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Research article

Modulation of hepatic and renal glucocorticoid receptors during aging of mice

H.S. Ranhotra & R. Sharma*
*Department of Biochemistry, North Eastern Hill University, Shillong 793 022, India; *Author for correspondence (e-mail: rsharma@nehu.ac.in; fax: +91-364-250076)*

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Abstract

The regulation of hepatic and renal glucocorticoid receptors (GRs) in young (4 weeks) and senescent (120 weeks) mice was studied. Significant changes in the level of GRs from liver and kidney were detected, whereas the affinity (K_d) for the hormone [^{3}H]dexamethasone did not change in young and old mice. The concentration of GRs was markedly decreased in liver (25%) and kidney (33%) of old mice as compared to young ones. The magnitude of heat activation of GR complexes was more pronounced in both the tissues at young age compared to old. In addition, we have found changes in the heat activation-inhibition studies of GRs, using polyunsaturated fatty acids (PUFAs) as measured by binding to DNA-cellulose and purified nuclei. Linoleic acid (C18:2) exhibited significant decrease in heat activation of both hepatic and renal hormone-bound GR, with higher magnitude of inhibition in young liver (64%) and kidney (68%) as compared to old (41% and 43%, respectively). Arachidonic acid (C20:4) was also found to be an inhibitor of activation causing significant decrease in hepatic GR activation, with greater inhibition in young liver with respect to old, however, without any such difference in the kidney of young and old mice. Furthermore, DNase I digestion and extraction of nuclear-bound GR-complexes from both the tissues reveal lesser extraction in old liver (26%) and kidney (24%) compared to young tissues, indicating chromatin condensation with aging, thereby controlling the accessibility to such transcription factors as GRs. These findings indicate that the changes in the GR concentration, activation-modulation by PUFAs and chromatin organization, that take place during aging, may contribute to functional changes in glucocorticoid action mechanisms in senescent animals.

Introduction

It is now widely believed that senescence operates at the cellular and molecular levels (Luckinbill and Foley 2000). During this phase, the ability to adapt to internal as well as external variations declines, leading to fall in homeostatic balance. Steroid hormones, in particular, are known to act at the gene level through binding to their specific intraceullar receptors in the target cells, thereby directly influencing the physiological status of animals. Glucocorticoids (GCs) exert their cellular effects through high affinity binding to their cognate cytoplasmic receptors, the glucocorticoid receptors (GRs) (Kellendonk et al. 1999). GCs play myriad physiological and biochemical roles, inlfuencing almost every tissue. GRs are hetero-oligomeric complex, consisting of a steroid binding protein (94 kDa), two Hsp90, one each of Hsp70 and 56 and a p23 acidic protein that keep receptors in an inactive, yet hormone binding conformation (Biola and Pallardy 2000; Prima et al. 2000). This complex dissociates upon non-covalent interaction of hormone to the steroid binding protein, by a process called 'activation' (Tsai and O'Malley 1994; Pratt and Toft 1997). The activated GR migrates to the nucleus and binds to the glucocorticoid response elements

(GREs), specific DNA sequences, ultimately modulating responsive gene expression (McNally et al. 2000). *In vivo*, GR activation is a critical step in GC action, as it is a rate limiting step for nuclear or chromatin binding. *In vitro*, activation can be achieved by heat (25 °C) treatment of cytosol containing hormone-receptor complexes, which causes dissociation of bound chaperones and subsequent activation (Meshinchi et al. 1990) with increased binding affinity towards DNA-cellulose and/or purified nuclei.

We have investigated possible changes in GR level, activation-modulation processes and chromatin-bound GR extraction using DNase I in the liver and kidney of young and old mice. In liver, GCs are primarily gluconeogenic, whereas they regulate Na^+ reabsorption and K^+ and H^+ elimination in kidney. Many workers have reported alterations in GR level during aging, some showing a decreased concentration in rat liver (Djordjevic-Markovic et al. 1999), and elevated level in the rat brain (Martin et al. 1999). However, there is a general agreement that GR level increases during early developmental period and gradually declines as they enter the post-weanling stage in Sprague-Dawley rats (Kalimi 1984). Polyunsaturated fatty acids (PUFAs) are known inhibitors of steroid binding to GRs (Sumida et al. 1993; Haourigui et al. 1994). However, their modulatory role on in vitro GR activation has not been ascertained. Hence, we studied the modulatory effect of linoleic acid and arachidonic acid on heat activation of hormone-bound GR as assessed by binding to DNA-cellulose and purified nuclei. Our study reveals an inhibitory effect of both PUFAs on GR activation in an age- and tissue-specific manner. It has been reported that chromatin condensation occurs with increasing age (Kanungo 1994). GR being a transcription factor must bind to chromatin to regulate gene expression. To elucidate the notion that, perhaps the binding of heat activated [^{3}H]dexamethasone-receptor complexes to chromatin may be altered in tissue- and age-specific manner, we employed DNase I, which can digest nuclei and extract nuclear-bound GR. The magnitude of extraction would be an indication of compactness of chromatin. Interestingly, we found lesser bound-GR extraction from both the tissues in old mice compared to young ones. Taken together, present data indicate that senescence causes major adaptibility changes in GRs concentration, activation-modulation and chromatin compactness that may play an important role in GR-mediated gene expression.

