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The effect of indomethacin on serum and skeletal muscle enzyme activities of dystrophic hamsters

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Recently, it has been shown that administration of prednisone [1, 2] or diethylstilbestrol [3] to patients with Duchenne muscular dystrophy produces a marked reduction of the raised serum levels of creatine kinase (CPK EC 2.7.3.2) found in this condition, and in the case of diethylstilbestrol of lactate dehydrogenase (EC 1.1.1.30) also. Moreover, Drachman *et al.* [4] have claimed that prednisone induced significant, though limited, clinical improvement. High serum enzyme activities in muscle disease appear to be a leakage phenomenon [5] so that, if agents which reduce serum enzyme activities, act on tissue outflow rather than on clearance, they may also affect enzyme concentrations in the muscle. Consequently, since effects on muscle rather than serum do not seem to have been reported previously and, if present, may be highly relevant to the problem of understanding drug action in muscle disease, we have studied the effect of indomethacin on both muscle and serum enzyme activities in an hereditary muscle disorder.

Eppenberger *et al.* [6] have shown that, as in human Duchenne dystrophy, serum CPK is greatly elevated in the hereditary muscular dystrophy of the Syrian hamster. The present study confirms that serum enzyme activities are raised in this disorder and provides evidence that skeletal muscle as well as serum enzyme activities can be altered by drug administration.

In hamster dystrophy, there are histological changes such as interfibrillar round cell infiltration [7], which suggest the presence of an inflammatory reaction, no doubt secondary in nature. Because of this and because of previous reports using anti-inflammatory steroids, e.g. prednisone, we decided to see if administration of an anti-inflammatory agent would modify the biochemical changes which occur in this disorder. Indomethacin, rather than a steroid, was chosen to minimise complications from general metabolic changes and the possible development of a steroid myopathy.

As a basis for comparison, we assayed CPK, lactate dehydrogenase (LDH) and hydroxy butyrate dehydrogenase (EC 1.1.1.30) in muscle and serum, together with muscle succinate: (INT) oxidoreductase (EC 1.3.99.1) as a representative of a particle-bound (mitochondrial) enzyme in four groups of animals. These were normal untreated, normal treated, dystrophic untreated and dystrophic treated hamsters.

Dystrophic hamsters were supplied by Mr. D. I. Roberts, The Coombehurst Breeding Establishment, Baughurst, nr. Basingstoke, Hants, England. They were derived by brother–sister mating from animals of the Bio. 14.6 strain originally imported from Telaco, Boston, Mass., U.S.A. Since all these animals are homozygous, with respect to the dystrophic gene, it was necessary to use animals from another strain as controls.

Indomethacin was given orally in drinking water. The concentration was estimated from the desired dose, i.e. 1 mg/k, body wt/day, the average daily *ad lib.*, water intake and body weight, at a particular stage of the experiment. Since indomethacin was only very sparingly soluble at the slightly acid pH, due to disssolved CO_2, of our distilled water, the following procedure was adopted: The required amount was dissolved in 100 ml of 150 mM $NaHCO_3$ and diluted to 1 litre with distilled water. Control animals received 15 mM $NaHCO_3$.

Blood was removed by heart puncture under ether anaesthesia and allowed to clot before separation of the serum. In rats, clotting releases CPK from platelets [8] making it necessary to measure plasma rather than serum levels. By contrast, this does not occur in hamsters [9] and serum can be used.

Homogenates were prepared from three different muscles, biceps femoris, gastrocnemius and tibialis anterior. The muscles were dissected out, as far as possible free of visible nerve, fat and connective tissue, and cut into small pieces with a scalpel. 100 mg of tissue was homogenized in ice cold 0.1 M Tris–HCl buffer, pH 7.4, containing 0.25 M sucrose with an Ultra Turrax (Janke & Kunkel, K. G., Staufen i Breisgau, W. Germany) homogenizer at approximately 75 per cent full speed for two periods of 30 sec and filtered through Green's Lens Tissue (Reeve Angel & Co. Ltd., London SE1 6BD). The volume was made up to a total of 10 ml.

The succinate. (INT) oxidoreductase activity of the homogenates was determined immediately by the method of Pennington [10]. LDH and hydroxybutyrate dehydrogenase (HBD) were measured after overnight storage at 4°, using the methods of Wroblewski an La Due [11] (LDH) and Rosalki and Wilkinson [12] (HBD) respectively. Creatine phosphokinase (CPK) was assayed, using a sulphydryl-activated, coupled enzyme procedure (Boehringer, u.v.-activated kit, Boehringer Corp. (London) Ltd). Samples of homogenates for CPK assay were stored at −18° for not more than 1 month. Changes in optical density at 340 μm in the methods for LDH, HBD and CPK were measured with a Gilford Reaction Rate Analyser, Type

Table 1. The effect of indomethacin treatment on serum enzymes in normal and dystrophic hamsters

Enzyme	Nc	Nt	Dc	Dt
CPK	970 ± 38 (9)	335 ± 92 (9)*	11900 ± 980 (12)	6500 ± 1250 (10)*
LDH	141 ± 44 (9)	192 ± 61 (6)	1241 ± 484 (7)	1094 ± 226 (7)
HBD–LDH	0.476 ± 0.050 (7)	0.502 ± 0.030 (6)	0.341 ± 0.017 (6)	0.403 ± 0.030 (6)*

Values are given as mean ± S.D. and the numbers of animals in parentheses. Nc—normal control, Nt—treated control, Dc—dystrophic control, Dt—treated dystrophic.

