Effect of the Ornithine Decarboxylase Inhibitor Di-fluoro Methyl-Ornithine on Damage in Electrically Stimulated Muscles

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Abstract

Di-fluoro methyl-ornithine (DFMO), an inhibitor of the enzyme ornithine decarboxylase (ODC), can prevent some types of tissue damage associated with increased membrane permeability. In this study DFMO was given orally and subcutaneously to rabbits in which the left tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were subjected to continuous indirect electrical stimulation at 10 Hz for 3 days. DFMO treatment blocked the increase in $^{99}Tc^{m}$-pyp uptake into TA muscles and into some (although not all) of the EDL muscles, and reduced markedly the amount of damage quantified morphologically in both TA and EDL muscles. These results are strongly suggestive of a direct causal relationship between membrane permeabilization and low levels of muscle damage. DFMO may be a useful tool with which to investigate further the mechanisms underlying muscle damage.

Key words: skeletal muscle, technetium 99m pyrophosphate, electrical stimulation, histology, necrosis.

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Continuous low-frequency electrical stimulation of the tibialis anterior (TA) muscle in rabbits can be used to induce transformation from fast-twitch to slow-twitch characteristics [16; 17]. One feature of the transformation process is a marked increase in the resistance of the muscle to fatigue, and this has led to an upsurge of interest in the use of grafts of transformed skeletal muscle for cardiac assistance [3; 18] and for the construction of neoanal sphincters [20]. Stimulation is, however, associated with some muscle damage [8; 9; 11], and although the proportion of fibres affected is small it is important to understand the aetiology of such damage and to minimize it in muscles used for clinical applications.

Stimulation-induced muscle damage appears to be preceded by an increase in the permeability of the muscle fibre membrane: within a few days of the onset of stimulation there is an increase in the uptake of ^{99}technetium pyrophosphate ($^{99}Tc^{m}$- pyp) into the muscle [1].

Stimulation of fast-twitch muscles at 10 Hz leads to a very large, transient increase in the activity of ornithine decarboxylase (ODC), the enzyme that catalyses the rate-limiting step in the production of the polyamines from ornithine. The activity reaches a peak after about 30 hours [12]. Such an increase has been seen in other experimental models, in which the rise in ODC activity was found to be biphasic, the first peak being detectable just 1 minute after application of testosterone to renal cells [5] or administration of mitogenic antibodies to T-cells [6], the second peak 12-24 hours later. These early increases have been attributed to mobilization of ODC that is normally bound, in an inactive form, directly to cell membranes via phospholipids [14; 15].

ODC activity can be inhibited by the ornithine analogue α-di-fluoro methyl-ornithine (DFMO), which binds non-competitively and irreversibly to the active site of the enzyme. The following evidence indicates that DFMO can prevent some types of acute tissue damage associated with increased membrane permeability. Koenig *et al.* [7], working with perfused hearts, showed that the abnormally large calcium uptake that follows a period of calcium-free perfusion was associated with an early peak in ODC activity. The amount of calcium taken up, and the resultant morphological damage, were both greatly reduced by perfusion with DFMO. The same group showed that breakdown of the blood-brain barrier induced by hypertonic infusion into the carotid arteries of rats *in vivo* was reduced when DFMO was included in the infusion fluid [6]. These findings suggest the possibility that DFMO may also prevent the permeabilization of the muscle fibre membrane that is associated with electrical stimulation. If so, muscle dam-

age resulting from increased activity, and possibly damage with other aetiologies, might be preventable by administration of this compound.

The purpose of this study was therefore to examine the levels of ODC activity during the early stages of electrical stimulation of rabbit TA and extensor digitorum longus (EDL) muscles and the influence on muscle damage of administration of DFMO.

Materials and methods

Operative procedures

All procedures were carried out under the British Home Office (Animals Scientific Procedures) Act, 1986. New Zealand White rabbits with body weights between 3 and 5 kg were operated under fentanyl/fluanisone anaesthesia (Hypnorm, Janssen Pharmaceutica, fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}, 0.3 ml kg^{-1} intramuscularly) after premedication with atropine sulphate (Sigma Chemical Co. Ltd., 3 mg kg^{-1}) and diazepam (Roche Products Ltd.; 5 mg kg^{-1}) given subcutaneously. Full aseptic precautions were taken. Electrodes were implanted in the hind limb close to the common peroneal nerve, which supplies the muscles of the anterior compartment of the hind limb and in particular the TA and EDL muscles. The electrodes were connected to a miniature stimulator [4] which was placed under the skin of the flank. The muscles themselves were not surgically disturbed. Animals were allowed to recover from the operation for 2 weeks before the stimulators were turned on via an optical link; in this way the effects of stimulation could be dissociated from any systemic response to anaesthesia or surgery. The stimulators delivered impulses at supramaximal intensity and a constant frequency of 10 Hz.

