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THE EFFECT OF BETA-ADRENERGIC AGONISTS AND AN ANTAGONIST ON PROTEIN METABOLISM IN CULTURED MYOTUBES

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Peter Thomas Anderson

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W. G. Bergen
Major professor

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THE EFFECT OF BETA-ADRENERGIC AGONISTS AND AN ANTAGONIST ON PROTEIN METABOLISM IN CULTURED MYOTUBES

By

Peter Thomas Anderson

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ABSTRACT

THE EFFECT OF BETA-ADRENERGIC AGONISTS AND AN ANTAGONIST ON PROTEIN METABOLISM IN CULTURED MYOTUBES

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The effects of beta-adrenergic agonists (BAA; including ractopamine (RAC) and clenbuterol (CLEN)) on protein metabolism in cultured muscle cells were evaluated, as was the ability of a beta-adrenergic antagonist (propranolol, PROP) to interfere with observed BAA effects. ELC5 myoblasts were grown in DMEM and 10% fetal bovine serum (FBS) and allowed to differentiate to form myotubes before treatments were added or experiments performed. For measurement of protein synthesis, myotubes were cultured in methionine deficient media and 10% FBS with 5 μCi of ^{35}S L-methionine per well. After 6 h incubation, media were removed and cells washed, removed from plates and protein quantity and incorporated radioactivity determined. Following electrophoresis, radioactivity incorporated into 43 kd proteins (including actin) and myosin heavy chain (MHC; 200 kd band) was determined. RAC increased ($P<.01$) apparent protein synthesis rate in incubations beginning at 24, 48, 72 and 96 h of treatment. RAC also increased apparent synthesis rate of 43 kd

proteins and MHC. CLEN treatment (48 h) increased total and specific protein synthesis ($P<.05$) but to a lesser extent than RAC. BAA effects on protein synthesis were partially blocked by inclusion of PROP. Neither BAA, nor PROP had any effect on rate of degradation of total or myofibrillar proteins ($P>.1$). Experiments with leupeptin and increased calcium demonstrated that these cells are responsive to protein degradation altering agents. RNA extracted from RAC-treated and control cells hybridized to a beta actin cDNA probe but hybridization to a skeletal muscle alpha actin cDNA probe was negligible. In summary, BAA increase protein synthesis in ELC5 myotubes in culture, an effect that can be partially blocked by PROP, but BAA do not affect protein degradation.

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Introduction

A goal of much current animal science research is to obtain means to improve carcass composition (lean/fat ratio) of food producing animals. Two factors provide impetus for this research. First, consumer demand has shifted toward meat products with lower fat and calorie content. This is a reflection of recommendations by the medical profession that intake of saturated fat and cholesterol should be reduced and increased weight consciousness on the part of consumers. It is critical that means be obtained to produce leaner animals, rather than simply trimming fat after livestock have been slaughtered. This is because deposition, and then removal, of fat is biologically and economically inefficient and because some fat depots (i.e., inter- and intra-muscular fat) are difficult or impossible to remove. The second contributing factor is the competitive economic environment that faces meat animal producers. Since deposition of muscle requires less feed energy (per unit of weight deposited) than fat, production of lean animals is more efficient than production of fat.

One means of improving carcass composition is administration of beta-adrenergic agonists (BAA). Whether administered orally, intraperitoneally, intravenously or intranasally, these compounds, which mimic some actions of

epinephrine and norepinephrine, induce greater carcass protein deposition and reduced carcass fat deposition in food producing or laboratory animals.

Current research is focused on understanding the mechanism(s) of BAA action. Understanding the biology involved in BAA-induced alterations in animal growth may lead to development of more effective products or improved management strategies for BAA-fed livestock. This knowledge could also allow producers to avoid potential problems with BAA use or lead to other uses of the products.

A Review of the Literature

I. Introduction

The sympathetic branch of the autonomic nervous system is normally associated with acute responses to stress. The sympathetic nervous system exerts its effects on the cardiovascular, respiratory and gastrointestinal systems, mobilizes energy reserves through glycogenolysis and lipolysis and increases heat production. These effects result from the release of catecholamines, epinephrine (EPI) and norepinephrine (NOREPI).

NOREPI is the adrenergic neurotransmitter and is synthesized and stored in peripheral sympathetic nerve endings. When released in response to efferent nerve impulses, it acts via stimulation of adrenergic receptors within the immediate vicinity and does not normally circulate in sufficient quantity to act as a hormone. EPI is a circulating hormone from the adrenal medulla and exerts its effects at adrenergic receptors throughout the body.

Adrenergic receptors are divided into 4 subclasses: α1, α2, ß1 and ß2. Classification into α and ß subtypes was originally based on their ability to cause contraction (α effect) or relaxation (ß effect) in smooth muscle. The response of a tissue will depend on the the presence and proportion of α and ß receptors, and the ability of the agonist to bind to them. The α1 and α2 subdivisions refer to post and presynaptic events, while the ß1 and ß2 subdivisions

are based on tissue responses.

ß1 and ß2 receptors, through the action of a guanine nucleotide binding (G) protein, Gs (Gilman, 1987), stimulate adenylate cyclase to raise intracellular cyclic AMP (cAMP) concentration. While ß1 and ß2 receptors have great sequence homology (54%, Lefkowitz and Caron, 1988), they are products of distinct genes (Frielle et al., 1987) which could be regulated separately. α2 receptors inhibit adenylate cyclase via the intermediation of G_i (an inhibitory G protein). α1 receptors, through an uncharacterized G protein, activate phospholipase C, which, through diacylglycerol, activates protein kinase C and inositol triphosphate, ultimately mobilizing intracellular calcium (Berridge and Irvine, 1984). Table 1, taken from Stock and Rothwell (1986), gives examples of tissue responses to NOREPI and isoprenaline.

In adipocytes, the main effects of activation by ß-adrenergic agonists (BAA) are stimulation of lipolysis, stimulation of glycogenolysis, inhibition of glycogen synthesis and stimulation of glucose oxidation (for review see Garcia-Sainz and Fain, 1982). The effects of ß activation on lipid and energy metabolism will not be discussed further in this review except to state that these shifts in metabolism increase energy available for protein synthesis.

In recent years, BAA have drawn considerable attention because of their potential to act as growth promotants or carcass modifiers in livestock or antiobesity agents in humans.

Table 1. Examples of Tissue Receptors and Responses to Norepinephrine and Isoprenaline.

	Response (receptor)	
Tissue	Norepinephrine	Isoprenaline
Smooth muscle		
Vascular	Contraction (α)	Relaxation (ß)
Bronchial	Relaxation (α2)	Relaxation (ß2)
Uterine	Contraction (α) or relaxation (ß2)	Relaxation (ß2)
Heart	Inotropic (α1)	Inotropic (ß1)
	Chronotropic (α1)	Chronotropic (ß1)
Liver	Glycogenolysis (ß2)	Glycogenolysis(ß2)
Adipose tissue	Lipolysis (ß1)	Lipolysis (ß1)

II. The Effect of BAA on Growth, Efficiency and Carcass Composition of Meat and Laboratory Animals

Beginning with the report of Cunningham et al. (1963), synthetic and naturally occurring BAA have been demonstrated effective in improving the performance and carcass composition of meat and laboratory animals. Table 2 summarizes the effects of BAA on full-fed market weight (when applicable) animals.

