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REGULATION OF GLUCOCORTICOID RECEPTORS IN THE KIDNEY OF IMMATURE AND MATURE MALE RATS

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Abstract—1. Specific binding of [^{3}H]dexamethasone to cytosol and the activation of bound hormone–receptor complexes were studied in the kidney of immature (3-week) and mature (26-week) Long–Evans male rats.

2. The concentration of specific binding sites was significantly higher (25%) in the kidney of immature rats as compared with mature, while dissociation constants (K_d) remain unaltered at both ages.

3. Heat activation (25°C for 45 min) significantly enhanced the binding of [^{3}H]dexamethasone–receptor complexes to DNA-cellulose and purified nuclei at both ages to the same extent. Cross-mixing experiments (i.e. binding of activated cytosol from mature rats to nuclei of immature and vice versa) gave similar results to the non-mixed groups.

4. Ca^{2+} activation (0°C for 45 min with 20 mM Ca^{2+}) also enhanced the nuclear and DNA-cellulose binding at both ages but to a greater magnitude in immature rats.

5. Differences in the number of specific binding sites and some of the physicochemical properties of kidney glucocorticoid receptors presented here between immature and mature rats may underlie the functional changes in tissue response with age.

INTRODUCTION

Glucocorticoids have a multitude of effects on a variety of animal tissues including kidney where they influence glomerular filtration rate, ion-transport and other metabolic functions. High affinity glucocorticoid receptors have been demonstrated in rat kidney (Funder *et al.*, 1973; Fanestil and Park, 1981). Following entry into the target cells, glucocorticoids interact with intracellular receptors to form complexes which subsequently undergo activation and translocate to the nucleus, where they interact with specific acceptor sites on chromatin and modulate gene expression (Yamamoto, 1985). This nuclear binding capacity can be achieved *in vitro* by incubating the hormone–receptor complexes at 25°C (Kalimi *et al.*, 1973; Milgrom *et al.*, 1973) and under high ionic conditions (Higgins *et al.*, 1973; Bailly *et al.*, 1978); this action is achieved possibly by exposure of positively charged amino acid residues on the surface of the receptor molecule (Milgrom *et al.*, 1973; Disorbo *et al.*, 1980) and this exposure, in turn enhances the affinity for nuclei and polyanions such as DNA and DNA-cellulose (Milgrom *et al.*, 1973; Kalimi *et al.*, 1975). The activation of the glucocorticoid receptor occurs *in vivo* under physiological conditions and is thought to be rate limiting for nuclear binding (Munck and Foley, 1979; Markovic and Litwack, 1980; Miyabe and Harrison, 1983).

Adaptive responsiveness to hormones are age-related phenomena as are changes in induction of many enzymes (Kanungo, 1980). These hormone-mediated responses are controlled by binding of the hormone to specific intracellular receptors, by activation of hormone–receptor complexes and translocation of these complexes to nuclear acceptor sites. Age-related changes in glucocorticoid receptor binding sites have been reported in most animal tissues, the liver being more extensively studied (Kalimi, 1984). Information on age-related changes in renal glucocorticoid receptors are very limited. Kalimi (1983) reported no age-related change in the receptor binding sites in kidney of male Sprague–Dawley rats. Recent literature suggests change in the physicochemical properties of glucocorticoid receptors from rat liver (Kalimi, 1984; Sharma and Timiras, 1987b) and skeletal muscle (Sharma and Timiras, 1987a). In the present paper we report a significantly higher number of specific binding sites using glass fibre filter assay and certain changes in the activation of [^{3}H]dexamethasone–receptor complexes in the kidney of immature (weanling) Long–Evans male rats as compared to mature rats.

MATERIAL AND METHODS

Animals

Immature (3-week old, just weaned) and mature (26-week old, adult) Long–Evans male rats, maintained at 24 ± 2°C on a 12/12 light/dark period, were fed Purina rat chow pellets and water *ad libitum*. The animals were bilaterally adrenalectomized and were given 0.9% NaCl instead of water for 3 days following adrenalectomy.

