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### Abstract

The specific binding of [<sup>3</sup>H] dexamethasone to its receptor, activation of the hormone-receptor complexes and DNase I digestion of nuclear bound hormone-receptor complexes were studied in the liver of mice during dietary restriction (alternate days of feeding for 3 months) compared to animals fed *ad libitum*. Results indicate an increase of receptor level (fmol/mg protein) in the diet-restricted (DR) animals as compared to those fed *ad libitum* (AL). Scatchard analyses confirm the increase in the level of receptors in DR animals, while the affinity (Kd) remained same

in both groups of mice. Protein slot-blot analysis also depicts the increase of the receptor level in DR fed compared to the AL fed animals. The extent of temperature- and salt-dependent activation of receptors showed no marked difference in AL- and DR-fed mice. DNase I extraction of bound hormone-receptor complexes from nuclei revealed similar pattern of digestion in both groups of animals.

### Key words

Hepatic glucocorticoid receptor · Dietary restriction · Mice

### Introduction

Glucocorticoids (GCs) are synthesized and secreted from the adrenal cortex and are responsible for the control of many metabolic processes. They are the key regulators of homeostasis and adaptation in animals, and have several effects on various animal tissues including liver, where they enhance the expansion of hepatic enzymes and increase protein and glycogen content [1,2]. GCs play an important role in energy metabolism regulation, immune response, inflammatory reaction, development and aging processes [3–5]. At a cellular level, GCs exert their actions by modulating gene expression through a cascade of regulatory events initiated by high-affinity interaction with a ~94 kDa intracellular protein – the glucocorticoid receptor (GR), belonging to a phylogenetically conserved super family of ligand-inducible transcription factors [6]. All members of this family bear essential structural and functional features – an amino terminal transactivation domain, a central zinc finger DNA-binding domain, and a carboxy terminal ligand-binding domain [7–9]. Unligand-

ed GR resides predominantly in the cytoplasm as part of a heterocomplex comprising at least three different heat shock proteins, hsp 90, 70 and 56 [10]. These proteins play key chaperone roles in the GR ability to bind steroid, protein conformation and nuclear transport [10–12]. Hormone binding induces a conformational change leading to the dissociation of all the receptor associated proteins leading to its activation. The activated GR translocates to the nucleus and binds to specific glucocorticoid response elements (GREs) [10,13]. Depending on the promoter structure of the gene, hormone-receptor complex lead to either increased or decreased gene transcription [10,14].

Dietary restriction (DR), a reduction in calorie intake without malnutrition, plays an important role in immunological, general protein and amino acid metabolism as well as on neuroendocrinological systems [15–18]. Glucocorticoid hormones also partly regulate all such effects. It is also known that the level of this hormone is elevated during dietary restriction in animals [19]. It has been observed that dietary interventions selectively regulate GR

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expression in different groups of animals; DR reduces the level of GR in the hippocampus and cerebral cortex of rat brain [20]. Maternal undernutrition during early to mid-gestation has been reported to increase the expression of GR in various tissues of neonatal sheep [21]. DR has been reported to potentiate immune system, delay immunosenescence, reduce pathology of diseases and extends life in rodents and primates [19,22–26]. Keeping in view these diverse roles of DR, we have studied the effect of DR on the hepatic GR level, its activation and interaction with chromatin, which will provide insight into the action mechanisms of glucocorticoids during such interventions.

## Materials and Methods

### Animals and diet

Swiss albino (Balb/c strain) male mice aged 8–10 weeks maintained under normal laboratory conditions were used. They were fed with standard pellet diet (Amrut Laboratory, Pune) and water *ad libitum* as per experimental schedule. DR mice were fed on alternate days for a period of three months [27]. Animals were sacrificed at the end of the feeding day. Previous studies have shown that mice maintained on such an alternate day feeding schedule will consume 30% less food over time and live up to 30% longer compared to animals fed *ad libitum* [20].

### Chemicals

[1, 2, 4, 6, 7-<sup>3</sup>H] dexamethasone, a synthetic glucocorticoid (specific activity 91 Ci/mmol) was from Amersham, UK. 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 68% efficiency for tritium.

### Buffers

These were as follows: (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>.

