Age-Dependent Decrease in Renal Glucocorticoid Receptor Function Is Reversed by Dietary Restriction in Mice

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ABSTRACT: The effects of age and dietary restriction (alternate days of feeding for 3 months) on the concentration, activation, and DNase I digestion of nuclear-bound glucocorticoid receptors (GRs) in the kidney of male mice at two different ages (5 months as adult and 20 months as old) were investigated. A significant decrease (30%) in the concentration of renal GRs was observed in older *ad libitum* (AL)-fed mice as compared to the adult mice. Dietary restriction (DR) of older mice significantly increased (28%) the level of GRs as compared to the AL-fed control animals. The affinity of the receptor for the hormone remained the same for both AL- and DR-fed animals at both ages. Scatchard and slot blot analyses of the data confirmed the decreased level of renal GRs in older mice compared to the adult mice as well as an increased level of receptor in older DR mice. Activation studies of GRs by both salt and heat indicated a decreased (15–20%) activation of renal GRs in older animals compared to the adult mice in the AL-fed group. It was further observed that DR significantly enhanced (30%) the degree of both salt- and heat-dependent activation of GRs in older animals compared to the AL-fed animals of the age-matched group. DNase I digestion and extraction of nuclear-bound GR complexes showed a lower degree (26%) of extraction in older AL-fed animals compared to the adult animals. However, DR did not alter the pattern of digestibility of bound GR complexes. These above findings indicate that DR could reverse the decrease of GR function in older animals and may provide better adaptability of kidney in water and electrolyte balance.

KEYWORDS: renal glucocorticoid receptor; mice; aging; dietary restriction

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INTRODUCTION

One of the serious consequences of aging is the loss of the organism's ability to cope with a variety of stresses due to a failure of cellular homeostasis.[1] Glucocorticoids (GCs) play a pivotal role in regulating basal and stress-related homeostasis. These hormones influence a number of biological processes in different tissues, including the kidney, where they affect glomerular filtration rate, ion transport, and electrolyte balance.[2–4] Most of the cellular effects of glucocorticoids are mediated by binding to a 94-kDa intracellular protein, the glucocorticoid receptor (GR), predominantly localized in the cytoplasm. The GR belongs to a superfamily of ligand-activated transcription factors.[5] The domain structure of the GR consists of an amino terminal transactivation domain, a central Zn-finger domain, and a carboxy terminal ligand-binding domain. Glucocorticoids, upon binding to the GR, translocate into the nucleus through a process called activation and interact with specific DNA sequences known as glucocorticoid response elements (GREs), resulting in increased or decreased gene expression.[6,7]

Dietary restriction (DR) commonly refers to a lowering of calorie intake without a reduction in the micronutrients essential for normal growth and development.[8,9] After McCay's initial studies[10] on DR, various laboratories have shown that DR can significantly extend life span in different groups of animals.[11,12] Emerging data on primate studies suggest a similar effect of DR on nonhuman primates.[13] DR delays a variety of diseases such as renal disorders, neoplasias, autoimmune diseases, and diabetes in different experimental animals.[14] It retards various pathophysiological changes associated with aging,[12,15–17] reduces age-associated neurodegenerative disorders in rodents,[18,19] and is also known to have protective effects against several types of cancer through the inhibition of cell proliferation and the induction of apoptosis.[20,21] Currently, DR is the only experimental method to retard aging in laboratory animals, exhibiting an increase of both mean and maximum life span.[22] Some of the effects of DR, such as protection against insulin-dependent diabetes in rodents, impaired tissue growth and regeneration, neurological impairments, and reproductive senescence are parallel to the consequences of elevated levels of glucocorticoids. The anti-inflammatory and antineoplastic effects of DR are also consistent with the same effects of an elevated level of glucocorticoids.[23] The exact mechanism of glucocorticoid-dependent mediation of the above processes is not fully elucidated. However, the action of GCs depends on the level of their receptors and also on post-receptor events. Keeping in view that these studies may provide basic knowledge of the mechanism of action of GCs during late onset of DR, we have studied the effect of such a restriction on the GR level, its activation, and DNase I digestion of nuclear-bound GR in the kidney of old male mice. There has been a fruitful debate on the age of onset of DR in experimental animals[24] vis-à-vis its feasibility for human practices. Herein, we have shown that the decrease in renal glucocorticoid receptor function is reversed by DR even when performed in older animals.

