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BINDING OF GLUCOCORTICOIDS TO A SOLUBLE FRACTION FROM RAT SKELETAL MUSCLE

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Abstract—The half-lives in rat skeletal muscle of triamcinolone acetonide, triamcinolone, betamethasone and prednisolone were measured after intraperitoneal injection of fixed doses (1 and 5 mg/kg body wt). $T_{\frac{1}{2}}$ of triamcinolone acetonide was 158 min compared with values ranging between 31 and 59 min for the other steroids. The concentration of dexamethasone in skeletal muscle unlike that of triamcinolone acetonide could not be estimated directly by the g.l.c. method, but both these steroids, which show duration of biological activity up to 48 hr after intraperitoneal injection, were bound more strongly to a soluble cellular component than were corticosterone, prednisolone or betamethasone, none of which showed biological activity for longer than 18 hr. Evidence is presented which suggests that this soluble component may be a protein.

IT WAS previously shown that the catabolic potencies of a range of glucocorticoids correlated with their duration of biological action in skeletal muscle.[1] The action of triamcinolone acetonide and dexamethasone extended beyond 48 hr compared with corticosterone, prednisolone, triamcinolone and betamethasone which were not effective for more than 6, 12 and 18 hr. In order to understand the reasons for these discrepancies studies on half-lives and protein binding were undertaken.

EXPERIMENTAL

Materials

Radioactive steroids. [1,2-^{3}H]Prednisolone (spec. act. 12·5 Ci/mmole); [1,2,4-^{3}H] betamethasone (spec. act. 5·0 Ci/mmole); [1,2,4-^{3}H]dexamethasone (spec. act. 5·6 Ci/mmole); [1,2,4-^{3}H]triamcinolone (spec. act. 5·0 Ci/mmole), and [1,2,4-^{3}H]triamcinolone acetonide (spec. act. 9·5 Ci/mmole) were obtained from Centre Etude Nuclear, Department des Radioisotopes, Mol-Donk, Belgium. [1,2,-^{3}H]corticosterone (spec. act. 36 Ci/mmole) was obtained from The Radiochemical Centre, Amersham, Bucks.

Chemicals and enzymes. Bovine serum albumin was obtained from Sigma (London) Chemical Co., Ltd., and Nagarse from Hughes & Hughes (Enzymes) Ltd. Cab-o-Sil (Thixotropic gel powder) was obtained from Packard Instruments Ltd., prednisolone was obtained from CIBA Laboratories, Horsham, corticosterone from Steraloids Ltd., Croydon, betamethasone from Glaxo Laboratories Ltd., Greenford, and triamcinolone and triamcinolone acetonide from E. R. Squibb & Sons Ltd., Speke,

Liverpool. *Animals.* Rats were white Wistar (250–300 g), CFHB strain from Carworth Europe, Alconbury, Huntingdon, U.K.

Muscles. The vastus lateralis, vastus medialis and gluteus medius were used.

Methods

Administration of steroids. Fixed doses of 1 and 5 mg/kg body wt were administered. These were injected intraperitoneally as a fine suspension in 0·5 ml of 0·9% (w/v) NaCl. Control animals received saline only.

Purity of steroids. Unlabelled steroids were dissolved in tetrahydrofuran to give solutions at a concentration of 1 mg/ml which were stored at 4°. These solutions gave a single spot on t.l.c. using the lower phase of chloroform–dioxan–water (2:1:1) as the mobile phase. Tritiated steroids were diluted in tetrahydrofuran to a concentration of 100 μCi/ml. The labelled steroids had the same R_f as the unlabelled compounds as shown by a single peak of radioactivity when run in similar t.l.c. systems. A Panax t.l.c. scanner was used.

Nitric acid digestion. This was based on the method of Pfeffer *et al.*[2] Skeletal muscle (1 g) was completely dissolved in 1 ml of concentrated nitric acid (s. g. 1·42) by heating in a water bath at 90°.

Extraction of steroids from skeletal muscle and measurement of tissue concentration. Samples of muscle (4 g) were finely chopped and placed in large centrifuge tubes together with either 32 ml of water, saturated solutions of $CaCl_2$ or Na_2SO_4, phosphate buffer, or phosphate buffer saturated with respect to $CaCl_2$ or Na_2SO_4. In all experiments the tissue was homogenized with the Ultra Turrax for 2 min at 8000 rev/min while the tube was maintained at 4°. The suspension was then extracted twice with ethyl acetate (125 ml). The organic phase was evaporated nearly to dryness on a rotary evaporator, at 25°, and was transferred with 16 ml ethyl acetate to a separatory funnel where it was washed successively with 2 ml each of aqueous NaOH (0·1 N), acetic acid (0·5% v/v) and distilled water. All aqueous solutions were saturated with respect to Na_2SO_4. When labelled steroids were administered to animals the amount of radioactivity in the organic phase was measured directly. In experiments involving unlabelled steroids the ethyl acetate solution was processed further in order to estimate the steroid concentrations by g.l.c. as described elsewhere.[3]

