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QUANTITATION OF GLUCOCORTICOID RECEPTORS IN BOVINE SKELETAL MUSCLE: TOPOGRAPHICAL DISTRIBUTION, SEX EFFECT AND BREED COMPARISONS

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Summary—The concentration of the cytosolic glucocorticoid receptor (GR) was determined in skeletal muscles of calves in order to study possible differences in individual muscles from different parts of the body as well as the influence of sex and breed. In male and female Simmental calves the topographical distribution of GR was similar: the lowest concentrations were seen in abdominal muscle, whereas in neck, shoulder and hindleg the GR concentrations were higher; this difference was more pronounced in male than in female calves. In general, female calves had about 2-fold higher GR concentrations than males. The cytosolic cortisol concentrations were differing neither between individual muscles nor between sexes. The cortisol secretion during a 24-h sampling period 1 week prior to slaughter showed no sex difference. GR concentrations in neck muscle of female calves of four different German cattle breeds (Holstein Friesian, Brown Swiss, Simmental and German Gelbvieh) were rather similar; however, when Brown Swiss with the highest GR levels were compared to Holstein Friesian calves with the lowest concentrations, a significant difference was evident ($P < 0.05$).

INTRODUCTION

Muscle growth is a very complex process in which steroids and many growth factors have regulatory functions. It is well established that muscle tissue consists of varying proportions of muscle fibres belonging to distinct metabolic types. The classification of the muscle fibres is based on their different metabolism as measured by ATPase, glycolytic and mitochondrial enzyme activities [1–3]. In meat producing animals the enzymatic activities in this context are rather constant within defined individual muscles and because no sex, breed or age differences have been observed [4], the thereby defined metabolic activity seems to be independent of those hormones commonly related with muscle growth, i.e. sex steroids and growth hormone. However, the described typification of muscles exclusively takes glucose utilization into account; it does not allow any statements on protein metabolism. In order to investigate hormonal systems that are involved in the regulation of protein accretion and protein degradation we studied the sensitivity of bovine skeletal muscle for steroids. For the anabolic estrogens and androgens we found differing receptor concentrations in individual muscles of prepubertal calves, that were correlated with the allometric growth in certain muscles seen under androgen or estrogen stimulation [5]. In contrast to the anabolic effect of the sex steroids, the increase of circulating concentrations of glucocorticoids leads to a degradation of protein in skeletal muscle and a reduction in protein mass of the body musculature [6]. In the study reported herein we investigated the sensitivity of bovine skeletal muscle to glucocorticoids in order to provide data on the distribution of glucocorticoid receptors in different muscles of the body as well as on the concentration of the glucocorticoid receptor in muscle of different sexes and breeds.

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EXPERIMENTAL

Animals and tissues

Five female and 5 male Simmental calves were used to study the topographical distribution and the effect of sex on glucocorticoid receptor. They were kept in individual cages and were fed with standard milk replacer, the

average daily body weight gain was 1280 g in males and in females. 1 week before slaughter blood samples were taken every 30 min over a period of 24 h. The calves were fitted with chronically indwelling catheters in the jugular vein. At least 10 days were allowed for each calf to recover from surgery before the frequent blood sampling. To 5 ml of blood, 0.1 ml of 0.3 M EDTA, pH 7.0 was added. After centrifugation (15 min, 4°C) plasma was stored at −20°C until analyzed. The calves were slaughtered at a final weight of 155 kg. Samples from neck muscle (m. trapezius), from shoulder (m. biceps brachii), abdomen (m. oliquus internus or m. transversus) and hind leg (m. rectus femoris) were taken. Within 15 min after exsanguination muscle specimens were dissected, minced and placed into liquid nitrogen until further processing.

Additionally samples of m. trapezius from female calves of the following breeds were taken at the local abattoir: German Gelbvieh ($n = 25$, mean life weight 193 kg), Simmental ($n = 27$, 175 kg), Brown Swiss ($n = 22$, 158 kg) and Holstein Friesian ($n = 19$, 213 kg).

Preparation of cytosols

Muscle samples were homogenized and processed as described previously [5]. Basically a 280,000 ***g*** supernatant was prepared and used as cytosol for further analysis.