Chemicals

All the chemicals used were of analytical grade, and biochemicals were purchased from Sigma Chemical Co., U.S.A. [1,2,4,6,7-^{3}H]Dexamethasone (sp. act. 78.7 Ci/mmol)

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was obtained from Amersham with radiochemical purity of 96.1% by HPLC. Non-radioactive dexamethasone was purchased from Sigma. Whatman glass microfibre filters (GF/A) were obtained from Fisher Scientific Co., U.S.A. Complete counting cocktail (3a 70B) was purchased from Research Products International Corporation, IL, U.S.A. DNA-cellulose (1.9 mg of native calf thymus DNA/ml of cellulose) was from Pharmacia Molecular Biology Division.

Tissue and cytosol preparation

The rats were killed by decapitation at a fixed time of day (1100 hr) to avoid any possible circadian variability and kidneys were quickly removed, washed in ice-cold normal saline and freed of fat and connective tissues. About 2 g of pooled kidney samples were minced with scissors and homogenized in 4 vol. (w/v) of TEGBN040 buffer [10 mM Tris–HCl, (pH 8.1)–1 mM Na_2EDTA–10% glycerol–1 mM 2-mercaptoethanol–100 μg of crystalline bovine serum albumin/ml–200 μM phenylmethylsulfonyl fluoride–40 mM NaCl] at 0°C using a Teflon homogenizer. The homogenates were centrifuged at 105,000 ***g*** for 60 min at 0°C in a Beckman L3-40 ultracentrifuge. The clear fat-free cytosols were removed and used for receptor assay.

Glucocorticoid binding assay

Clear cytosols (100 μl) were incubated at 0°C for 2 hr with 1–80 nM [^{3}H]dexamethasone alone or with 1000-fold excess of non-radioactive dexamethasone. Each assay was done in triplicate. Saturation of specific binding occurred during this time. Following incubation, the entire reaction mixture was spotted onto dry 2.4-cm glass microfibre filter (GF/A) (Cousens and Eskin, 1982; Goldfeld *et al.*, 1983). After 10 min of incubation at room temperature, filters were washed 3 times (15 min each) in 20 ml of NET buffer [10 mM Tris–HCl (pH 8.1)–1 mM Na_2EDTA–40 mM NaCl] per filter at 0–4°C with continuous shaking. Excess liquid was removed from each filter by keeping briefly under heat lamp on aluminum foil. Radioactivity in the dried filter was counted in a complete counting cocktail (3a 70B) using Beckman LS-100C liquid scintillation counter with efficiency of 51.5% for tritium. The filter assay takes advantage of the strong affinity for the glucocorticoid receptors to the glass fibre filter. The background of free [^{3}H]dexamethasone binding to the filters is approx. 0.05% of the added radioactivity. Specific saturable binding was calculated by subtracting the radioactivity bound in the presence of the labeled hormone alone. The method of Scatchard (1949) was employed to determine the number and affinity of specific dexamethasone binding sites.

Preparation of glucocorticoid–receptor complexes

Pooled kidney samples were minced and homogenized in 4 vol. (w/v) of TS buffer (10 mM Tris–HCl–0.25 M sucrose (pH 7.6)] at 0°C. The homogenates were centrifuged for 10 min at 2000 ***g*** at 0°C in a Sorvall SS 34 rotor to sediment nuclei. The resulting supernatant was further centrifuged at 105,000 ***g*** for 60 min at 0°C in a Beckman L3-40 ultracentrifuge. The clear fat-free cytosol was incubated for 2 hr at 0°C with 40 nM [^{3}H]dexamethasone alone or 1000-fold excess of non-radioactive dexamethasone. Free hormones were removed by adding dextran coated charcoal (3% charcoal, 0.3% dextran T-70 in TS buffer). After incubation at 0°C for 10 min, the contents were centrifuged at 2000 ***g*** for 5 min at 0°C. The clear supernatants were used for DNA-cellulose and nuclear binding assays.

DNA-cellulose binding assay

The binding of [^{3}H]dexamethasone–receptor complexes to DNA-cellulose was determined (Kalimi *et al.*, 1975) with slight modification. Briefly, 200 μl aliquots of hormone–receptor complexes were added in duplicate to washed pellet of DNA-cellulose (100 μg DNA) and incubated at 0°C for 1 hr. The pellets were gently mixed on a vortex machine at 10–15-min intervals. The reaction was stopped by addition of 1.0 ml cold TS buffer and centrifuged at 2000 ***g*** for 10 min at 0°C. The samples were then washed twice in cold TS and finally suspended in 0.5 ml of TS and transferred to vials containing 4.0 ml of complete counting cocktail. The radioactivity was counted as described above.

