Indian Journal of Biochemistry & Biophysics
Vol. 32, June 1995, pp. 125-129

Postnatal changes in kidney glucocorticoid receptor of mice

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Received 9 January 1995; revised 13 March 1995

The specific binding of [^{3}H]dexamethasone to its receptor and the activation of the hormone-receptor complexes from the kidney of mice at various postnatal ages were investigated. The results indicated that the level (fmol/mg protein) of glucocorticoid receptors remained unaltered at 10-, 15- and 30-day, increased significantly at 45-day and thereafter declined to its earlier level in 60-day old mice. Scatchard analysis of binding data confirmed the increased level of receptors at 45-day compared to day 15, without any change in the hormone affinity to its receptor at these two ages. The extent of temperature- and salt-dependent activation of the receptor showed no marked differences in 10- and 60-day old mice, as determined by DNA-cellulose binding assays. However, the nuclear binding of temperature- and salt-activated glucocorticoid-receptor complexes was significantly higher in 10-day old mice. Cross-mixing experiments showed nuclear specificity in higher binding of thermally activated receptor at day 10 of postnatal age. Molybdate, tungstate and *N*-ethylmaleimide inhibited the temperature- and salt-dependent activation of glucocorticoid-receptor complexes similarly at both the ages studied. DNase I extraction of bound hormone-receptor complexes from nuclei showed higher extractability at day 10 (63%) compared to day 60 (43%). These findings indicated changes in glucocorticoid receptor concentration together with chromatin organization that might play an important role in glucocorticoid-mediated responses during postnatal development of mice.

Glucocorticoids, the key regulators of homeostasis and adaptation in developing animals, have several effects on various animal tissues including kidney where they influence glomerular filtration rate and ion-transport[1-3]. These hormones exert cellular and molecular actions by modulating gene expression through a cascade of regulatory events initiated by high affinity interaction with their intra-cellular receptors[4]. The steroid binding possibly triggers an allosteric change in receptor structure, involving dissociation of non-steroid binding proteins (heat shock proteins, hsp 90). This results in a conformational change which exposes the DNA-binding domain of the receptor protein allowing it to bind to the DNA-cellulose and/or nuclei[5]. The process is termed as activation or transformation and is ligand dependent[6]. Interaction of activated glucocorticoid-receptor complexes with nuclear acceptor sites (glucocorticoid regulatory elements, GREs, usually located upstream from the transcription start site) alters the local configuration of DNA and/or chromatin and modulate the expression of target genes[4,7]. There are several known activators (temperature, salt and dilution) of glucocorticoid receptors together with inhibitors (molybdate, tungstate, *N*-ethylmaleimide) of activation process[8-11].

Although numerous studies have been made on the age-related changes in glucocorticoid receptor concentration in different mammalian tissues[12-14], reports on activation/transformation changes in receptors as well as on their binding to nuclear chromatin during postnatal development are limited. The present study describes changes in the level of kidney glucocorticoid receptor and also in the magnitude of the activation/inhibition of the hormone-receptor complexes during development. Conformational changes in the chromatin organization during postnatal ages which may be involved in glucocorticoid regulation of gene expression in developing animals are also examined.

Materials and Methods

Male Swiss albino mice (Balb/c strain) of different postnatal ages (10-, 15-, 30-, 45- and 60-day) were used. They were maintained under normal laboratory conditions at 24 ± 2°C and fed standard pellet diet (Amrut Laboratory, Pune) and water *ad libitum*. [1, 2, 4, 6, 7-^{3}H]dexamethasone, a synthetic glucocorticoid (sp. activity 89 Ci/mmol) was from Amersham, England. Unlabelled dexamethasone and other biochemicals were from Sigma Chemical Co., USA. All other chemicals used were of analytical grade.

Buffers—(A): 0.25 *M* sucrose, 10 m*M* Tris-HCl (*p*H 7.5), 1 m*M* EDTA, 10 m*M* sodium molybdate,

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10% glycerol, 1 m*M* DTT and 10 m*M* NaCl; (B): 0.25 *M* sucrose and 10 m*M* Tris-HCl (*p*H 7.6); (C): 0.25 *M* sucrose, 10 m*M* Tris-HCl (*p*H 7.6) and 0.5% Triton X-100, and (D): 0.25 *M* sucrose, 10 m*M* Tris-HCl (*p*H 7.6) and 4.2 m*M* $MgCl_2$.