### Glucocorticoid receptor (GR) preparation and radio receptor assay (RRA)

Animals were sacrificed by cervical dislocation at a fixed time of day (1 p.m.). Their liver was 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 40000 × g for 45 min at 2 °C to obtain a clear fat free cytosol used for RRA. Aliquots (100 μl) of cytosol were incubated with 40 nM of [<sup>3</sup>H] dexamethasone alone or with a 500-fold excess of non-radioactive dexamethasone for 4 h at 0 °C to get maximum saturable binding. 5–120 nM [<sup>3</sup>H] dexamethasone was used in a similar manner for Scatchard analysis. 50 μl of DCC (4% activated charcoal + 0.4% dextran T-70 in buffer A) [28] was added to remove any unbound steroid. Specific saturable binding was obtained by subtracting the radioactivity (CPM) bound in presence of unlabeled hormone from that bound in presence of labeled dexamethasone. The number of specific binding sites

and the dissociation constants (Kd) were calculated according to the Scatchard method [29].

### GR slot blotting

Polyclonal anti-GR-ab raised against amino acid sequence (SVF SNG YSS PGM RPD VS) from the N-terminal region of the rat-GR was a gift from Profs. N. Katunuma and H. Kido, Japan. Goat anti-rabbit IgG-HRP conjugate was obtained from Bangalore Genei, India. 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. 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 μl of (×10) cytosol adjusted to equal protein (50 μg) from both AL and DR was applied in the center, and was allowed to filter through the membrane by gentle vacuum. The nitrocellulose membrane was then placed in blocking solution (5% non-fatty milk in TBS) for an hour. It was then washed twice in TTBS (20 mM Tris-HCl, pH 7.5/500 mM NaCl/0.05% Tween-20) with gentle agitation and kept for overnight incubation with the anti-GR-ab solution (1 : 1500). The membrane was again washed twice in TTBS to remove unbound abs. After this, the membrane was transferred to goat anti-rabbit IgG-HRP (1 : 3000) conjugate solution and kept for 3 hours. 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) over the membrane and after the development of color, the reaction was stopped by washing the membrane in ddH<sub>2</sub>O and photographed using a digital HP Photosmart 315 camera.

### Preparation of activated glucocorticoid receptor complexes

A 20% (w/v) homogenate of liver was prepared in buffer B. It was centrifuged at 2000 × g for 10 min at 2 °C to sediment the nuclei. The supernatant was centrifuged at 40000 × g for 45 min at 2 °C and the clear cytosol was incubated for 4 h at 0 °C with 40 nM [<sup>3</sup>H] dexamethasone alone or with a 500-fold excess of non-radioactive dexamethasone; bound hormone-receptor (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> at 0 °C) activation for 45 min to obtain activated complexes [30]. Aliquots of the complexes were also kept at 0 °C for 45 min to provide the inactivated receptor complexes as controls.

### DNA-cellulose binding assay

Commercially available DNA-cellulose was suspended overnight in buffer B at 2 °C. From the slurry, aliquots containing 100–150 μg DNA was 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 × 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 × g for 10 min at 2 °C [31]. The cellulose pellets were washed twice with buffer B and the final pellet was suspended in cocktail-W and transferred to scintillation vial. The radioactivity bound was counted and expressed as CPM/100 μg DNA.

## Nuclear binding assay

The crude nuclear pellets obtained as above were further processed [32]. Chilled buffer C was added to the pellet, and the contents were gently homogenized and centrifuged at  $2000 \times g$  for 10 min at  $2^\circ\text{C}$ . 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 which containing 100–150  $\mu\text{g}$  of DNA were pipetted in micro-fuge tubes and washed in buffer B to obtain purified nuclear pellets. Aliquots of the activated H-R complexes were added to it and the reaction was stopped by adding buffer B (1.0 ml) after an hour, the pellets were subsequently processed as above, and the bound radioactivity was counted as described for DNA-cellulose binding assay.

