

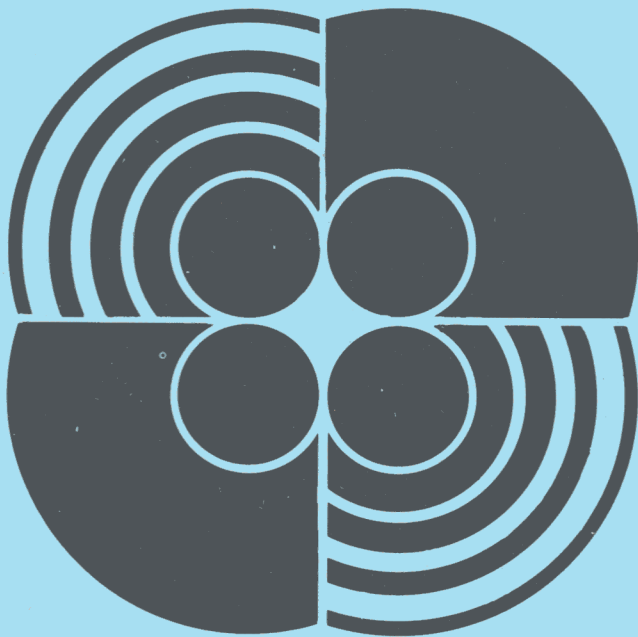
**REGULATORY CHANGES IN GLUCOCORTICOID RECEPTORS IN THE SKELETAL MUSCLE OF IMMATURE AND MATURE MALE RATS**

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**mechanisms of ageing and development**

## REGULATORY CHANGES IN GLUCOCORTICOID RECEPTORS IN THE SKELETAL MUSCLE OF IMMATURE AND MATURE MALE RATS

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### SUMMARY

Specific binding of [<sup>3</sup>H]dexamethasone to cytosol and translocation of bound receptor complexes to purified nuclei were studied in the skeletal muscle of immature (3-week) and mature (26-week) Long-Evans male rats. A marked decrease (57%) in the specific binding sites with no apparent change in dissociation constant ( $K_d$ ) was observed in the skeletal muscle of mature rats compared to immature. Heat activation (25°C for 45 min) significantly enhances the nuclear binding of steroid-receptor complexes in the skeletal muscle of rats of both the ages at almost similar level. Cross-mixing experiments (i.e. binding of activated cytosol from mature rats to nuclei of immature and *vice-versa*) gave similar values. Interestingly, Ca<sup>2+</sup>-activated (0°C for 45 min with 20 mM Ca<sup>2+</sup>) nuclear translocation was significantly higher (27%) in the skeletal muscle of immature rats compared to mature. Our results indicate that glucocorticoid receptor level and some physicochemical properties change with age in the skeletal muscle of rats.

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*Key words:* Glucocorticoid receptors; Skeletal muscle; Aging

### INTRODUCTION

Glucocorticoids exert their effects on a variety of cellular and metabolic processes of animal tissues. In liver, they stimulate enzymes and increase protein and glycogen content. In many tissues, including muscle, their catabolic actions decrease synthesis and increase degradation of protein and RNA [1]. However, knowledge of the molecular

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mechanisms of these catabolic effects on muscle tissue is still limited. Many reports have demonstrated that skeletal muscle from rats contains glucocorticoid receptor proteins [2–4]. Alterations in the adaptive responsiveness to hormonal and other biochemical stimuli are age-related [5] as are changes in the induction of many enzymes [6–8]. These hormone-mediated responses are controlled partly by its binding to specific intracellular receptors and then by translocation of hormone-receptor complexes to nuclear acceptor sites [9]. All of the molecular events in hormone actions subsequent to receptor binding are subject to alteration with age [5]. There are many variable reports on age-related changes in this steroid receptor binding sites in most of the tissues, liver being more extensively studied [10]. Recent literature suggests many changes in the physicochemical properties of hepatic glucocorticoid receptors as a function of age [10]. Roth [3] noted a decrease in the number of glucocorticoid binding sites in the skeletal muscle of aging male Sprague–Dawley rats. In contrast, Kalimi [11] reported no significant age-related changes in receptor binding sites in the same tissue of Sprague–Dawley rats. In the present paper, we report a significant age-related decrease in the specific cytosolic binding sites, using a glass fibre filter assay, and certain changes in the nuclear translocation of [<sup>3</sup>H]dexamethasone-receptor complexes in the skeletal muscle of reproductively mature Long-Evans male rats as compared to immature rats.

