Effect of inhibitors of arachidonic acid metabolism on efflux of intracellular enzymes from skeletal muscle following experimental damage

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The role of arachidonic acid metabolism in the efflux of intracellular enzymes from damaged skeletal muscle has been examined *in vitro* using inhibitors of cyclo-oxygenase and lipoxygenase enzymes. Damage to skeletal muscle induced by either calcium ionophore A23187 (25 μ M) or dinitrophenol (1 mM) caused an increase in the efflux of prostaglandins E_2 and $F_{2\alpha}$ together with a large efflux of intracellular creatine kinase. Use of a cyclo-oxygenase inhibitor completely prevented the efflux of prostaglandins, but had no effect on creatine kinase efflux. However, several agents having the ability to inhibit lipoxygenase enzymes dramatically reduced creatine kinase efflux following damage. These data suggest that a product or products of lipoxygenase enzymes may be mediators of the changes in plasma membrane integrity which permit efflux of intracellular enzymes as a consequence of skeletal muscle damage.

INTRODUCTION

Elevated plasma levels of enzymes normally found at high concentration in skeletal muscle are well described in patients with various muscle disorders (Pennington, 1980) and in patients with Duchenne muscular dystrophy the plasma levels of creatine kinase are often two orders of magnitude greater than normal. It is generally assumed that this release of intracellular constituents reflects a change in the permeability of the surface membrane of the muscle fibres in disease states, but elevated levels may also be found in normal subjects during or following particularly strenuous or unaccustomed exercise (Griffiths, 1966; Shumate *et al.*, 1979). Elevated levels of intramuscular calcium can cause damage to skeletal muscle (Duncan, 1978; Publicover *et al.*, 1978) and increased intramuscular calcium levels have been implicated in the pathogenic mechanisms underlying muscular dystrophy (Bodenstein & Engel, 1978; Maunder-Sewry *et al.*, 1980). In addition, damage to isolated skeletal muscle following excessive contractile activity has been shown to be associated with an increase in total muscle calcium (Claremont *et al.*, 1984) and is prevented by removal of external calcium from the incubation medium (Jones *et al.*, 1984).

Various studies have been undertaken to try and elucidate the mechanism by which an increase in intracellular calcium induces damage to skeletal muscles (e.g. see Wroghmann & Pena, 1976; Ebashi & Sugita, 1979; Rodemann *et al.*, 1981; Baracos *et al.*, 1986). Our previous inhibitor studies (Jackson *et al.*, 1984) have suggested that elevated intracellular muscle calcium is deleterious to membrane integrity by activation of muscle phospholipase (probably phospholipase A_2) leading to release of membrane-bound fatty acids and the formation of lysophospholipids. In addition, a possible role of free radical metabolites in the process of damage has been suggested by studies demonstrating that the vitamin E content of muscle influences the extent of

enzyme efflux which occurs following a given amount of contractile activity (Jackson *et al.*, 1983). Further support for this hypothesis comes from e.s.r. studies in which an increase in the amount of stable free radical in muscle was found following damaging excessive contractile activity *in vivo* (Jackson *et al.*, 1985a).

One of the consequences of an activation of phospholipase A_2 during damage would be a release of membrane-bound arachidonic acid, allowing its conversion to bioactive metabolites via cyclo-oxygenase or lipoxygenase enzymes. Since many of the products of these two enzyme systems are well known to have significant biochemical and physiological effects on cells, we have used inhibitors of these two enzyme systems to examine the possible roles of arachidonic acid metabolites in enzyme efflux from experimentally damaged skeletal muscle *in vitro*.

MATERIALS AND METHODS

Female Wistar rats (100–200 g) maintained on a standard laboratory diet were killed by cervical dislocation and the soleus muscles were rapidly dissected and removed. The muscles were mounted in special holders and incubated in 3.0 ml of bicarbonate-buffered Ringer at 37 °C. The apparatus and conditions used were as previously described (Jones *et al.*, 1983). After one or two initial 30 min incubation periods, the medium was exchanged and during the treatment period the agents known to damage the muscles and induce intracellular enzyme efflux were added to the medium. After 30 min the medium was exchanged and this was repeated every 30 min until the end of the experiment (a maximum of 90 min). The creatine kinase activity of the incubation media and muscles were analysed as previously described (Jones *et al.*, 1983). The calcium content of incubated muscles was measured by atomic absorption spectrometry (Jackson *et al.*, 1985b) and the contents of prostaglandins

Fig. 1. Efflux of prostaglandins E₂ (■, □) and F_{2α} (◆, ◇) and creatine kinase (●, ○) from rat soleus muscles treated with calcium ionophore (A23187) in the absence (filled symbols) or presence (open symbols) of biphenylacetic acid

All muscles were treated with calcium ionophore during the 30 min period represented by the shaded area. When biphenylacetic acid was used, it was added throughout the experiment.