## DNase I digestion studies

DNase I digestion studies were performed on purified nuclei [33]. Heat-activated hormone-receptor complexes were allowed to interact with their respective purified nuclei as described for nuclear binding assay. After washing off the unbound complexes, the pellets were incubated with 100  $\mu\text{l}$  of DNase I (prepared in buffer D) at a concentration of 150 units/100  $\mu\text{g}$  DNA. The control tubes received 100  $\mu\text{l}$  of buffer only [34]. The digestion was stopped by the addition of 1.0 ml buffer B, followed by centrifugation at  $2000 \times g$  for 10 min at  $2^\circ\text{C}$ . The pellets were washed twice with the same buffer and after suspending in cocktail, the bound radioactivity was counted. The results were expressed as % [ $^3\text{H}$ ] dexamethasone-receptor complexes bound to nuclei. Controls were taken as 100% bound.

## Protein and DNA estimation

Protein content of the receptor preparation was measured according to the dye-binding method of Bradford [35] using bovine serum albumin (BSA) as standard. The concentration of DNA in purified nuclear suspension was determined by the Burton method [36]. 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

### Changes in glucocorticoid receptor level

The initial observations on the body weight of mice during alternate days of feeding for 3 months exhibited a significant decrease ( $-34\%$ ;  $p < 0.001$ ) in weight of DR mice ( $19.9 \pm 1.1$  g;  $n = 8$ ) as compared to AL-fed mice ( $30.4 \pm 1.2$  g;  $n = 8$ ). Our findings indicate that the level of glucocorticoid receptor (GR) is increased significantly in the liver of dietary restricted (DR) animals as compared to the *ad libitum* (AL)-fed mice (Table 1). Scatchard analyses (plots not shown) confirm a higher (41%) concentration of receptor binding sites (173.0 fmol/mg protein) in the liver of DR animals as compared to that of AL-fed mice (122.2 fmol/mg protein). On the other hand, the affinity (Kd) of GR for hormone remained the same (2.3 nM) in both groups of animals. The slot blot analyses of receptor preparation corroborate the increased level of GR in the liver of DR mice compared to AL-fed mice (Fig. 1).

Table 1 Specific binding sites ( $B_{\text{max}}$ ) and dissociation constant ( $K_d$ ) of glucocorticoid receptors in the liver of dietary restricted (DR) and *ad libitum* (AL) fed mice<sup>a</sup>

Conditions	$B_{\text{max}}$ (fmol/ mg protein)	$K_d$ (nM)
AL	$122.2 \pm 7.3$	$2.3 \pm 0.3$
DR	$173.0 \pm 5.7^*$	$2.3 \pm 0.2$

<sup>a</sup> The data were obtained from 5–6 mice for each group and analyzed using Scatchard plot as mentioned in Materials and Methods. The results are expressed as the mean  $\pm$  standard deviation of four separate experiments for each group. \* Statistically significant ( $p < 0.001$ ) with respect to AL fed mice.

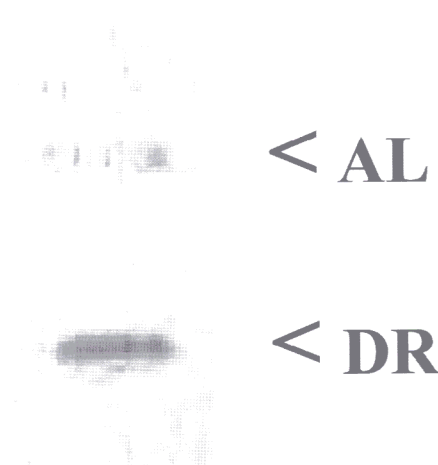


Fig. 1 Slot blot analysis of liver glucocorticoid receptor from *ad libitum* (AL) and diet-restricted (DR) mice.

The details of experimental conditions are described in "Materials and Methods" section. Equal amount of liver 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 animals.

### Activation studies on glucocorticoid receptors

The activation process of glucocorticoid receptors (GR) complexes was studied in both AL and DR fed animals using DNA-cellulose and purified nuclear binding assays. Results show that both temperature ( $25^\circ\text{C}$  for 45 min) and salt (20 mM  $\text{Ca}^{2+}$  at  $0^\circ\text{C}$  for 45 min) significantly enhance (2–2.5 fold) the GR activation as judged by their DNA-cellulose (Fig. 2) and purified nuclear (Fig. 3) binding assays. However, there was no significant change in the magnitude of activation (either by heat or salt) of GR in either group. The results indicate no difference in the *in vitro* activation of hormone-receptor complexes from the liver of AL and DR mice under the conditions mentioned above.