Table 2. The effect of BAA on Growth, Efficiency and Carcass Composition of Meat and Laboratory Animals [a]

Reference (compound)	ADG [b] min max	G/F [c] min max	LDA [d] min max	Fat [e] min max	Pro[f] Fat [g] min max	min max
			CATTLE			
Ricks et al. 1984	79	100	111	65	112	80
clenbuterol	92	100	116	64	113	75
Williams et al. 1987	-	-	-	-	112	83
clenbuterol	-	-	-	-	122	63
Allen et al. 1987	106	108	141	82	-	66
cimaterol	130	131	142	79	-	60
Boucque et al. 1987	117	105	-	-	110	64
cimaterol						
Miller et al. 1988	-	-	118	60	-	-
clenbuterol						
Rust et al. 1989	119	120	103	100	-	-
ractopamine	145	147	104	100	-	-
			SWINE			
Cunningham et al. 1963	111	111	-	-	103	90
epinephrine	119	119	-	-	107	77
Jones et al. 1985	101	109	107	94	-	97
cimaterol	105	112	114	88	-	87
Moser et al. 1986	100	100	104	93	105	-
cimaterol	100	100	109	90	105	-
Bekaert et al. 1987	100	100	111	96	103	-
cimaterol	100	100	114	94	105	-
Cole et al. 1987	104	106	-	86	107	86
salbutamol	108	110	-	77	107	86
vanWeerden et al. 1987	110	110	-	-	-	-
clenbuterol						
Wallace et al. 1987	-	-	112	94	104	98
L-644969	-	-	129	73	112	97

SHEEP						
Beermann et al. 1986	100	106	126	68	-	-
cimaterol	100	108	132	34	-	-
Duquette et al. 1987	114	110	101	86	102	100
L-644969	116	122	113	57	111	88
Hanrahan et al. 1987	106	100	103	-	100	87
cimaterol (wethers)	122	119	124	-	106	80
Hanrahan et al. 1987 cimaterol (rams)	121	127	124	72	109	-
Kim et al. 1987 cimaterol	129	116	139	77	102	95
Kim et al. 1989 cimaterol	111	111	-	-	117	83
Warriss et al. 1989						
cimaterol	125	127	121	61	115	71
clenbuterol	121	126	129	64	115	77
POULTRY						
Dalrymple and Ingle	99	100	-	-	100	95
1987 cimaterol	102	102	-	-	101	93
Duquette et al. 1987	102	101	-	-	101	96
L-640033	102	102	-	-	103	91
RATS						
Emery et al. 1984 clenbuterol	127	98	-	-	111	84
McElligott et al. 1987 clenbuterol	124	121	-	-	-	-
Reeds et al. 1988	-	-	-	-	106	94
various	-	-	-	-	129	61

[a] all values expressed as percent of control
[b] average daily gain
[c] gain to feed ratio
[d] longissmus dorsi muscle cross-sectional area
[e] carcass fat thickness
[f] carcass percent protein (may be percent muscle in some cases)
[g] carcass percent fat

The BAA-induced improvement in protein deposition appears to be confined to skeletal (and occasionally cardiac) muscle since in rats (Reeds et al., 1986) and calves (Williams et al., 1987) no increase in non-muscle protein deposition was observed. Indeed, increased muscle nitrogen retention may be at the expense of other tissues. In the study of Williams et al. (1987), carcass nitrogen gain was .45 kg while nitrogen retention increased only .31 kg. In the study of Reeds et al. (1986) after 21 d of clenbuterol (CLEN) treatment, liver and kidney weight was lower than control.

The dramatic effects of BAA on growth and composition make them useful for evaluation of nutrient utilization in models other than full-fed animals. In a study using ad libitum and limit-fed sheep, cimaterol (CIM) increased daily carcass protein deposition 33.6% and decreased daily carcass fat deposition 17.0% in lambs allowed to eat ad libitum (Kim et al., 1989). Control lambs fed to maintain their weight gained 12.1 g/d of carcass fat and lost 2.3 g/d carcass protein. When fed to maintain weight, CIM-treated lambs gained 4.9 g/d carcass fat and gained 4.1 g/d carcass protein. Among full fed lambs, nitrogen retention was increased 130% in response to CIM. CIM treatment also increased daily energy expenditure (8%), fasting heat production (14%) and metabolizable energy required for maintenance (19%). Hovell et al. (1989) have also observed reduced nitrogen loss in CLEN and CIM-treated sheep.

Winter (1983; cited by Stock and Rothwell, 1986) pair fed

rats such that the feed intake of CLEN-treated rats was equal to control. Treated rats gained 17% less weight and had 14% greater heat production, estimated as energy intake minus energy gain. Gross and net energetic efficiency was reduced 40 and 32%, respectively. Despite lower rates of weight and energy gain, protein and water gain of treated rats was equal to controls. Thus, the greater heat production was at the expense of fat gain, which was limited to 38% of controls.

Recently the effects of BAA on animals with differing genetic merit for lean growth have been reported. Eisen et al. (1988) fed CIM to selected (for growth rate) and unselected mice and observed a significant strain*treatment interaction for growth rate. CIM increased rate of gain in unselected mice but did not increase growth of the selected strain. The authors suggested that the selected line of mice deposit protein at a maximum rate that cannot be stimulated, whereas protein deposition and weight gain of mice from the unselected line could be increased by CIM.

Chromiak et al. (1989) used rats from strains selected for large or small body size to examine the effects of CIM injection. In terms of weight or protein gain, neither strain responded positively to CIM. In fact, at the highest doses (4 or 8 $mg*kg^{-1}d^{-1}$) growth rate of both strains was reduced, the large strain to a greater extent than the small. The authors speculated that either tissue energy reserves or energy intake of the rats was inadequate to allow CIM-increased muscle growth. Thereby, the effects of CIM on weight gain are a

direct result of effects on carcass fat accumulation. While purely speculative, this explanation is reasonable as neonatal suckling rat pups were used, which would have low carcass energy reserves and would be growing rapidly. Furthermore, since Emery et al. (1984) observed a 26% increase in energy expenditure of CLEN-treated rats, it would be expected that less dietary energy would be available for growth of treated rats. It may also be that CLEN increased the essential amino acid requirement of the pups such that normal rat milk represented a protein deficient diet for the treated rats.

CIM treatment for 3 or 9 weeks stimulated whole body energy expenditure and brown adipose tissue thermogenic activity comparably in genetically normal and obese mice (Walker and Romsos, 1988). However, efficiency of energy retention was lower in treated obese mice than treated normal mice. Hindlimb muscle gain was stimulated in obese mice but did not equal that of normal mice.

Mersmann et al. (1987) fed CIM to pigs from 10 to 60 kg to determine if CIM effects similar to those observed in market weight animals would be observed in pigs much lighter than market weight. No measures of growth performance or carcass composition were affected by CIM. The authors proposed several theories to explain the lack of effect. The most plausible of these include: 1) lack of receptors, low affinity of receptors for CIM or poorly coupled intracellular events in young pigs compared to market weight pigs. 2) age-related differences in metabolic regulation of responsive tissue do

not allow typically observed BAA effects in young animals. 3) 18% protein diets were inadequate to allow expression of increased capability for lean deposition, despite meeting or exceeding NRC recommendations. Based on reports that BAA decrease protein degradation, these authors speculated that CIM would have a protein sparing effect in young pigs, which require expensive, high protein diets. To examine this in the study just discussed, they also fed 14% protein diets with and without CIM, to pigs from 10 to 60 kg. Pigs fed 14% protein, below NRC recommendations, performed poorer than those fed 18% but CIM did not have a protein sparing effect.

Similarily, Williams et al. (1987) observed no effect of CLEN on nitrogen balance in small (60 kg) veal calves. At 120 kg CLEN increased nitrogen balance in these calves.

With a hypothesis opposite to that of Mersmann et al. (1987), Anderson et al. (1987) investigated whether ractopamine (RAC) treatment would increase protein and lysine requirements of finishing pigs due to increased protein deposition. Pigs fed ractopamine grew faster and more efficiently than controls only when their diet contained 18% protein or 15% protein plus added lysine, although these levels were above NRC recommendations. Performance of pigs fed 12 or 15% protein was not improved by RAC, although treated pigs exhibited typical leanness response, regardless of dietary protein or lysine.

Some conclusions can be drawn from reported studies of BAA effects on growth, efficiency and composition of anim-

als. In market weight, full-fed animals BAA typically improve leanness and muscling by 10-30%, with similar improvement in nitrogen retention. Despite increased energy wastage, efficiency of growth (gain/feed) is improved. This improvement is probably related to altered composition of gain, as the greater water content of muscle, compared to fat, dictates that muscle gain requires less feed energy than fat deposition. Effects on growth rate are variable. In young animals, however, especially those selected for growth or size, BAA have little effect. For the most part, effects of BAA on protein deposition are diet dependent while lipid response to BAA is diet independent.

III. Mechanism of BAA Action in Muscle

Since the effects of BAA on growth and composition of animals are well established, much current research is focused on the mechanism of action of these compounds. An increase in muscle size would be the result of increased muscle cell size (hypertrophy), increased muscle cell number (hyperplasia) or both. BAA could affect either of these functions. Yang and McElligot (1989) have recently reviewed current literature regarding mechanisms of BAA action.

