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Protein synthesis and degradation in isolated muscle

Effect of ω3 and ω6 fatty acids

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The ability of derivatives of the essential fatty acids linoleic acid ($C_{18:2}$,ω6) and α-linolenic acid ($C_{18:3}$,ω3) to stimulate rates of protein synthesis and degradation was investigated in isolated intact muscles from fasted rabbits. Both ω6 derivatives examined, arachidonic acid ($C_{20:4}$, ω6) and dihomo-γ-linolenic acid ($C_{20:3}$, ω6), when added at concentrations up to 1 μM, stimulated the rate of protein synthesis and the release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Metabolites of the ω6 series, namely eicosapentaenoic acid ($C_{20:5}$,ω3) and docosahexaenoic acid ($C_{22:6}$,ω3), were without effect on the rate of protein synthesis and resulted in a decrease in the release of $PGF_{2\alpha}$. None of the fatty acids had a significant effect on the rate of protein degradation. Although insulin (100 μunits/ml) also stimulated rates of protein synthesis when added alone, none of the ω3 or ω6 fatty acids, when added with insulin at concentrations of 0.2 μM, potentiated the effect of the hormone.

INTRODUCTION

Arachidonic acid has been shown to stimulate rates of protein synthesis in isolated rabbit muscles (Smith *et al.*, 1983). Two inhibitors of PG synthesis, namely indomethacin and meclofenamic acid, diminished the release of $PGF_{2\alpha}$ from the muscles and blocked the stimulatory effect on protein synthesis of arachidonic acid and intermittent stretching (Smith *et al.*, 1983). Insulin (100 μunits/ml) also stimulated protein synthesis in muscles from fasted rabbits, and indomethacin inhibited this effect as well (Reeds & Palmer, 1983).

A second prostaglandin, PGE_2, has been implicated in the control of protein degradation in both normal (Rodemann & Goldberg, 1982) and pathological states (Goldberg *et al.* 1984).

These observations have given rise to the hypothesis that two metabolities of arachidonic acid, namely PGE_2 and $PGF_{2\alpha}$, are involved in the control of protein accretion in skeletal muscle by influencing the processes of degradation and synthesis respectively. The present series of experiments were designed to investigate the role of other metabolites of the essential fatty acids in the stimulation of rates of protein synthesis and degradation and to examine the effect of these metabolites on the stimulation of protein synthesis by insulin.

EXPERIMENTAL

Materials

ARA, DGLA, EPA and DHA were all obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) Actrapid monocomponent pig insulin was purchased from Farillon (Romford, Essex, U.K.). [^{3}H]$PGF_{2\alpha}$ radioimmunoassay kits were purchased from Steranti Research Ltd. (St. Albans, Herts., U.K.). L-[2,6-^{3}H]Phenylalanine was purchased from Amersham International (Amersham, Bucks., U.K.). All other chemicals, including those used in the preparation of the incubation medium and in the β-phenethylamine assay, were purchased from Sigma or from BDH (Poole, Dorset, U.K.).

Animals and incubation conditions

Male New Zealand White rabbits (600–950 g) were used in all experiments. The animals were fasted for 18 h before removal of the forelimb digit extensor muscles (Palmer *et al.*, 1981). The muscles were 2–2.5 mm in diameter, 2–2.5 cm in length and weighed 35–70 mg. They were dissected intact and with tendons attached, a suture tied to the distal tendon was attached to the base of the incubation vessel, and a 10 g weight was attached to the suture on the proximal tendon (Palmer *et al.*, 1981). The medium was based on that described by Trowell (1959), modified to contain both non-essential and essential amino acids at approximately the concentration found in rabbit blood (Reeds *et al.*, 1980). To facilitate the measurement of tyrosine release, the tyrosine content of the medium was decreased to 40 μM (Palmer *et al.*, 1985). Each incubation vessel contained approx. 2 ml of medium, which was sterilized before use by filtration through a 0.2 μm-pore-size Acrodisc (Gelman Sciences, Northampton, U.K.) and was maintained at pH 7.4 by continuous gentle bubbling with oxygen/CO_2 (19:1). Incubation vessels were covered with Parafilm throughout the experiments. Fatty acids were stored as stock solutions in chloroform/methanol (1:1, v/v) under oxygen-free N_2 at −20 °C. Portions were evaporated to dryness under a stream of oxygen-free N_2 and redissolved in ethanol at a concentration such that 10 μl of the ethanol solution added to the incubation vessel achieved the required molarity; 10 μl of ethanol was added to the controls. Incubations lasted for 4.5 h, the essential fatty acids being present in the appropriate incubation vessels throughout this time. After 4 h the medium was replaced with medium of a similar composition but containing 1.5 mM-L-phenylalanine and L-[2,6-^{3}H]phenylalanine (final sp. radioactivity 1500 d.p.m./nmol). At the end of the incubation

