper binding. After placing the NC membrane in the slot blot apparatus, the slots used were rehydrated with ddH<sub>2</sub>O for uniform binding. To each slot, 100  $\mu$ l of ( $\times 10$ ) cytosol adjusted to equal protein (50  $\mu$ g) from both AL and DR fed mice of both age groups were applied in the center and was allowed to filter through the membrane by means of a gentle vacuum. The NC membrane was then placed in a blocking solution (5% non-fat 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 rabbit anti-GR Ab solution (1:1500). After washing the membrane twice in TTBS, it was transferred to the goat-antirabbit-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<sub>2</sub>O<sub>2</sub>) 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<sub>2</sub>O. It was then photographed using a digital HP Photosmart 315 camera.

#### *Preparation of activated GR complexes*

A 20% (w/v) homogenate of the liver was prepared in buffer B. It was centrifuged at 2000  $\times g$  for 10 min at 2 °C to sediment the nuclei. The supernatant was then centrifuged at 40,000  $\times g$  for 45 min at 2 °C, and to the clear cytosol was added [<sup>3</sup>H] dexamethasone to a final concentration of 40 nM; bound H-R complexes were separated by DCC (in buffer B) treatment. Aliquots of these complexes were then subjected to heat (25 °C) and salt (20 mM Ca<sup>2+</sup>) activation for 45 min to obtain activated 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 (Sigma Chemical Co., USA) was suspended overnight in buffer B at 2 °C. From the slurry, aliquots containing 100–150  $\mu$ g DNA were transferred to microfuge tubes, and 1.0 ml of chilled buffer B was added to each tube and the cellulose was pelleted by centrifuging at 2000  $\times g$  for 10 min at 2 °C. Aliquots of the activated H-R complexes were added to the cellulose pellets and mixed well. After an hour, the reaction was stopped by adding buffer B (1.0 ml) followed by centrifugation at 2000  $\times g$  for 10 min at 2 °C (Kalimi et al. 1975). The cellulose pellets were washed twice with buffer B, and the final pellet was suspended in cocktail-W and transferred to a scintillation vial. The radioactivity bound in pellets was counted and expressed as CPM/100  $\mu$ g DNA.

#### *Nuclear binding assay*

The crude nuclear pellets obtained as above were further processed (Eberhardt et al. 1978). To the pellet was added chilled buffer C, and the contents were gently homogenized and centrifuged at 2000  $\times g$  for 10 min at 2 °C. The pellet was then washed thrice with buffer B followed by centrifugation, and the final pellet thus obtained was suspended in buffer B to give a homogeneous slurry. Aliquots of this containing 100–150  $\mu$ g of DNA were pipetted into microfuge tubes and washed in buffer B to obtain purified nuclear pel-

lets. Aliquots of the activated H–R complexes were added to it, and after an hour, the reaction was stopped by adding buffer B (1.0 ml). The pellets were subsequently processed as above, and the bound radioactivity was counted as described for the DNA–cellulose binding assay.

#### *DNase I digestion studies*

DNase I digestion studies were performed on purified nuclei (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  $\mu$ l at 2 °C for 45 min. The DNase I was dissolved in buffer D and used at a concentration of 150 units/100  $\mu$ g DNA. The control tubes received 100  $\mu$ l of buffer only (Ranhotra and Sharma 2001). The nuclear pellets were properly mixed, and the reaction was stopped by adding 1.0 ml buffer B followed by centrifugation at 2000  $\times$  *g* for 10 min at 2 °C. The pellets were processed, and the bound radioactivity determined as described above. The results were expressed as % [<sup>3</sup>H] dexamethasone–receptor complexes bound to nuclei. Controls were taken as 100% bound.

#### *Protein and DNA estimation*

The protein content of the receptor preparation was measured according to the dye-binding method of Bradford (1976), using bovine serum albumin (BSA) as standard. The concentration of DNA in the purified nuclear suspension was determined by the method of Burton (1968). Data obtained from different sets of experiments were analyzed statistically. The level of significance

(*P*-value) between two sets of data was calculated according to Student's *t*-test.

## **Results**

#### *Body weight*

Both adult and old animals maintained on DR for 3 months exhibited a decrease (–34%; *P* < 0.001 in adult and –23%; *P* < 0.001 in old mice) in the body weight as compared to the AL fed ones.

#### *Changes in the GR level*

Our study of receptor levels indicates a decrease (25%) of receptors in the liver of old mice compared to the adult ones in AL fed mice, whereas the dietary restricted animals of both the age groups showed a marked increase in the receptor concentration (37% in adult and 31% in the old animals) as compared to the AL fed ones (Table 1). However, the affinity of the GR for its ligand remained unchanged in both AL and DR fed animals from both the age groups. Scatchard analyses (plots not shown) and the slot blot analyses of receptor preparation confirm the increased level of GR in the liver of DR mice as compared to the AL fed ones in both the age groups (Figure 1).