Preparation and assay of glucocorticoid receptors—Animals were killed by cervical dislocation at a fixed time of the day (11:00 hr), kidneys were quickly removed, freed of fat and connective tissues, washed in chilled normal saline and blotted dry. A 20% (w/v) homogenate was prepared in ice-cold buffer A using teflon-glass motor driven homogenizer at 0-4°C. The homogenate was centrifuged at 27,500 × *g* for 60 min at 2°C in a Hitachi model CR20B2 high speed refrigerated centrifuge. Aliquots (100 µl) of clear, fat free cytosol were incubated with 40 n*M* [^{3}H]dexamethasone alone or with 500-fold excess unlabelled dexamethasone for 4 hr at 0°C, to get maximum saturable specific binding. For Scatchard analyses[15], 2.5-120 n*M* [^{3}H]dexamethasone was used along with parallel control tubes containing 500-fold excess unlabelled dexamethasone. Unbound steroid at the end of the incubation period was removed using 50 µl dextran-coated charcoal (4% activated charcoal + 0.4% dextran T-70 in buffer A as described in ref. 9). After 10 min, the charcoal mixture was centrifuged and 100 µl of clear supernatants were transferred to scintillation vials and 4 ml scintillation fluid (cocktail W from SRL) added to each vial. Bound radioactivity was measured in a Beckman LS1801 counter with efficiency of 65% for tritium. Specific saturable binding was calculated by subtracting the radioactivity bound in presence of unlabelled dexamethasone from that found when labelled dexamethasone was used alone.

DNA-cellulose and nuclear binding assays—Pooled kidney tissues from two ages (10- and 60-day) were homogenized (20% w/v) separately in buffer B and centrifuged at 2000 × *g* for 10 min at 2°C, to sediment the nuclei. The supernatant thus obtained was further centrifuged at 27,500 × *g* for 60 min at 2°C. Fat free cytosol was incubated with 40 n*M* [^{3}H]dexamethasone for 4 hr at 0°C. Unbound steroid was removed using dextran coated charcoal (prepared in buffer B). Bound hormone-receptor complexes were then subjected to activation by heat (25°C) and salt (20 m*M* Ca^{2+} at 0°C), separately for 45 min (ref. 9). The magnitude of activation was determined by incubating aliquots (200 µl) of hormone-receptor complexes with DNA-cellulose pellets (pre-washed in buffer B) for 60 min at 0°C. The tubes were gently vortexed at regular intervals to ensure proper binding. The reaction was stopped by adding 1 ml of buffer B and the unbound complexes were removed by washing the pellets twice with buffer B. The final pellets were suspended in 4 ml cocktail W and the bound radioactivity measured. For nuclear binding assay, crude nuclei obtained as above were further purified using buffer C, washed twice with buffer B and suspended in the same buffer. Nuclear pellets containing 150-200 µg DNA were obtained and incubated with activated hormone-receptor complexes for 60 min at 0°C. Further processing was done as described for DNA-cellulose binding assay.

Activation inhibition studies—Inhibition of both temperature- and salt-dependent activation was studied using inhibitors (molybdate, tungstate and *N*-ethylmaleimide) at a final concentration of 20 m*M*. These were added to aliquots of the receptor preparation prior to the start of the activation process. The magnitude of activation-inhibition was determined both by nuclear and DNA-cellulose binding assays.

DNase I digestion studies—Temperature activated glucocorticoid receptor complexes bound to nuclei were extracted using DNase I (prepared in buffer D; 150 units/100 µg DNA) for 45 min at 0°C. After washing with buffer B, the resultant pellets were suspended in cocktail and the bound radioactivity was counted.

Protein and DNA estimations—Protein content was determined by the method of Bradford[16] and that of DNA by the method of Burton[17]. The data were statistically analyzed and the level of significance (*p*) between two sets of data was calculated according to student's t-test.

Results

Developmental changes in glucocorticoid receptor level

Specific saturable binding of [^{3}H]dexamethasone to its receptor remains unaltered in the kidney of mice upto day 30, increased significantly at day 45 and then declines to a level similar to earlier postnatal ages at day 60 (Fig. 1A). Our data on scatchard analysis confirms a higher concentration of hormone binding sites (236 fmol/mg protein) at 45 day compared to that at 15 day (180 fmol/mg protein) of postnatal development (Fig. 1B). From the slope of the curves, the apparent dissociation constant (*K*d) were found to be similar (2.90 n*M* for day 15 and 2.86 n*M* for day 45), indicating that the affinity of the hormone for the receptor remains unchanged during development.