* Differences between treated and control groups significant with $P < 0.001$

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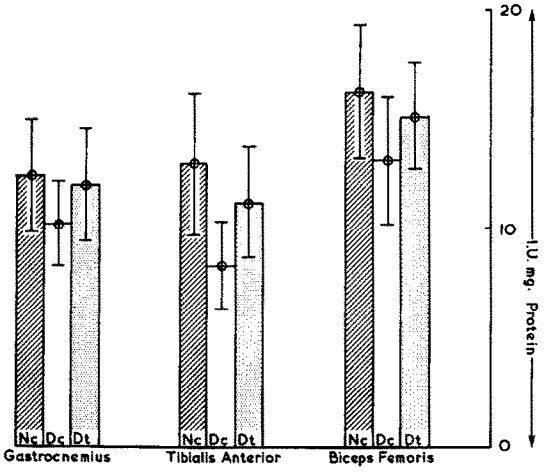

Fig. 1. Muscle CPK activities in Control (Nc), dystrophic untreated (Dc), and treated dystrophic (Dt) hamsters. Results are depicted as means ± S.D. Differences between the means of Nc and Dc and Dt and Dc are significant with $P < 0.02$ (Student's t-test).

300N, with automatic recorder (Gilford Instruments Laboratories Inc., Oberlin, Ohio). Units of enzyme activity are expressed as μmole of substrate utilized per min, per litre or per mg protein under the defined conditions, i.e. International Units (L.U.).

Protein was measured by the Lowry technique [13], using human serum albumin (Dade) as standard. Since in this procedure protein soluble in dilute NaOH is estimated, the result is equivalent to non-collagen protein.

The significance of the difference between mean values for the various groups was evaluated using Student's t-test.

Our results confirm the very high serum activity of CPK in dystrophic hamsters noted by Eppenberger *et al.* [16] and show that total serum LDH is also greatly elevated. The isoenzyme pattern of LDH, as indicated by HBD–LDH ratio, differs from normal in that the proportion of muscle type (M) subunits is increased. The same change in isoenzyme pattern is seen in the muscle and, in this respect, the alteration found in hamster dystrophy is somewhat unusual, since in human Duchenne dystrophy [14] and in denervation atrophy [16] the trend is in the other direction, so that the isoenzyme pattern becomes more like that of immature or foetal muscle, i.e. a higher proportion of heart type (H) subunits are present. However, a similar finding has been reported by Pearson *et al.* in the gastrocnemius of patients with sex-linked dystrophy of the benign Becker type [16].

The fact that the serum HBD/LDH ration in both normal and affected animals is appreciably higher than the ratios in the three muscles studied suggests a considerable contribution to the serum activity from some tissue other than skeletal muscle; nevertheless, the lowered serum HBD–LDH ration in the diseased animals is consistent with the increase in serum LDH being due mainly to leakage from skeletal muscle.

Administration of indomethacin (1 mg/k body wt per day) produces a highly significant fall in serum CPK, both in normal and dystrophic animals (Table 1). In the case of LDH, although the serum activity is somewhat lower in the treated dystrophic hamsters by comparison with the dystrophic controls, the difference is not significant. However, the HBD–LDH ratio in the treated group is significantly higher, i.e. it is closer to that observed in the normal group.

Before considering the effects of indomethacin on tissue enzymes in the particular muscles studied, it is pertinent to compare differences between these muscles in the untreated normal and dystrophic animals, particularly since information on biochemical changes in hamster dystrophy is relatively limited. In the normal group, gastrocnemius and tibialis anterior are very similar, on a non-collagen protein basis, in their content of all three enzymes assayed, whereas the biceps femoris contains significantly less succinate: (INT) oxidoreductase but more CPK activity (Fig.

Table 2. Muscle enzyme levels in normal, untreated dystrophic and treated dystrophic hamsters (I.U./mg protein)