E₂ and F_{2α} in the incubation media were measured with radioimmunoassay kits purchased from New England Nuclear (prostaglandin E₂) and Travenol Laboratories (prostaglandin F_{2α}).

All the agents were of AnalaR grade or the highest grade commercially available. Phenidone (1-phenyl-3-pyrazolidone) and NDGA were obtained from the Sigma Chemical Co. Biphenylacetic acid was a kind gift from Lederle Laboratories, and BW755C was kindly supplied by Wellcome Research Laboratories Ltd.

All results are presented as the means $\pm$ S.E.M. for four to six muscles.

RESULTS

Damage to isolated skeletal muscles was initiated by the use of either the calcium ionophore, A23187, an agent known to cause increased intracellular calcium levels leading to both ultrastructural damage (Publicover *et al.*, 1978) and enzyme efflux (Jones *et al.*, 1984) or by

Fig. 2. Efflux of prostaglandins E₂ (■, □) and F_{2α} (◆, ◇) and creatine kinase (●, ○) from rat soleus muscles treated with DNP in the absence (filled symbols) or presence (open symbols) of biphenylacetic acid

treatment with DNP which causes both substantial enzyme loss and ultrastructural damage to muscle (Jackson *et al.*, 1984).

Effect of a cyclo-oxygenase inhibitor

Treatment with the calcium ionophore (25 μ M) induced a large increase in the efflux of prostaglandin E₂ from incubated muscles (Fig. 1) and a smaller, but significant, increase in prostaglandin F_{2α} efflux. This was accompanied by a large efflux of creatine kinase from the muscles. Treatment of the muscles throughout the experiment with biphenylacetic acid (95 μ M) completely prevented the calcium ionophore-induced release of prostaglandins E₂ and F_{2α} (Fig. 1), but had no effect on the extent of the calcium ionophore-induced loss of creatine kinase.

Similar effects were seen following treatment with DNP (1 mM). This also induced a large efflux of prostaglandin E₂ and a small but significant increase in the efflux of prostaglandin F_{2α} from control muscle samples (Fig. 2) and caused a substantial release of creatine kinase from the muscle samples. Biphenylacetic acid was again found to prevent this rise in prostaglandin release without influencing creatine kinase efflux.

Fig. 3. Efflux of creatine kinase from rat soleus muscles treated with calcium ionophore (a) or DNP (b) in the presence (□) or absence (■) of phenidone (100 μ M)

Effect of joint cyclo-oxygenase and lipoxygenase inhibitors

The effect of two compounds capable of inhibiting both the cyclo-oxygenase- and lipoxygenase-catalysed conversion of arachidonic acid were tested. Muscles were treated with either calcium ionophore or DNP with and without either 100 μ M-phenidone (Fig. 3) or 400 μ M-BW755C (Fig. 4). Phenidone almost completely inhibited the efflux of creatine kinase from both calcium ionophore- and DNP-treated muscles (Fig. 3), whereas BW755C appeared to permit the initial rise in creatine kinase efflux which occurred during calcium ionophore treatment, but prevented the further increase which occurred after removal of the ionophore (Fig. 4a). BW755C significantly reduced the creatine kinase efflux from the DNP-treated muscles (Fig. 4b).

Effect of NDGA on enzyme efflux from damaged muscles

NDGA is a compound which has significant inhibitory effects on lipoxygenase activity and also has an ability to scavenge certain types of free radicals produced by non-enzymic systems. NDGA at a concentration of 5 μ M was found to have a significantly inhibitory effect on creatine kinase efflux from muscles treated with calcium ionophore (Fig. 5a) and at 50 μ M completely inhibited creatine kinase efflux from muscles treated with DNP (Fig. 5b).