MATERIALS AND METHODS

Animals and Diets

Swiss albino (Balb/c strain) male mice of two different age groups (5- and 20-months old), maintained under normal laboratory conditions, were used for experimentation. The animals were fed with a standard pellet diet (Amrut Laboratory, Pune, India) and water *ad libitum*. Old mice subjected to DR were fed on alternate days for a period of 3 months.[25,26] However, they had free access to water on all days.

Chemicals and Buffers

[1, 2, 4, 6, 7-^{3}H] dexamethasone, a synthetic glucocorticoid (specific activity 91 Ci/mmol), was purchased from Amersham, UK. Nonradioactive dexamethasone was from Sigma Chemical Co., USA. All the other chemicals used were of highest analytical grade. The radioactive counting (cpm) was carried out using a Wallac 1409 liquid scintillation counter having 68% efficiency for tritium. The buffers used 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; and (D) 0.25 M sucrose/10 mM Tris–HCl, pH 7.6/4.2 mM $MgCl_2$.

Cytosol Preparation and Glucocorticoid Receptor Assay

Glucocorticoid receptors were assayed in the kidney as described elsewhere.[3,27–30] The number of specific binding sites (fmol/mg protein) and the dissociation constants (K_d) were calculated according to the method of Scatchard.[31]

GR Slot Blot Analysis

Polyclonal anti-GR-AB, raised against amino acid sequence (SVFSNGYSSPGMRPDVS) from the N-terminal region of the rat GR was a gift from Profs. N. Katunama and H. Kido, Japan. The experiment was performed on Bio-Rad Bio-Dot® SF Micro filtration apparatus following the instructions given in the user's manual and as done previously.[29,32]

Preparation of Activated GR Complexes

A 20% (w/v) homogenate of kidney was prepared in buffer B. It was centrifuged at 2,000 × *g* for 10 min at 2°C to sediment the nuclei. The supernatant was then centrifuged at 40,000 × *g* for 45 min at 2°C and to the clear cytosol [^{3}H]dexamethasone was added to a final concentration of 40 nM; bound hormone–receptor (H-R) complexes were separated by DCC (in buffer B) treatment. Aliquots of these complexes were then subjected to salt (20 mM Ca^{2+}) and heat (25°C) activation for 45 min to obtain activated complexes.[28,29,33] Aliquots of the cytosols were also kept at 0°C for 45 min to provide the unactivated receptor complexes as controls.

DNA Cellulose and Nuclear Binding Assays

The magnitude of activation of renal GR was studied using DNA cellulose and nuclear binding assays as described earlier.[3,28,29,34,35] The radioactivity bound in pellets was expressed as CPM/100 μg DNA.

DNase I Digestion Studies

DNase I digestion studies were performed on purified nuclei obtained from the kidney of AL- and DR-fed mice of both the age groups as detailed earlier.[28,29,36] The results were expressed as percentages of [^{3}H]dexamethasone-receptor complexes bound to nuclei. Controls were taken as 100% bound.

Protein and DNA Estimations

The protein content of the receptor preparation was measured according to the dye-binding method of Bradford,[37] using bovine serum albumin (BSA) as standard. The concentration of DNA in a purified nuclear suspension was determined by the method of Burton.[38] 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

A significant decrease in the body weight (23%; $P < 0.001$) was observed in old dietary-restricted mice compared to the AL-fed mice (data not shown), signifying the impact of DR on those animals.