Enzyme	Muscle	Nc	Dc	Dt	P*
Succinate: (INT) Oxidoreductase	Gastrocnemius	0.36 ± 0.06 (28)	0.41 ± 0.08 (17)	0.39 ± 0.09 (14)	N.S.
	Tibialis Ant.	0.39 ± 0.07 (27)	0.42 ± 0.07 (19)	0.41 ± 0.07 (15)	N.S.
	Biceps femoris	0.20 ± 0.05 (26)	0.23 ± 0.06 (20)	0.22 ± 0.06 (18)	N.S.
LDH	Gastrocnemius	2.44 ± 0.27 (17)	2.02 ± 0.30 (16)	2.27 ± 0.16 (10)	$0.05 > P > 0.02$
	Tibialis Ant	2.51 ± 0.21 (15)	2.04 ± 0.35 (15)	2.10 ± 0.06 (15)	N.S.
	Biceps femoris	2.59 ± 0.26 (18)	2.11 ± 0.27 (17)	2.17 ± 0.16 (16)	N.S.
HBD	Gastrocnemius	0.70 ± 0.07 (17)	0.53 ± 0.07 (16)	0.60 ± 0.04(10)	$0.01 > P > 0.001$
	Tibialis Ant	0.68 ± 0.07 (15)	0.54 ± 0.07 (15)	0.55 ± 0.02 (7)	N.S.
	Biceps femoris	0.68 ± 0.09 (18)	0.51 ± 0.05 (17)	0.56 ± 0.06 (8)	N.S.
HBD–LDH	Gastrocnemius	0.289 ± 0.015 (17)	0.262 ± 0.016 (16)	0.262 ± 0.026 (10)	N.S.
	Tibialis Ant	0.266 ± 0.010 (15)	0.264 ± 0.016 (15)	0.263 ± 0.008 (7)	N.S.
	Biceps femoris	0.263 ± 0.020 (15)	0.243 ± 0.014 (17)	0.257 ± 0.015 (8)	$0.05 > P > 0.02$

Values are given as mean ± S.D. and the numbers of animals in parentheses. Nc—normal untreated, Dc—dystrophic untreated, Dt—dystrophic treated.

* Significance of difference between means for untreated and treated dystrophic hamsters

1) than the other two. This pattern is maintained in the dystrophic group. When the muscles in untreated normal and untreated dystrophic animals are compared, the dystrophic muscles contain less CPK, LDH and HBD, but the activity of succinate: (INT) oxidoreductase, except in the gastrocnemius, is not significantly altered. Depending on which muscle is considered, the loss in activity of CPK and LDH is between 14 and 18 per cent for LDH and between 17 and 33 per cent for CPK. The HBD–LDH ratio is also reduced by 5–7 per cent except in the tibialis anterior, the lowered value indicating a higher proportion of M-subunits.

The effect of indomethacin treatment on muscle enzyme activities in dystrophic hamsters is complex and depends upon which muscle is examined (Table 2). However, firstly, muscle enzyme levels in the treated normal group, in contrast to serum, were unaffected by the drug so that data for the normal treated group is omitted. When the dystrophic treated group is compared with the dystrophic controls, the mean tissue enzyme activities generally show a trend towards normal, but only in certain instances is the difference significant. The most striking effect is on CPK, data for which is presented separately (Fig. 1). In the gastrocnemius, for example, indomethacin treatment causes a significant rise in CPK, LDH and HBD. In all three muscles, although CPK activity in the treated dystrophic hamsters remains lower than that in the normals, the difference is no longer significant. The rise in gastrocnemius in LDH and HBD is similar, so that the ratio HBD–LDH is not significantly altered.

In the biceps femoris, there is an increase in HBD unaccompanied by any overall increase in LDH; consequently, the ratio HBD–LDH rises.

At present, there is no proof that these effects are related to the known pharmacological action of indomethacin, e.g. inhibition of prostaglandin synthetase [17] or of lysosomal enzyme release [18], so that the chief interest of the present study is the demonstration that the biochemical changes in muscle, as well as in serum, which occur in an hereditary muscle disease can be partially reversed by drug administration.

We must emphasise that, although indomethacin treatment leads to a partial restoration of intra-cellular enzyme levels to normal, we have no evidence, as yet that there is any corresponding increase in survival time of the affected animals. A pilot study now in progress may provide evidence on this point.

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Correlation between inhibition by anti-inflammatory substances, of arachidonic acid-induced hypotension and of prostaglandin biosynthesis *in vitro**

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According to Vane [1], the nonsteroidal anti-inflammatory drugs act as inhibitors of prostaglandin biosynthesis. Various studies have sought to find a parallel between the inhibition of prostaglandin biosynthesis and their antiphlogistic action, but marked differences in the inhibitory action were observed according to the organs providing prostaglandin synthetases. Therefore, no strict parallel can be drawn between synthesis inhibition and antiphlogistic effect [2, 3].

On the other hand, the hypotensive action of arachidonic acid, precursor of PGE_2 and $PGF_{2\alpha}$, is enhanced by high doses of heparin [4], whereas this hypotension is inhibited by eicosatetraynoic acid, an inhibitor of prostaglandin synthesis [5, 6]. The arachidonic acid-induced hypotension, which depends on endogenous formation of prostaglandins, could be considered as evidence of prostaglandin synthesis *in vivo*, facilitated in the presence of heparin [4]. Therefore, a study of the hypotensive activity of arachidonic acid in animals treated with nonsteroidal anti-inflammatory compounds could be used as an indicator of their inhibitory property towards prostaglandin synthetases *in vivo*. A measure of the total inhibitory activity of the anti-inflammatory drugs using intact animals could be a better approach to the actual mechanism