Nuclear binding assay

The crude nuclear pellet was dissolved in 5 vol. of TS buffer, filtered through a double layered cheesecloth and centrifuged at 2000 ***g*** for 10 min at 0°C. Nuclei were further purified using 1.8 M sucrose in 10 mM Tris–HCl buffer (pH 7.6) containing 25 mM KCl and 3 mM $MgCl_2$ as previously described (Beato *et al.*, 1969; Kalimi and Gupta, 1982). The nuclei were washed and resuspended in TS buffer. Aliquots of the nuclear suspension containing 100 μg of DNA were centrifuged at 2000 ***g*** for 10 min at 0°C and the supernatant fractions were discarded. [^{3}H]Dexamethasone-labeled cytosol (200 μl) was added in duplicate to above nuclear pellets. The samples were gently mixed on a Vortex machine and incubated at 0°C for 1 hr. At the end of the incubation period, 1.0 ml of cold TS buffer was added and the suspension gently mixed on a Vortex machine. The nuclei were pelleted and washed twice in TS buffer. The final nuclear pellets were resuspended in 0.5 ml of TS buffer and radioactivity measured in a liquid scintillation counter as described earlier.

Determination of protein and DNA

Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albimun as standard. DNA content was measured by the method of Burton (1956). All the data were statistically analyzed (Garrett, 1966). The level of significance (P) between two sets of data was calculated according to Student's t-test.

RESULTS

Specific binding of [^{3}H]dexamethasone to the kidney cytosol of immature and mature rats

The specific binding of [^{3}H]dexamethasone to kidney cytosols was measured at increasing concentrations (0.1–8 $\times$ 10^{-8} M) of steroid at 0°C for 2 hr. Saturation of specific binding occurred within 2 hr at 4×10^{-8} M [^{3}H]dexamethasone in the kidney of rats of both ages. The values were plotted for Scatchard analysis. Our data (Table 1 and Fig. 1) on Scatchard binding plot for [^{3}H]dexamethasone show that the concentration of specific binding sites is significantly higher (25%) in the kidney of immature as compared with mature rats. From the slope of the linear regression curves the apparent dissociation constants (K_d) were found to be similar in rats of both ages.

Specific binding of kidney [^{3}H]dexamethasone–receptor complexes to DNA-cellulose

In the present experiment, we have compared the *in vitro* binding of glucocorticoid–receptor complexes to DNA-cellulose. Our results (Fig. 2) show that heat activation (25°C for 45 min) significantly enhances the DNA-cellulose binding of steroid–receptor complexes in rats of both the ages to a similar extent. The results indicate no marked age-related difference in the *in vitro* thermal activation of kidney glucocorticoid receptors. DNA-cellulose binding of hormone–receptor complexes is almost similar at 0°C at both ages. Ca^{2+} activates at 0°C the binding of both immature and mature hormone–receptor complexes to DNA-cellulose. The extent of Ca^{2+} activated

Table 1. Concentration of cytosolic [^{3}H]dexamethasone receptors in the kidney of immature (3-) and mature (26-week) male rats

Age (weeks)	Specific [^{3}H]dexamethasone binding sites (fmol/mg protein)	K_d (nM)
3	107.13 ± 3.85*	5.45 ± 0.44
26	85.39 ± 2.41	6.12 ± 0.34

The data were collected from 4–5 rats of each age group. The results are mean ± SD of three separate assays for each age group.
*Statistically significant ($P < 0.01$) with respect to 26-week rats.

DNA-cellulose binding is significantly higher (113%) in immature as compared with mature rats.