Abbreviations used: ARA, arachidonic acid ($C_{20:4}$,ω6); DGLA, dihomo-γ-linolenic acid ($C_{20:3}$,ω6); EPA, eicosapentaenoic acid ($C_{20:5}$, ω3); DHA, docosahexaenoic acid ($C_{22:5}$,ω3); PG, prostaglandin.

the muscles were weighed, washed briefly in ice-cold NaCl (9 g/l) and frozen at −20 °C until required for analysis. Subsequent treatment of the muscles to determine the specific radioactivity of protein-bound phenylalanine was described previously (Smith *et al.*, 1983).

Tyrosine released into the medium during the first 4 h of the incubation was measured by the method of Waalkes & Udenfriend (1957), modified as described by Palmer *et al.* (1985). $PGF_{2\alpha}$ release was measured by radioimmunoassay. Tyrosine release had previously been shown to remain constant during a 5 h incubation (Palmer *et al.*, 1985).

Phenylalanine content of the medium and the muscle protein was determined by fluorescence assay, after conversion of the phenylalanine into β-phenethylamine (Garlick *et al.*, 1980), with an Aminco–Bowman spectrophotofluorimeter. Radioactivity in the β-phenethylamine extracts was measured in a Packard 460CD liquid-scintillation counter with NE265 (A. and J. Beveridge, Edinburgh, Scotland, U.K.) as the scintillant.

Calculation of fractional rates of protein synthesis and degradation

Fractional rates (k_s) of protein synthesis were calculated from the specific radioactivity of the phenylalanine in the medium and in the muscle protein by using the formula:

$$k_s\ (\%/\text{day}) = \frac{S_b}{S_a} \times \frac{100}{t}$$

where S_b and S_a are the specific radioactivities of the protein-bound and free phenylalanine respectively and t is time in days (Garlick *et al.*, 1980). It had previously been shown that the homogenate pool of free phenylalanine attained a specific-radioactivity value that was 96% of that in the medium within 2 min of the addition of [^{3}H]phenylalanine when the medium contained 2.5 mM-phenylalanine (Smith *et al.*, 1983). Subsequently this rapid equilibration has been shown to be maintained with concentrations of phenylalanine from 2.5 to 1.0 mM. At 1.5 mM, the concentration used here, homogenate specific radioactivities of 1518±35 d.p.m./nmol ($n = 5$) were found 2 min after the addition of phenylalanine to the medium (final sp. radioactivity 1580 d.p.m./nmol).

RESULTS AND DISCUSSION

Rates of protein synthesis (Table 1) were significantly increased by both the ω6 fatty acids (ARA and DGLA) at a concentration of 0.2 μM. Both were also effective at higher concentrations (up to 1 μM). Neither of the ω3 derivatives (EPA and DHA) had any effect on protein synthesis, either at 1 μM (Table 1) or at lower concentrations down to 0.2 μM ($k_s = 1.6 \pm 0.3$ and 1.7 ± 0.5 respectively, $n = 4$).

$PFG_{2\alpha}$ release was increased by both ARA and DGLA (+56% and +75% respectively) and was significantly reduced by DHA and EPA (−46% and −49% respectively).

This reduction in $PGF_{2\alpha}$ release by DHA and EPA is probably due to competition by these ω3 precursors for the enzymes involved in eicosanoid synthesis, which reduce overall PG synthesis (Culp *et al.*, 1979) and do not give rise to prostaglandins of the '2' series (Dyerberg *et al.*, 1978). It also appears that, whereas increases in PG release resulting from intermittent mechanical stretching (Smith *et al.*, 1983) or insulin (Reeds & Palmer, 1983) or the addition of ARA or DGLA (Table 1) are associated with a stimulation in the rate of protein synthesis, the converse is not true, i.e. the basal rate of protein synthesis observed in control muscles incubated without mechanical or hormonal stimuli is not further diminished by a reduction in the release of PGs. The inability of indomethacin and meclofenamic acid to reduce the basal rate of protein synthesis in control muscles while having a marked inhibitory effect on hormonally elevated rates of protein synthesis (Smith *et al.*, 1983) is an analogous observation. Thus is appears that the maintenance of a basal rate of protein synthesis either requires extremely