Hyperplasia. In vitro, BAA stimulate proliferation of non-muscle cells (Selye et al., 1961; Brown-Grant, 1961;

Barka, 1965; Baserga, 1966; Bybee and Tuffery, 1988; Dumont et al., 1989) as well as satellite cells from embryonic chick breast muscle (Grant et al., 1989). It is unknown if BAA enhance proliferation of satellite cells from postnatal muscle or in vivo. If hyperplasia in vivo is increased in response to BAA, muscle DNA content would be increased through addition of satellite cell DNA to existing muscle fibers. Indeed, increased DNA is a prerequisite to protein accumulation in normal growth (Burleigh, 1974; Allen et al., 1979), compensatory growth (Howarth and Baldwin, 1971; Beerman, 1983) or stretch-induced muscle growth (Barnett et al., 1980).

The effect of BAA treatment on muscle DNA content of growing animals is unclear. Beermann et al. (1986) reported 25% lower DNA concentration in the muscle of CIM treated (for 7 weeks) lambs, Kim et al. (1987) and Reeds et al. (1986) observed similar results. However compared to control lambs, lambs fed CIM for 12 weeks in the study of Beermann et al. (1986) had similar DNA/protein ratios. Ultimately the treated lambs accumulated DNA to the same degree as protein. The 7 week CIM group of Beermann et al. (1986) had 5.9% more DNA per muscle, but 35% larger muscles. Since DNA/protein ratios were lower at 7 weeks, but not after 12 weeks of treatment, it seems that increased DNA is not a prerequisite to increased muscle accumulation. Based on data from the 12 week group, however, it can be concluded that increased DNA will result from BAA treatment, although it seems to trail increased muscle in time.

Hypertrophy. While effects of BAA on hyperplasia are still under debate, it is clear that BAA treatment increases muscle cell hypertrophy. Since skeletal muscle is a dynamic tissue, the increased muscle cell hypertrophy caused by BAA administration could be the result of increased protein synthesis, decreased protein degradation, or both.

Early workers concluded that BAA decrease protein degradation in in vitro muscle systems. Garber et al. (1976) incubated isolated rat epitrochlaris with physiological levels of EPI and observed inhibition of alanine and glutamine release. This result was reproduced by isoproterenol treatment and blocked by propranolol (PROP; a nonselective beta adrenergic antagonist). These authors concluded that EPI diminished protein degradation although an increase in protein synthesis could have given the same result. Li and Jefferson, (1977) used 14C phenylalanine as a marker and observed a 20% reduction in protein degradation in perfused rat hemicorpus with isoproterenol treatment. Protein synthesis was unchanged, although the authors point out that in this system hemicorpus preparations from normal fed rats become insulin deficient during the time course of the experiments. Since hypoinsulinemia would dramatically reduce protein synthesis and likely affect degradation, the conclusions from this study must be interpreted with caution.

More recently, Reeds et al. (1986) and Maltin et al. (1986) have suggested that feeding CLEN to rats decreased in vivo protein degradation. This conclusion was based on

unchanged synthesis rates coupled with increased protein accumulation since protein degradation was calculated by difference (eg. degradation = synthesis - accretion), not measured directly. Since that time, workers in the same group have pointed out that calculation of degradation by difference, may lack accuracy since a vital assumption, that protein pool size increases linearly, is invalid in CLEN-treated rats (Maltin et al., 1989). Reeds et al. (1986) made little mention of an increase in RNA content of the muscle of treated rats, which would suggest increased capacity for protein synthesis.

Eadara et al. (1988) observed a transient decrease in protein degradation (3-methyl histidine excretion per unit of muscle) in rats fed CIM. In this study, protein degradation was decreased 33% on the first d of treatment, this effect persisted for 4 d, after which no effect was observed. The mean CIM-induced decrease in protein degradation was 25% for 1 week of treatment.

In culture, protein degradation decreased 10-15% when primary chicken muscle cell cultures were treated with CIM for 6 d but no difference was observed in cultures treated for 2 h (Young et al., 1987). Observation of chronic, but not acute effects led these authors to conclude that CIM-decreased degradation is not due to direct inhibition of protease action. Forsberg and Merrill (1986) have also noted reduced protein degradation in BAA-treated muscle cell cultures.

If BAA reduce protein degradation, quantity or activity of proteolytic enzymes should be diminished in treated

animals. Calcium dependent proteinases (CDP; purified by Dayton et al., 1976a) are thought to be important in myofibrillar protein turnover at neutral pH (Dayton et al., 1976b). Wang and Beermann (1988) have reported that cimaterol treatment reduced activity of the μM form of CDP (requires uM concentrations of calcium for maximum activity, a mM form of CDP also exists) in lambs. Physiological importance of mM CDP in muscle protein turnover is questionable since the free calcium concentration in muscle cytosol in the normal physiological state is much lower than that required by mM CDP (Jobis and O'Connor, 1966; Dayton et al., 1976b). Activity of the mM form of CDP was unaffected by CIM (Wang and Beermann, 1988).

Bergen et al. (1989) observed no difference in total (μM + mM) CDP activity in semitendinosus muscles of RAC-treated pigs which had elevated synthesis and degradation rates, compared to control. Cathepsins B and H were also unaffected by RAC while cathepsin L activity increased in response to RAC (Bergen et al., 1989). McElligot et al. 1987 observed an increase in cathepsin B (but no change in cathepsin D) in soleus of CLEN-treated rats. Cathepsin B of gastrocnemius and extensor digitorum longus was not different from control and these authors did not measure protein metabolism. In contrast, Forsberg et al. (1987) observed a 45% decrease in cathepsin B in the semitendinosus muscle of sheep fed CIM for 90 d.

While some have concluded that decreased protein

breakdown can account entirely for BAA enhanced skeletal muscle growth (Scanes et al., 1988), others have shown increased synthesis to be involved. Deshaies et al. (1981) injected radioactively labeled amino acids intraperitoneally in rats that recieved daily subcutaneous injections of isoproterenol and observed increased amino acid incorporation of up to 64% in tibialis muscle in response to 5 days of treatment. Synthesis rates returned to control levels after 24 h of isoproterenol withdrawal.

Emery et al. (1984) have shown increased protein synthesis in rats injected with CLEN. Eadara et al. (1988) reported a 32% increase in fractional rate of protein synthesis as well as increases in amount and concentration of RNA in muscle of rats fed CIM.

Few studies have examined the effects of BAA on protein synthesis of food producing animals. Fractional protein synthesis rate was increased in semitendinosus muscle of pigs fed RAC (Bergen et al., 1989). Helferich et al. (1988) investigated the effects of RAC on a specific muscle protein (skeletal muscle alpha actin; SKMAA) in vivo. They observed that synthesis of SKMAA is increased 50% and abundance of mRNA coding for SKMAA is 2-3 times higher in the longissmus dorsi muscle of RAC-treated pigs than control. When the RNA was translated in an in vitro cell free system, all identifiable myofibrillar proteins were increased. Claeys et al. (1989) have reported increased muscle protein synthesis in young lambs treated with CLEN.

Eisemann et al. (1988) have demonstrated decreased uptake of αNH_2 N in hindquarters of steers after acute (1 d) CLEN treatment but increased αNH_2 N uptake in the same steers after 9 d of treatment. These authors speculated that CLEN initially decreased protein degradation but later effected an increase in protein synthesis. Maltin et al. (1989) have also pointed out that timing of BAA experiments may significantly affect results.

In primary chicken muscle cells cultured with cimaterol, Young et al. (1987) observed an 11% increase in synthesis rate of myosin heavy chain, a major myofibrillar protein, with no increase in total protein synthesis. In the study of Forsberg and Merrill (1986), protein synthesis in mouse myotubes cultured with 100 μM CIM was 129% of controls although not significantly different. However, when rat myotubes were used, protein synthesis was not affected by treatment. Roeder et al. (1987) did not observe differences in protein synthesis or degradation in L6 myotubes treated with isoproterenol, EPI, NOREPI or CIM, although positive responses to insulin indicate that the cells were responsive. These workers treated cultures with BAA for 18 h before measuring protein synthesis. Perhaps longer BAA treatment is required to alter protein metabolism.

Nutting (1982) observed that EPI and NOREPI (at higher doses) stimulate amino isobutyric acid uptake and protein synthesis in hypophysectomized rats. These events were not increased above levels achieved with growth hormone (GH) or thyroxine treatment of hypophysectomized rats.

Zeman et al. (1987) observed that CLEN treatment decreased atrophy in denervated rat skeletal muscle. Babij and Booth (1988) denervated the soleus and gastrocnemius muscle of rats and showed that CLEN inhibited atrophy and diminished loss of mRNA coding for SKMAA. When atrophy was induced by suspension instead of denervation, the loss of SKMAA mRNA was diminished by CLEN treatment but atrophy was not prevented.