Materials and methods

Animals and chemicals

Swiss albino (Balb/c) male mice of two different age groups, young (4 weeks) and old (120 weeks), kept under standard laboratory conditions were used in all the experiments. [1,2,4,6,7 ^{3}H]dexamethasone (specific activity, 91 Ci/mmol) was obtained from Amersham, UK. Nonradioactive dexamethasone, dimethyl sulfoxide (DMSO), linoleic acid (C18:2), arachidonic acid (C20:4) [both free acids], tris, EDTA, sodium molybdate, DNA-cellulose, DNase I, activated charcoal, dextran T-70 and Triton X-100 were purchased from Sigma, USA. All the other chemicals used were of highest purity analytical grade. All experiments were carried out at 0–2 °C, unless otherwise mentioned. All radioactive counting (CPM) was carried out using Wallac 1409 liquid scintillation counter having 68% efficiency for tritium.

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 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 $MgCl_2$.

Preparation and assay of glucocorticoid receptors

The mice were sacrificed by cervical dislocation at a fixed time of the day (10:00 h), their livers and kidneys were quickly removed, washed in chilled normal saline (0.9% NaCl) and blotted dry. A 20% homogenate of these tissues were prepared in buffer A and centrifuged at 27,500 × *g* for 60 min at 2 °C. Aliquots of clear fat-free cytosol (100 μl) were incubated at 0 °C for 4 h with 1–120 nM [^{3}H]dexamethasone alone or with 500-fold excess of non-radioactive examethasone. Saturation of specific binding occurred during this period. 50 μl dextran-coated charcoal (4% activated charcoal + 0.4% dextran T-70) prepared in buffer A was added to remove any unbound hormone. Specific binding was obtained by subtracting the radioactivity (CPM) bound in the presence of unlabeled dexamethasone (non-specific binding) from that bound in the presence of labeled dexamethasone alone (total binding). The number of

specific binding sites (fmol/mg protein) and the dissociation constant (K_d) were calculated from Scatchard plot (Scatchard 1949).

Preparation and activation of glucocorticoid-receptor complexes

Pooled tissues from 4–5 mice of each age group were homogenized in buffer B and centrifuged at 2,000 × *g* for 10 min at 2 °C to sediment nuclei. Supernatant thus obtained was further centrifuged at 27,500 × *g* for 60 min at 2 °C. Finally, the fat-free clear cytosol was incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 0 °C, during which maximal saturation binding occurred; bound hormone-receptor complexes were separated using dextran-coated charcoal (prepared in buffer B).

Bound glucocorticoid-receptor complexes were heat activated at 25 °C for 45 min in the presence or absence of linoleic and arachidonic acids separately to a final concentration of 160 μM (prepared as 1 mM stock in DMSO). Control tubes received equal volume of DMSO without the fatty acids. The magnitude of activation-modulation was determined by incubating hormone-receptor complexes with pre-washed DNA-cellulose pellet containing 100–150 μg DNA for 60 min at 0 °C (Borbhuiya and Sharma 1995a, b). DNA-cellulose-bound hormone-receptor complexes were obtained by washing the pellets twice with buffer B. The final pellets were suspended in 4 ml of cocktail T and bound radioactivity counted. For the nuclear binding assay, crude nuclei obtained as above was further purified using buffer C and finally suspended in buffer B. Pellets containing 100–150 μg DNA were incubated with hormone-receptor complexes and processed in the same manner as described for DNA-cellulose. The results are expressed in terms of [^{3}H]dexamethasone-receptor complex bound to DNA-cellulose or nuclei (CPM/100 μg DNA).