Activation studies on glucocorticoid receptor

We studied the activation/transformation process of the glucocorticoid receptor complexes at two ages (day 10 and 60) using DNA-cellulose and purified nuclei. Results show that both temperature and salt enhance, significantly the DNA-cellulose binding of

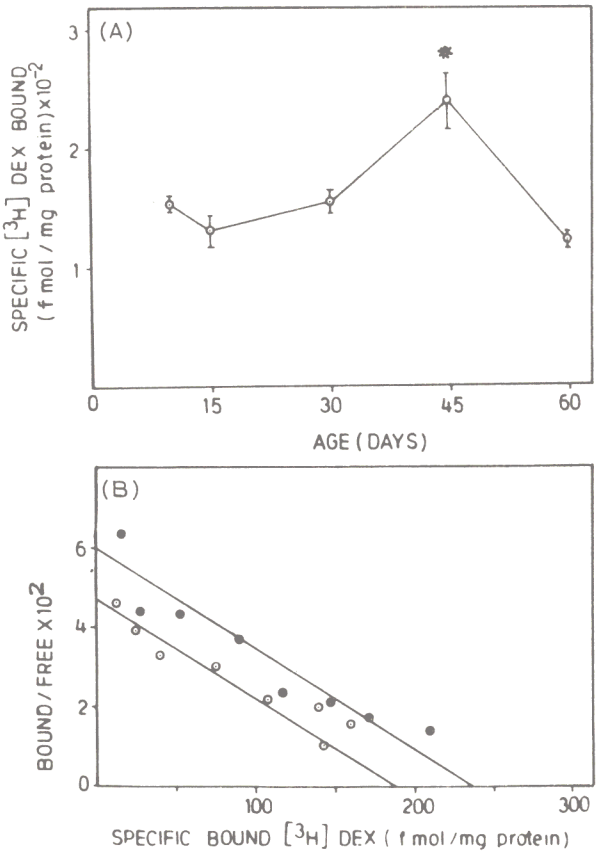

Fig. 1—(A): Specific saturable binding of [^{3}H]dexamethasone (DEX) in the kidney of mice at different postnatal ages. [Fractionation and receptor assay conditions are described in Materials and Methods. Values are mean for 5-6 mice of each age group. Bars, S.D. *Statistically significant ($p < 0.001$) with respect to day 30. (B): Scatchard plot of the specific binding of [^{3}H]dexamethasone to its receptor in the kidney of 15-(○ − ○), and 45- (● − ●) day old mice. Ratio of bound to free hormone concentrations is plotted against specific bound hormone receptor/mg protein. Curves depict the mean values for three separate assays. Plots gave the number of specific receptor binding sites 180 ± 5.4 and 236 ± 8.2 fmol/mg protein at 15- and 45-day of age, respectively. Slopes indicated dissociation constant (K_d) values of 2.90 ± 0.17 and 2.86 ± 0.42 nM for receptor at 15- and 45-day of postnatal age].

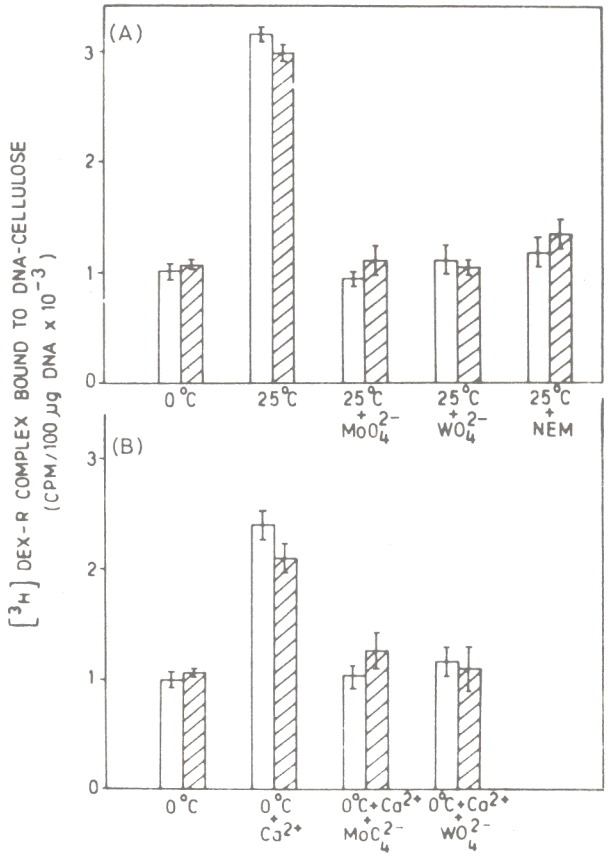

Fig. 2—Specific binding of kidney [^{3}H]dexamethasone-receptor complexes to DNA-cellulose upon (A), heat; and (B), calcium activation in (□), 10- and (▨), 60-day old mice. [Activation and inhibition of activation conditions are described in Materials and Methods section. Results are mean ± SD for three separate assays with 4-6 mice of each age group. Inhibitors used were 20 m*M* each of molybdate (MoO_4^{2-}), tungstate (WO_4^{2-}) and *N*-ethylmaleimide (NEM)].