Fig. 4. Efflux of creatine kinase from rat soleus muscles treated with calcium ionophore (a) or DNP (b) in the presence (□) or absence (■) of BW755C (400 μ M)

Table 1. Muscle calcium contents after calcium ionophore treatment in the presence or absence of phenidone or NDGA

Muscles were assayed at the end of the experiment, i.e. 90 min after the completion of calcium ionophore treatment. Results are presented as μ mol/g dry wt. and given as the means $\pm$ S.E.M. for four to six muscles.

'Protective' agent	Calcium content (μ mol/g dry wt.)	
	Muscles treated with calcium ionophore and 'protective' agent	Control muscles treated with calcium ionophore alone
Phenidone	20.4 $\pm$ 2.4	18.2 $\pm$ 1.6
NDGA	23.9 $\pm$ 4.1	19.5 $\pm$ 2.5

In some experiments the NDGA was removed from the incubation fluid 60 min after the end of DNP treatment. This caused a rapid and substantial release of creatine kinase over the next 30 min (Fig. 5b).

Effect of NDGA on muscle calcium content following experimental damage

Any possible protective effect of NDGA caused by inhibition of calcium accumulation by muscle during

Fig. 5. Efflux of creatine kinase from rat soleus muscles treated with calcium ionophore (a) or DNP (b) in the presence (□) or absence (■) of NDGA

Eight muscles were examined in each group and at 60–90 min post-treatment NDGA was removed from the medium surrounding four of the muscles in the treated group (◇).

damage was checked by analysis of muscle calcium content following calcium ionophore treatment with and without the presence of phenidone or NDGA (Table 1). Since fresh rat soleus muscle has a calcium content of approx. 5 $\mu\text{mol/g}$ dry wt., the results here demonstrate a large rise in total muscle calcium following ionophore treatment both with and without the presence of phenidone or NDGA.

All putative protective agents utilized in this study (i.e. biphenylacetic acid, BW755C, phenidone and NDGA) were also tested to ensure that they did not inhibit creatine kinase activity *in vitro* and, in addition, the total creatine kinase content of muscle treated with calcium ionophore with or without protective agents was also examined (results not shown). The ionophore was found to induce a loss of less than 10% of total muscle creatine kinase, which was almost entirely accounted for by the creatine kinase found in the incubation fluids.

DISCUSSION

The plasma activities of intracellular muscle enzymes are utilized as an index of the rate of muscle damage in

clinical monitoring of the progress of muscle diseases. There are various features of the process of enzyme efflux which are not well understood. In particular, it is not known whether *in vivo* viable and intact cells lose significant amounts of intracellular enzymes or whether the efflux seen is only from cells which have lost plasma membrane integrity. The process of enzyme efflux from skeletal muscle following both excessive contractile activity and treatment with various poisons appears to involve an influx of external calcium (Jones *et al.*, 1984; Claremont *et al.*, 1984; Jackson *et al.*, 1984) and has been proposed to involve calcium activation of phospholipase A enzymes (Jackson *et al.*, 1984). The work reported here has demonstrated unequivocal release of prostaglandins E_2 and $F_{2\alpha}$ during damage induced by the calcium ionophore (A23187) or DNP (Figs. 1 and 2) which must result from release of arachidonic acid from its esterified storage form. This provides further support for our hypothesis that calcium is acting via activation of phospholipase to induce membrane changes leading to enzyme efflux (Jackson *et al.*, 1984).

A role for prostaglandin E_2 in mediating calcium-induced protein degradation has been proposed by Rodemann *et al.* (1982) and release of prostaglandin $F_{2\alpha}$ has been proposed to stimulate protein synthesis (Reeds & Palmer, 1983; Smith *et al.*, 1983), but prostaglandins do not appear to be involved in the process of enzyme efflux. Biphenylacetic acid is the active metabolite of the drug Lederfen and is an extremely efficient cyclo-oxygenase inhibitor (Sloboda & Osterberg, 1976). In the present study it was found to decrease the non-stimulated basal production of prostaglandin E_2 and $F_{2\alpha}$ by isolated muscles, and completely prevented the increased release of prostaglandins associated with treatment of the muscles with calcium ionophore or DNP (Figs. 1 and 2). However, biphenylacetic acid had no effect on the enzyme efflux following calcium ionophore or DNP treatment.