Administration of $^{99}Tc^{m}$-pyp

$^{99}Tc^{m}$-pyp was obtained from the Radiopharmacy Department of the Royal Liverpool University Hospital. It was freshly prepared each day. Most of the rabbits received only a single dose of 200 MegaBequerels (MBq), which was injected via the marginal ear vein 3 hours prior to termination. For the rabbits that received $^{99}Tc^{m}$-pyp daily, doses of between 50 and 200 MBq were used, the dose being increased each day to minimize the influence of residual activity.

The half-life of $^{99}Tc^{m}$-pyp is only 6 hours. Therefore all the measurements of $^{99}Tc^{m}$-pyp activity were corrected for the time taken to perform the counting.

Administration and efficacy of DFMO

The DFMO administration protocol was originally suggested to us by Dr A. Pegg (Hershey Medical Centre, Pennsylvania, U.S.A.). For each experimental animal treated with DFMO there was a control, weight-matched rabbit that received injections of a saline placebo for the duration of the stimulation (3 days). Treatment with DFMO began 12 hours before the onset of muscle stimulation. Rabbits (n=5) were given subcutaneous injections of 200 mg/kg body weight DFMO in saline 12-hourly, supplemented by 4.5 g of the drug in their daily drinking water (approximately 250 ml). DFMO has a bitter taste, so the water consumption of the rabbits was monitored to ensure that the DFMO-treated animals did not become dehydrated.

Terminal procedures

Rabbits were deeply anaesthetized with 30% urethane solution (300 mg.kg^{-1}) and Sagatal (60mg/ml pentobarbitone, given to effect). The TA and EDL muscles were then excised and the animal killed by anaesthetic overdose. Blocks 5 mm thick were cut from the whole cross-section of each muscle. The middle block was used for morphological analysis. It was mounted on a cork disc, frozen in isopentane cooled over liquid nitrogen, and stored at -70^{0}C pending use. The adjacent blocks on either side were weighed and counted in an LKB Wallac 1270 Rack Gamma counter to determine the isotope content.

Quantitative morphological assessment of damage

Transverse sections 10 µm thick were cut in a cryostat and air dried for 1 hour. A standard haematoxylin and eosin staining protocol was then used. The quantitative morphology technique has been described previously [8]. Briefly, a grid of 1 mm squares was placed over the muscle section. Every fourth 1 mm square was brought into register with an eye-piece graticule containing a square 10 by 10 grid, and the percentage of damaged fibres, volume fraction of damaged fibres and volume fraction of interfibrillar tissue were determined by point counting.

Assay of ODC content of muscle samples

The ODC assay was based on the method of Fuller *et al.* [2]. The 1-^{14}C analogue of ornithine is decarboxylated by the enzymic activity of ODC in the sample to form $^{14}CO_2$, which can then be trapped and counted.

Conical-bottomed test tubes were used. The plastic tops to these tubes were pierced by a pin that held a small plastic well. Into this was placed a circle of filter paper onto which 20 µl of Optisolv (LKB) was absorbed in order to trap the $^{14}CO_2$. Muscle samples were homogenized in a freshly prepared buffer of 0.05M Tris HCl (pH 7.5), 0.02 mM pyridoxal phosphate, 1.0 mM dithiothreitol and 0.1 mM EDTA. Homogenates were spun at 6,500 g for 10 minutes at 4^{0}C. Duplicate 190 µl samples of the supernatant were placed into ODC assay tubes on ice. The remaining solution was used for protein assay [10]. Samples of the assay fluid were used as blanks for the ODC assay. 10 µl of 50 µM [1-^{14}C] ornithine (Amersham, specific activity 56 mCi/mmol) was added to each assay tube. The mixture was incubated at 37^{0}C for 1 hour. The reaction was then stopped by injection of 0.5 ml of 1 M citric acid through the lid. After a further 1 hour the filter papers were removed and placed in a scintillation vial with 4 ml of Optiphase X scintillation fluid (LKB). The vials were counted for 1 minute each in an LKB Wallac 1215 Rack-Beta II liquid

