Biochimica et Biophysica Acta 930 (1987) 237–243
Elsevier

BBA 12097

Age-dependent regulation of glucocorticoid receptors in the liver of male rats

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(Received 20 March 1987)
(Revised manuscript received 29 May 1987)

Key words: Glucocorticoid receptor; Aging; (Rat liver)

Specific binding of [^{3}H]dexamethasone to cytosol and the activation of bound hormone-receptor complexes were studied in the liver of immature (3 weeks old) and mature (26 weeks old) Long-Evans male rats. The concentration of specific binding sites was significantly higher (33%) in the liver of immature rats as compared to mature, while dissociation constants (K_d) remain unaltered at both ages. Heat activation (for 45 min at 25 °C) significantly enhances the binding of [^{3}H]dexamethasone-receptor complexes to DNA-cellulose and purified nuclei at both the ages, with a greater magnitude in mature rats. Cross mixing experiments (i.e., binding of activated cytosol from mature rats to nuclei of immature and vice-versa) show receptor specificity. Ca^{2+} activation (20 mM Ca^{2+} for 45 min at 0 °C) also enhances the nuclear and DNA-cellulose binding at both the ages, but to a similar extent. Differences in the number of specific binding sites and some of the physicochemical properties of glucocorticoid receptors presented here between immature and mature rats may underlie the functional changes in tissue response with age.

Introduction

Glucocorticoids exert their multitude of effects on a variety of cellular and metabolic processes. Upon entry into the target cells, they 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 [1]. This nuclear binding capacity can be achieved in vitro at an elevated temperature [2,3] and under high ionic conditions [4,5] possibly by exposing positively charged amino acid residues on the surface of the receptor molecule [2,6], which in turn enhances the affinity for nuclei and polyanions such as DNA and DNA-cellulose [2,7]. This phenomenon occurs in vivo under physiological conditions and is thought to be rate limiting for nuclear binding [8–10].

Alterations in the adaptive responsiveness to hormones are age-related as are changes in the induction of many enzymes [11]. These hormone-mediated responses are controlled by binding to specific intracellular receptors, by activation of hormone-receptor complexes and translocation of these complexes to nuclear acceptor sites. There are many variable reports on age-related changes in this steroid receptor binding sites in most of the animal tissues [12]. The apparent variability may be due to the age, sex, species/strain, environmental and physiological conditions (intact vs. adrenalectomized) and the use of synthetic vs. naturally occurring ligands for binding assays. Recent literature suggests changes in the

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physicochemical properties of hepatic glucocorticoid receptors as a function of age [12]. Roth [13] noted an increase in the number of glucocorticoid binding sites in the liver of 12-months compared to 3-month-old male Sprague-Dawley rats. In contrast, Kalimi [14] reported no significant age-related changes in the receptor binding sites in the same tissue of male Sprague-Dawley rats. Weaning is accompanied by physiological changes which permit the animals to pass from essentially maternal environment to a more independent state. Adjustments (to diet, sexual maturity and behavior) are necessary for continuing growth and changes in glucocorticoids, the major adaptive hormones, play a key role in endowing the maturing individual with the ability to survive. While many studies have focused on the two ends of the lifespan, early preweaning development and late ages, in the present study we have compared glucocorticoid receptor concentration and physicochemical properties at weaning and in adulthood. 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 liver of immature (weanling) Long-Evans male rats as compared to mature rats.

Materials and Methods

Animals. Immature (3-week-old, just weaned) and mature (26-week-old, adult) Long-Evans male rats, maintained at $24 \pm 2°C$ on a 12/12 light/dark period, were fed Purina 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 (spec. act. 78.7 Ci/mmol) was obtained from Amersham with radiochemical purity of 96.1% by high-pressure liquid chromatography. 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, 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 h) to avoid any possible circadian variability and livers were quickly removed, washed in ice-cold normal saline and freed of fat and connective tissues. About 2 g of pooled liver samples were minced with scissors and homogenized in 4 vols. (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 $105\,000 \times g$ for 60 min at 0°C in a Beckman L3-40 ultracentrifuge. The clear fat-free cytosol were removed and used for receptor assay.