#### MATERIALS AND METHODS

##### *Animals*

Immature (3-week) and mature (26-week) Long-Evans male rats, maintained at  $24 \pm 2^\circ\text{C}$  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., USA. [1,2,4,6,7-<sup>3</sup>H]dexamethasone (spec. act. 82.4 Ci/mmol) was obtained from Amersham with radiochemical purity of 95% by HPLC. Non-radioactive dexamethasone was purchased from Sigma. Whatman glass microfibre filters (GF/A) were obtained from Fisher Scientific Co., USA. Complete counting cocktail (3a 70B) was purchased from Research Products International Corporation, Illinois.

##### *Tissue and cytosol preparation*

The rats were killed by decapitation at a fixed time of day (1100 h) and leg muscles (gastrocnemius) were quickly removed, washed in ice-cold normal saline and freed of fat and connective tissue. About 2 g of pooled muscle 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<sub>2</sub>EDTA/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 an

Ultra-Turrax blender for  $2 \times 15$  s at 50 V. The homogenates were centrifuged at 105 000  $g$  for 60 min at  $0^\circ\text{C}$  in a Beckman L5-65 ultracentrifuge (Type 40.3 rotor). The clear fat-free cytosol was removed and used for receptor assay.

#### *Glucocorticoid binding assay*

Clear cytosols (100  $\mu\text{l}$ ) were incubated at  $0^\circ\text{C}$  for 2 h with 0.5–80 nM [ $^3\text{H}$ ]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) [12,13]. 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  $\text{Na}_2\text{EDTA}$ /40 mM NaCl) per filter at  $0$ – $4^\circ\text{C}$  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 (3a70B) 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 [ $^3\text{H}$ ]dexamethasone binding to the filters is approximately 0.05% of the added radioactivity. Specific saturable binding was calculated by subtracting the radioactivity bound in the presence of a 1000-fold excess of the unlabeled dexamethasone from that bound in the presence of the labeled hormone alone. The method of Scatchard [14] was employed to determine the number and affinity of specific dexamethasone binding sites.

#### *Nuclear binding assay*

Pooled muscle samples were minced and homogenized in 4 vols. (w/v) of TS buffer (10 mM Tris-HCl/0.25 M Sucrose, pH 7.6) at  $0^\circ\text{C}$  using an Ultra-Turrax blender for  $2 \times 15$  s at 50 V. The homogenates were centrifuged for 10 min at 2000  $g$  at  $0^\circ\text{C}$  in a Sorvall SS 34 rotor to sediment nuclei. The resulting supernatant was further centrifuged at 105 000  $g$  for 60 min at  $0^\circ\text{C}$  in a Beckman L5-65 ultracentrifuge. The clear fat free cytosol was incubated for 2 h at  $0^\circ\text{C}$  with 20 nM [ $^3\text{H}$ ]dexamethasone alone or 1000-fold excess of non-radioactive dexamethasone and used for nuclear binding assay. The crude nuclear pellet was dissolved in 5 vols. of TS buffer, filtered through a double layered cheese cloth and centrifuged at 2000  $g$  for 10 min at  $0^\circ\text{C}$ . 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  $\text{MgCl}_2$  as previously described [15,16]. The nuclei were washed and resuspended in TS buffer. Aliquots of the nuclear suspension containing 100  $\mu\text{g}$  of DNA were centrifuged at 2000  $g$  for 10 min at  $0^\circ\text{C}$  and the supernatant fractions were discarded. [ $^3\text{H}$ ]dexamethasone-labeled cytosol (200  $\mu\text{l}$ ) was added to above nuclear pellet. The pellets were gently mixed on a Vortex machine and incubated at  $0^\circ\text{C}$  for 1 h. 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 pellet was resuspended in 0.5 ml of TS buffer and radioactivity 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.* [17] using bovine serum albumin as standard. DNA content was measured by the method of Burton [18]. All the data were statistically analyzed [19]. The level of significance (*P*) between two sets of data was calculated according to Student's *t*-test.