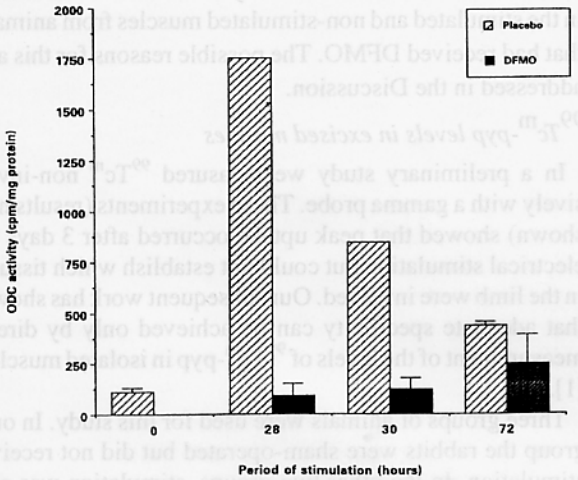

Figure 1. ODC activity in TA muscles stimulated for different durations at 10 Hz. Animals received concurrent treatment with either DFMO or placebo. Values are mean ± SEM where n=2; for other points n=1.

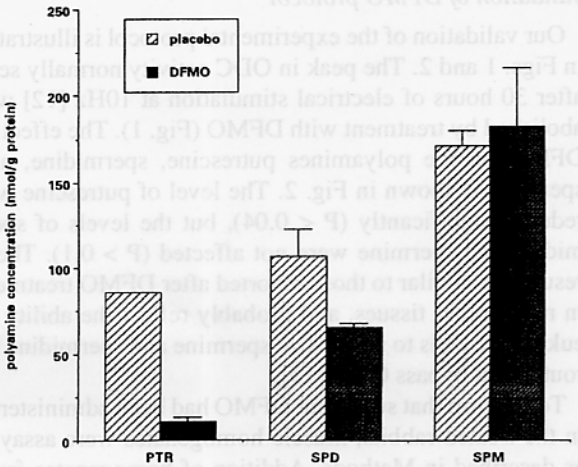

Figure 2. Levels of polyamines putrescine (ptr), spermidine (spd) and spermine (spm) in TA muscles stimulated at 10 Hz for 3 days. Animals received concurrent treatment with DFMO or placebo. Values are mean ± SEM where n=2; for other points n=1.

scintillation beta counter. Values of ODC activity were expressed as dpm.hour $^{-1}$.mg^{-1} protein.

Assay of free DFMO in muscle samples

For each set of assays a supernatant of mouse kidney homogenate (which has a naturally high level of ODC activity) was prepared from frozen kidney samples. Aliquots of this were placed in conical-bottomed tubes, together with an equal volume of the muscle homogenate to be assayed for DFMO activity. The ODC assay was then carried out as above. The decrease in ODC activity caused by the addition of the homogenate was compared to the decrease caused by the addition of known concentrations of DFMO solution. Blanks had ODC assay fluid added in place of the muscle homogenate or DFMO solution.

*Table 1. Quantitative morphology of muscle samples from animals in which muscles of the left hind limb were stimulated at 10 Hz with concurrent injections of placebo or DFMO. Results are expressed as mean ± SEM; n=l0 for placebo-treated and n=5 for DFMO-treated animals. *P < 0.05, comparison with contrlateral muscles; **P < 0.05, comparison with corresponding placebo-treated muscles. TA/L=left TA; TA/R=right TA; EDL/L,=left EDL; EDL/R=right EDL.*

Volume fraction of damaged tissue (%)

	TA/L	TA/R	EDL/L	EDL/R
Placebo	0.43±0.20*	0±0	1.32±0.53*	0.01±0.01
DFMO	0.05±0.05**	0±0	0.12±0.07**	0±0

Percentage of fibres showing damage

	TA/L	TA/R	EDL/L	EDL/R
Placebo	0.72±0.33*	0.01±0.01	1.62±0.58*	0.04± 0.02
DFMO	0.10+0.10*	0±0	0.39±0.18**	0.50+0.11

Volume fraction of non-muscle tissue (%)

	TA/L	TAIR	EDL/L	EDLIR
Placebo	7.32±0.56	6.14±0.74	7.99±1.11	7.49±2.49
DFMO	6.65±0.62	7.75±0.75**	9.03±1.25	7.67±0.33