The alternative enzyme system for conversion of arachidonic acid to active metabolites (i.e. lipoxigenase enzymes) has been described in several tissues and cell types, e.g. platelets (Nugteren, 1975), pancreatic acinar cells (Rubin *et al.*, 1982), kidney glomeruli (Sraer *et al.*, 1983), uterus (Morgan *et al.*, 1984) and liver (Hewertson *et al.*, 1984), although not as far as we are aware in skeletal muscle. Phenidone and BW755C have been shown to inhibit both the cyclo-oxygenase and lipoxigenase enzymes (Higgs & Vane, 1983) and have also been found to prevent intracellular enzyme efflux from the isolated skeletal muscle preparation (Figs. 3 and 4). BW755C was however only found to inhibit the process at relatively high concentrations (i.e. > 350 μM) and it has been suggested that it may have an alternative action as a weak calcium channel blocker at this concentration (Higgs *et al.*, 1985). However, since extremely potent calcium channel blockers, such as verapamil and nifedipine, are ineffective in reducing enzyme efflux from experimentally damaged skeletal muscle (Jones *et al.*, 1984) this is unlikely to be the mode of action of BW755C in this system. Further evidence for a protective effect due to the inhibition of lipoxigenase comes from studies using NDGA (Higgs & Vane, 1983). At a concentration of 5 μM this substance effectively prevented enzyme efflux induced by the calcium ionophore (Fig. 5a) and at 50 μM inhibited enzyme efflux induced by DNP. NDGA is also known to be an inhibitor of non-enzymic free-radical-

mediated processes (Halliwell & Gutteridge, 1985) but other substances having this property (propyl gallate or butylated hydroxytoluene) have previously been shown to have no protective effect in this system (Jackson & Edwards, 1986).

Table 1 demonstrates that the apparent protective effects of phenidone and NDGA are not via inhibition of calcium accumulation during muscle damage. It therefore seems likely that lipoxygenase products are involved in the process of calcium-induced enzyme efflux from skeletal muscle and furthermore the data presented here suggest that lipoxygenase enzymes are likely to be present in skeletal muscle, although the possibility that the effects seen are due to lipoxygenase in contaminating white cells cannot be completely discounted. BW755C has been shown to reduce the damage to cardiac muscle which occurs following experimental myocardial infarction (Mullane *et al.*, 1984). Those authors attributed the effect to inhibition of the toxic effects of invading leucocytes, but were nevertheless able to demonstrate lipoxygenase activity in control myocardium.

Removal of NDGA from the incubation fluid surrounding the skeletal muscle preparation following treatment with DNP caused a large and rapid efflux of creatine kinase (Fig. 5b), demonstrating that the inhibition of lipoxygenase is readily reversible and that the phospholipase A-mediated release of arachidonic acid and other fatty acids alone is insufficient to induce enzyme efflux from skeletal muscle. This implies that a product or products of lipoxygenase activity must be capable of causing changes in muscle membrane permeability.

Several different forms of mammalian lipoxygenase have been described that catalyse the conversion of arachidonic acid to 5-HETE (Borgeat *et al.*, 1976), 12-HETE (Nugteren, 1975) and 15-HETE (Sraer *et al.*, 1983), but in other muscle tissues, i.e. smooth muscle and cardiac muscle, the 12-HETE lipoxygenase seems to predominate (Morgan *et al.*, 1984; Mullane *et al.*, 1984). Vitamin E has recently been shown to inhibit lipoxygenase enzymes (Grossman & Waksman, 1984; Reddanna *et al.*, 1985), which may explain the reported protective effect of Vitamin E against skeletal muscle damage (Jackson *et al.*, 1983). There is some suggestion that the unstimulated basal efflux of creatine kinase from the incubated muscles may also occur by similar mechanisms to that seen with damage, since the creatine kinase activity during the first 30 min of incubation prior to administration of toxic agents was also found to be decreased by NDGA (Fig. 5a) or phenidone (Fig. 3a).

In summary, the inhibitor studies reported here have suggested that lipoxygenase products are mediators of the intracellular enzyme efflux seen following experimental damage to skeletal muscle. Lack of suitable lipoxygenase inhibitors for use in man currently prevents studies to determine the relevance of this to intracellular enzyme efflux seen in patients with muscle diseases and in normal subjects following excessive exercise; studies to examine this aspect in animal models are required.