DNase I digestion of glucocorticoid receptor bound nuclei

Temperature activated [^{3}H]dexamethasone-receptor complexes from both the tissues and ages, were allowed to interact with their respective purified nuclei, as described above. After washing off the unbound complexes, the pellets were incubated with DNase I (prepared in buffer D) at a concentration of 100 U/100 μg DNA; control tubes received the buffer only (Borbhuiya and Sharma 1995a, b). The digestion was stopped by the addition of 1 ml of buffer B, followed by centrifugation at 2,000 × *g* for 10 min. The pellet was processed and the bound radioactivity determined (expressed as % [^{3}H]dexamethasone-receptor bound to nuclei) as described earlier. Controls were attributed 100% bound.

Protein and DNA estimations

Protein concentration of the receptor preparation was estimated according to the dye-binding method of Bradford (1976), using bovine serum albumin (BSA) as standard. DNA content of purified nuclear suspension was determined by the method of Burton (1956). 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 and discussion

GCs regulate several physiological and biochemical activities in a wide range of target tissues such as liver, kidney, heart, lungs, brain, and immune cells etc. The responsiveness of tissues and cells to certain hormonal modulators has been shown to be altered during development and aging (Kanungo 1994; Singh and Sharma 1995). All the known actions of GCs involve high-affinity interaction with the specific cytoplasmic receptors, subsequent, *activation* and translocation of receptors to the nucleus, where they interact with cognate acceptors' sites (GREs) in the promoter region of responsive genes and modulate their expression.

Our study of Scatchard analyses indicate a decreased level of GR in the liver (25%) and kidney (33%) of old (120 weeks) mice compared to young (4 weeks) ones (Table 1). However, slopes of the plots (not shown) exhibit no alteration in the affinity (K_d) of GR for its ligand at these two different ages. The higher level of GR in the liver and kidney of young mice may be a contributory factor for the role of this hormone in early growth and developmental phase of animals life span. This phase involves appearance of several new proteins and enzymes performing specialized functions, few of them are under the genetic regulation of GCs. The level of these proteins exhibits alterations with advancing age (Kanungo 1994). Significant decrease in the GR level of old mice may impair metabolic functions, which may be one of the reasons for reduced ability to maintain homeostatic balance during this phase of life span.

Table 1. Specific binding sites (B_{max}) and dissociation constant (K_d) of glucocorticoid receptors in the liver and kidney of young (4 weeks) and old (120 weeks) mice.[a]

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

[a]The data were obtained from 4–5 mice of each age group and analyzed using Scatchard plot as given in 'Materials and methods'. The results are mean ± standard deviation of four separate experiments for each age group. *Statistically significant ($P < 0.001$) with respect to 4-week-old (young) mice.

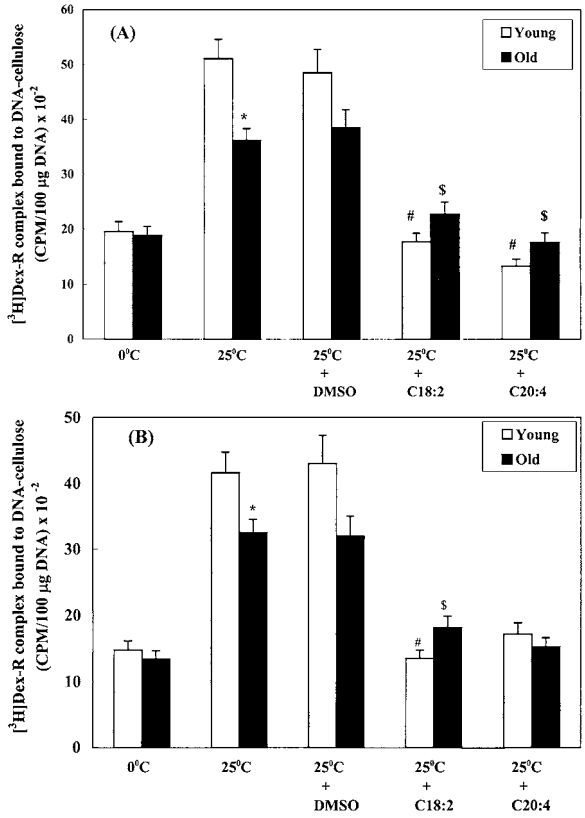

Figure 1. Binding of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes to DNA-cellulose in young (4 weeks) and old (120 weeks) mice. Cytosol from these tissues were prepared in buffer without molybdate and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H]dexamethasone for 4 h at 0 °C. The hormone-receptor complexes were then subjected to heat (25 °C) activation for 45 min alone or in presence of dimethyl sulfoxide (DMSO) as control, linoleic (C18:2) and arachidonic (20:4) acids, which were added to a final concentration of 160 μM. DNA-cellulose binding and further processing of the pellets were performed as described in 'Materials and methods'. The results are mean ± standard deviation of four separate experiments with 4–5 mice of each age group. *Statistically significant ($P < 0.01$) as compared to young mice. #,$Statistically significant ($P < 0.01$) to control for their respective age.