Most reports to date have focused on synthesis and degradation of total muscle protein, which includes cytoplasmic, stromal and myofibrillar proteins. These protein pools have different turnover rates (Young, 1970) and different metabolic functions. Hence, assessment of total turnover may not accurately reflect myofibrillar protein turnover. Direct measures of protein breakdown are practically impossible to achieve in whole animals or in muscle strip experimental systems (Skjaerlund et al., 1988); hence, there is considerable uncertainty about reported data on the mode of action of BAA in muscle protein metabolism.

Some workers have suggested that the effects of BAA are dependent on muscle fiber type. In CIM-fed rats, Kim et al. (1985) observed a 34.2% increase in the cross-sectional area of Type II fibers while cross-sectional area of Type I fibers was increased 17.5%. However, Beermann et al. (1986) reported equal increases in the size of the two fiber types in the semitendinosus of lambs but CIM reduced the percentage of Type I fibers from 10.7% to 3.7% of the total. CIM did not affect percentage of the fiber types in longissmus muscle of

lambs but Type II fibers increased in size while Type I did not (Kim et al., 1987). These results are similar to Deshaies et al. (1981), who reported that isoproterenol induced hypertrophy of the tibialis, a mixed fiber type muscle, while the soleus, primarily Type I was unaffected. In contrast, Maltin et al. (1986), Reeds et al. (1986) and Zeman et al. (1987) have reported increased weight gain in soleus muscle of CLEN-treated rats. Maltin et al. (1986) also reported an increase in size of Type II fibers in the soleus of rats, without a change in the size of Type I fibers. Maltin et al. (1989) have described a series of experiments which led them to conclude that in denervated phasic (predominately fast, Type I fibers) muscles, CLEN treatment induces an increase in protein synthesis, while in CLEN-treated tonic muscles, degradation is decreased. These authors suggest that this is due to near maximal RNA content in tonic muscles which makes these muscles unable to respond by increasing rate of protein synthesis while RNA content, and thus protein synthesis, of phasic muscles can be augmented.

BAA could affect muscle protein metabolism through direct action on the muscle cell or by indirect action, mediated through endocrine or paracrine effects of hormones, growth factors or metabolites produced by other cells. In skeletal muscle BAA bind to ß receptors, stimulating adenylate cyclase activity resulting in increased cAMP and activation of cAMP-dependent protein kinase (Bowman and Nott, 1969). These events result in phosphorylation of proteins (Gard and

Lazarides, 1982) but the ultimate effect is unclear. BAA affect calcium transport across the plasma membrane and intracellular calcium movement (Schmid et al., 1985). Calcium concentrations are involved in regulation of protein degradation (Zeman et al., 1985; Rodemann et al., 1982; Silver and Etlinger, 1985) and involved in protein synthesis (Lewis et al., 1982; Kameyama and Etlinger, 1979).

Indirect effects could involve insulin, GH or insulin-like growth factors (IGF). In lambs, chronic administration of CIM lowered circulating insulin and IGF-1 and raised circulating thyroxine and GH (Beermann et al., 1987). Insulin has anabolic effects on muscle protein turnover (Jefferson et al., 1974; Fulks et al., 1975; Jefferson et al., 1977). BAA increased circulating levels of insulin in acute studies (Bassett, 1970; Imura et al., 1971; Loubatieres et al., 1971; Smith et al., 1985), but not in an 8 d study (McElligot et al. (1987). Circulating hormone concentrations may not tell the whole story. BAA increased insulin binding to receptors in vivo (Webster et al., 1986a) and in in vitro membrane preparations (Webster et al., 1986b). The effects on insulin binding are blocked by addition of propranolol (Webster et al., 1986b). These effects of BAA on receptor function could affect intracellular protein metabolism without affecting circulating insulin.

In vivo, BAA decreased GH secretion (Blackard and Heidingsfelder, 1968) and inhibited GH secretory response to arginine (Hertelendy et al., 1969), nicotinic acid (Hertel-

endy and Kipnis, 1973) and prostaglandin E1 (Hertelendy et al., 1972). ß-adrenergic blockade increased GH (Collu et al., 1975, 1978; Chihara et al., 1984, 1985). In vitro, however, BAA directly stimulated GH release from perfused pituitary cells (Perkins et al., 1983, 1985; Krieg et al., 1986). To an extent, this apparent conflict was resolved by Krieg et al. (1988) who showed that isoproterenol can stimulate a release of GH in vivo, but isoproterenol also induced release of somatostatin which rapidly returns GH to pretreatment levels and transiently prohibits further GH release.

Role of the beta receptor in protein metabolism. While it is clear that BAA exert their effects on lipid metabolism through the beta receptor (Garcia-Sainz and Fain, 1982), involvement of the beta receptor in protein metabolism has received only preliminary study. PROP did not inhibit CLEN-stimulated protein accretion but did reduce the increase in muscle fiber size in the study of Maltin et al. (1987; as cited by Maltin et al., 1989). Neither PROP nor atenolol blocked the effect of CLEN on increased protein accumulation in rats (Reeds et al., 1988). From these findings in animal studies, Maltin et al. (1989) concluded that the beta receptor is not involved in BAA-induced alterations in protein metabolism. This conclusion, however, generates the obvious question of how BAA could affect metabolism without binding to beta receptors. Data from in vitro studies indicates beta receptor involvement. Garber et al. (1976) were the first to demonstrate

inhibition of BAA effects on protein metabolism by PROP. Recently, Harper and Buttery (1989) have released a preliminary report of work with rat and mouse derived myogenic cell lines in which 100-fold excess of PROP blocked completely CIM-induced increases in protein synthesis. Although relative concentrations of BAA and antagonists are much easier to control in in vitro experiments than in vivo, conflicting results from in vitro experiments compared to animal studies lead to the larger question of the usefullness of in vitro studies.

IV. Use of Muscle Cell Culture in Studies of Protein Metabolism

Cell culture can be a powerful research tool. Growth biologists, muscle biologists and endocrinologists can utilize muscle cell culture to study regulation of muscle cell proliferation and differentiation, receptor binding and regulation, protein synthesis and degradation, and molecular events associated with these functions. Excellent reviews on the merits of muscle cell culture have been written by Allen (1987) and Dayton and Allen (1987). The advantages of muscle cell culture include opportunity to study metabolism in a relatively controlled environment and ability to use a pure population of cells to examine direct treatment effects on muscle cells. Performing experiments in culture often facil-

itates measurement of variables that may be difficult or impossible in vivo, such as direct measurement of protein degradation. Further, many experiments may be prohibitive in cost when performed in animals but affordable in culture.

However, caution must be exercised when interpreting results from cell culture experiments. Although controlled, the environment in cell culture is artificial and results cannot be extrapolated to the whole animal. Absence of circulating hormones, growth factors and metabolites, lack of innervation and other differences from the in vivo state must temper conclusions from muscle cell culture experiments. In addition, cell culture conditions and materials, especially serum, can vary from one laboratory to another or between experiments within the same laboratory. Within laboratory variation can be minimized but comparison of results from various laboratories can be risky.

Muscle cell cultures can be divided into two broad categories, based on the origin of the cells. Primary cultures are those taken directly from living organisms. Cell line cultures are established from cells that have been chemically treated and/or selected for their ability to grow continuously in culture. Many cell lines have retained some or most of their native functions.

Primary cells and cell lines each have distinct advantages and disadvantages. Theoretically, because they are derived directly from animals or embryos, primary muscle cells more nearly mimic in vivo functions. Since primary cultures

do not survive in culture for extended periods they are not prone to the considerable genetic drift occasionally observed in cell lines. Primary cells can be taken from animals in various physiological states and their in vitro response observed.

Primary cells have distinct disadvantages when compared to cell lines. Primary cells are more difficult and expensive to prepare and cell quantity may be limiting for some experiments. It has only recently become possible to obtain cultures from animals beyond the neonatal stage. In addition, it is virtually impossible to obtain a pure culture of primary cells. Contamination of a culture by non-muscle cells can affect results of experiments due to paracrine action or due to binding of ligands to receptors of fibroblasts or other cell types. The effects of contaminant cell types may be difficult or impossible to identify and may differ from one culture to another.

In contrast, cell lines arise from cloned populations and are not contaminated by non-muscle cells. Another advantage of cloned lines is the relative homogeneity of the cell population which allows for uniform, repeatable response to treatment. In comparison to primary cells, establishment of experimental cultures from stock cultures of a cell line is quick and easy.