hormone-receptor complexes at both the ages to a similar extent (Fig. 2A & B). Binding of glucocorticoid-receptor complexes, incubated at 0°C for 45 min as a control also shows similar binding at both the ages. The results indicate no postnatal difference in the *in vitro* activation of hormone receptor under the conditions mentioned above.

Since DNA-cellulose by virtue of it being a non-specific assay system, could not implicate any age-related differences in the activation of glucocorticoid receptors, purified nuclei were used to provide a more physiological assay system. The results indicate that nuclear binding of both thermal- and salt-activated glucocorticoid-receptor complexes is significantly higher in immature (10 day) compared to that of mature (60 day) mice (Fig. 3A & B). Data from cross-mixing experiments (nuclei of 10 day and thermally activated hormone-receptor complexes of 60 day and *vice versa*) showed similar results compared to that of the non-mixed group. These findings indicate higher binding capacity of immature nuclei to activated hormone-receptor complexes.

Activation inhibition studies

Data show that the magnitude of inhibition of thermal activation by molybdate, tungstate and *N*-ethylmaleimide and that of salt activation by molybdate and tungstate, studied using both DNA-cellulose (Fig. 2A & B) and nuclei (Fig. 3A & B) is similar in both the ages. The results reveal that the physicochemical properties responsible for inhibition of the activation process of kidney glucocorticoid receptor remain unchanged during development of mice.

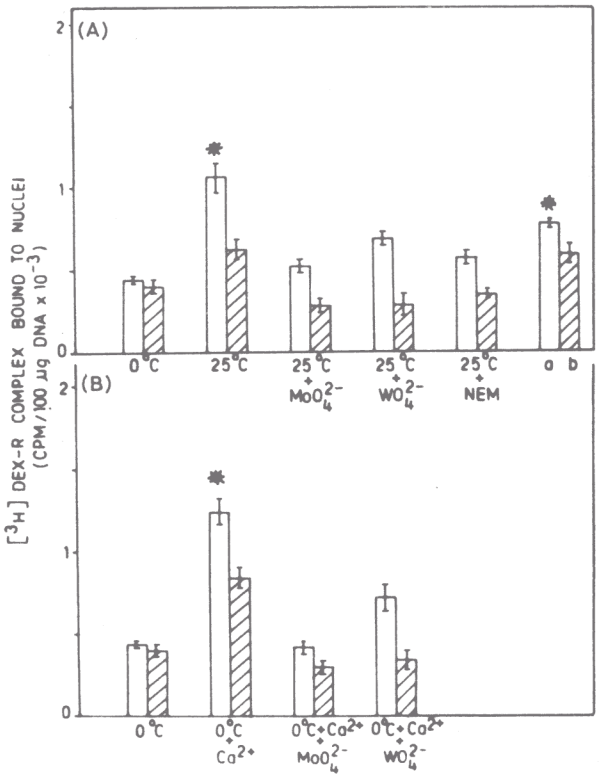

Fig. 3—Specific binding of kidney [^{3}H]dexamethasone-receptor complexes to purified nuclei upon (A), heat; and (B), calcium activation in (□),10- and (▨), 60-day old mice. Experimental procedures are same as for Fig. 2. In cross-mixing experiments, nuclei of 10-day and activated cytosol receptor of 60-day and *vice versa*, respectively were used. *Statistically significant ($p<0.01$) with respect to 60-day].

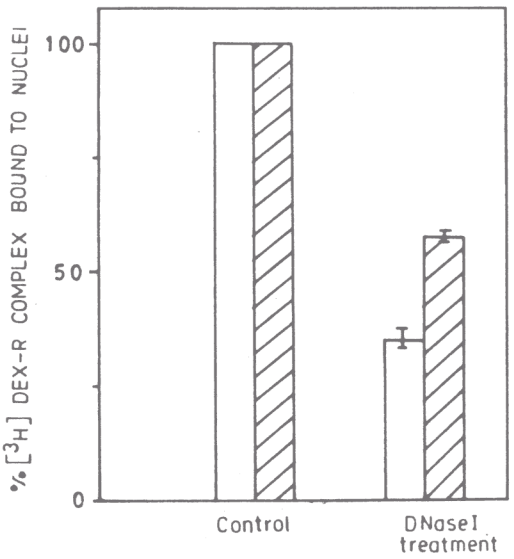

Fig. 4—DNase I extractability of bound [^{3}H]dexamethasone-receptor complexes from the kidney nuclei of (□), 10- and (▨), 60-day old mice. DNase I digestion procedures are given in the text. Values are mean ± SD of three separate experiments].