Results

Validation of DFMO protocol

Our validation of the experimental protocol is illustrated in Figs. 1 and 2. The peak in ODC activity normally seen after 30 hours of electrical stimulation at 10Hz [12] was abolished by treatment with DFMO (Fig. 1). The effect of DFMO on the polyamines putrescine, spermidine, and spermine is shown in Fig. 2. The level of putrescine was reduced significantly ($P < 0.04$), but the levels of spermidine and spermine were not affected ($P > 0.1$). These results are similar to those reported after DFMO treatment in many other tissues, and probably reflect the ability of eukaryotic cells to synthesize spermine and spermidine by routes that bypass ODC [13].

To confirm that sufficient DFMO had been administered in the treated rabbits, muscle homogenates were assayed as described in Methods. Addition of homogenates from TA muscles of placebo-treated rabbits to a mouse kidney homogenate had no significant effect on ODC activity: ODC activity remained at 98.0 ± 10.6% of the level measured in the presence of assay fluid alone (mean ± SEM, n=3). In contrast, homogenates from the TA muscles of DFMO-treated animals produced a fall in ODC activity to only 43.0 ± 10.4% of the level for the assay fluid alone (mean ± SEM, n=3, $P < 0.02$). This effect of the muscle homogenate on the ODC activity was dose dependent (regression coefficient $P < 0.05$). A similar decrease in levels could be produced with a solution of DFMO at a concentration of about 0.15 mM. Thus, DFMO was present in excess in the muscles from the treated rabbits. Despite this, some residual ODC activity could still be measured in the stimulated and non-stimulated muscles from animals that had received DFMO. The possible reasons for this are addressed in the Discussion.

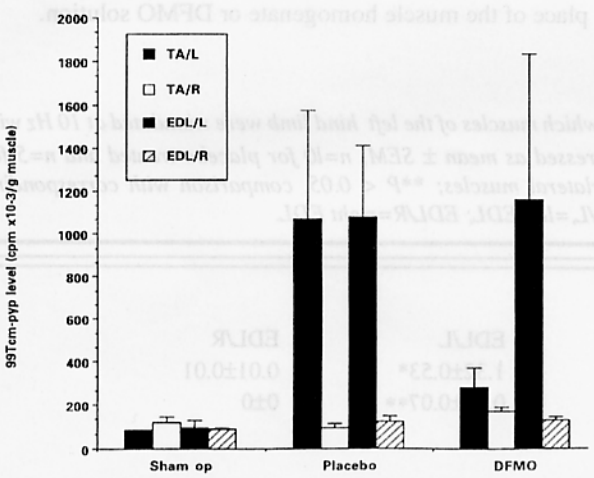

Figure 3. Levels of $^{99}Tc^{m}$-pyp in excised muscles. In both the placebo-treated and DFMO-treated groups the TA and EDL muscles of the left side received stimulation at 10 Hz for 3 days. All values are mean ± SEM; n=2 for sham-operated animals, n=10 for placebo-treated animals; n=5 for DFMO-treated animals. Abbreviations as for Table 1.

$^{99}Tc^{m}$-pyp levels in excised muscles

In a preliminary study we measured $^{99}Tc^{m}$ non-invasively with a gamma probe. These experiments (results not shown) showed that peak uptake occurred after 3 days of electrical stimulation but could not establish which tissues in the limb were involved. Our subsequent work has shown that adequate specificity can be achieved only by direct measurement of the levels of $^{99}Tc^{m}$-pyp in isolated muscles [1].

Three groups of animals were used for this study. In one group the rabbits were sham-operated but did not receive stimulation. In the other two groups, stimulation was applied to the left common peroneal nerve at 10 Hz for 3 days. One group received DFMO treatment during stimulation, and the other group received injections of a saline placebo. The mean $^{99}Tc^{m}$-pyp levels in muscles from these three groups of animals are shown in Fig. 3.