Studies on the level of GR revealed a decreased (30%) receptor level in the kidney of older animals as compared to the adult animals, whereas, the dietary-restricted old animals showed a significant increase (28%) in the receptor con-

TABLE 1. Concentration and affinity of [^{3}H]dexamethasone–receptor in the kidney of adult, old, and old dietary-restricted male mice

Animals	Concentration (fmol/mg protein)	Affinity (nM)
Adult	111.2 ± 6.91	2.41 ± 0.03
Old	77.37 ± 6.27*	2.36 ± 0.11
Old-DR	99.50 ± 7.08**	2.46 ± 0.08

*Statistically significant ($P < 0.001$) with respect to adult mice.
**Statistically significant ($P < 0.001$) with respect to old mice.

of the data confirmed both the age-specific decrease of receptors in AL-fed mice, as well as the increase of receptors in DR animals compared to the AL-fed animals. Slopes of the plot did not exhibit any alteration in the affinity of the GR for its ligand in the two different age groups in the kidneys of DR-fed mice (FIG. 1). Slot blot analyses of the receptor preparation in both the age groups also confirmed the decreased level of GR in old mice, as well as an increased level of GR protein in old DR animals compared to the AL-fed animals (FIG. 2).

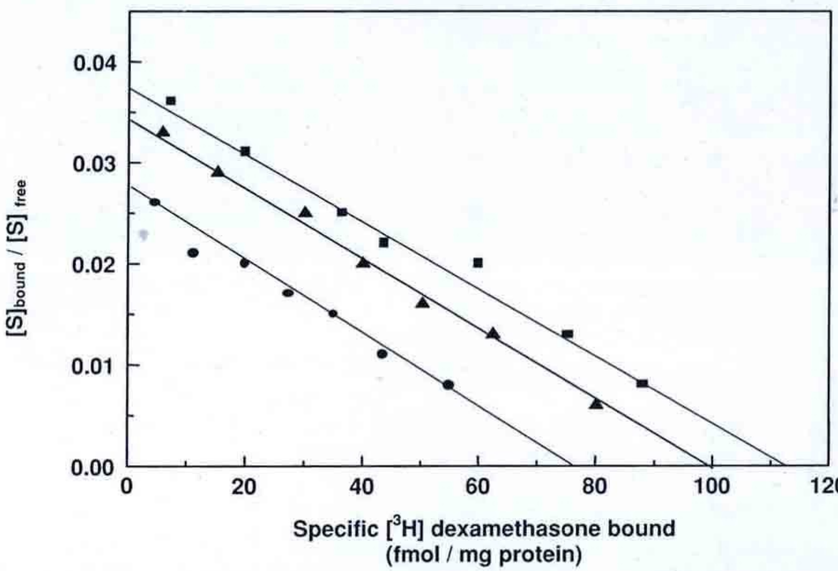

FIGURE 1. Scatchard plot of [^{3}H]dexamethasone binding in the kidney of adult (■), old (●), and old dietary-restricted (▲) male mice. Cytosols were incubated with 5–120 nM [^{3}H]dexamethasone ± 500-fold excess cold dexamethasone for 4 h at 0°C. Specific binding at each concentration was calculated by subtracting nonspecific binding from total binding, and the data obtained were analyzed by the Scatchard method. The slope of the curve gave the dissociation constant (K_d), and the intercept on the x-axis gave the maximum receptor binding sites. Each point is the mean of four separate experiments with 5–6 mice of each

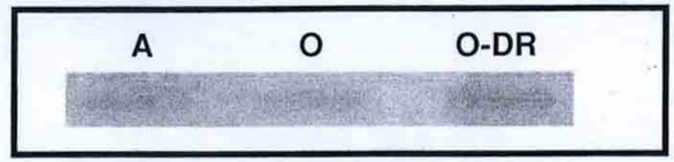

FIGURE 2. Slot blot analysis of kidney glucocorticoid receptor from adult (A), old (O), and old dietary-restricted (O-DR) male mice. Equal amount of kidney cytosol containing GR from each group of mice was applied onto each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-HRP conjugate.