Specific binding of kidney [^{3}H]dexamethasone–receptor complexes to purified nuclei

Experiments using DNA-cellulose binding ability as a measure of the number of receptors activated could not unequivocally implicate age-related differences in the activation of glucocorticoid receptors. Therefore, purified kidney nuclei were substituted in place of DNA-cellulose to provide a more physiological assay system than the DNA-cellulose binding measurements. The results (Fig. 3) were almost similar to those obtained with DNA-cellulose. The heat activation significantly enhances the nuclear binding of steroid–receptor complexes in rats at both ages to a similar extent. Nuclear binding of hormone–receptor complexes at 0°C is also similar at both ages. In cross-mixing experiments, we studied the binding of heat-activated hormone–receptor complexes of mature rats to nuclei of immature and vice versa to identify any age-related changes in nuclear translocation of [^{3}H]dexamethasone–receptor complexes. Our data show that both mixed groups responded to heat-activation as did the non-mixed group (Fig. 3), indicating no age-specificity of nuclei in binding of heat-activated hormone–receptor complexes. In addition, Ca^{2+} activation significantly enhances the nuclear binding of [^{3}H]dexamethasone–receptor complexes in the kidney of rats of both the ages with a greater magnitude (55%) in immature as compared with mature rats. This low-temperature-dependent activation of glucocorticoid receptors with Ca^{2+} is more pronounced using DNA-cellulose than isolated nuclei. These results indicate that some of the physicochemical properties such as heat activation of kidney glucocorticoid receptor remain unchanged, while others, like Ca^{2+} activation, changed at these ages of the lifespan of rats.

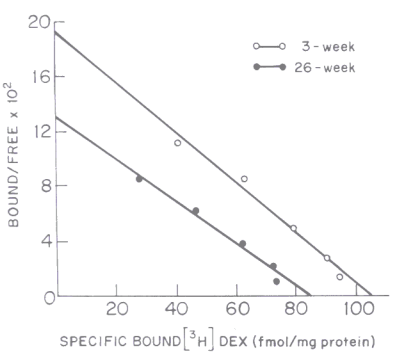

Fig. 1. Scatchard plot of the *in vitro* specific binding of [^{3}H]dexamethasone to kidney cytosol of immature (○) and mature (●) male rats. Ratio of bound to free (B/F) hormone concentrations is depicted as a function of bound receptors/mg protein. Curves represent the mean values for three separate assays. Slopes and abscissa intercepts were determined by the method of linear regression analysis.

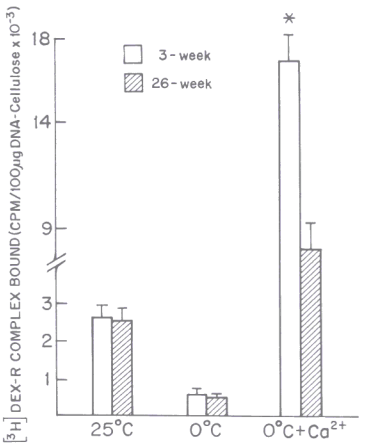

Fig. 2. Specific binding of kidney [^{3}H]dexamethasone–receptor complexes to DNA-cellulose in immature (3-) and mature (26-week) male rats. Cytosols were incubated with 40 nM [^{3}H]dexamethasone in presence or absence of 1000-fold excess of unlabeled dexamethasone for 2 hr at 0°C. The cytosol were further incubated at: (i) 25°C for 45 min; (ii) 0°C for 45 min; and (iii) 0°C with 20 mM Ca^{2+} for 45 min. Following incubation, specific DNA-cellulose binding was determined in duplicate as mentioned in Materials and Methods. The results are mean ± SD for three separate experiments with 4–5 rats of each age group. *Statistically significant ($P < 0.01$) with respect to 26-week rats.

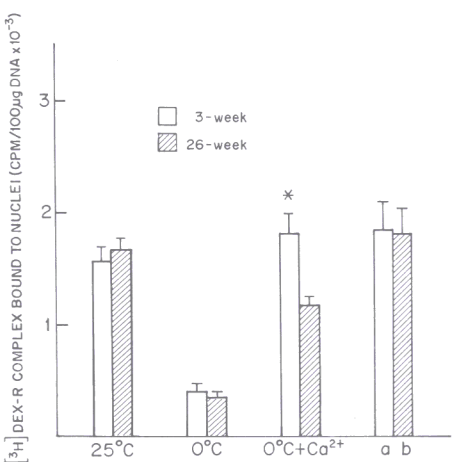

Fig. 3. Specific binding of kidney [^{3}H]dexamethasone–receptor complexes to purified nuclei in immature (3-) and mature (26-week) male rats. Experimental procedures are same as for Fig. 2. *Statistically significant ($P < 0.01$) with respect to 26-week rats. The (a), (b) barogram represents mixing experiments in which 25°C activated cytosol of 26-week rats incubated with the nuclei of 3-week rats (a) and 25°C activated cytosol of 3-week rats with the nuclei of 26-week rats (b).