Preparation of soluble muscle extracts for binding studies. Samples of frozen muscle (0·5 g) taken from animals injected with labelled steroids were ground to a fine powder, with liquid N_2, in a mortar and transferred to a centrifuge tube. Phosphate buffer (10 ml) was added and the content of the tube were well mixed. The tubes were centrifuged at 2000 *g* for 10 min and the supernatant was removed by decantation. The residue was washed with buffer (2 ml) by centrifugation and the combined supernatants were centrifuged at 105,000 *g* for 1 hr. The supernatant (soluble muscle extract) from this centrifugation was decanted and stored at 4°. Not more than 1 hr was allowed to elapse before it was used.

Assessment of protein-binding. This was carried out by the batch gel filtration method.[4]

The method was validated by comparison with equilibrium dialysis using albumin as the binding protein. Sephadex G-25 (coarse grade) was used. The gel (200 mg) was weighed into small tubes. Phosphate buffer (1·0 ml) was added and the gel was allowed to swell at 4° overnight. Samples of soluble muscle extract (1·0 ml) obtained from animals treated with labelled steroids 5 min prior to killing were then added to the

tubes. These were kept at 4° for 1 hr with shaking every 10 min. Aliquots (0·1 ml) of the supernatant fluid were then assayed for radioactivity.

Calculation. Volume of fluid outside gel,

$$V_E = \frac{E_1}{E_2} \times V$$

where E_1 = extinction of Blue Dextran (0·01 % in phosphate buffer) before the addition of Sephadex.

E_2 = extinction of Blue Dextran solution after swelling 200 mg of Sephadex in it.

V = volume of Blue Dextran solution to which Sephadex was added (2 ml).

Volume of fluid inside gel, $V_1 = V - V_E$.

If K = distribution coefficient of labelled steroid between Sephadex and phosphate buffer in the absence of binding molecules,

$$K = \frac{x}{a - x}$$

where x = dis/min in external volume, V_E

a = total dis/min.

In the presence of binding molecules:

if S_b = total bound steroid (dis/min)

$$S_b = x' - S_u$$

where x' = dis/min in external volume, V_E

S_b = total bound steroid (dis/min)

S_u = total free steroid (dis/min)

$$S_u = K(a - x')$$

$$S_b = x' - K(a - x')$$

$$\text{per cent binding} = \frac{S_b}{a} \times 100.$$

Tritiated water in muscle homogenates. A sample of muscle (1 g) was homogenized in water (5 ml) using the Ultra Turrax and the water was distilled off at 50–60° under vacuum. The distillate was collected in a tube cooled in a mixture of acetone and solid CO_2 and redistilled under the same conditions. The total radioactivity in the distillate was measured by liquid scintillation counting.

RESULTS

Concentration of corticosteroids in skeletal muscle. When the concentrations of triamcinolone acetonide, triamcinolone, betamethasone and prednisolone were compared in skeletal muscle at various times after intraperitoneal injection, the rates of entry and removal of the unchanged substances from the tissues varied considerably (Fig. 1). Triamcinolone acetonide reached its maximum concentration in the muscle 15 min after injection, whereas the highest concentrations of triamcinolone, betamethasone and prednisolone were reached at 90, 60 and 30 min, respectively. Similarly the half-lives of the substances, measured from peak concentrations, were 158, 59, 27