### DNase I extraction of bound GR from nuclei

The sensitivity of liver nuclear chromatin digestion by DNase I was compared in both AL- and DR-fed animals. This was performed to ascertain the chromatin organization and its possible role in nuclear binding of activated glucocorticoid receptor complexes during dietary restriction. The data show similar magnitude (60–61.5%) of extraction of GR bound to hepatic nuclei upon DNase I digestion in both the groups of animals (Fig. 4).

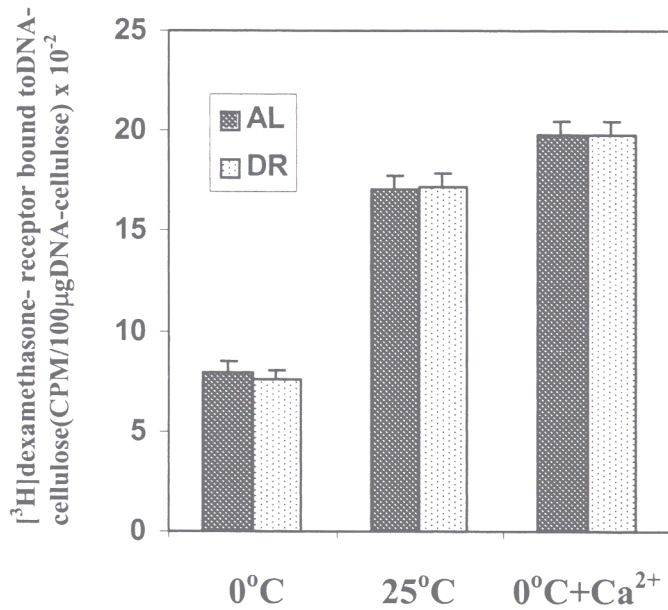


Fig. 2 Specific binding of liver [<sup>3</sup>H] dexamethasone receptor complexes to DNA-cellulose from the AL and DR mice. Liver cytosols were prepared in buffer B and the hormone-receptor complexes obtained by incubating with 40 nM [<sup>3</sup>H] dexamethasone for 4 h at 0 °C. The hormone-receptor complexes were then subjected to heat (25 °C) and Ca<sup>2+</sup> (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 the mean ± standard deviation of four separate experiments with 5–6 mice of each group.

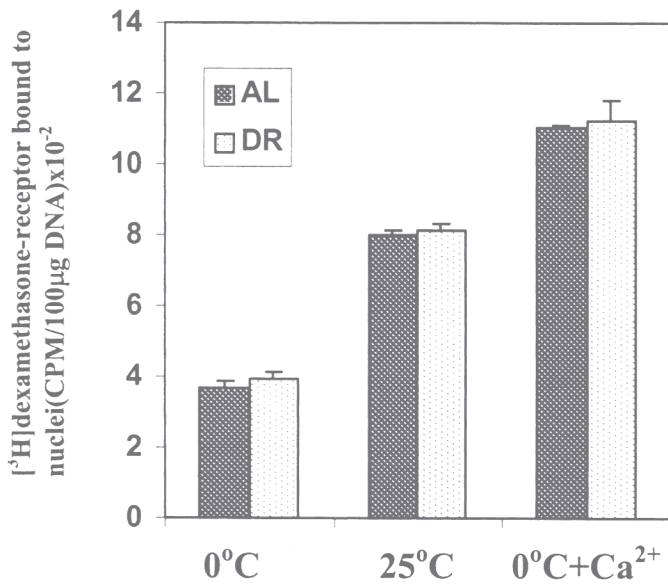


Fig. 3 Specific binding of liver [<sup>3</sup>H] dexamethasone receptor complexes to purified nuclei from the AL and DR mice. The hormone-receptor complex preparation and activation conditions are same as given in Fig. 2. Activated hormone-receptor complexes were incubated with purified nuclei instead of DNA-cellulose as mentioned in Materials and Methods. The results are the mean ± standard deviation for four separate experiments with 5–6 mice of each group.