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REFERENCES

- Baracos, V., Greenberg, R. E. & Goldberg, A. L. (1986) *Am. J. Physiol.* **250**, E702-E710.
- Bodenstein, J. B. & Engel, A. G. (1978). *Neurology* **28**, 429-446
- Borgeat, P., Hamberg, M. & Samuelsson, B. (1976). *J. Biol. Chem.* **251**, 7816-7820
- Claremont, D., Jackson, M. J. & Jones, D. A. (1984). *J. Physiol. (London)* **353**, 57p
- Duncan, C. J. (1978) *Experientia* **34**, 1531-1535
- Ebashi, S. & Sugita, H. (1979) in *Current Topics in Nerve and Muscle Research* (Aguayo, A. J. & Karpatis, G., eds.), pp. 73-84, Excerpta Medica, Amsterdam
- Griffiths, P. D. (1966) *Clin. Chim. Acta.* **13**, 413-420
- Grossman, S. & Waksman, E. G. (1984) *Int. J. Biochem.* **16**, 281-289
- Halliwell, B. & Gutteridge, J. M. C. (1985) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford
- Hewertson, S., McDonald-Gibson, R. G. & Slater, T. F. (1984) *Biochem. Soc. Trans.* **12**, 835-836
- Higgs, G. A., Muirgidge, K. G. & Moncada, S. (1985) in *Calcium Entry Blockers and Tissue Protection* (Godfraind, T., ed.), pp. 51-58, Raven Press, New York
- Higgs, G. A. & Vane, J. R. (1983) *Br. Med. Bull.* **39**, 265-270
- Jackson, M. J. & Edwards, R. H. T. (1986) in *Reactive Oxygen Species in Chemistry, Biology and Medicine* (Quintanilha, A., ed.), NATO Publications, in the press
- Jackson, M. J., Jones, D. A. & Edwards, R. H. T. (1983) *Ciba Found. Symp.* **101**, 224-234
- Jackson, M. J., Jones, D. A. & Edwards, R. H. T. (1984) *Eur. J. Clin. Invest.* **14**, 369-374
- Jackson, M. J., Edwards, R. H. T. & Symons, M. C. R. (1985a) *Biochim. Biophys. Acta* **847**, 185-190
- Jackson, M. J., Jones, D. A. & Edwards, R. H. T. (1985b) *Clin. Chim. Acta* **147**, 215-221
- Jones, D. A., Jackson, M. J. & Edwards, R. H. T. (1983) *Clin. Sci.* **65**, 193-201
- Jones, D. A., Jackson, M. J., McPhail, G. & Edwards, R. H. T. (1984) *Clin. Sci.* **66**, 317-322
- Maunder-Sewry, C. A., Gorodetsky, R., Yaron, R. & Dubowitz, V. (1980) *Muscle Nerve* **3**, 502-508
- Morgan, A., McDonald-Gibson, R. G. & Slater, T. F. (1984) *Biochem. Soc. Trans.* **12**, 837-838
- Mullane, K. M., Read, N., Salmon, J. A. & Moncada, S. (1984) *J. Pharmacol. Exp. Ther.* **228**, 510-522
- Nugteren, D. H. (1975) *Biochim. Biophys. Acta* **380**, 299-307
- Pennington, R. J. T. (1980) *Br. Med. Bull.* **36**, 123-126
- Publicover, S. J., Duncan, C. J. & Smith, J. L. (1978). *J. Neuropathol. Exp. Neurol.* **37**, 544-557
- Reeds, P. J. & Palmer, R. M. (1983) *Biochem. Biophys. Res. Commun.* **116**, 1084-1090
- Reddanna, P., Rao, M. K. & Reddy, C. C. (1985) *FEBS Lett.* **193**, 39-43
- Rodemann, H. P., Waxman, L. & Goldberg, A. L. (1982) *J. Biol. Chem.* **257**, 8716-8723
- Rubin, R. P., Kelly, K. L., Halenda, S. P. & Laychock, S. G. (1982) *Prostaglandins* **24**, 179-193
- Schumate, J. B., Brooke, M. H., Carroll, J. E. & Davis, J. E. (1979) *Neurology* **29**, 902-904
- Sloboda, A. E. & Ostberg, A. C. (1976) *Inflammation* **1**, 415-438
- Smith, R. H., Palmer, R. M. & Reeds, P. J. (1983) *Biochem. J.* **214**, 153-161
- Sraer, J., Rigaud, M., Bens, M., Rabinovitch, H. & Ardaillon, R. (1983) *J. Biol. Chem.* **258**, 4325-4330
- Wrognmann, K. & Pena, S. J. G. (1976) *Lancet* **ii**, 672-674