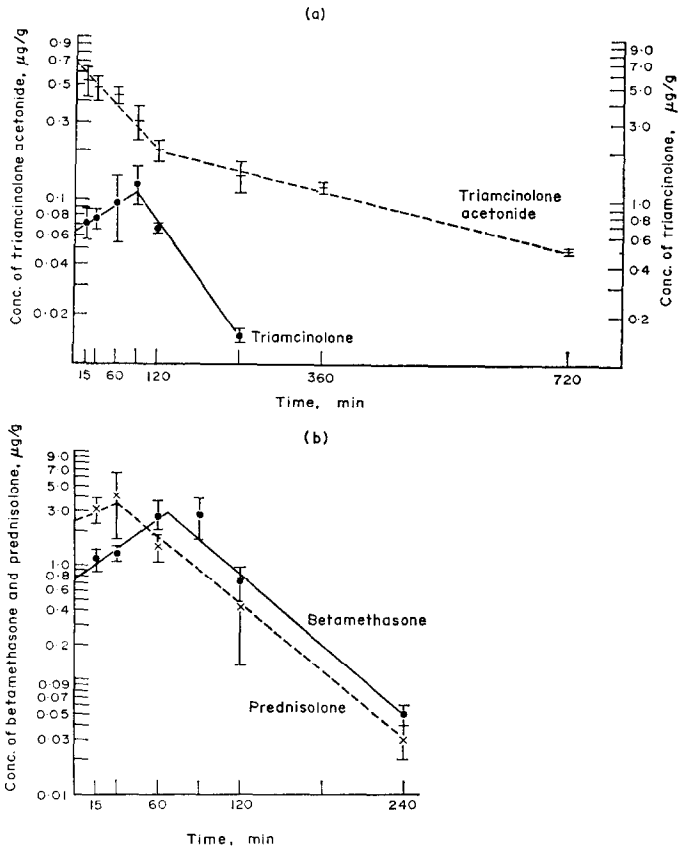

FIG. 1. Graphs showing decline of concentrations of; (a) triamcinolone acetonide and triamcinolone and, (b) betamethasone and prednisolone in skeletal muscle of rats after administration of 20 mg/kg doses. Points represent the mean of the values found in five animals, with two standard deviations.

and 31 min for triamcinolone acetonide, triamcinolone, betamethasone and prednisolone, respectively. The half-life of triamcinalone acetonide between 2 and 12 hr after giving the drug was 300 min.

The use of labelled steroids administered at 1 and 5 mg/kg gave total extractable radioactivity which was within 10 per cent of the total radioactivity (Table 1) for betamethasone, triamcinolone, prednisolone and corticosterone. Distillation of water from homogenates containing these steroids as described in Methods, showed that the small balance was accounted for as T_2O. However, where dexamethasone and triamcinolone acetonide were used there was a much greater discrepancy between extractable radioactivity and total radioactivity and this was not accounted for as T_2O. On the assumption that all the steroids were only metabolized to a small extent after 5 min these results suggested that dexamethasone and triamcinolone acetonide might be bound to the muscle in a way which rendered them difficult to extract with the solvent, a feature of glucocorticoid binding previously noted in HTC cells.[5] This view was supported when standard binding experiments were carried out on extracts from frozen muscle of animals previously treated with labelled glucocorticoids. The

TABLE 1. CONCENTRATION OF GLUCOCORTICOIDS IN SKELETAL MUSCLE

Steroid	Dose (mg/kg)	Concn (μg/g)	Tissue radioactivity in ethyl acetate extracts (%)
Corticosterone	1	0·04 ± 0·01	
	5	0·22 ±	98·5
Prednisolone	1	0·15 ± 0·03	
	5	0·68 ± 0·16	90·0
Betamethasone	1	0·08 ± 0·01	
	5	0·35 ± 0·05	93
Triamcinolone	1	0·07 ± 0·02	
	5	0·21 ± 0·09	97
Dexamethasone	1	0·15 ± 0·03	
	5	0·55 ± 0·06	74
Triamcinolone acetonide	1	0·10 ± 0·01	
	5	0·73 ± 0·10	78·5

Steroids (5 mg/kg and 1 mg/kg, 10 μCi/rat) were administered to 5 animals 5 min before they were killed. The concentrations represent the mean of five experiments ± S.E.M., and are derived from the total counts extracted into ethyl acetate from samples of muscle homogenized in water. Total radioactivity was obtained in two experiments where further samples of muscle were completely digested in nitric acid.

results showed (Table 2) that dexamethasone and triamcinolone acetonide appear to be more strongly bound to a constituent of the supernatant than the other steroids tested and that of these two substances triamcinolone acetonide was more strongly bound than dexamethasone. When labelled triamcinolone acetonide was added *in vitro* to frozen and crushed muscle in amounts predicted from *in vivo* experiments

TABLE 2. THE BINDING OF GLUCOCORTICOIDS TO RAT MUSCLE *in vivo*

Steroid	Animal	Binding/mg tissue protein (%)
Corticosterone	a	12
	b	15
Prednisolone	a	13
	b	12
Betamethasone	a	11
	b	10
Dexamethasone	a	17
	b	20
Triamcinolone acetonide	a	26
	b	34

Each steroid (5 mg/kg, 200 μCi/mg) was administered to 2 rats 5 min before killing. Duplicate measurements of tissue binding were carried out on each muscle sample as described in Methods.

