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EFFECT OF DENERVATION OR CASTRATION ON STEROID RECEPTORS IN RAT BULBOCAVERNOSUS/LEVATOR ANI MUSCLES*

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Summary—Alterations in cytosolic glucocorticoid (GR) and androgen receptors (AR) in atrophic rat bulbocavernosus/levator ani muscles (BCLA) were investigated. The BCLA was removed 15 days after denervation (DEN) of the right part of BCLA or castration (CAS) and compared with the innervated left part of BCLA (INN) or the BCLA from sham-operated rats (SHAM). Receptor analyses were performed using charcoal adsorption or agar-gel electrophoresis. The main results were: (1) no alterations in K_D were observed; (2) GR were increased in DEN compared to INN when expressed per mg cytosolic protein ($P < 0.0001$) or g tissue ($P < 0.0002$) as well as in DEN compared to SHAM when expressed per mg protein ($P < 0.0002$) or g tissue ($P < 0.0003$); (3) GR were increased in CAS compared to SHAM when calculated per mg protein ($P < 0.05$) or g tissue ($P < 0.04$); (4) no differences between DEN and INN or SHAM were noted when results were expressed per mg DNA; (5) AR were increased in CAS compared to SHAM only when expressed per mg protein ($P < 0.003$); (6) GR/AR was increased in DEN compared to INN ($P < 0.0001$) or SHAM ($P < 0.0006$), but unaltered in CAS compared to SHAM. The data reflect differences in the behaviour of GR and AR in the atrophic BCLA and suggest a relative increase in sensitivity to glucocorticoids compared to androgens in the DEN muscle.

INTRODUCTION

Despite the obvious increase in protein degradation relative to protein synthesis accompanying muscular atrophy, it remains unclear whether this imbalance results from an absolute increase in degradation, decrease in synthesis or a combination of the two [1]. Increased responsiveness to catabolic or decreased responsiveness to anabolic processes are therefore both plausible mechanisms.

The marked atrophy of skeletal muscle which follows exposure to excessive amounts of glucocorticoids led to the hypothesis that muscular atrophy may be mediated by increased sensitivity to endogenous glucocorticoids [2]. The observations of increased glucocorticoid receptors (GR) in denervated [2] or immobilized [3] rat gastrocnemius muscles and in dystrophic chicken muscle [4] lent support to the concept that increased cytosolic binding sites may confer this sensitivity. Using similar logic, a decrease in receptor sites for anabolic hormones, e.g. cytosolic androgen receptors (AR), may be indicative of decreased sensitivity to these hormones.

The present studies were undertaken to better establish the role of steroid receptors in muscular atrophy. In particular, we sought to determine whether the previously reported increases in GR in atrophic muscle were merely a general phenomena among steroid receptors in atrophic muscle, regardless of the proposed function of these receptors. We have investigated alterations in GR and AR in rat bulbocavernosus/levator ani muscles (BCLA) which were undergoing atrophy induced by either denervation or castration. The BCLA was chosen as a model because it is known that both glucocorticoids and androgens play a role in the metabolic regulation of this muscle [5–8] and readily measurable quantities of GR [8, 9] and AR [10, 11] are present.

EXPERIMENTAL

Chemicals

[6,7-^{3}H]dexamethasone (sp. act. 48.9 Ci/mmol), [17α-methyl-^{3}H]methyltrienolone (R1881) (sp. act. 87 Ci/mmol) and radioinert R1881 were purchased from NEN Chemicals (Dreieichenhain, F.R.G.). Radioinert dexamethasone was purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Other radioinert steroids were purchased from Sigma or Serva (Heidelberg, F.R.G.). Norit A charcoal was purchased from Serva; fine particles were removed prior to use by repeated washing with distilled water.

Animals

Male Wistar rats weighing between 325 and 425 g were used. Assay validation was performed using

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Table 1. Treatment schedule for male Wistar rats

	Group 1 (15-day denervated)	Group 2 (15-day castrated)	Group 3 (15-day sham-operated)
GR assay	3-day ADX	3-day ADX	3-day ADX
AR assay	24 h OX	—	24-h OX

ADX = adrenalectomy, OX = castration, day/h = days/hours post-operative.