## RESULTS AND DISCUSSION

Glucocorticoids are involved not only in cellular growth and differentiation but also in the metabolic functions of various tissues of animals [20]. Development and aging of animals may partly be characterized by changes in responsiveness of tissues and cells to certain hormonal modulators. It has been demonstrated that the degree of tissue responsiveness to steroid hormones is directly proportional to the amount of specific receptor molecules. In the present study a synthetic glucocorticoid, dexamethasone, was used for receptor assay because of its very little interaction with plasma transcortin. The binding reaction described in this paper using the glass fibre filter rather than the charcoal assay takes advantage of its strong affinity for glucocorticoid receptors. Our data (Table I and Fig. 1) on Scatchard binding plot for [<sup>3</sup>H]dexamethasone show that the concentration of specific binding sites are significantly higher (57%) in the skeletal muscle of immature as compared to mature rats. From the slope of the curves the apparent dissociation constants (*K<sub>d</sub>*) were found to be similar in rats of both the ages. The higher level of receptor protein may be a contributory factor for the role of glucocorticoid in the development and growth of skeletal muscle during early phases of the life span. The higher level of receptors in the rat skeletal muscle may also be correlated with weaning and changes in diet resulting in a different metabolic status at this phase of the life span. A marked decrease in the concentration of binding sites for [<sup>3</sup>H]dexamethasone has been reported in the skeletal muscle of mature (12-month) rats as compared to 3-month-old rats [3]. Mayer *et al.* [21] reported development and age-related reduction in the concentration of specific binding sites with no change in the ability of microfibrillary protease to respond to exogenous doses of glucocorticoid.

TABLE I

CONCENTRATION OF CYTOSOLIC DEXAMETHASONE RECEPTORS IN THE SKELETAL MUSCLE OF IMMATURE (3-WEEK) AND MATURE (26-WEEK) MALE RATS

Age (weeks)	Specific [ <sup>3</sup> H]dexamethasone binding sites (fmol/mg protein)	<i>K<sub>d</sub></i> (nM)
3	22.36 ± 0.57	1.62 ± 0.25
26	9.46 ± 0.61*	1.67 ± 0.24

The data were collected from 4 to 5 rats of each age group. The results are means ± S.D. of three separate assays for each age group. \*Significant statistically (*P* < 0.001).

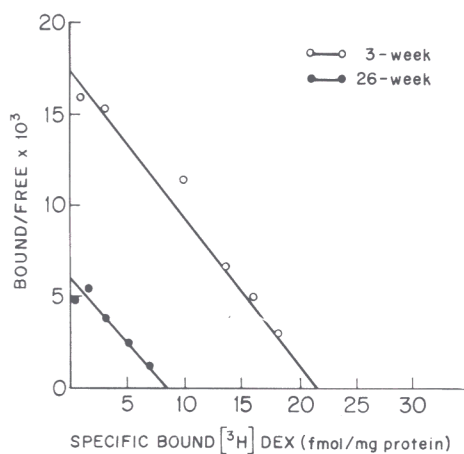


Fig. 1. Scatchard plot of the *in vitro* specific binding of [<sup>3</sup>H]dexamethasone to skeletal muscle cytosols of immature (○—○) and mature (●—●) male rats. Ratio of bound to free (B/F) hormone concentrations is depicted as a function of bound receptors/mg protein.

This suggests that even the diminished receptor content is sufficient for the expression of maximum response in skeletal muscle to glucocorticoid. While our present investigation supports the view of Roth [3] and Mayer *et al.* [21], it contradicts Kalimi's report [11] of no age-related differences in the cytosolic receptor concentration of skeletal muscle of Sprague–Dawley rats. However, our results are in agreement with the report of Kalimi [11] that there is no apparent age-related change in the receptor dissociation constant. Our results also suggest that a decrease in the concentration of binding sites for [<sup>3</sup>H]dexamethasone does not occur in very old age but after reaching reproductive maturity.

It is now well documented that the ability of cells and tissues to respond to glucocorticoids is tissue specific and altered during development [20] and aging [22] at both the receptor and post-receptor levels. A very important aspect of the steroid-induced alterations in gene transcription is the mechanism(s) by which the steroid-receptor complex binds to nuclear acceptor sites. There are at least three different levels of events: binding of hormone to its receptor, its activation and then binding of hormone-receptor complex to the nucleus. Under physiological conditions, activation of the hormone-receptor complex appears to be modulated by specific cellular factors and is rapidly achieved once the steroid has penetrated the cell [23]. In the present experiment, we have compared binding of the glucocorticoid receptor to isolated nuclei from the skeletal muscle of both immature and mature rats to determine physicochemical changes in receptors at activation and nuclear translocation level. Our data (Fig. 2) show that heat activation (for 45 min at 25°C) significantly enhances the nuclear binding of steroid-receptor complexes in rats of both the ages at a similar level. In cross experiments we studied the binding of heat activated cytosol from mature rats to nuclei of immature and *vice-versa* to see any age-related difference in nuclear translocation of dexametha-