Receptor analysis

For the routine analysis of glucocorticoid receptors 1 ml of cytosol was incubated overnight on ice with 20 nM [^{3}H]dexamethasone (40 Ci/mmol, Amersham, Braunschweig, Fed. Rep. Germany) with or without 1 μM dexamethasone (Sigma, Deisenhofen, Fed. Rep. Germany). Equilibrium was reached during the incubation period. Incubation was terminated with a charcoal treatment of the cytosols (0.4% final charcoal concentration). 0.1 ml aliquots of the charcoal treated cytosols were then counted directly and further 0.4 ml aliquots were incubated in duplicate for 10 min at 0°C on 0.4 g of DEAE–Sephacel (Pharmacia, Uppsala, Sweden) in small disposable columns (Analytichem International, Harbour City, Calif., U.S.A.) fitted with a frit. After washing with 5 ml buffer (10 mM NaH_2PO_4, 3 mM DTT, 0.5% Surfynol (Biesterfeld, Hamburg, Fed. Rep. Germany) pH 7.5), the entire gel volume was transferred with 1 ml of water directly into scintillation cuvettes. [^{3}H]-Steroids were counted after extraction with 3 ml Xylofluor (Baker Chemicals, Deventer, The Netherlands).

For the determination of the equilibrium constant, cytosol was incubated with 1 nM [^{3}H]dexamethasone with or without a range of concentrations of unlabeled dexamethasone from 0.2 to 100 nM final concentration. After charcoal treatment the cytosols were further analyzed by adsorption to DEAE–Sephacel as described above. Scatchard analysis of displacement curves was done with the LIGAND program [7].

For the control of the reproducibility of the glucocorticoid receptor assay, muscle samples from several calves were pooled together, homogenized and aliquots of this sample were processed and analyzed in each assay. The coefficient of variation was 15%.

Hormone determinations

Cortisol (4-pregnene-11β-17α-21-triol-3,20-dione; Steraloids, Wilton, N.H., U.S.A.) concentrations in blood plasma and in cytosols were measured with a competitive enzyme immunoassay (EIA). Cortisol-21-glucuronide (Steraloids) was labeled with horseradish peroxidase as described for other steroids earlier [8]. The antibody used was a generous gift of Dr H. Grill, University of Mainz, Fed. Rep. Germany. This antibody (code "51") was raised in rabbits against cortisol-21-hemisuccinate-BSA; its crossreactivities are described as follows: cortisol 100%, cortisone 8%, corticosterone 9.5%, prednisolone 18% and dexamethasone <0.1%. The antibody dilution was 1:40,000. The EIA is performed on microtitration plates and uses a double antibody technique [8]; the calibration curve ranged from 0.5 to 125 pg cortisol per well. 10 ng of the tracer conjugate were applied per well. Before analysis, blood plasma and cytosol samples were extracted with a 10-fold volume of tert-butyl methyl ether by shaking for 15 min. After freezing at −60°C, extracts were decanted, evaporated and reconstituted aliquots (equalling 4 μl plasma) were analyzed in the EIA. For the determination of recoveries, aliquots of plasma or cytosol were spiked with [^{3}H]cortisol and extracted analogically. The mean recovery in 16 assays was 86.5% ± 2.7. Interassay coefficient of EIA variation was 14, 10 and 7% at 0.8, 1.5 and 3.5 ng/ml, respectively.

Table 1. Cytosolic glucocorticoid receptor concentrations (fmol/mg protein) in four different muscles from male and female calves

	Neck	Shoulder	Abdomen	Hind leg
Males	9.88 ± 1.45^{a}	6.70 ± 1.22^{a}	$1.32 \pm 1.18^{b**}$	$5.70 \pm 1.13^{a*}$
Females	18.21 ± 1.23^{a}	9.28 ± 1.31^{ab}	5.88 ± 1.49^{b}	9.80 ± 1.24^{ab}

Values are back-transformed $\log_e$ means $\pm$ SEM; values with different superscript letters are different within a row ($P < 0.005$).
** = $P < 0.01$ compared to females (*$P < 0.1$).

Estradiol-17β in plasma was determined by immunoaffinity chromatography and a biotin–streptavidin amplified EIA as described earlier in detail [9]. The detection limit was 0.5 pg/ml plasma. For the determination of testosterone, plasma samples were extracted as described for cortisol and then measured with a RIA as specified earlier [10].