3-day adrenalectomized rats for GR and 24-h castrated rats for AR determinations. Animals for other experiments were divided into groups and treated according to the plan outlined in Table 1. Muscles from 5 rats were generally pooled for each assay, except in those experiments performed to determine biological variation. Pentobarbital (50 mg/kg i.p.) anaesthesia was used in all experiments. Adrenalectomized rats were provided with 0.9% NaCl instead of tap water during the post-operative period. Unilateral denervations of the BCLA were performed by sectioning the pudendal nerve where it crossed the bulbourethral gland; prior to sectioning the nerve was electrically stimulated and contraction of the BC visually observed. Sham operations were similar to denervations with the omission of nerve section.

Preparation of cytosol and binding assays

Animals were decapitated and muscles quickly removed and placed in ice-cold buffer (0.01 M Tris–HCl, 0.0015 M EDTA, 0.01 M Na molybdate, pH 7.4). All subsequent procedures were performed near 0°C. Muscles were dissected free of all extraneous tissue, minced with scissors and pulverized in a porcelain mortar chilled in liquid nitrogen. The powder was transferred to centrifuge tubes and 1 or 4 vols buffer added to samples for agar-gel electrophoresis (AGE) or dextran-coated charcoal adsorption (DCC), respectively. Time from mixing after addition of buffer to beginning of centrifugation was always 1 h. After centrifugation at 100,000 ***g*** for 60 min, the supernatant was removed by aspiration and used as cytosol for the binding assays. An aliquot of cytosol and pellets were stored at −20°C for subsequent determination of protein and DNA, respectively.

The BCLA of the rats with unilateral denervation was separated into denervated (DEN) and innervated (INN) halves prior to homogenization. Despite the preclusion of measurement of accurate muscle weights without loss of tissue due to difficulties with exact dissection and associated delays, the success of denervation was easily verified by the smaller and redder appearance of the DEN BCLA (Fig. 1). In some sham-operated animals (SHAM), BCLA was separated into right and left halves; since no differences were noted in GR and AR between the two halves, in later experiments right and left SHAM were pooled.

Incubations were carried out in duplicate for 4 h, except when the time-dependent association of $[^3H]$DEX or $[^3H]$R1881 was determined. Receptor determinations by AGE and DCC were performed by addition of cytosol aliquots to tubes containing varying concentrations of $[^3H]$DEX (0.12–18 nM) or $[^3H]$R1881 (0.10–22 nM) to determine total binding

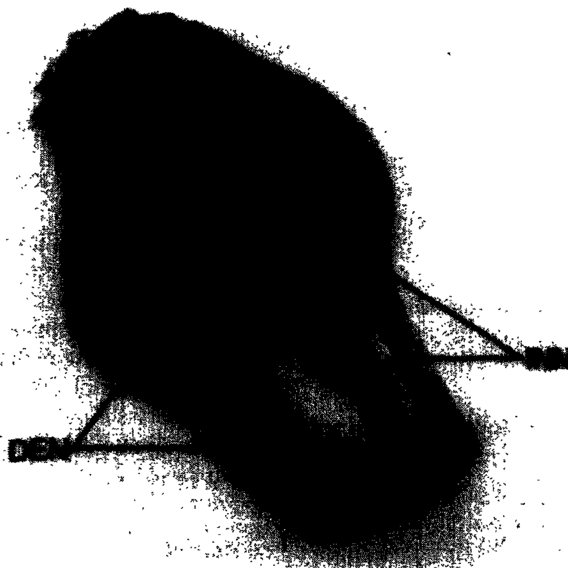

Fig. 1. Denervated (DEN) and innervated (INN) parts of 15-day unilaterally denervated bulbocavernosus/levator ani muscle.

and to a similar set of tubes containing a 100-fold excess of radioinert dexamethasone or R1881 to determine non-specific binding. In the case of AGE, steroids were added in 0.02 ml of a 5% (GR) or a 10% (AR) ethanol-buffer solution to incubation tubes immediately prior to addition of 0.1 ml cytosol. AGE was performed according to Wagner[12], as previously described [13]. Steroids were added to DCC tubes in 0.1 ml of a 3% (GR) or 6% (AR) ethanol-buffer solution prior to addition of 0.2 ml cytosol. Aliquots of 0.1 ml were taken to determine total amount of labelled steroid added. To the remaining 0.2 ml, 0.3 ml DCC [0.625% (w/v) charcoal and 0.0625% (w/v) dextran T70 in buffer] was added. Following 20 min incubation, tubes were centrifuged at 1500 g for 10 min. Radioactivity was counted in 10 ml Biofluor using a liquid scintillation counter (Packard Tri-Carb 2450). In some AGE and DCC experiments competitors were added in a 100-fold excess to determine binding specificity.