#### *Activation studies of the GR*

Temperature (25 °C for 45 min) and salt (20 mM Ca<sup>2+</sup> at 0 °C for 45 min) dependent activation of the GR were studied in both AL and DR fed animals at both adult and old ages using DNA–cellulose and nuclear–binding assays. Results indicate a lower (15%–20%) activation of H–R complexes in older mice compared to the AL fed

Table 1. Concentration (fmol/mg protein) and affinity (nM) of [<sup>3</sup>H] dexamethasone–receptor in the liver of adult (5 months) and old (20 months) DR and AL fed male mice.

Age (months)	AL		DR	
	<i>B</i> <sub>max</sub>	<i>K</i> <sub>d</sub>	<i>B</i> <sub>max</sub>	<i>K</i> <sub>d</sub>
5	122.05 ± 1.21	2.61 ± 0.07	167.21 ± 5.97 <sup>a</sup>	2.65 ± 0.14
20	91.18 ± 4.34	2.62 ± 0.02	119.93 ± 5.03 <sup>a</sup>	2.55 ± 0.08

<sup>a</sup> Statistically significant with respect to AL fed mice.

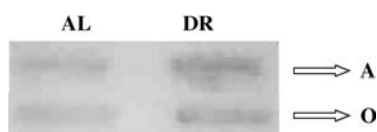


Figure 1. Slot blot analysis of liver GR from adult (A) and old (O) AL and DR fed mice. The details of experimental conditions are described in the 'Materials and methods' section. An equal amount of liver cytosol containing GR from AL and DR fed mice was applied onto each slot and processed for immunoblotting 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.

adult mice. Although, there was no change in the magnitude of activation during AL and DR in adult animals, it was significantly higher (30%–40%) in the old animals subjected to DR as compared to the AL fed ones (Figures 2 and 3).

#### DNase I extraction of GR

DNase I extraction of bound hepatic GR revealed a significantly higher extraction in the adult mice (57%–59%) as compared to the old (32%–33%) mice (Figure 4). However, no significant change was observed in DR subjected mice as compared to the AL fed ones.

## Discussion

GCs play an essential role in maintaining basal and stress-related homeostasis, and the lack of GC's action is incompatible with life. As GR is expressed in a vast majority of tissues, it is reasonable to assume that GCs affect almost all tissues in the body. Depending upon the tissue, its effect could either be anabolic or catabolic. The magnitude of a tissue's response to glucocorticoid depends on both the level of hormone and its receptor sensitivity (Bamberger et al. 1996). Several metabolic adjustments during DR could be achieved of which many of them are controlled by glucocorticoid and its action mechanism. Most glucocorticoid actions are mediated by its intracellular receptors and their interaction with specific DNA sequences termed GREs (Barnes 1998; Adcock and Ito 2000).

We have studied the long-term effect of DR on the endogenous level, activation properties and nuclear binding of the GR in the liver of two different age groups of AL and DR fed mice. In our experimental schedule, it has been observed that DR for 3 months significantly reduces the body weight of both adult and old mice. However, the

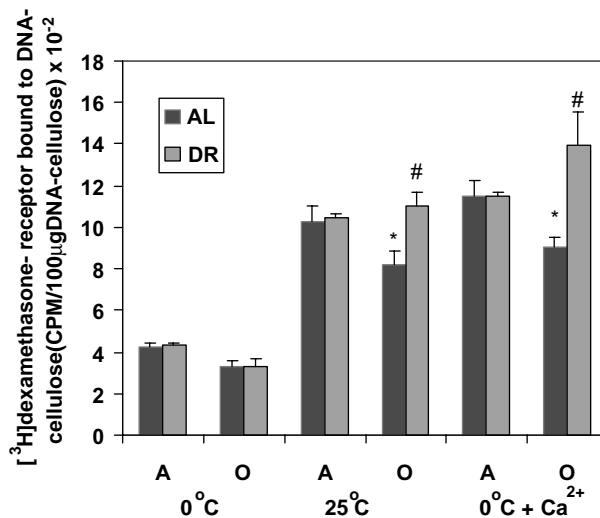


Figure 2. Specific binding of liver [<sup>3</sup>H] Dexamethasone–receptor complexes to DNA–cellulose from the AL and DR fed mice of both adult (A) and old (O) ages. Liver cytosols were prepared in buffer B and the H–R complexes obtained by incubating with 40 nM [<sup>3</sup>H] dexamethasone for 4 h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