Salt- (20 mM Ca^{2+} at 0°C for 45 min) and temperature- (25°C for 45 min) dependent activation of the GR was studied in the kidney of both adult and old mice using DNA cellulose and purified nuclear binding assays. Results indicated a lower activation of receptors (15–20%) in older animals compared to the adult animals. A higher level of activation by both salt and heat in older DR animals was observed compared to the AL-fed animals as judged by both the DNA cellulose (FIG. 3) and nuclear (FIG. 4) binding assays.

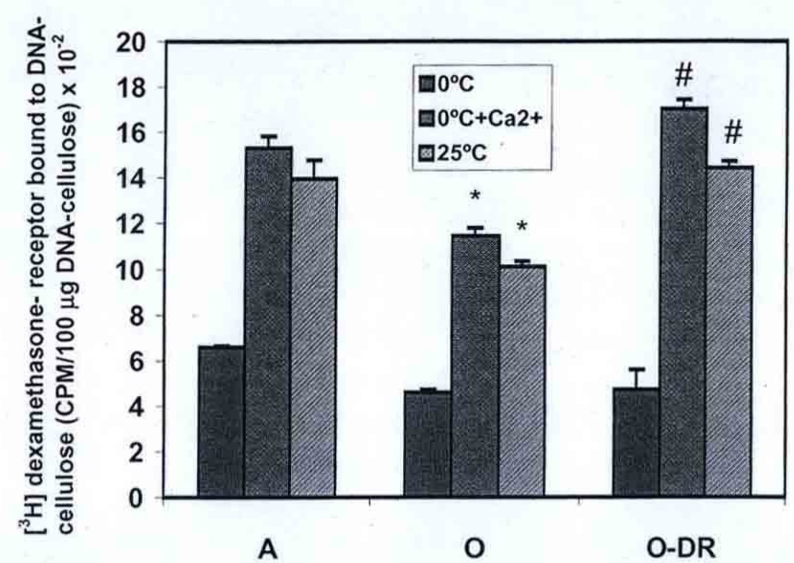

FIGURE 3. Specific binding of kidney [^{3}H]dexamethasone–receptor complexes to DNA cellulose from the adult (A), old (O), and old dietary-restricted (O-DR) mice. Cytosols were prepared in buffer B and the hormone–receptor complexes obtained by incubating with 40 nM [^{3}H]dexamethasone for 4 h at 0°C. The hormone–receptor complexes were then subjected to Ca^{2+} (20 mM at 0°C) and heat (25°C) activation for 45 min as against 0°C control.

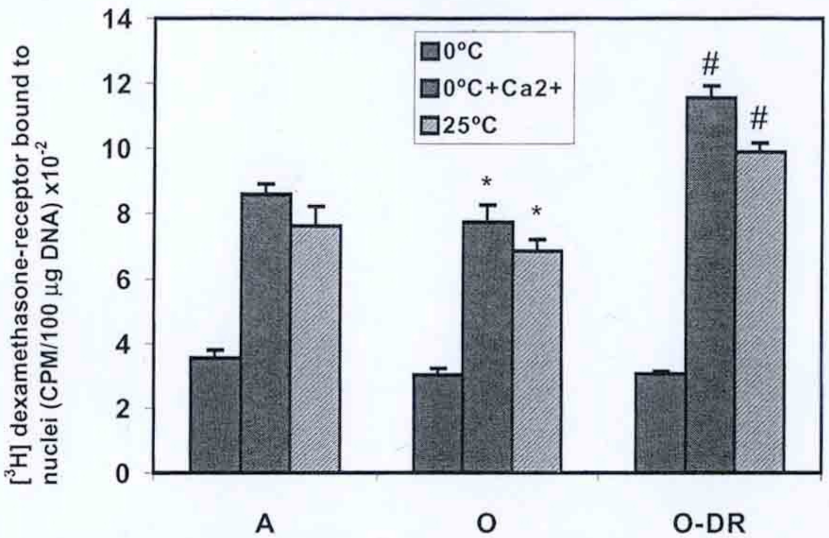

FIGURE 4. Specific binding of kidney $[^3H]$dexamethasone–receptor complexes to purified nuclei from the adult (A), old (O), and old dietary-restricted (O-DR) mice. The hormone–receptor complex preparations and activation conditions are the same as given in FIGURE. 3. Activated hormone–receptor complexes were incubated with purified nuclei instead of DNA cellulose. The results are mean ± standard deviation for four separate experiments with 5–6 mice of each group. *Statistically significant ($P < 0.05$) compared to the adult. #Statistically significant ($P < 0.001$) compared to the old mice.