It has been reported earlier that GR level in liver is increased in senescent rats as compared to young/adult animals (Kalimi et al. 1988), with similar observation in rat brain (Martin et al. 1999), however without any apparent change in the affinity for the hormone. Few reports, however, suggest a decrease in receptor level with aging in rat liver (Djordjevic-Markovic et al. 1999) and brain (H. Ozawa, personal communication), whereas in rat adipocytes there was no such alterations (Kalimi and Banerji 1981). Our results clearly indicate that there is no apparent age-associated alteration in the binding affinity of GR in both the tissues of mice as reported in other animal models. These findings also reveal that the level of GR exhibits tissue- and age-specific correlation, which may modulate GC responsiveness and thus may play an important role in adaptive responses as a function of age.

Free fatty acids (FFAs) have been shown to modulate receptor function of many steroidal hormones including GCs (Bresnick et al. 1990). In mammals, PUFAs have been attributed to play important roles in growth and development (Simopoulous 1991). Pilot experiments performed by us reveal that heat (25 °C for 45 min) significantly enhanced the activation of hormone-bound receptor complexes from liver (2–2.5-fold) and kidney (1.5–2.5-fold) in both the ages, albeit the magnitude of activation was higher (24–29%) in young hepatic and renal GR with respect to old (Figures 1, 2A, B). PUFAs have been earlier shown to reduce the binding of glucocorticoid to its receptor. However, the modulatory role of PUFAs on receptor activation has not been ascertained. Hence, it was interesting to carry out studies on the role of PUFAs on *in vitro* GR heat activation to reveal any modulatory effect. Our study reveals that PUFAs (linoleic and arachidonic acid) inhibit the heat activation of [^{3}H]dexamethasone-receptor complexes in a dose-dependent manner (data not shown). Both these PUFAs were most effective at a concentration of 160 μM, exhibiting 40–75% maximal inhibition of receptor activation. Linoleic acid caused significant magnitude of inhibition in the liver (64%) and kidney (68%) of young mice as compared to old (41% and 43%, respectively) (Figures 1A, B). Arachidonic acid also showed a similar extent of inhibition of hepatic GR activation in young compared to old animals

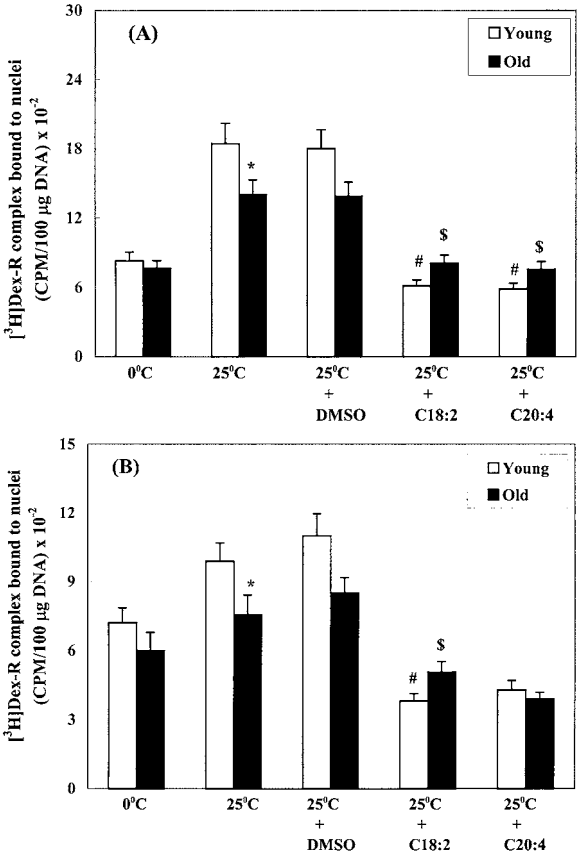

Figure 2. Binding of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes to purified nuclei in young (4 weeks) and old (120 weeks) mice. Purified nuclei from respective tissues were utilized instead of DNA-cellulose for activation-inhibition studies. Other experimental procedures are same as mentioned for Figure 1. *Statistically significant ($P < 0.01$) compared to young mice. $^{\#,\$}$Statistically significant ($P < 0.01$) to control for their respective ages.