Protein determination

Cytosolic protein was determined with a bromophenol assay [11] on microtitration plates using bovine serum albumin as a standard in the range 0.5–20 mg/ml.

Statistical calculations

Glucocorticoid receptor concentrations were analyzed by analysis of variance (ANOVA) after log transformation. The significance of difference between groups was assessed by Bonferroni statistics. To study the episodic cortisol secretion during the 24-h sampling period the data were analyzed with the PULSAR program, a computerized algorithm by Merriam and Wachter [12]. The calculated parameters were then compared by analysis of variance. Cytosolic cortisol concentrations were also compared by analysis of variance.

RESULTS

Scatchard analysis of the binding of dexamethasone in bovine skeletal muscle cytosol revealed linear plots; the apparent dissociation constant was 4.9 nM, the coefficient of variation in 4 experiments was 5%. For standard type glucocorticoid receptor (GR) quantitations sufficiently saturating [^{3}H]dexamethasone concentrations were used. The specific binding determined in this approach was in the same range as the B_{max} determined by Scatchard analysis.

Table 2. Cytosolic cortisol concentrations (ng/ml) in four different muscles from male and female calves

	Neck	Shoulder	Abdomen	Hind leg
Males	0.56 ± 0.08	0.52 ± 0.10	0.55 ± 0.12	0.57 ± 0.11
Females	0.56 ± 0.17	0.54 ± 0.14	0.55 ± 0.17	0.57 ± 0.16

Values are means $\pm$ SEM, $n = 5$.

Nonspecific binding was in the range of 0.5–1.5 fmol/mg protein, which was equivalent to about 5% of total binding. Adsorption of GR to DEAE–Sephacel was superior to direct counting of charcoal treated cytosols because the nonspecific binding was markedly reduced (5% vs 30% of total binding in directly counted charcoal treated cytosols) and the reproducibility was better.

In Table 1 a comparison of GR concentrations in four individual muscles from neck, shoulder, abdomen and hind leg of male and female calves is given. In both sexes the lowest GR concentrations were found in abdominal muscle. In female calves the GR number in neck muscle was higher than in abdomen; this difference was more pronounced in male calves where the GR concentrations in hindleg, shoulder and neck were markedly higher than in abdominal muscle. Comparing all GR concentrations in both sexes, i.e. the overall means of the four different muscles taken together, 1.9-fold higher levels were seen in females than in males ($P = 0.007$). This difference was mainly based on lower concentrations in abdomen and hind leg of the male calves, whereas there was no significant sex difference in neck and shoulder muscle. The cortisol concentration in muscle cytosols was constant in different muscles within one animal. Comparing the cortisol concentrations in individual muscles as well as overall muscles in both sexes no differences were seen (Table 2). The cortisol secretion during a 24-h sampling period was episodic in both sexes. Overall mean, baseline, peak amplitude and peak frequency as calculated with PULSAR are given in Table 3. There was no sex difference in any of these parameters. The male calves had

Table 3. Parameters of cortisol secretion during 24 h in male and female calves

	Overall mean (ng/ml)	Baseline (ng/ml)	Peak amplitude (ng/ml)	Peak frequency (n/24 h)
Males ($n = 5$)	2.14 ± 0.56	1.19 ± 0.49	3.77 ± 1.02	6.22 ± 2.25
Females ($n = 4$)	2.20 ± 0.72	1.14 ± 0.21	3.37 ± 1.25	8.24 ± 1.65

Values are means $\pm$ SD of the parameters as calculated with the PULSAR program.

Table 4. Glucocorticoid receptor concentrations (fmol/mg protein) in neck muscle of female calves of four different breeds

German Gelbvieh ($n = 15$)	$7.18 \pm 1.02^{a,b}$
Simmental ($n = 27$)	$8.80 \pm 1.00^{a,b}$
Brown Swiss ($n = 22$)	10.56 ± 1.10^{a}
Holstein Friesian ($n = 19$)	6.18 ± 0.39^{b}

Values are means ± SEM; means with the same superscripts are not significantly different, $P < 0.05$.

testosterone plasma concentrations of maximal 0.5 ng/ml. The secretion was episodic in nature. In female calves the testosterone concentrations were below the detection limit of the assay (0.04 ng/ml). Estradiol-17β concentrations were between 0.5 and 2.7 pg/ml in female and in male calves, respectively.