Glucocorticoid binding assay. Clear cytosols (100 μl) were incubated at 0°C for 2 h with 1–80 nM [^{3}H]dexamethasone alone or with a 1000-fold excess of non-radioactive dexamethasone. Each assay was performed 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) [15,16]. After 10 min of incubation at room temperature, filters were washed three 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 a heat lamp on aluminum foil. Radioactivity in the dried filter was counted in a complete counting cocktail (3a70B) using a Beckman LS-100C liquid scintillation counter with efficiency of 51.5% for tritium. The binding reaction described in this paper, using glass-fibre filter rather than charcoal assay, takes advantage of the strong affinity of the glucocorticoid receptors to the glass fibre filter [17,18]. The background of free [^{3}H]dexamethasone binding to the filters is approx. 0.05% of the added radioactivity. This indicates that the use of glass-fibre filter also exploits the complete removal of nonbound hormone. Specific saturable binding was calculated by subtracting the radioactivity bound in the presence of a 1000-fold excess of the unlabeled

dexamethasone from that found in the presence of the labeled hormone alone. The method of Scatchard [19] was employed to determine the number and affinity of specific dexamethasone binding sites.

Preparation of glucocorticoid-receptor complexes. Pooled liver samples were minced and homogenized in 4 vols. (w/v) of Tris-sucrose buffer (10 mM Tris-HCl/0.25 M Sucrose, pH 7.6) at 0 °C. This buffer was used instead of TEGBN040 to avoid any effect of salt and other ions on the activation of glucocorticoid receptors. 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 h at 0 °C with 40 nM [^{3}H]dexamethasone alone or with a 1000-fold excess of non-radioactive dexamethasone. Free hormones were removed by adding dextran coated charcoal (3% charcoal, 0.3% dextran T-70 in Tris-sucrose 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 [7] 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 h. 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 Tris-sucrose buffer and centrifuged at 2000 × *g* for 10 min at 0 °C. The samples were then washed twice in cold Tris-sucrose buffer and finally suspended in 0.5 ml of Tris-sucrose buffer 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 vols. of Tris-sucrose buffer, filtered through a double-layered cheese cloth 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 [20,21]. The nuclei were washed and resuspended in Tris-sucrose 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 h. At the end of the incubation period, 1.0 ml of cold Tris-sucrose buffer was added and the suspension gently mixed on a Vortex machine. The nuclei were pelleted and washed twice in Tris-sucrose buffer. The final nuclear pellets were resuspended in 0.5 ml of Tris-sucrose buffer and radioactivity was measured in a liquid scintillation counter as described earlier.

Estimation of protein and DNA. Protein content was determined by the method of Lowry et al. [22] using bovine serum albumin as standard. DNA content was measured by the method of Burton [23]. All the data were statistically analyzed [24]. 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 liver cytosols of immature and mature rats

The specific binding of [^{3}H]dexamethasone to liver cytosols was measured with increasing concentrations ($0.1–8 \cdot 10^{-8}$ M) of steroid at 0 °C for 2 h. Saturation of specific binding occurred within 2 h at $6 \cdot 10^{-8}$ M [^{3}H]dexamethasone in the liver of rats of both the ages. The values were plotted for Scatchard analysis. Our data (Table I) on

TABLE I

CONCENTRATION OF CYTOSOLIC [^{3}H]DEXAMETHASONE RECEPTORS IN THE LIVER OF IMMATURE (3 WEEKS) AND MATURE (26 WEEKS) MALE RATS

The data were collected from four to five rats of each age group. The results are mean ± S.D. of three separate assays for each age group.

* Statistically significant ($P < 0.01$).

Age (weeks)	Specific [^{3}H]dexamethasone binding sites (fmol/mg protein)	K_d ($\times 10^{-8}$ M)
3	237.41 ± 11.25 *	1.33 ± 0.065
26	177.50 ± 9.61	1.41 ± 0.035