(Figure 1A). However, the magnitude of inhibition does not show any significant difference in renal GR activation from young and old animals (Figure 1B). It seems that linoleic acid induced age-specific difference in inhibition of GR activation is found in both the tissues. However, arachidonic acid showed its age-specific inhibitory effect only in the liver of mice. Since DNA-cellulose being a non-specific assay system, it could not unequivocally implicate differences in the inhibitory effects of PUFAs on acceptor binding by activated hormone-receptor complexes. Hence, we utilized purified nuclei from both the tissues of respective ages to provide a more relevant physiological assay system. Nuclear binding assay results show linoleic acid as being equally effective in causing inhibition of GR heat activation, with greater magnitude of inhibition from young liver (66%) and kidney (65%) as compared to old tissues (42% and 40%, respectively) (Figures 2A, B). Again, arachidonic acid showed tissue-specificity in causing greater inhibition of young (68%) hepatic GR activation as compared to old (45%) (Figure 2A). The age-specific difference in arachidonic acid-mediated inhibition of activation was not significant in case of kidney where the inhibition was ~57% at both the ages of mice (Figure 2B). Hence, DNA-cellulose and nuclear binding assays revealed a similar pattern of inhibition in terms of age- and tissue-specificity on activation by these two PUFAs. Earlier experiments performed by us reveal that saturated fatty acids [palmitic (C16:0) and stearic (C18:0) acid] were unable to inhibit the heat activation of glucocorticoid-receptor complexes, indicating an involvement of unsaturated moiety. The exact mechanism of these inhibitory effects is unclear as yet. The particular domain in the GR responsible for the inhibition of activation has not been delineated, nor the group(s) in the PUFAs that interact with such domain is known. Probably, PUFAs influence a conformational change in the receptor molecule through the involvement of their unsaturated moieties, thereby discouraging the dissociation of bound heat shock proteins, that keeps the receptor in an unactivated form. The tissue- and age-specific differences in the inhibitory effect on GR activation may be attributed to such alterations in the receptor site(s), that may be modulated by these PUFAs. Greater inhibition of GRs activation in young animal tissues may indicate the status of physicochemical properties of GRs and other associated factors those modulate the receptor activity. Previous studies showing the inhibitory effects of PUFAs in the binding of [^{3}H]dexamethasone to hepatic GR in fish (Lee and Struve 1992) suggest the unsaturated moiety (ies) as the likely candidate, but this remains to be elucidated.

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

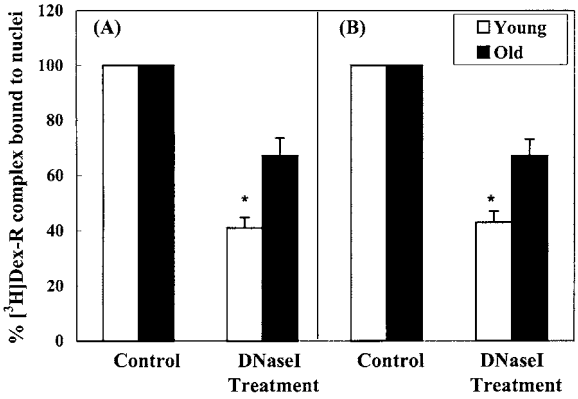

Figure 3. DNase I digestion-extraction of hepatic (A) and kidney (B) nuclear-bound [^{3}H]dexamethasone-receptor complexes of young (4 weeks) and old (120 weeks) mice. Heat-activated, nuclear-bound hormone-receptor complexes were extracted using DNase I which was used at a concentration of 100U/100 μg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

Kanungo (1983) and Chaurasia and Thakur (1995), who reported reduced digestibility of chromatin by DNase I of old rat brain compared to young and adult. DNase I cuts the DNA where it is maximally exposed. These findings reveal a more compact chromatin organization in old mice as compared to young, thereby limiting the access to DNase I and probably also to transcription factors like GRs. These differences in chromatin compactness may play an important role in determining tissue- and age-specific responsiveness to GCs by the animals. Also, there are earlier reports on age-dependent decline in nuclear binding efficiency of estradiol-receptor complexes in rat liver (Konoplya et al. 1986) and uteri (Chuknyiska et al. 1985; Belisle et al. 1986).

In conclusion, our study indicates changes in the GR concentration, activation-modulation by PUFAs and chromatin organization during aging in mice. These alterations may contribute towards functional changes in glucocorticoid action and responsiveness in aged animals.

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