The difference in radioactivity between total bound and non-specific bound tubes was taken as a measure of specific binding. To validate the measurement of a single class of high-affinity, saturable binding sites, Scatchard plots [14] of data were initially employed. In later experiments single-point analyses using 16 nM [^{3}H]DEX or 22 nM [^{3}H]R1881 were performed. While the B_{max} was generally higher when measured by AGE compared to DCC, the tendencies between the treatment groups obtained by the two methods were similar. Therefore, DCC alone was used in later experiments.

Other analytical procedures

Cytosolic protein was determined by the biuret reaction. Bovine serum albumin was used as standard. DNA determinations were performed using a modified Burton method [15] as described by Bartsch *et al.*[16], except that the frozen pellets were first pulverized in liquid nitrogen. Salmon sperm DNA was used as standard. Statistical differences were assessed by analysis of variance followed by the LSD test.

RESULTS

In castrated or adrenalectomized animals, specific binding approached a plateau of maximum binding within 1 h for [^{3}H]R1881 and 2 h for [^{3}H]DEX. Appreciable alterations in binding were not noted up to 27 h for either. Therefore, 4 h incubation time was considered adequate. When competitors were added to determine binding specificity, T and DHT competed effectively for [^{3}H]R1881 binding sites; neither interfered with binding of [^{3}H]DEX. Similarly TAC, corticosterone and cortisol competed for [^{3}H]DEX, but not [^{3}H]R1881, binding sites.

In one series of experiments, muscles from 1 to 2 animals were pooled and one-point assays performed using DCC. A B_{max} of 9.3 ± 2.8 ($\bar{X} \pm$ SD), with a range of 4.9–12.7, fmol/mg protein ($n = 6$) was obtained for [^{3}H]R1881 binding; values for [^{3}H]DEX binding were 23.4 ± 12.1 ($n = 7$) with a range of 6.1–41.9 fmol/mg protein. The interassay C.V. ($n = 5$), including cytosol preparation, was less than 9% for both assays. The intraassay C.V. ($n = 10$) for [^{3}H]DEX binding, including homogenization and cytosol preparation, was less than 8%.

The K_D for neither GR nor AR was altered by DEN or castration (CAST). The K_Ds ranged from 2.2 to 5.6 nM and 0.1 to 0.4 nM for [^{3}H]DEX and [^{3}H]R1881 binding, respectively. Sample Scatchard plots from one experiment for GR are shown in Fig. 2.

The B_{max} for GR and AR in the various treatment groups, as well as GR/AR, are listed in Table 2. No differences in cytosolic protein were observed between groups. The mean ($\pm$SD) DNA concentrations (mg/g pellet) were DEN 1.44 ± 0.28, INN 0.89 ± 0.17, SHAM 0.90 ± 0.18 and CAST 1.03 ± 0.16. DNA was increased in DEN compared to INN, SHAM ($P < 0.0001$) and CAST ($P < 0.003$). In Table 3 significance levels between groups for GR, AR and the GR/AR ratio are shown. Increases in GR were present in DEN compared to INN or SHAM as well as in CAST compared to SHAM when results were expressed as fmol/mg cytosolic protein or g tissue, but no differences were noted when results

Table 2. Maximum number of binding sites (B_{max}) in BCLA cytosol of the various treatment groups

		DEN	INN	CAS	SHAM
		$n = 5$	$n = 5$	$n = 5$	$n = 5$
GR	fmol/g Tissue	3924 ± 802	1893 ± 362	2896 ± 594	2033 ± 479
	fmol/mg Protein	85 ± 16	42 ± 7	61 ± 11	45 ± 11
	fmol/mg DNA	2778 ± 553	2229 ± 539	2916 ± 436	2496 ± 182
		$n = 6$	$n = 6$	$n = 4$	$n = 4$
AR	fmol/g Tissue	644 ± 183	551 ± 66	683 ± 93	468 ± 89
	fmol/mg protein	12 ± 2	11 ± 2	16 ± 5	10 ± 1
	fmol/mg DNA	459 ± 153	627 ± 131	676 ± 54	495 ± 137
GR/AR	fmol/g Tissue	6.1	3.4	4.2	4.3
	fmol/mg Protein	7.1	3.8	3.8	4.5
	fmol/mg DNA	6.1	3.6	4.3	5.0

Values are means $\pm$ SD. GR/AR for each group was calculated from the mean values.