DNase I digestion of bound GR from the kidney revealed a significantly higher (26%) extraction of nuclear-bound heat-activated $[^3H]$dexamethasone–receptor complexes from adults as compared to the old animals. However, no significant change in the magnitude of extraction was observed between old AL and DR animals (FIG. 5).

DISCUSSION

Aging is a progressive accumulation of changes that are associated with ever-increasing susceptibility to diseases and death. Investigators at a number of laboratories are striving to understand the mechanistic interactions between nutrition and aging.[39] Experiments have purportedly shown that DR provides a variety of protective mechanisms during aging.[8] Alterations in the adaptive responses to hormones and other biochemical stimuli, including a decreased ability to respond to stress, are characteristics of aged animals. The mecha-

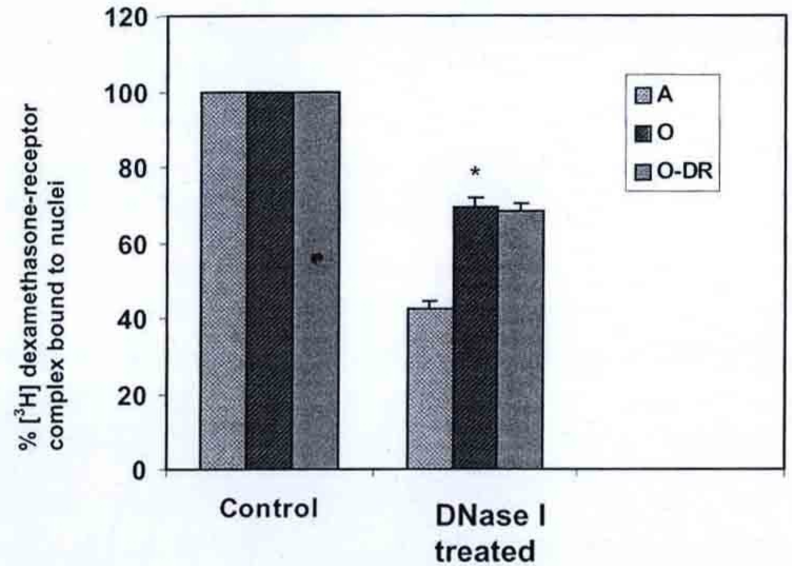

FIGURE 5. DNase I extractability of bound $[^3H]$dexamethasone–receptor complexes from the kidney nuclei of adult (A), old (O), and old dietary-restricted (O-DR) mice. Heat-activated, nuclear-bound hormone–receptor complexes were extracted using DNase I as per experimental protocols. The results are mean ± standard deviation of four separate experiments performed for each group. *Statistically significant ($P < 0.001$) compared to the adult.

proliferation and apoptosis, stress responses, inflammation, and repair systems.[23] All such effects in one way or another are regulated by glucocorticoids (GCs). GCs exert their cellular and molecular actions by binding to GRs, which ultimately modulate the expression of genes by a cascade of regulatory events.[4,40,41] GR concentration and properties change during aging and this decrease in receptor number has been observed in numerous tissues.[42–44] Herein, we have studied the long-term effect of DR on the endogenous level of GR, its activation, and digestion of nuclear-bound receptors by DNase I in the kidney of older animals. In our experimental schedule, we followed the 3-month dietary restriction regimen that gave the maximum stable change in GR expression. It was observed that 3-month DR significantly reduced the body weight of old mice. This observation of body weight reduction confirmed that the animals were indeed subjected to dietary restriction and that is consistent with earlier reports.[29,45]