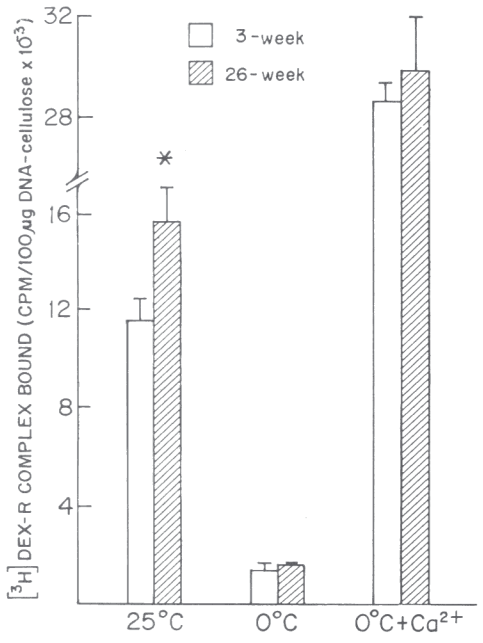

Fig. 1. Specific binding of liver [^{3}H]dexamethasone-receptor complexes to DNA-cellulose in immature (3-weeks old) and mature (26-weeks old) male rats. Cytosols were incubated with 40 nM [^{3}H]dexamethasone in presence or absence of a 1000-fold excess of unlabeled dexamethasone for 2 h at 0 °C. The cytosols were further incubated at: (i) 25 °C for 45 min; (ii) at 0 °C for 45 min; and (iii) at 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 ± S.D. for three separate experiments with four or five rats of each age group. * Statistically significant ($P < 0.05$) with respect to the 3-week-old group.

Scatchard binding plot for [^{3}H]dexamethasone show that the concentration of specific binding sites is significantly higher (33%) in the liver of immature as compared to 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 the ages.

Specific binding of liver [^{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. 1) show that heat activation (for 45 min at 25 °C) significantly enhances the DNA-cellulose binding of steroid-receptor complexes from both the ages with a greater magnitude in mature (34%) rats as compared to immature. DNA-cellulose binding of hormone-receptor complexes is almost similar at 0 °C at both ages. Ca^{2+} activates (17 to 18-fold) at 0 °C the binding of both immature and mature hormone-receptor complexes to DNA-cellulose to a similar degree. The results indicate a marked age-related difference in the in vitro thermal activation of liver glucocorticoid receptors.

Specific binding of liver [^{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 liver nuclei were substituted in place of DNA-cellulose to provide a more physiological assay system than the DNA-cellulose binding measurements. The results (Fig. 2) were similar to those obtained with DNA-cellulose. The heat activation significantly

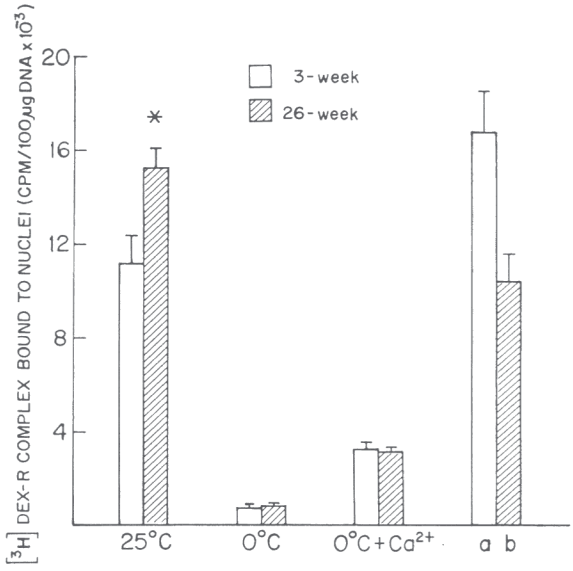

Fig. 2. Specific binding of liver [^{3}H]dexamethasone-receptor complexes to purified nuclei in immature (3-weeks old) and mature (26-weeks old) male rats. Experimental procedures are as those described in Fig. 2. * Statistically significant ($P < 0.05$) with respect to the 3-week-old group. The a, b bar represents cross-mixing experiments in which 25 °C activated cytosol of 26-week-old rats incubated with the nuclei of 3-week-old rats (a) and 25 °C activated cytosol of 3-week-old rats with the nuclei of 26-week-old rats (b).

enhances the nuclear binding of steroid-receptor complexes in rats at both ages with a greater magnitude in mature (36%) than in immature rats. Nuclear binding of hormone-receptor complexes at 0 °C is 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 (Fig. 2) do not show any age-related changes of nuclei in translocation of heat activated hormone-receptor complexes. Thus, the age-related changes we observed in the binding of hormone-receptor complexes to nuclei are due to changes in receptor specificity at these phases of the lifespan. In addition, Ca^{2+} activation significantly enhances the nuclear binding of [^{3}H]dexamethasone-receptor complexes in the liver of rats to the same degree at both ages (Fig. 2). This low-temperature-dependent activation of glucocorticoid receptors with Ca^{2+} is more pronounced using DNA-cellulose (17 to 18-fold) than isolated nuclei (3.5 to 3.7-fold). Our results indicate that some of the physicochemical properties such as heat activation of glucocorticoid receptor change, while others, like Ca^{2+} activation, remain unchanged at these ages of the lifespan of rats.