Muscle cell lines have disadvantages that must be considered when interpreting data obtained from experiments in which they are used. A major concern is that cell lines may

have become transformed during the process of adapting to life in culture. In fact the cells that originally comprise the cell line are selected based primarily on their ability to survive in culture. This is an artificial state that is farther removed from the in vivo state than primary cultures. If possible, results from studies using cell lines should be confirmed in primary cultures from an appropriate target species, although a response in a primary culture is not necessarily indicative of the in vivo condition either. Because they can be kept alive indefinitely, cell lines are susceptible to considerable genetic drift after numerous passages. Consequently, proliferation and differentiation of the cells must be continually observed and cells should be recloned occasionally.

Nonetheless, many cell lines are quite useful for studies of protein metabolism since their properties closely resemble those of normal cells. The most widely used and best characterized muscle cell line is the L6 line, originally isolated by Yaffe (1968). This cell line originated from rat thigh muscle primary cultures which were treated with methylcholanthrene and selected for long-term survival in culture. L6 cells, which can be frozen and stored in liquid nitrogen, are useful because they divide, differentiate and fuse to form myotubes and have beta receptors and receptors for insulin and insulin-like growth factors I and II.

OBJECTIVES

The initial objective of the work reported here was to develop a cell culture system useful for evaluation of the direct effects of various agents on protein metabolism of the muscle cell. After development of the culture system, specific objectives were:

1. To determine if RAC affected protein metabolism of the muscle cell. If so:

2. To determine the effects of RAC and CLEN on total and myofibrillar protein synthesis and degradation.

3. To determine if observed effects could be blocked by addition of an antagonist (PROP).

Once these objectives were met, an attempt would be made to investigate intracellular molecular mechanism of BAA effect on protein synthesis by observing response of the SKMAA mRNA in BAA-treated cells.

Chapter 1

RACTOPAMINE INCREASES TOTAL AND MYOFIBRILLAR PROTEIN SYNTHESIS IN CULTURED MYOTUBES

Abstract

The ability of the phenethanolamine ractopamine (RAC; 10^{-6}M) to stimulate protein synthesis in cultured muscle cells was evaluated. ELC5 myoblasts were grown in DMEM and 10% fetal bovine serum (FBS) and allowed to differentiate to form myotubes. At 4, 24, 48, 72 and 96 h after addition of RAC treatment or control media, protein synthesis was assessed in 8 wells of myotubes per treatment in each of four replicate experiments. For measurement of protein synthesis, myotubes were cultured in DMEM without methionine (MET- DMEM) with 10% FBS and 5 μCi of ^{35}S L-methionine per well. After 6 h, media were removed and cells washed, removed from plates, pelleted and solubilized in protein denaturing buffer. Protein was precipitated by addition of trichloroacetic acid and solubilized in 1 N NaOH for determination of protein and incorporated radioactivity. Following electrophoresis, radioactivity incorporated into 43 kd proteins (including actin) and myosin heavy chain (MHC; 200 kd) band was determined. RAC increased apparent protein synthesis rate in incubations beginning at

24, 48, 72 and 96 h of treatment. RAC also increased apparent synthesis rate of 43 kd proteins and MHC ($P<.05$). These results were confirmed in experiments using 1 mM of added non-radioactive methionine. From these data we conclude that RAC stimulates total protein, 43 kd protein and MHC synthesis in ELC5 myotubes.

KEY WORDS: Ractopamine, Myotubes, Protein Synthesis, Myofibrillar Proteins

Introduction

Beta-adrenergic agonists (BAA) increase muscle protein accumulation and depress fattening when fed to food producing or laboratory animals (for review see Hanrahan, 1987). Increased muscle protein deposition in response to BAA treatment is dependent on the essential amino acid content of the diet (Anderson et al., 1987). Since muscle is a dynamic tissue, increased muscle protein deposition could result from increased synthesis or decreased degradation of protein or both. The mechanism(s) involved in BAA enhanced muscle protein deposition are not well understood (Maltin et al., 1989). Increased protein synthesis in response to BAA treatment in vivo has been reported (Deshaies et al., 1981; Emery et al., 1984; Claeys et al., 1989; Bergen et al., 1989), but others have observed unaltered protein synthesis in vitro (Li and Jefferson, 1977) or in vivo (Reeds et al., 1986; Bohorov et al., 1987). Discrepancies between reported data may be due to differences in treatment compound, length of treatment or experimental methods.

Our previous work has demonstrated that ractopamine (RAC; a phenethanolamine known to act as a BAA in adipose tissue; Coutinho et al., 1989) increases fractional synthesis rate (FSR) of porcine skeletal muscle protein. Increased abundance of mRNA coding for skeletal muscle alpha actin observed in RAC treated pigs (Helferich et al., 1988) indicates that RAC effects are pretranslational.

The data of Helferich et al. (1988) do not indicate whether RAC stimulates synthesis of sarcoplasmic proteins or preferentially enhances synthesis of myofibrillar proteins. Furthermore, in vivo studies have not been designed to determine if BAA exert their action through direct interaction with the muscle cell or whether their effects are mediated by circulating growth factors, metabolites, etc.

This study was designed with two objectives. First, to evaluate the direct effect of RAC treatment for various lengths of time on total protein synthesis in muscle cells. Use of a cell line, ELC5 myoblasts, precluded presence of non-muscle cell types. The second objective was to determine the effect of RAC on synthesis rate of specific myofibrillar proteins.

Materials and Methods

Cell culture. Cells used were ELC5, a myogenic cell line subcloned from the L6 line of Yaffe (1968) by Lilly Research Laboratories, Greenfield, IN. Myoblasts, multiplied at 37 C in a humidified atmosphere of CO_2/air (1:19) in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), were plated at a density of $10^4/cm^2$ in six well tissue culture clusters. Upon confluency, FBS was reduced to 2% for 24 h to synchronize cells within the cell cycle, then returned to 10% to allow uniform fusion. Parallel cultures were stained and nuclei counted. Typically, 50-60% fusion was observed.

Two experiments were conducted. The first experiment was designed to examine the time course of synthesis of total protein and two specific proteins (43 kd proteins, including actin, and myosin heavy chain, MHC, 200 kd). Protein synthesis was measured as described below during incubations of 1, 2, 3, 4, 5, 6, 8, and 12 h in 6 wells of myotubes per incubation period.

The second experiment was designed to assess the effects of RAC. RAC (10^{-8} M) was included in treated cultures beginning 24 h after fusion. At 4, 24, 48, 72 and 96 h of RAC exposure, one-fifth of the control and treated clusters were randomly selected for measurement of protein synthesis. At 48 h, media were replaced in control and treated cultures designated for measurement at 72 and 96 h of exposure to RAC. At each time point, 8 treated and 8 control wells were used in each of 4 replicate experiments (blocks).

Measurement of protein synthesis. To measure apparent protein synthesis, media were replaced with DMEM that contained no methionine (met- DMEM) with 584 mg/liter L-glutamine. Ten % FBS and 5 μCi of ^{35}S L-methionine per well were included as the only sources of methionine. After the designated time of exposure to ^{35}S methionine (1-12 h in time course experiment, 6 h in other experiments), radioactive media were removed, cells washed twice with DMEM and trypsinized briefly to detach them from the wells. In preliminary experiments it was determined that trypsin exposure for 5 min or less was

sufficient to detach cells from culture dishes without degrading myofibrillar proteins (data not shown). To ascertain whether methionine concentration in the protein synthesis incubation medium would influence ^{35}S methionine incorporation or treatment effects, protein synthesis was also assessed in RAC treated and control cultures in media containing additional 1 mM non-radioactive methionine.

Immediately after removal from culture dishes, DMEM, with 10% FBS was added to inactivate trypsin. Cells from two wells were combined to form a single sample, considered to be the experimental unit, pelleted by centrifugation and frozen immediately. Cell pellets were solubilized by boiling in 300 µl electrophoresis loading buffer (62.5 mM Tris-Cl, 5% ß-mercaptoethanol, 2% sodium dodecyl sulfate and 10% glycerol). Protein in a 50 µl aliquot was precipitated with 20% (w/v) trichloroacetic acid (TCA), resuspended in 300 µl 1 N NaOH. Protein content of the NaOH solution was determined by the method of Lowry et al. (1951) and radioactivity incorporated into TCA-insoluble protein was determined by liquid scintillation analysis.