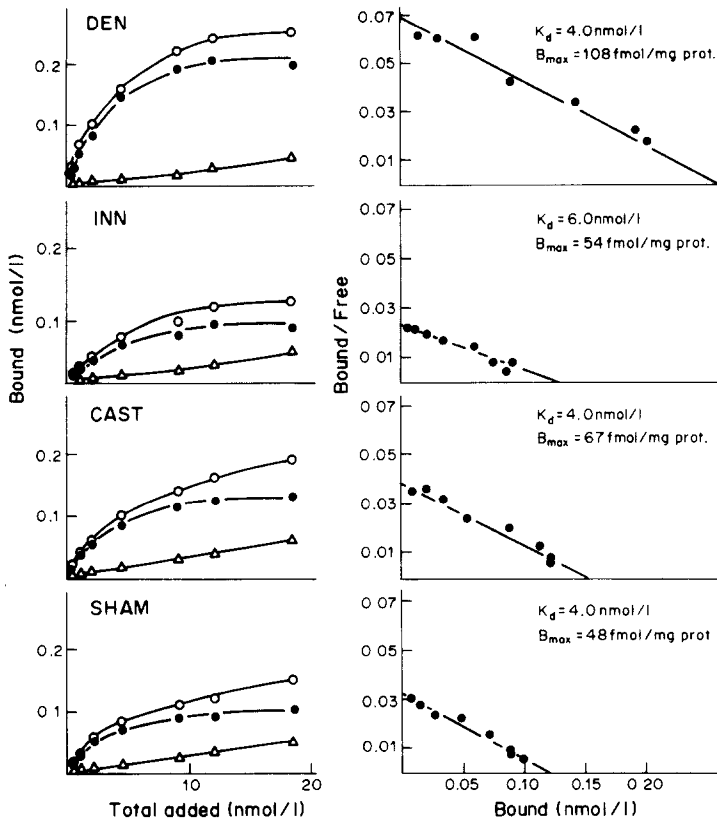

Fig. 2. Saturation analysis and Scatchard plots of the [^{3}H]DEX binding in the various treatment groups. Receptor assay: DCC. All data were obtained from a single experiment.

were expressed as fmol/mg DNA. The only observed alteration in AR was an increase in CAST vs SHAM when results were reported as fmol/mg protein. The GR/AR was increased in DEN and decreased in INN compared to SHAM.

DISCUSSION

Since the biological effects of both glucocorticoids and androgens appear to be largely mediated through hormone interaction with receptor proteins [17], the presence of GR and AR in BCLA should be essential for hormone action. It is logical to assume that one point of modulation of sensitivity to endogenous hormones may be alterations in the number of steroid receptors, even though the presence of these receptors alone is not an adequate indicator of the ability of the hormone to mediate its biological effects due to possible post-receptor defects.

One difficulty associated with interpretation of the observed differences in GR and AR between the treatment groups is the high variation inherent in GR and AR determinations in the BCLA. However, considering the relatively low intra- and interassay coefficients of variation, we are satisfied that the quantitative differences between experiments utilizing muscles from one or two animals largely reflected biological variation independent of the experimental techniques. In order to minimize this problem, muscles from 5 rats were pooled for each determination and all animals for a single experiment were carefully age-matched and shipped together; in one

Table 3. Significance levels for differences in B_{max} between the various treatment groups

		DEN vs INN	DEN vs SHAM	CAST vs SHAM	INN vs SHAM
GR	fmol/g Tissue	0.0002 (↑)	0.0003 (↑)	0.04 (↑)	NS
	fmol/mg Protein	0.0001 (↑)	0.0002 (↑)	0.05 (↑)	NS
	fmol/mg DNA	NS	NS	NS	NS
AR	fmol/g Tissue	NS	NS	NS	NS
	fmol/mg Protein	NS	NS	0.003 (↑)	NS
	fmol/mg DNA	NS	NS	NS	NS
GR/AR		0.0001 (↑)	0.0006 (↑)	NS	0.02 (↓)

↑, Increased; ↓, decreased.

assay, BCLA from DEN, INN, CAST and SHAM were always included. Therefore, the procedures employed should be reliable for detection of trends in alteration of B_{max} and K_D for GR and AR.