Discussion

Glucocorticoids are involved not only in the metabolic functions of various animal tissues but also in cellular growth and differentiation [25]. Development and aging of animals may partly be characterized by changes in the responsiveness of tissue and cells to certain hormonal modulators [26]. The occurrence of quantitative changes in receptor molecules is well demonstrated [26]. 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 liver of immature rats as compared to mature, we observed a change in the thermal activation of glucocorticoid-receptor complexes at these two phases of the lifespan of rats.

The higher levels of receptor protein in the liver of immature rats may be a contributing factor for the role of glucocorticoid in the development and growth of the rats during early phases of the life span. The higher level of receptors in the liver may also be correlated with weaning and changes in diet resulting in a different metabolic status at this phase of the lifespan. Our findings are consistent with the earlier reports [27] of greater binding of [^{3}H]cortisol in the liver cytosol of weaning rats compared to mature. The higher level of [^{3}H]dexamethasone receptors has been reported in vivo in the liver cytosol of weaned rats [28]. The magnitude of the induction of rat liver cytosolic aspartate aminotransferase and phospho*enol*pyruvate carboxykinase by hydrocortisone is maximum at the age of 6 weeks and decreases by 20–30% at the age of 30 weeks [29,30]. This decrease in the inductibility of enzymes by hydrocortisone may be correlated with the decrease in the glucocorticoid receptors. Our results are in agreement with the reports [14,21] that there is no apparent age-related change in the receptor dissociation constant (K_d). Although Roth [13] reported an increase in the number of glucocorticoid binding sites in the liver of 12-month-old Sprague-Dawley rats compared to 3-month-old, Kalimi [14] reported no change in the receptor concentration at these ages in the same strain. Our findings of a greater number of binding sites for glucocorticoid receptors at the weanling stage in Long-Evans rats may be species specific.

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 ill-defined, conformational change which enables the complexes to interact with specific acceptor sites on chromatin and modulate gene expression [1]. We have studied the activation of glucocorticoid-receptor complexes using DNA-cellulose and purified nuclei from the liver 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 glucocorticoid receptor complexes is more pronounced in mature rats as compared to immature. It has been reported earlier that the binding of heat-activated

[^{3}H]dexamethasone-receptor complexes to nuclei was almost similar comparing the liver of neonatal to adult and of adult to aged Sprague-Dawley rats [14,21]. Although the number of binding sites decrease, the greater activation of hormone-receptor complexes in mature rats may compensate for the low receptor level and support for the role of glucocorticoids at this phase of the lifespan. These maturational changes in activation of glucocorticoid receptors may be species/strain specific in rodents. The observed age-related changes in activation of hepatic glucocorticoid receptors may be due to the differences in the endogenous modulators and/or translocation inhibitor such as pyridoxal phosphate [31]. Interestingly, rat skeletal muscle glucocorticoid-receptor complexes do not show age-related differences in heat activation [18]. Our findings indicate tissue-specific changes in the physicochemical properties of glucocorticoid receptors. Our cross-mixing experiments do not show any changes in nuclei at these two ages, with respect to binding of heat-activated hormone-receptor complexes. This finding is consistent with the report of no appreciable differences in the liver nuclei for binding of glucocorticoid-receptor complexes in rats of 1.5 month and 6 months. In contrast, efficiency of liver nuclei to bind estrogen- and progesterone-receptor complexes declines gradually as a function of age [32]. The decrease in efficiency of nuclei to bind estradiol-receptor complexes has also been reported in aged rat uteri [33,34]. This decrease in nuclear efficiency is correlated with the reduction in nuclear acceptor sites and/or changes in chromatin structure in aged rats. In addition, low-temperature-dependent activation of hormone-receptor complexes by Ca^{2+} remains unchanged at both the ages studied. The exact mechanisms of this 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 a 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 [35]. The low-temperature-dependent activation of glucocorticoid-receptor complexes with Ca^{2+} was more pronounced using DNA-cellulose than that of purified nuclei. This may be due to the open DNA binding sites in DNA-cellulose compared to intact nuclei.

Our findings indicate that the glucocorticoid receptor level and some of its physicochemical properties differ at these phases of the lifespan of rats. The observed differences in glucocorticoid receptors may lead to functional changes in the tissue response as a function of age.

Acknowledgements

R.S. offers thanks to the Ministry of Human Resources and Development, Government of India for a National Scholarship, and the University of Manipur for the study leave.

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