Table 1. Effect of metabolites of essential fatty acids on the rates of prostaglandin $F_{2\alpha}$ release and fractional rates of protein synthesis and degradation in isolated muscle

Muscles were incubated for a total of 4.5 h. After 4 h, isotope-free medium was replaced with medium of a similar composition, but containing 1.5 mM-phenylalanine and [^{3}H]phenylalanine (final sp. radioactivity 1500 d.p.m./nmol). Samples of the medium removed after 4 h were assayed for tyrosine and $PGF_{2\alpha}$ release. Protein synthesis was measured from the incorporation of phenylalanine during the final 30 min incubation. Stock solutions of the fatty acids in ethanol were prepared so that 10 μl of the appropriate stock solution added to the medium gave the required concentration of fatty acid; 10 μl of ethanol was added to the controls. The fatty acids were present in the appropriate vessels throughout the entire 4.5 h period. Values are means ±S.E.M. for at least six observations. By Student's t test (paired values), significant differences from control (no additions) are shown by: $^*P < 0.05$; $^{**}P < 0.01$.

Addition to medium	Fractional rate of protein synthesis (k_s) (%/day)	Fractional rate of protein degradation (k_d) (%/day)	$PGF_{2\alpha}$ release ($pg \cdot h^{-1} \cdot mg^{-1}$)
None	1.5±0.2	5.9±0.7	14.9±1.6
ARA (0.2 μM)	2.4±0.4**	4.6±1.0	23.2±2.5*
ARA (1 μM)	2.3±0.7**	7.4±1.1	19.7±2.5
DGLA (0.2 μM)	2.2±0.6**	6.2±0.8	26.1±3.9*
DGLA (1 μM)	2.1±0.5**	6.0±0.7	23.2±2.9*
EPA (1 μM)	1.6±0.3	5.3±0.7	7.5±1.2*
DHA (1 μM)	1.6±0.4	6.1±1.1	8.1±1.4*

Table 2. Effect of metabolites of essential fatty acids on the action of insulin in isolated muscles

Experimental conditions were as described in Table 1. Insulin was added from a stock solution in medium to achieve a final concentration of 100 μunits/ml as determined by radioimmunoassay of medium after 4 h of incubation. Insulin was present in the appropriate vessel throughout the 4.5 h incubation period. Values are means ±S.E.M. for at least six observations. By Student's *t* test (paired values), significant differences from control values (Table 1) were as follows: $*P < 0.05$; $**P < 0.01$.

Addition to medium	Fractional rate of protein synthesis (k_s) (%/day)	Fractional rate of protein degradation (k_d) (%/day)	$PGF_{2\alpha}$ release ($pg \cdot h^{-1} \cdot mg^{-1}$)
None	1.5±0.2	5.9±0.7	14.9±1.6
Insulin (100 μunits/ml)	2.4±0.3**	7.0±0.9	21.2±0.4*
+ARA (0.2 μM)	2.2±0.3**	6.3±0.9	16.4±1.8
+ARA (1.0 μM)	1.8±0.1	6.9±0.8	13.9±1.8
+DGLA (0.2 μM)	2.1±0.2*	5.0±0.7	17.7±1.4
+DGLA (1.0 μM)	1.9±0.3	4.9±0.8	15.7±1.8
+EPA (1.0 μM)	1.9±0.2	5.6±0.8	17.2±3.4
+DHA (1.0 μM)	2.1±0.5	5.8±1.4	15.3±2.5

low levels of $PGF_{2\alpha}$ or is independent of any PG-mediated control mechanism.

The stimulatory effect of DGLA could arise from two mechanisms, as this fatty acid is both a precursor for the synthesis of ARA (and of the PGs of the '2' series) and can also be metabolized directly to PGs of the '1' series. To examine the possibility that protein synthesis may be stimulated by '1'-series PGs, muscles were incubated with $PGF_{1\alpha}$ at concentrations of 0.25–15 μM. No significant stimulation of the rate of protein synthesis was observed, values of 1.2, 1.3 and 1.4%/day being obtained at $PGF_{1\alpha}$ concentrations of 0.25, 2.5 and 15 μM respectively. It has been observed previously that $PGF_2\alpha$ is effective in stimulating the rate of protein synthesis at a concentration of approx. 2.5 μM (Smith *et al.*, 1983).