Thirty µg of protein were electrophoresed for 12 h at constant current of 20 milliAmperes through 2 cm stacking gels (4% acrylamide, linker:polylinker ratio = 37.5) followed by 12 cm separating gels (12.5%, 37.5). After staining with Coomassie Brilliant Blue and destaining, the 43 kd and MHC bands were sliced from the gels and incorporated radioactivity determined by liquid scintillation analysis. Al-

though counting efficiency is low, counting bands allows precise measurement for comparison of treatments (data not shown).

Statistical analysis. Data were analyzed by analysis of variance with treatment, time and block effects included in the model. Means were separated using Bonferroni t test statistics (Gill, 1978). The separate responses of the 43 kd proteins, MHC and total protein to RAC were compared by calculating the mean percentage increase due to RAC within each block. Student's t tests were used to separate these means.

Results

Time course of protein synthesis. A linear increase in dpm/μg in total protein, the 43 kd proteins and MHC during incubations for up to 12 h is depicted in Fig 1. The R^2 values, calculated separately for total protein, 43 kd proteins and MHC were .65 for each ($P<.05$).

Effect of ractopamine. No significant block effects were observed for any variable. Treatment did not affect quantity of protein (data not shown). RAC increased ($P<.01$) the incorporation of ^{35}S into total (TCA-insoluble) protein in 6 h incubations beginning at 24, 48, 72 and 96 h of treatment (Table 1). Response to RAC treatment (48 h) was similar

whether the incubation medium included met- DMEM (18% increase in total protein; $P<.05$) or met- DMEM with 1 mM added non-radioactive methionine (21%; $P<.01$; data not shown).

Synthesis of the 43 kd proteins and MHC also were increased in response to treatment at most time points after 4 h (Table 2). Incorporation of ^{35}S methionine into total or specific proteins was not increased by RAC in ELC5 myotubes exposed to RAC for 4 h.

Based on block means at 24, 48, 72 and 96 h of treatment, synthesis of 43 kd proteins was stimulated to a greater ($P<.05$) extent than total protein or MHC synthesis (RAC = 146, 124 and 126% of control for 43 kd, total protein and MHC, respectively). While not compared statistically, it appears that protein synthesis in these cultures was greatest 48 h after treatments were added.

Discussion

Results of these cell culture experiments are consistent with results from our previous studies in pigs (Bergen et al., 1989; Helferich et al., 1988). Increased muscle protein synthesis is likely involved in the increased muscle cell hypertrophy of animals fed RAC.

The effects of RAC on protein metabolism in cell or tissue culture systems have not been reported previously. Few studies of the effects of BAA other than RAC on protein

metabolism in cell culture have been reported. Young et al. (1987) observed increased synthesis of the myofibrillar protein fraction and MHC specifically in cultured chicken myotubes treated with cimaterol. In the work of Forsberg and Merrill (1986), protein synthesis in mouse myotubes cultured with 100 μM cimaterol was 129% of controls although not significantly different. However, when rat myotubes were used, protein synthesis was not affected by treatment. Perhaps a lower concentration of cimaterol would have given different results. In our laboratory 100 μM RAC is toxic to myotubes (Parkhill and Anderson, unpublished data). Roeder et al. (1987) did not observe differences in protein synthesis or degradation in L6 myotubes treated with BAA including isoproterenol, epinephrine, norepinephrine or cimaterol, although positive responses to insulin indicate that the cells were responsive. Roeder et al. (1987) treated cultures with BAA for 18 h before measuring protein synthesis. Perhaps a longer treatment would have significantly altered protein metabolism. In the work reported here, RAC did not affect protein synthesis after 4 h of treatment, but did increase protein synthesis after 24 h of treatment. Treatment periods between 4 and 24 h were not studied.

These data, along with our in vivo data (Bergen et al. 1989, Helferich et al. 1988) support reports of increased protein synthesis in response to BAA. Deshaies et al. (1981) observed increased protein synthesis in rats treated with isoproterenol. Emery et al. (1984) also observed increased

protein synthesis in rats given subcutaneous injections of clenbuterol and Eadara et al. (1988) reported a 32% increase in synthesis of 3-methylhistidine-containing proteins in rats fed cimaterol.

Our results differ from reports of unaltered protein synthesis in vitro (Li and Jefferson, 1977) and in vivo (Reeds et al., 1986; Bohorov et al., 1987) in response to BAA other than RAC. Failure to demonstrate an increase in protein synthesis, despite large increases in RNA content which would facilitate increased protein synthesis, led Reeds et al. (1986) and Bohorov et al. (1987) to conclude that reduced muscle protein degradation was the means by which BAA induce muscle hypertrophy. It should be noted, however, that the protein degradation data of Reeds et al. (1986) and Bohorov et al. (1987) were calculated by difference (eg. degradation = synthesis - accretion) instead of by direct measurement.

An obvious difference between the work reported here and that of Reeds et al. (1986) and Bohorov et al. (1987) is our choice of RAC as the treatment compound. Few others have evaluated the effect of RAC on protein metabolism, although Smith et al. (1987) have observed increased mRNA coding for myosin light chain in cattle fed RAC and Helferich et al. (1988) observed increased mRNA coding for skeletal muscle alpha actin in RAC-treated pigs. While not direct evidence, increased mRNA abundance of major myofibrillar protein(s) would be indicative of increased protein synthesis. Furthermore, workers investigating the other compounds did not

evaluate synthesis or degradation of specific myofibrillar proteins.

It would be expected that quantity of protein would have been increased in response to RAC, given the increase in rate of protein synthesis. The failure of RAC to increase protein content of the cultures could be a result of parallel increases in protein synthesis and degradation. In the work of Bergen et al. (1989) RAC increased both synthesis (1.7%/d) and degradation (1.5%/d) of muscle protein in pigs with a net increase in muscle protein deposition. It is likely that the length of the experiments was insufficient for RAC to evoke an observable change in protein quantity. In these cultures, and in those of Young et al. (1987), protein content increased only slightly after several days in culture. Thus, when fractional synthesis rates are low, a 24% increase in synthesis, as observed in these experiments, may not be sufficient to noticeably affect protein quantity in short term studies in culture. Indeed, if protein degradation was 80% of synthesis, protein accumulation would only increase by 4.8%. A 4.8%/d enhancement in the fractional accretion rate of muscle protein would not be a detectable difference in this system but would greatly affect ultimate protein content of meat animals over a several week feeding period.

In both treated and control cultures, rates of protein synthesis were diminished at 72 and 96 h of treatment, compared to 48 h. After 96 h of treatment in this system, which is 144 h after myoblasts reached confluency, myotubes

begin to lift off plates and cell death increases. Protein degradation may exceed synthesis under these conditions. If that is so, the methods described above may be insufficient to detect increased protein content in response to RAC treatment.

It is difficult to reconcile that synthesis of the 43 kd proteins was stimulated to a greater extent than total protein or MHC synthesis. The 43 kd band does not contain only alpha actin, the actin specific to the myofibril, but beta and gamma actin as well, which are constitutive forms indigenous to all cells including myoblasts and myotubes. This band undoubtably also includes the cytoplasmic proteins creatine kinase (E.C. 2.7.3.2; which is known to increase as differentiation and myotube formation progress) and aldolase (E.C. 4.1.2.b). Hence, ^{35}S-methionine incorporated into cytoplasmic proteins likely contributed to reported values. Indeed, the 43 kd band was larger than the MHC band in the gels and contained more radioactivity, although MHC is known to be more abundant than actin in muscle. This suggests that proteins other than actin were included in the 43 kd band. Since sarcoplasmic and myofibrillar proteins have different turnover rates in vivo (Young, 1970), the possibility that actin and creatine kinase turnover at different rates in vitro should be considered in interpretation of these data. MHC is the only significant component of the 200 kd band, so it may be a more appropriate indicator of myofibrillar protein synthesis in the system described here.

Gulve and Dice (1989) observed increased protein synthesis as the quantity of non-radioactive amino acid was increased. These authors suggested that under some conditions, quantity of the indicator amino acid could limit protein synthesis. In work reported here, RAC treatment increased protein synthesis whether assayed in cultures with or without added non-radioactive methionine. This finding indicates that methionine-limiting conditions did not account for observed RAC effects.

Figure 1. Time course of total protein, 43 kd protein and myosin heavy chain synthesis.