Data on GR level suggest an overall decrease (30%) in the receptor level in the kidneys of old mice compared to the adult mice. The high level of receptors in the kidneys of the adult animals may be a contributory factor for the role of this hormone during the early growth and development of the organ.[2,3]

Decrease in the kidney GR level of old mice may impair renal functions, which may be one of the reasons for the reduced ability to maintain renal filtration rate and water and electrolyte homeostasis during old age.[28] Other reports also suggest a decrease in the GR level with aging in rat liver and kidney.[2,29,46] Lowered GR concentration is also observed in the presence of normal plasma GCs in aged humans. The reduction may indeed be an inherent part of the aging process.[47,48] Our data also exhibited an increase in the level of receptors in kidney during DR in old animals compared to the AL-fed animals. Such an increase in the receptor concentration may help the old animals control the GC-mediated responses during DR. GCs are known to influence glomerular filtration rate, ion transport, Na^+ uptake, and other metabolic functions in the kidney.[2,3,49,50] Higher level of GR in the kidney of older DR mice may help the animals to maintain electrolyte balance and a kidney filtration rate more efficiently, which would otherwise be affected by the aging process. 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 in the two different age groups. DR rodents are more resistant to a variety of stresses on account of increased production of stress proteins that may increase the resistance of cells.[51] In old animals, an elevated level of GR by dietary restriction might help to enhance different biological activities and quite likely provide better adaptability to the environment.[29]

Activation studies of GR were carried out in the kidney of old mice during dietary restriction to see any change in the magnitude of salt- (Ca^{2+} at 0°C for 45 min) and temperature- (25°C for 45 min) dependent activation. A significant decrease (15–20%) was observed in the magnitude of activation in renal GR in old animals compared to the adult animals in the AL-fed mice. The differences in the endogenous modulators of GR functioning at two different phases of life span and/or alterations in the physicochemical properties of GRs might play a role in lowering the activation in older mice.[52] Low-temperature Ca^{2+}-dependent activation of the H–R complexes was more pronounced than temperature-mediated activation in the kidney of older animals. The exact mechanisms for this activation are not well understood. However, Ca^{2+} enhancement of nuclear and DNA cellulose binding of GR may be due to a direct interaction of Ca^{2+} with the receptor molecule and/or receptor-transforming factor(s), which results in a conformational change capable of exposing the DNA- and/or chromatin-binding domain.[2,53] Low-temperature Ca^{2+}-dependent activation was more pronounced when DNA cellulose rather than purified nuclei is used. This may be due to the open DNA binding sites in DNA cellulose compared with intact nuclei.[2] A greater activation (30%) of renal receptors was observed in old mice subjected to DR compared to the AL-fed mice. This increase in the receptor activation in aged DR animals may help them achieve better GC action at that stage of the life span.

The digestion and extraction of nuclear-bound GR by DNase I was studied in the kidney of adult, old, and old dietary-restricted mice in order to reveal the differences, if any, in the extraction of nuclear-bound receptors. Aging increases the compactness of chromatin and reduces its digestibility by DNase I.[54] DNase I cuts the DNA where it is maximally exposed and that depends on the degree of chromatin condensation. Our observation revealed a higher degree of extraction of nuclear-bound hormone–receptor complexes in the adult kidney as compared to the old, whereas DR did not show any effect on such extractability. It relates to the fact that there may not be any appreciable change in the organization of chromatin in DR animals compared to the AL-fed animals.[29] Our findings of reduced extractability by DNase I in aged animals corroborate with the observations of others who also reported reduced digestibility of chromatin by DNase I of the old rat brain compared to that of the young and the adult.[36,55]

In conclusion, our findings indicate a decreased level of GRs in the kidney of old mice and that long-term DR results in an increase in the GR level in those animals. DR also increases the magnitude of activation of GRs in older mice that is otherwise reduced. Hence, DR could be used to elevate the GR concentration in older animals whose receptor level is already reduced during that period of the 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 renal responses and for adapting to stress in old age.[29]

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