Forelimb muscles of rabbits weighing 590–800 g had rates of protein synthesis *in vitro* of 11.2–6.8%/day and calculated rates of protein degradation of 5.7–2.9%/day (Palmer *et al.*, 1985). Similar rates of protein degradation were found in muscles from the same animals *in vitro*, whereas rates of protein synthesis were reduced in the isolated tissues to 26% of the rate found *in vitro* (Palmer *et al.*, 1985). Although the present study used rabbits which were fasted overnight, a treatment which reduces rates of protein synthesis *in vitro* in the rat (Garlick *et al.*, 1983) and in isolated muscles of the rabbit (Reeds & Palmer, 1983; Palmer *et al.*, 1985), it appears that the principal reason for the catabolic state of isolated muscles is the reduction in the rate of protein synthesis. The reasons for this are not clear, but both the application of mechanical stimuli (Palmer *et al.*, 1981) and the addition of insulin (Reeds & Palmer, 1983) partially restore the rate of protein synthesis, and, together, mechanical stimulation and insulin resulted in a rate of protein synthesis of 5.4%/day (Palmer *et al.*, 1985). A third reason for the reduction in the overall rate of protein synthesis may be a reduction in glycogen content and in the rate of protein synthesis in a central core of anoxic fibres, such as occurs within 1 h in isolated rat muscles (Maltin & Harris, 1985). However, isolated muscles from rabbits weighing less than 1 kg do not appear to develop anoxic cores (Harris *et al.* 1985) and have previously been shown to have an increased glycogen content after a 6 h incubation and to maintain constant ATP levels throughout this time (Palmer *et al.*, 1981). Whatever the reason for the reduced rate of protein synthesis in isolated tissues, it is apparent that the addition of ω6 fatty acids stimulates the rate of protein synthesis, and the addition of ω3 fatty acids does not. Furthermore, the stimulatory effect of the $C_{20:3}$,ω6 fatty acid DGLA appears to be dependent on its metabolism firstly to the $C_{20:4}$,ω6 fatty acid (ARA) and subsequently to PGs of the '2' series (specifically $PGF_{2\alpha}$), suggesting that skeletal muscle contains an active Δ^5-desaturase system.

Insulin, added at a concentration of 100 μunits/ml of incubation medium, stimulated protein synthesis by approx. 60% and also increased the release of $PGF_{2\alpha}$ (Table 2). Neither of the ω3 fatty acids (EPA and DHA) when added together with insulin stimulated the rate of protein synthesis further; in fact both resulted in a non-significant decrease in the rate of protein synthesis and in the rate of release of $PGF_{2\alpha}$.

These observations are not surprising in view of the apparent mediation of the stimulatory action of insulin on protein synthesis by metabolites of ARA, since both the ω3 fatty acids may compete with endogenous ARA for the cyclo-oxygenase enzyme that converts ARA into PGs (Culp *et al.*, 1979).

More surprisingly, neither ARA nor DGLA potentiated the effect of insulin when added at a concentration of 0.2 μM (Table 2). In fact, in the presence of insulin +1 μM-ARA or -DGLA the rate of protein synthesis was not significantly increased above that observed in control muscles, and $PGF_2\alpha$ release was reduced, indicating that the stimulation elicited by insulin is attenuated by exogenous fatty acids.

Insulin is known to induce a cascade of changes in the membrane phospholipids as an early event after binding to its receptor. These include the release of phosphatidylinositol 4,5-bisphosphate and diacylglycerol from the phosphoinositides (Berridge, 1984; Farese *et al.*, 1985) and an increase in the release of $PGF_{2\alpha}$ (Reeds & Palmer, 1983; Table 2). All of these metabolites are considered

important in the stimulation by insulin of cellular metabolism. Thus any interference in the cascade of hormonally induced changes in membrane phospholipid metabolism may interfere with the production of potential second messengers and thereby with the subsequent stimulation in the rate of protein synthesis. Alternatively, the stimulatory effect of the ω6 fatty acids may be concentration-dependent, as a comparison of the effects of 0.2 and 1.0 μM-ARA and -DGLA on the action of insulin in stimulating both $PGF_{2\alpha}$ release and protein synthesis tend to suggest. If the release of ARA is already optimally stimulated by insulin, the addition of further, exogenous, ARA may in itself be inhibitory, as evidenced by the reduced $PGF_{2\alpha}$ release, or it may be metabolized by a pathway involving enzymes other than cyclo-oxygenase, i.e. the lipoxygenase and cytochrome *P*-450 ω-hydroxylation pathway, to produce potentially inhibitory metabolites, such as the leukotrienes, lipid peroxides or epoxides (Samuelsson, 1983).

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