DNase I extraction of bound glucocorticoid receptor from nuclei

The sensitivity of kidney nuclear chromatin digestion by DNase I was compared at two ages (10- and 60-day). It was done to ascertain the chromatin organization and its possible role in developmental changes in the nuclear binding of activated glucocorticoid-receptor complexes. The data show higher extractability of bound complexes from nuclei of 10-day (63%) than that of 60-day (43%) mice (Fig. 4).

Discussion

Glucocorticoid receptors are a group of trans-acting factors whose activity is controlled by specific binding of the hormone[18]. Several studies have suggested that the glucocorticoid receptor level in the cell is a limiting factor in the process of gene induction by the hormone[19]. However, the post-receptor events such as activation/transformation, nuclear translocation and chromatin organization might influence the magnitude of biological effects[20-22].

Our findings of higher level of glucocorticoid receptors at day 45 of postnatal age may possibly be associated with changing dietary and metabolic adjustments and also be a contributory factor for the role of this hormone in the development and maturation of kidney's glomerular filtration rate and ion-transport during this phase of lifespan. A similar developmental change in the rat pancreatic glucocorticoid receptors has been reported[23]. Using scatchard analysis of hormone binding we confirm that higher level of glucocorticoid receptor at day 45 is because of higher concentration and not because of its higher affinity towards the hormone. It was earlier reported that the affinity of glucocorticoid receptor for hormone does not change as a function of age in different tissues[12,13].

The heat- and salt-dependent activation of glucocorticoid-receptor complexes were studied at day 10 and 60 (pre- and post-weaning) to determine the physicochemical changes, if any, in the receptor molecule at activation/translocation level. Although no age-related changes were observed in the magnitude of activation using DNA-cellulose binding assay, the use of purified nuclei as a binding medium, however, showed that binding of both heat- and salt-activated glucocorticoid-receptor complexes is significantly lower in 60-day old mice. The higher binding of activated receptors in 10-day nuclei compared to 60-day was confirmed by cross-mixing experiments. It was observed that higher binding of glucocorticoid receptor is because of the nuclei and not because of the receptor. A similar trend was observed in the liver of mice (data not shown). Higher binding of hormone-receptor complexes may be because of more open bin-

ding sites on chromatin at day 10 compared to day 60 in these tissues.

The inhibition of activation of glucocorticoid receptors by known inhibitors of phosphatase activity (molybdate and tungstate) reflects that dephosphorylation may play an important role in hormone receptor activation/transformation, which converts them to DNA binding form[24]. The inhibition of thermal activation by *N*-ethylmaleimide, a sulfhydryl group modifier indicates that these group(s) are required for the binding of hormone-receptor complexes to DNA[11,25]. The extent of inhibition is similar at both the ages studied indicating that the mechanistic requirement of receptor activation is not altered during development of mice.

In order to elucidate the higher binding of hormone-receptor complexes in nuclei of 10-day old mice, we studied the extraction of bound glucocorticoid-receptor complexes from nuclei at two ages by digesting with DNase I. The supercoiling of DNA double helix around histone core confers the specificity of digestion of DNA in chromatin by pancreatic DNase I[26]. It cuts chromatin DNA at 10-bp intervals and has been used to identify chromatin organization. Our data on higher digestion of chromatin in 10-day reflects a less condensed chromatin organization at this stage of postnatal development. This probably allows a higher *in vitro* binding of activated hormone-receptor complexes. However, at day 60, where extraction of bound complexes is less, chromatin might have acquired a more compact organization and hence a lower binding of activated complexes. Our findings corroborate with earlier reports that digestibility of chromatin by DNase I decreases as development proceeds[27]. In conclusion, our findings indicate that changes in glucocorticoid receptor concentration as well as chromatin organization in the kidney of mice may influence the competence of this tissue responsiveness to glucocorticoid action in developing animals.

Acknowledgement

This work was supported by Grant-in-aid (No. SP/SO/D-35/89) from the Department of Science and Technology, New Delhi.

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