sone-receptor complexes. Our data show that both mixed groups responded to heat activation as did the non-mixed group (Fig. 2) indicating no age specificity in nuclear binding of muscle glucocorticoid receptors. Our results indicate no marked age-related difference in the *in vitro* thermal activation of glucocorticoid receptor in the skeletal muscle of rats. Our present findings are in agreement with the earlier report of Kalimi [24] on hepatic nuclear translocation of heat activated glucocorticoid receptors. In addition,  $\text{Ca}^{2+}$  activation (20 mM  $\text{Ca}^{2+}$  for 45 min at  $0^\circ\text{C}$ ) significantly enhances the nuclear binding of [ $^3\text{H}$ ]dexamethasone-receptor complexes in skeletal muscle of rats of both the ages (Fig. 2). The extent of  $\text{Ca}^{2+}$  activated nuclear translocation is significantly higher (27%) in immature as compared to mature rats. The exact mechanisms of this low temperature  $\text{Ca}^{2+}$  activation of the glucocorticoid receptor complex are not well understood. However, it is possible that  $\text{Ca}^{2+}$  enhancement of nuclear binding may be due to direct interaction of  $\text{Ca}^{2+}$  with receptor molecule and/or receptor transforming factors which perhaps could cause conformational change that exposed the DNA- and chromatin-binding domain [23]. Similar to our observations, Kalimi [24] reported that  $\text{Ca}^{2+}$  dependent activation of rat hepatic glucocorticoid receptor decreases as a function of age. Our findings extend and corroborate the view that some of the

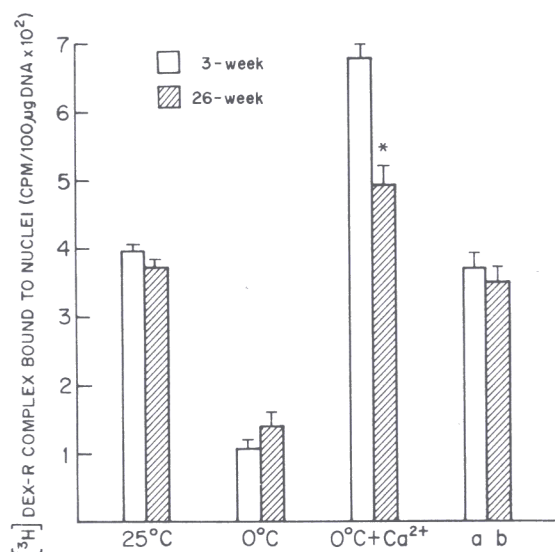


Fig. 2. Specific binding of [ $^3\text{H}$ ]dexamethasone-receptor complex to skeletal muscle nuclei from immature (3-week) and mature (26-week) male rats. Cytosols were incubated with 20 nM [ $^3\text{H}$ ]dexamethasone in the presence or absence of 1000-fold excess of unlabeled dexamethasone for 2 h at  $0^\circ\text{C}$ . The cytosols were further incubated at: (i)  $25^\circ\text{C}$  for 45 min; (ii) at  $0^\circ\text{C}$  for 45 min; and (iii) at  $0^\circ\text{C}$  with 20 mM  $\text{Ca}^{2+}$  for 45 min. Following incubation, specific nuclear binding was determined as mentioned in materials and methods. The results are mean  $\pm$  S.D. for 3–4 samples from 4 to 5 rats of each age group. \*Significant statistically ( $P < 0.001$ ). The a b barogram represents mixing experiments in which  $25^\circ\text{C}$  activated cytosol of 26-week rats incubated with the nuclei of 3-week rats (a) and  $25^\circ\text{C}$  activated cytosol of 3-week rats with nuclei of 26-week rats (b).

physicochemical properties of glucocorticoid receptors are similar while others change with age of rats. Recent literature [10] suggests changes in the physicochemical properties of hepatic glucocorticoid receptors during development and aging of rats. These impairments in the properties of receptors may lead to functional alterations in the tissue response as a function of age.

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