Most workers have reported cytosolic receptor values only on the basis of cytosolic protein content. However, since steroid action is mediated ultimately by nuclear receptor binding [17] and it is unclear whether the origin of these receptors is cytosolic or nuclear [18], it is possible that such measurements are not biologically relevant. Therefore, we compared our results on the basis of tissue weight, cytosolic protein and DNA. Differences in receptor concentration did indeed depend on the basis of expression. In an effort to circumvent this problem, we determined a GR/AR for each group; as expected, similar ratios were found for each treatment regardless of the basis of comparison. Further interpretation of the differences between the treatment groups is based on the assumption that an altered GR/AR reflects a potential upset in the balance between the two opposing steroidal systems, and that the direction of the alteration reflects the potential biological response. Therefore, an increased GR/AR suggests increased catabolic as opposed to anabolic potential, while a decreased GR/AR suggests decreased catabolic and increased anabolic potential.

The present results clearly demonstrate a differential response of GR and AR during muscular atrophy induced by denervation, and the increased GR/AR reflects a shift in hormonal sensitivity in the expected direction considering the known roles of glucocorticoids and androgens in the metabolic regulation of BCLA. Excess glucocorticoids induce atrophy of a similar magnitude to that observed in limb muscles [8]. The androgen dependence of this muscle is well-known, androgen administration resulting in hypertrophy and androgen withdrawal resulting in atrophy [5–7].

Interpretation of the GR/AR during castration-induced atrophy is more difficult. While increases in GR were noted, there was also a tendency to increased AR in CAST BCLA. Our results are in agreement with those from other laboratories, however. DuBois and Almon [8] found a post-castration increase in GR in BCLA. The observed increase in AR per mg protein was similar to that recently reported by Rance and Max [19]. Furthermore, post-castration increases in both GR and AR in rat limb muscles have been reported by Dahlberg *et al.*, suggesting a general regulation of both receptors by androgens [20]. Regardless, in the case of castration the balance between the catabolic and anabolic processes is clearly upset by removal of the anabolic stimulus (androgens), with the resultant biological balance shifted in favour of the catabolic influence irrespective of endogenous sensitivity in terms of receptor status.

The significance of the decreased GR/AR in INN compared to SHAM is unclear. This difference was not apparent when the GR and AR were considered independently, but may reflect a slight hypertrophic response in the INN BCLA. The occurrence of muscular hypertrophy in response to denervation of the contralateral muscle is well-known [22] and the anatomical positioning of the left and right BCLA increases the likelihood of stretch, and consequently stretch-induced hypertrophy, in the INN muscle. However, since highly significant differences between DEN and SHAM were also noted such a slight hypertrophic response could only minimally contribute to the differences observed between the DEN and INN BCLA.

The differential effects of denervation and castration on rat BCLA have been previously described. Greater decreases in contraction time and myofibrillar ATPase activity were observed in the DEN than CAST BCLA, a shift of enzyme pattern to that of oxidative metabolism was noted only in the DEN BCLA, the protein loss was preferential for myofibrillar proteins in the DEN but not CAST BCLA and an increase in DNA concentration was noted only in the DEN BCLA [5]. Denervation resulted in a larger weight loss than castration [6]. Fibre number alterations do not occur, however, during either the early stages of denervation atrophy [23] or castration atrophy of BCLA [24]. In light of these differences, it is clear that differences in the biochemical mechanisms underlying the atrophies must also exist. Similarities in the extent of atrophy produced by denervation or excess glucocorticoids initially led to the conjecture by DuBois and Almon [2] that changes in glucocorticoid sensitivity may be generally involved in regulation of muscle mass, implying a common mechanism of action. However, many of the effects produced by excess glucocorticoids, presumably via GR, are not observed in denervated muscle [24]. While the present results do suggest a relative increase in sensitivity to glucocorticoids in atrophic BCLA, it remains to be determined whether such a commonality as increased GR is causally associated with loss of muscle mass.

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