The 2 sham-operated animals showed low levels of $^{99}Tc^{m}$-pyp in both TA and EDL muscles. Ten placebo-treated rabbits that received electrical stimulation at 10 Hz all showed significantly higher levels of $^{99}Tc^{m}$-pyp in the stimulated (left) muscles than in the contralateral muscles. There was, however, a great deal of interindividual variation in the actual level of $^{99}Tc^{m}$-pyp uptake. In the 5 DFMO-treated animals there was a different pattern of $^{99}Tc^{m}$-pyp uptake. In the stimulated TA muscles the levels were not significantly different from those in the contralateral unstimulated muscles, and were significantly lower than those in the TA muscles that had received stimulation at 10 Hz with placebo. Thus the DFMO treatment appeared to have decreased the uptake of $^{99}Tc^{m}$-pyp in the TA muscles. The EDL muscles showed a much more variable response and mean levels of $^{99}Tc^{m}$-pyp were not significantly different between animals that received DFMO and those that received placebo.

Quantitative morphology of damage in muscle samples

The muscles from the animals in which stimulation at 10 Hz was combined with DFMO or placebo administration for 3 days were assessed morphologically; the data is shown in Table 1. In this Table the volume fraction of non-muscle tissue is a measure of the amount of interstitial tissue, including endomysial and perimysial connective tissue, in the muscle. This was not significantly different in any of the muscles examined.

In the animals that received electrical stimulation alone, the stimulated TA and EDL muscles both showed significantly more damage than their contralateral controls in terms of the volume fraction of damaged fibres and percentage of damaged fibres. Both TA and EDL muscles from animals that received DFMO as well as electrical

stimulation had significantly fewer damaged fibres than the corresponding muscles in the saline-treated group – in fact there was no significant difference between these muscles and their contralateral controls.

Discussion

In animals that had received DFMO treatment we were able to confirm that the inhibitor was present in the muscles in excess. Despite this, some ODC activity (approximately 60 dpm/mg protein) still remained in these muscles. The possibility was considered that a proportion of the ODC in the cell was resistant to DFMO, or was sequestered in a subcellular compartment inaccessible to the compound. However, the ODC activity in muscle homogenates from non-DFMO-treated animals could be reduced to the level of an assay fluid blank by adding DFMO (data not shown). This suggests that in a muscle which has not been exposed previously to DFMO all the ODC can be inactivated by the compound. Therefore in the muscles from the DFMO-treated animals either the ODC is different in nature or alternative routes for ornithine decarboxylation exist. Spermidine and spermine can be produced by pathways that bypass ODC [13] – for example by the action of arginine decarboxylase, which has a low affinity for ornithine. In the present study, DFMO did not depress levels of spermine in stimulated muscles (Fig. 2), despite a clearly demonstrated suppression of the ODC response (Fig. 1). We conclude that treatment of the animals with DFMO activated alternative routes to polyamine production, leading to significant amounts of non-ODC-catalysed decarboxylation of ornithine in the ODC assays.

The serum level of $^{99}Tc^{m}$-pyp in the rabbits was approximately 1 x 10^{6} dpm/ml. Since the concentration of $^{99}Tc^{m}$-pyp in TA and EDL muscles subjected to stimulation at 10 Hz was higher than this, the uptake of $^{99}Tc^{m}$-pyp could not be accounted for in terms of œdema or an increase in blood volume alone, and a proportion of the counts must have come from the intracellular compartment.

DFMO reduced the uptake of $^{99}Tc^{m}$-pyp in the TA muscles. However, it appeared to have a more variable effect on the $^{99}Tc^{m}$-pyp uptake of the EDL muscles. We have shown for a variety of stimulation regimes that EDL muscles are consistently more damaged than the ipsilateral TA muscles [1; 8; 9]. Stimulated EDL muscles also take up more $^{99}Tc^{m}$-pyp than the corresponding TA muscles [1]. It may be that the mechanism of $^{99}Tc^{m}$-pyp uptake in the EDL muscles includes a component that is qualitatively different from that in the TA and not susceptible to reduction by DFMO in the same way.

Although the fibre damage associated with stimulation is not fully developed until about 9 days of stimulation [8; 9; 11] a low level of damage (2% of fibres at maximum) was already detectable in these experiments at 3 days. DFMO treatment produced a significant decrease in the incidence of this damage, reducing it to control levels. Unlike the effect of DFMO on $^{99}Tc^{m}$-pyp uptake, this reduction in damage was observed consistently in both the TA and EDL muscles. This suggests that the inhibitory effects of DFMO may not be confined to membrane permeabilization.

There is still much to learn about the mechanisms responsible for muscle damage, particularly in regard to the reversibility or irreversibility of damage and the part played by membrane permeabilization [19]. The effects of DFMO reported here should encourage use of this enzyme inhibitor as an investigative tool with which to enlarge our understanding of these issues.

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