(0·1 μg/g) the amount of binding was 8·9 per cent/mg tissue protein. The bound steroid was reduced to 6·6 per cent by a 100-fold excess of non-labelled triamcinolone acetonide, dexamethasone or corticosterone. This *in vitro* binding appeared therefore to

be relatively non-specific. A preliminary indication that the substance to which triamcinolone acetonide is bound *in vivo* is proteinaceous in nature was obtained by extracting the muscle supernatant with ethyl acetate after various treatments, including high salt concentration and proteolytic enzyme (Table 3). The lowest recovery of

TABLE 3. EFFECT OF DIFFERENT PROCEDURES ON EXTRACTION OF A GLUCOCORTICOID FROM RAT MUSCLE

Homogenization medium	Incubation temp. (°C)	Enzyme	Salt	Dis/min/g
Water	18	–		2616
Water	18	–	Na_2SO_4	2535
Water	37	–	Na_2SO_4	2661
Phosphate buffer	18	–		2410
Phosphate buffer	18	–	Na_2SO_4	2481
Phosphate buffer	37	–	Na_2SO_4	2336
Phosphate buffer	37	+		2866
Phosphate buffer	37	+	Na_2SO_4	2752
Water	18	–	$CaCl_2$	2739

^{3}H-Triamcinolone acetonide (0·2 mg/kg, sp. act. 200 μCi/mg) was administered to animals 5 min before killing them. Muscle was pooled and 18 equal portions were used for nine different procedures. Each portion was prepared for extraction with ethyl acetate as described in Methods except that in different experiments the homogenate was incubated for 40 min at different temperatures with and without Nagarse (10 μg/ml).

steroid was achieved with those methods in which phosphate buffer (pH 7·4) was used for homogenization and no proteolytic enzyme was added to the homogenates. The highest recoveries resulted from the incubation of the supernatant with Nagarse, or the inclusion of $CaCl_2$ in the homogenizing medium.

DISCUSSION

The long half-life of triamcinolone acetonide agrees well with its long duration of biological activity. Ribosomes from skeletal muscle still had decreased ability to incorporate amino acids into protein *in vitro* 48 hr after injections (5 mg/kg) of triamcinolone acetonide or dexamethasone.[1] Unfortunately it was not possible to make derivatives of dexamethasone suitable for g.l.c. and therefore direct measurements of its concentration in muscle were not possible. Extrapolation of the decay curve (Fig. 1) for triamcinolone acetonide, however, gave an estimated concentration in muscle water of 10^{-9} M at 48 hr after a single injection (20 mg/kg), and concentrations of triamcinolone, betamethasone and prednisolone many orders of magnitude lower. Others[6] have demonstrated a long half-life for triamcinolone acetonide in humans, but our demonstration that there is a small level of tritium exchange as early as 5 min after administration of the steroids examined shows the need for caution in interpreting the data of Kusama *et al.*[6] and others[7] who have equated total activity with unchanged steroid some hours after the administration of tritiated compounds. Hackney *et al.*[8] showed that fibroblasts contain a cytoplasmic protein which binds triamcinolone acetonide and other glucocorticoids and that this binding protein is saturated at a concentration of triamcinolone acetonide between 1×10^{-8} M and 5×10^{-8} M. The amount of triamcinolone acetonide bound to macromolecular

material in a steroid-resistant sub-line was less than 20 per cent of the amount bound by the steroid-sensitive cells. Similarly in steroid-resistant strains of lymphosarcoma P1798 the amount of triamcinolone acetonide bound to a cytoplasmic protein was approximately 30 per cent of the amount bound in sensitive cells.[9] Triamcinolone acetonide appeared to protect the binding protein from breakdown during isolation.[10] Among other cell types which contain cytoplasmic receptors for glucocorticoids are hepatocytes[11] and thymocytes.[12–15] Our results indicate that triamcinolone acetonide and dexamethasone, which have a much longer biological action in muscle than corticosterone, prednisolone, betamethasone or triamcinolone, became bound to a macromolecule which may be of functional significance. Further experiments are necessary to tell whether this macromolecule is a protein and whether it is of cytoplasmic or nuclear origin.

In order to demonstrate that the long half-life of triamcinolone acetonide in muscle is dependent on the specific binding of the steroid it will be necessary to compare the proportion of steroid which is bound during the initial equilibrium period and during the slow release phase. This demonstration has not been achieved owing to methodological difficulties. Thus, tritium exchange from labelled triamcinolone acetonide was shown to occur *in vivo* in amounts which made it impossible to estimate the concentration of the steroid simply by counting the radioactivity in the tissue homogenates except during a very short time intervals after injection and therefore it was necessary to use direct estimation of the steroid concentration. The sensitivity of the g.l.c. method was sufficient to allow estimation of the steroids for some hours after i.p. administration but was not sufficient to allow the estimation of tissue concentrations after administration of smaller doses, comparable with those used clinically, nor of the small quantities of steroid available in the binding experiments. The use of ^{14}C-labelled steroids would overcome these problems.

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