The GR receptor concentrations in neck muscle of four different German cattle breeds are given in Table 4. The highest GR levels were seen in Brown Swiss calves, followed by Simmental and German Gelbvieh. Concentrations were lowest in Holstein calves. The only significant difference was between Brown Swiss and Holstein calves ($P < 0.05$), all other comparisons showed no difference.

DISCUSSION

Binding characteristics of glucocorticoid receptor (GR) in bovine skeletal muscle were in the same range as reported for other species, e.g. for pig or for rats [13, 14]. The linear Scatchard plots obtained indicate a single class of binding sites. The receptor concentrations measured represent free, unoccupied binding sites; occupied receptors are tightly bound in the nucleus and cannot be extracted in the cytosolic fraction with the preparation used in this study. Therefore the receptor concentrations determined can be considered as a reflection of the true sensitivity of the muscle to glucocorticoids.

Topography

The topographical distribution in muscle of different parts of the body was unequal, i.e. the GR concentration in individual muscles within one animal was different. These differences cannot be attributed to locally different cortisol concentrations in the muscles investigated; increased cortisol concentrations would result in decreased numbers of free binding sites but no correlation was found between the number of GR and the concentration of cortisol in cytosols. Therefore the different GR concentrations might be interpreted as indeed different glucocorticoid sensitivity in individual muscles. The physiological background for differing sensitivities to glucocorticoids in individual muscles might be, that depending on the relative significance of a single muscle for the statics of the body, it can be inappropriate to undergo major changes in muscle mass.

Sex

Our data demonstrate higher GR concentrations in muscle from female compared to male calves. As discussed for the topographical distribution, this can be interpreted as truly different sensitivity to glucocorticoids, because neither cortisol secretion in plasma nor the cortisol concentration in muscle showed any sex dependent relation. Sex differences of GR concentration in skeletal muscle have been discussed previously: Dahlberg *et al.* reported a higher concentration of GR in muscle cytosols from female rats as compared to male rats [15]. In contrast, DuBois and Almon have reported higher GR concentrations in male than in female rats [16]; in both studies postpubertal rats were used. Snochowski *et al.* [13] found no difference in the GR concentrations in skeletal muscle of female, male and castrated pigs. An upregulating effect of androgens on GR has also been reported for perineal muscle in rats [17]. The calves used in the study reported herein were prepubertal. Nevertheless the male calves had well detectable testosterone plasma concentrations. During the 24-h blood sampling their testosterone secretion showed the typical diurnal pattern (data not shown) as described for elder bulls [18]. Our data do not confirm the concept of an androgenic upregulation of GR. Estradiol has been reported to induce down regulation of GR mRNA in rat anterior pituitaries [19]. In the calves of the present study estradiol-17β plasma concentrations were very low but measurable in both sexes at equal concentrations. There was no relation between the estradiol plasma concentrations and the higher GR levels in the female calves.

Possibly the higher GR concentration in female calves might be an inherent sex difference that is not affected by the still low endogenous sex steroid secretion in that young calves although it cannot be excluded that the androgens present in the male calves do regulate the GR. Reduced GR levels and thus lower sensitivity to endogenous glucocorticoids might be a further explanation for the reduced protein

catabolism, the increased protein accretion and the well known better growth rate of young bulls producing anabolic androgens.

Breed

In contrast to our first working hypothesis, that dairy and beef type breeds might have different glucocorticoid sensitivities, the GR concentrations in neck muscle of four different cattle breeds were all in the same range and showed no relation with the breed type. Moreover, the only difference was seen between two dairy type breeds: the Holstein Friesians with lowest and the Brown Swiss with highest GR concentrations. Possibly the GR concentration in skeletal muscle is not necessarily a function of breed and is rather affected by the age or the weight of the animals: the mean live weights of the calves investigated were correlated with the mean GR concentrations ($r = -0.31$, $P > 0.001$). For rats it has been demonstrated that GR concentrations in skeletal muscle decrease with increasing age [16].

In conclusion GR concentration do not explain the different growth rates of the breeds investigated. Possibly other growth factors and their receptors or β-adrenergic receptors, as studied in pigs [20], have key positions in determining growth rates of different breeds.

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