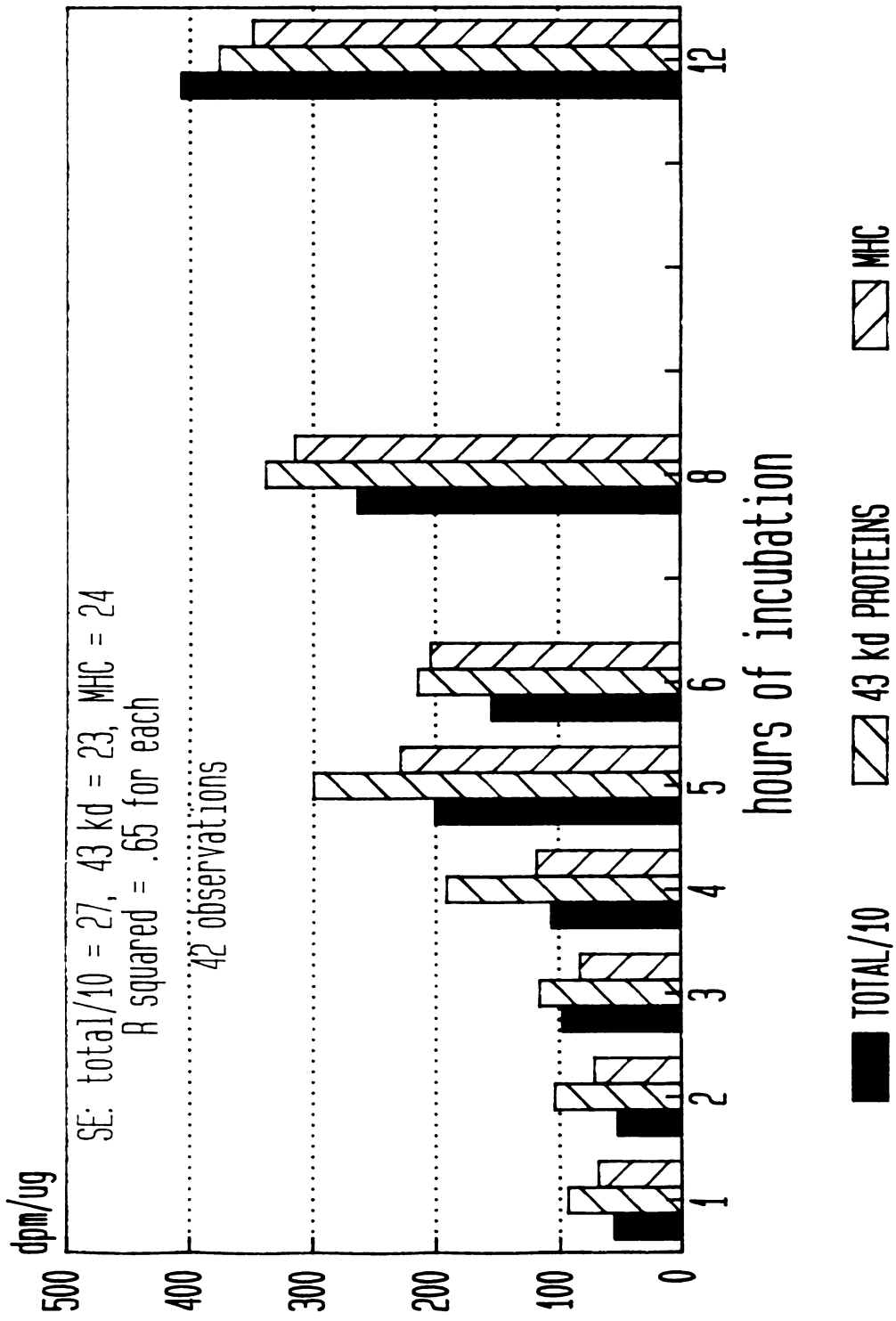

dpm/ug
500
400
300
200
100
0
SE: total/10 = 27, 43 kd = 23, MHC = 24
R squared = .65 for each
42 observations
1
2
3
4
5
6
8
12
hours of incubation
TOTAL/10
43 kd PROTEINS
MHC

Table 1. The effect of ractopamine on ^{35}S incorporation in ELC5 myotubes

Control	2159	2464	2862	1773	1838
Ractopamine	2414	3129[b]	3369[c]	2383[b]	2274[c]

[a]Values reported are least squares means (pooled SE = 112.4; n = 16/treatment/time)
[b]Differs from control (P<0.1).
[c]Differs from control (P<0.05).

Table 2. The effect of ractopamine on incorporation of ^{35}S methionine into 43 kd proteins and MHC in ELC5 myotubes[a]

	Hours of treatment				
Treatment	4	24	48	72	96
	dpm in 43 kd protein/μg total protein/6h				
Control	163.1	248.0	322.6	194.3	201.1
Ractopamine	174.9	317.3[b]	556.4[d]	226.5	315.9[c]
	dpm in MHC/μg total protein/6h				
Control	172.7	183.1	274.5	197.7	164.5
Ractopamine	163.8	185.3	394.8[d]	219.9	234.7[d]

[a]Values reported are least squares means (pooled SE = 21.1 for 43 kd proteins, 20.5 for MHC; n = 16/treatment/time)
[b]Differs from control (P<0.1).
[c]Differs from control (P<0.05).
[d]Differs from control (P<0.01).

Chapter 2

THE EFFECT OF BETA-ADRENERGIC AGONISTS AND AN ANTAGONIST ON PROTEIN SYNTHESIS AND DEGRADATION IN CULTURED MYOTUBES

Abstract

The effects of beta-adrenergic agonists (BAA; ractopamine (RAC) and clenbuterol (CLEN)) in the presence or absence of an antagonist (propranolol; PROP) on protein synthesis and degradation were evaluated. ELC5 myoblasts were grown in DMEM and 10% fetal bovine serum (FBS) and allowed to differentiate fully to form myotubes. Protein synthesis was assessed by incubating myotubes for 6 h in DMEM without methionine with 10% FBS and 5 μCi of ^{35}S methionine per well as methionine sources. To assess protein degradation, proteins were pre-labeled with ^{35}S L-methionine for 48 h, medium replaced and release of TCA-soluble counts into chase medium, as well as loss of label from TCA-insoluble protein were assessed at various time intervals. Following electrophoresis, radio-

activity incorporated into (synthesis) or lost from (degradation) the 43 kd protein (including actin) and myosin heavy chain (MHC; 200 kd) bands was determined. BAA increased ($P<.05$) synthesis of total and specific myofibrillar proteins, RAC to a greater extent than CLEN. PROP partially inhibited these increases. BAA did not affect ($P>.05$) rate of degradation of total or specific proteins although responses to leupeptin and to increased calcium indicate that the cells are responsive to protein degradation altering agents. In these cells BAA stimulate synthesis of total and specific myofibrillar proteins, but do not affect protein degradation. The effects of BAA on protein synthesis are mediated at least in part through the beta receptor.

KEY WORDS: Beta-adrenergic agonist, Beta-adrenergic antagonist, Myotubes, Protein Synthesis, Protein degradation, Myofibrillar Proteins

Introduction

Beta-adrenergic agonists (BAA) increase muscle protein accumulation and depress fattening when fed to food producing or laboratory animals (for review see Hanrahan, 1987). We have previously reported that ractopamine (RAC), a phenethanolamine known to act as a BAA in adipose tissue (Coutinho et al., 1989) increases total (Bergen et al., 1989) and myofibrillar (Helferich et al., 1988) protein synthesis in vivo and in muscle cell culture (Anderson et al., 1989; Chapter 1). The effect of RAC on protein synthesis is thought to be pretranslational (Helferich et al., 1988) and appears to be a direct effect on the muscle cell (Anderson et al., 1989).

The mechanism(s) by which BAA other than RAC enhance skeletal muscle hypertrophy is unclear (Maltin et al., 1989). While increased in vivo protein synthesis in response to BAA other than RAC has been reported (Deshaies et al., 1981; Emery et al., 1984; Clayes et al., 1989), and Young et al. (1987) observed increased protein synthesis in cimaterol-treated muscle cell cultures, others have observed unaltered protein synthesis in vitro (Li and Jefferson, 1977; Roeder et al., 1987; Forsberg and Merrill, 1988) or in vivo (Reeds et al., 1986; Bohorov et al., 1987). Discrepancies between reported data may be due to differences in treatment compound, length of treatment or experimental conditions.

The effects of various BAA on protein metabolism have been compared in only a few studies although comparisons of

effects of BAA on lipid metabolism have been reported (Mersmann et al., 1987; Coutinho et al., 1989). Mechanism(s) of RAC effects on protein metabolism have not been compared to that of more widely studied compounds such as clenbuterol (CLEN).

The objectives of the present study were to compare the effects of BAA RAC and CLEN on protein metabolism of cultured myotubes and to determine whether addition of a beta-adrenergic antagonist (propranolol; PROP) would interfere with BAA effects.