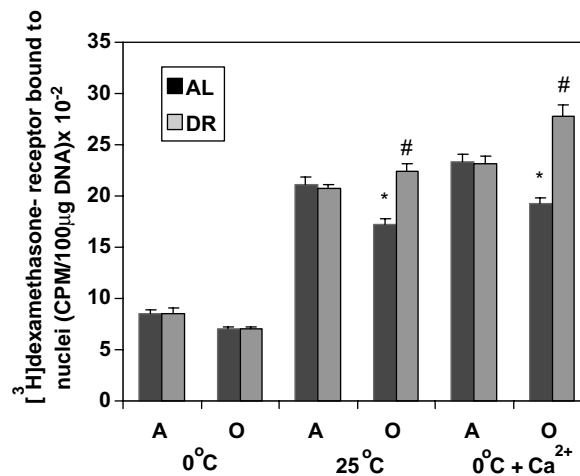


Figure 3. Specific binding of liver [<sup>3</sup>H] Dexamethasone-receptor complexes to purified nuclei from the AL and DR fed mice of both (A, adult; O, old) the ages. The H-R complex preparations and activation conditions are same as given in Figure 2. Activated H-R complexes were incubated with purified nuclei instead of DNA-cellulose as mentioned in 'Materials and methods'. The results are mean  $\pm$  standard deviation for four separate experiments 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.

decrease was more pronounced in adult animals as compared to older ones. Our observation of body weight reduction confirms that the animals have a reduced food intake and is consistent with an earlier report (Lee et al. 2000).

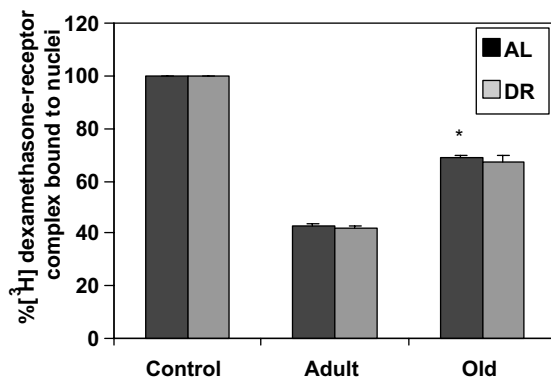


Figure 4. DNase I extractability of bound [<sup>3</sup>H] dexamethasone-receptor complexes from the liver nuclei of adult (A) and old (O) AL and DR fed mice. Heat-activated, nuclear-bound H-R complexes were extracted using DNase I as per experimental protocols given in the 'Materials and methods' section. The results are mean  $\pm$  standard deviation of four separate experiments performed for each group. \* Statistically significant compared to the adult mice.

Our data on GR level (Scatchard and slot blot analyses) suggest a decrease in the receptor level (25%) in the liver of old mice, without any alteration in the receptor affinity. The high level of receptors in 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). The decrease in the GR level of old mice may impair metabolic functions, which may be one of the reasons for the 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 (Djordjovic-Markovic et al. 1999), whereas there were no such alterations (Kalimi and Banarjee 1981) in rat adipocytes. Our studies indicate a significant increase in the receptor level during DR in both age groups as compared to the AL fed animals. Although the increase was almost similar in magnitude at both the ages, such an increase may help the animal in controlling the GC mediated responses of metabolic needs during DR. Rodents subjected to DR 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, it might play an important role by improving the

metabolic activities and helps the animals to adapt better to the environment. Maternal under-nutrition during early to mid-gestation has been attributed to an increase in the expression of the GR mRNA in the liver of neonatal sheep (Whorwood et al. 2000). DR has also been reported to selectively decrease the expression of GR in the hippocampus and cerebral cortex of rats (Lee et al. 2000).

Activation studies of GR were carried out from the liver of both adult and old mice during DR to detect any change in the magnitude of temperature (25 °C for 45 min) and salt (Ca<sup>2+</sup> at 0 °C for 45 min) dependent activation. Results show a significant decrease in the magnitude of activation of hepatic GR in old (15%–20%) animals compared to the adult ones in the AL fed mice. 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 GRs (Bodine and Litwack 1988). Surprisingly, we have also observed a greater activation (30%–40%) in old mice during DR as compared to the AL fed ones, whereas the adult group showed no such changes. This increase in the receptor activation in aged animals subjected to DR may help them to achieve better glucocorticoid action with a 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 of adult and old mice during DR in order to reveal differences 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 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 animals (Chaurasia and Thakur 1997). DNase I cuts the DNA where it is maximally exposed and thus depends on the degree of chromatin condensation. Our data showed a higher degree of extraction in the adult liver (57%–59%) as compared to that of the old mice (32%–33%), whereas there was no marked effect of DR on such extractability at either age. This relates to the fact that there may not be an appreciable change in the organization of chromatin in DR subjected ani-

mals as compared to the AL fed ones. Our finding of decreased extractability in aged animals corroborate with the observations of others (Chaturvedi and Kanungo 1983; Chaurasia and Thakur 1997) who also reported reduced digestibility of chromatin by DNase I of old rat brain compared to the young and adult mice. Taken together from these studies, it may be concluded that long-term DR results in an increase of GR in the liver of both adult and old mice. Hence, DR could be used to elevate the GR level in older animals whose receptor level gets already reduced during that period of life span. Further, 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.

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