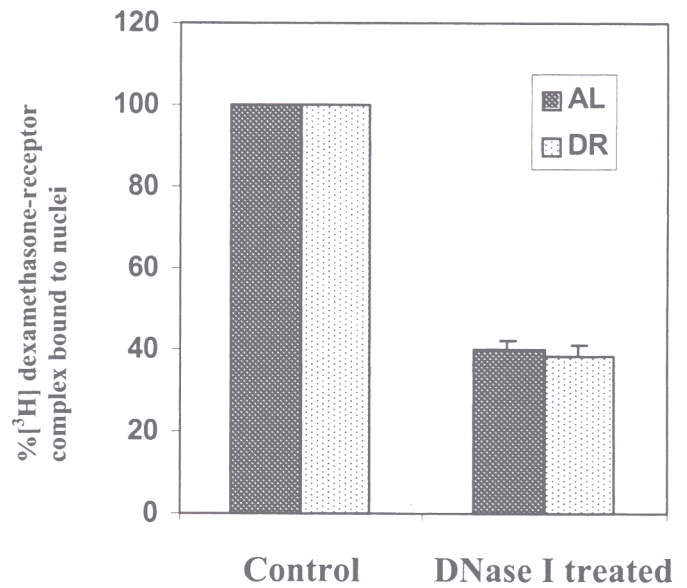


Fig. 4 DNase I extractability of bound [<sup>3</sup>H] dexamethasone-receptor complexes from the liver nuclei of AL and DR mice. Heat-activated, nuclear-bound hormone-receptor complexes were extracted using DNase I as per experimental protocols given in the Materials and Methods section. The results are mean ± standard deviation of four separate experiments performed for each group.

## Discussion

Glucocorticoids (GCs) have been known to influence multitude of processes within the body. The major effects are to reduce inflammation, stabilize blood glucose level, maintain muscle strength and promote fluid excretion [7,13]. Lack of GC action is incompatible with life in higher animals as it plays essential role in maintaining basal and stress related homeostasis. The tissue response to glucocorticoid is either anabolic or catabolic. It has anabolic effects on protein and RNA metabolism in liver and catabolic effects on other tissues like muscles, adipose tissues, skin and bone [1]. The action of GCs primarily depends on the level of its receptors and on the steps following hormone binding to receptor (post-receptor events). In the present study, we report the effect of long-term dietary restriction on the level and also on the activation and nuclear binding of activated glucocorticoid receptors in the liver of mice.

Our findings suggest the increase of the glucocorticoid receptor level (as judged by Scatchard analyses as well as slot blots) in the liver of DR fed mice as compared to *ad libitum* (AL)-fed mice. The increase in the level of GR may be a contributory factor in controlling the glucocorticoid-mediated response of metabolic need during long-term dietary restriction in DR mice. There are reports that DR rodents are more resistant to a variety of stresses such as trauma, heat shock and drug toxicity [37]. The increased resistance in DR rodents may be a result of the GCs' involvement in cellular protection, possibly by modulating GR functions. The increased GCs and their receptors may also help maintain the anabolic role of this hormone in metabolic demand of liver during dietary restriction. It has 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 [38]. We have also observed that the activity of tryptophan oxygenase, a glucocorticoid-inducible enzyme, is increased during DR in mice (data not shown). The upregulation of glucocorticoid receptors in the liver of mice might have functional role in inducing such enzymes for better metabolic regulation during dietary restriction. 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 [21]. DR has also been reported to selectively decrease the expression of GR in the hippocampus and cerebral cortex of rats [20].

Our findings also fail to indicate any significant difference in the magnitude of activation of liver glucocorticoid receptor in both DR and AL-fed mice. It reflects that receptor activation may not be altered during such dietary interventions. In order to study the liver 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 supercoiling of DNA around the histone core confers the specificity of digestion of DNA in chromatin by pancreatic DNase I [33]. It cuts chromatin DNA at 10-bp intervals and has been used to identify chromatin organization. Our data on digestion of liver chromatin in both AL and DR fed mice revealed no change in the extent of digestion of nuclear bound GR complexes. This shows that there may not be an appreciable change in the organization of chromatin in DR-fed mice as compared to the AL mice. In conclusion, these findings clearly entail that the long-term dietary restriction leads to a cumulative increase in the level of GR in the liver of mice. This is a significant report of its kind that the DR can be seen to regulate the level of GR in mice to adapt to metabolic need depending on the calorie intake.

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