DISCUSSION

Glucocorticoids are involved not only in the metabolic functions of various animal tissues but also in cellular growth and differentiation (Cake and Litwack, 1976). Development and aging of animals may partly be characterized by changes in the responsiveness of tissue and cells to certain hormonal modulators (Roth and Hess, 1982). The occurrence of quantitative changes in receptor molecules is well demonstrated (Roth and Hess, 1982). The possibility that qualitative changes occur as well is still uncertain. Our data clearly point out the presence of such qualitative changes. Indeed, in addition to significantly higher glucocorticoid receptor binding sites in the kidney of immature rats as compared to mature, we observed a change in the low-temperature-dependent activation of glucocorticoid–receptor complexes by Ca^{2+} at these two ages of the lifespan of rats.

The higher levels of receptor protein in the kidney of immature rats may be a contributing factor for the role of glucocorticoid in the development and maturation of the kidney during early ages. The higher level of receptors in the kidney may also be correlated with weaning and change in diet resulting in a different metabolic status at this phase of the lifespan. Our results are in agreement with the report (Kalimi, 1983) that there is no apparent age-related change in the receptor dissociation constant.

A very important aspect of the steroid-induced alterations in gene transcription is the mechanism(s) by which the steroid–receptor complex interacts with nuclear acceptor sites. The activation of steroid–receptor complexes has been described as a not-well-defined, conformational change which enables the complexes to interact with specific acceptor sites on chromatin and modulate gene expression (Yamamoto, 1985). We have studied the activation of glucocorticoid–receptor complexes using DNA-cellulose and purified nuclei from the kidney of both immature and mature rats to determine the physicochemical changes in receptor molecule at activation and nuclear translocation levels. We observed that thermal activation of kidney glucocorticoid–receptor complexes is almost similar at both ages. These findings agree with the earlier report (Kalimi, 1983) of no age-related difference in the thermal activation of glucocorticoid receptors in the kidney of male Sprague–Dawley rats. Interestingly, rat skeletal muscle glucocorticoid–receptor complexes do not show age-related differences in heat activation (Sharma and Timiras, 1987a). Recently, we have observed a greater heat activation of [^{3}H]-dexamethasone–receptor complexes in the liver of mature rats as compared to immature (Sharma and Timiras, 1987b). Our findings indicate tissue-specific changes in the physicochemical properties of glucocorticoid receptors. On the other hand, low-temperature-dependent activation of hormone–receptor complexes by Ca^{2+} is more pronounced in the kidney of immature rats as compared with mature. We have earlier reported (Sharma and Timiras, 1987a) that Ca^{2+}-dependent low-temperature activation of rat skeletal muscle glucocorticoid–receptor decreases as a function of age. In contrast, the low-temperature-dependent activation of hepatic glucocorticoid–receptor by Ca^{2+} does not show any change at these ages (Sharma and Timiras, 1987b). This differential activation of glucocorticoid receptor in different tissues of rats supports the concept of receptor polymorphism and evokes different glucocorticoid responses in different tissues (Webb *et al.*, 1985). The exact mechanisms of low-temperature Ca^{2+} activation of the glucocorticoid–receptor complexes are not well understood. However, Ca^{2+} enhancement of nuclear and DNA-cellulose binding may be due to direct interaction of Ca^{2+} with the receptor molecule and/or receptor transforming factor(s). This interaction could cause a conformational change capable of exposing the DNA- and chromatin-binding domain (Groday *et al.*, 1982). Low-temperature-dependent activation of glucocorticoid–receptor complexes with Ca^{2+} was more pronounced using DNA-cellulose than purified nuclei. This may be due to the open DNA binding sites in DNA-cellulose compared with intact nuclei.

In conclusion, our findings indicate that glucocorticoid receptor level and some of its physicochemical properties differ in the kidney of mature and immature rats. The observed differences in glucocorticoid receptors may lead to functional changes in the tissue response as a function of age.

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