Materials and Methods

Cell culture. Cells and culture conditions have been previously described (Anderson et al., 1989). Briefly, ELC5 myoblasts, multiplied at 37 C in a humidified atmosphere of CO^2/air (1:19) in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), were plated at a density of $10^4/cm^2$ in 6 well tissue culture clusters. Treatments (10^{-6} M RAC, 10^{-7} M CLEN, PROP at 10 x BAA concentration) were added 24 h after differentiation. Pen-Strep (fifty units/ml) was included in the media.

Measurement of protein synthesis. After 48 h of BAA treatment, protein synthesis was assessed by incubating myotubes

in DMEM without methionine (met- DMEM) containing 584 mg/l L-glutamine with 10% FBS and 5 μCi of ^{35}S L-methionine per well as sources of methionine. Observed RAC and CLEN effects on protein synthesis were also tested in cultures incubated with incubation medium as described above with the inclusion of 1 mM non-radioactive methionine.

After 6 h exposure to the label, media were removed, cells washed twice with DMEM and trypsinized to detach them from the wells. Immediately after removal from culture dishes, DMEM, with 10% FBS was added to inactivate trypsin. Cells were pelleted by centrifugation, media poured off and the cell pellet frozen immediately. Cell pellets were solubilized by boiling in electrophoresis loading buffer (62.5 mM Tris-Cl, 5% ß-mercaptoethanol, 2% sodium dodecyl sulfate and 10% glycerol). Protein in an aliquot was precipitated with 20% (w/v) trichloroacetic acid (TCA) and resuspended in 1 N NaOH. Protein content of the NaOH solution was determined by the method of Lowry et al. (1951) and radioactivity incorporated into TCA-insoluble protein was determined by liquid scintillation analysis.

Approximately 30 μg of protein were electrophoresed for 12-16 h at constant current of 15 mA per gel through 2 cm stacking gels (4% acrylamide, linker:polylinker ratio = 37.5) followed by 12 cm separating gels (12.5%, 37.5). After staining with Coomassie Brilliant Blue and destaining, the 43 kd and MHC bands were sliced from the gels and incorporated radioactivity determined by liquid scintillation analysis.

Measurement of protein degradation. Protein degradation was assessed in pulse-chase experiments using methods similar to those of Gulve and Dice (1989). Proteins were prelabeled by incubating myotubes for 48 h in DMEM with FBS and 5 μCi of ^{35}S L-methionine per well. BAA, but not PROP, were included in the pre-label medium. After labeling, media were removed, the cell monolayer washed twice with DMEM and chase medium (including 10 mM non-radioactive methionine) added. After 1 h media were replaced with fresh chase media, after washing the cell monolayer twice with DMEM, this was considered time 0. Appropriate BAA and PROP treatments were included in chase media. In preliminary experiments, media samples (50 μl) were taken at 2-4 h intervals, proteins in media samples were precipitated by addition of 20% (w/v) cold TCA and centrifugation, radioactivity in the TCA-soluble media fraction was assessed by liquid scintillation counting. At various times the cell monolayer was removed and remaining radioactivity of total and specific myofibrillar proteins determined as in synthesis experiments. TCA-soluble counts in the cell pellet were considered to comprise the intracellular free pool.

Experimental design. A 2x3 factorial design was utilized with PROP (absence or presence) and BAA (control, RAC or CLEN) as the factors. Protein synthesis was assessed in each of 6 replicate experiments (blocks), two or three 6 well tissue culture clusters were randomly assigned to each of 6 treatment combinations. Cells from 2 wells were combined to form

a single sample, considered to be the experimental unit. Preliminary protein degradation experiments utilized at least 18 wells per treatment with repeat media samples from each well; individual wells were the experimental units. Each well was sampled only once in experiments reported in Tables 3 and 4.

Statistical analysis. Protein synthesis data were analyzed by analysis of variance with treatment and block as main effects included in the model. In protein degradation experiments, regression analysis was used to calculate slope and intercept values. Dunnett's t test statistics were used to evaluate pre-planned comparisons between treatment means and control (Gill, 1978). Bonferroni t test statistics were used to evaluate comparisons not involving control means (Gill, 1978).

Results

Protein synthesis. Based on analysis of variance, the effects of RAC, CLEN, and PROP on total protein synthesis in these cultures were significant ($P<.05$) and a significant 2 factor (BAAxPROP) interaction was observed. BAA increased ($P<.01$) apparent synthesis rate of total protein in ELC5 myotubes, as assessed in 6 h incubations with ^{35}S L-methionine (Table 1). In cultures without PROP, RAC increased protein synthesis to a greater extent than CLEN (26.0 vs 14.4%).

Inclusion of PROP at 10 times the concentration of BAA partially blocked the BAA-induced increase in protein synthesis. The reduction in BAA effect was greater for RAC (26.0% increase reduced to 15.0% by PROP) than for CLEN (14.4% in absence of PROP, 10.3% in presence of PROP) but was significant for each.

In 4 of the 6 replicates of this experiment, PROP was added 1 h before BAA were added to allow occupation of receptors, in the other 2 replicates PROP and BAA were added simultaneously and competed directly for receptors. Pre-incubation with PROP did not contribute significantly to variation and was withdrawn from the model.

In general, 43 kd proteins and MHC synthesis responses to BAA and PROP treatment mirrored total protein synthesis responses (Table 1) although inhibition of CLEN effects by PROP was slight.

BAA increased protein synthesis in cultures with 1 mM non-radioactive methionine added to incubation medium (Table 2). In these cultures, RAC treatment increased total protein synthesis 16.8% ($P<.01$) and CLEN 10.2% ($P<.05$). In cultures with added non-radioactive methionine, incorporation of ^{35}S into 43 kd proteins and MHC was too low to quantify.

Protein degradation. In initial protein degradation experiments utilizing a 24 h chase period, BAA did not affect appearance of dpm in media (Fig 1). This was true when BAA were included in the incubation medium for the entire

pre-label period (as reported in Fig 1) as well as in cultures with brief or no prior exposure to BAA (data not shown).

Based on these data it was assumed that BAA effects on protein degradation in ELC5 myotubes, if any, were slight. Longer experiments were then designed with the intent of allowing degradation to continue for at least 1 half-life of the proteins. In these experiments cells were harvested at various times in order to directly assess disappearance of label from protein. This was done because protein in cell pellets at the end of previous experiments had higher specific radioactivity in BAA treated cultures than control. This method also removed concern about adherent radioactivity not completely washed from the cell monolayer which could appear in later media samples but would not appear in the TCA-insoluble fraction.

In a longer-term (72 h) experiment (Table 3), BAA treatment resulted in greater ($P<.05$) specific radioactivity of protein at time 0, greater ($P<.05$) dilution of specific radioactivity per unit of time and greater loss of radioactivity from the bound protein pool per unit of time but did not affect protein degradation expressed as the percentage of protein degraded/h, based on initial specific activity. The more rapid dilution of specific radioactivity in the BAA treated cultures does not reflect increased degradation since cellular proteins should be uniformly labeled. Since specific radioactivity would decline as a result of new protein synthesis, the more rapid decline is an indication of greater

protein synthesis in treated cultures. Measurement of protein synthesis by this means has been described previously (Goldberg, 1969) and relies on maintenance of constant protein pool size. In these cultures protein content per well did not change appreciably over the course of the chase period, indicating that relatively constant pool size was maintained.

Thus, in BAA-treated cultures, protein was degraded at the same rate as in control cultures. However, due to greater initial specific radioactivity of the bound protein pool in treated cultures, a greater quantity of radioactive methionine was released from the bound pool per unit of time. This was perplexing in light of equal appearance of radioactivity in the media (Fig 1). Thia apparent discrepancy was resolved by the discovery that the intracellular free amino acid pool, which was not quantified in preliminary experiments, contained more radioactivity in treated cultures.

Since PROP affected protein synthesis, its effect on protein degradation was assessed. PROP did not significantly affect degradation of total protein, the 43 kd band or MHC (Table 4).

Protein metabolism of ELC5 myotubes has not been described previously. Therefore, in order to strengthen the conclusion that BAA do not affect protein degradation in these cells, we sought to demonstrate that ELC5 myotubes are responsive to other protein degradation-affecting agents. Leupeptin, a tripeptide inhibitor of serine proteases, which is known to decrease protein degradation (Kameyama and

Etlinger, 1979) including degradation of myofibrillar proteins (Silver and Etlinger, 1985), decreased ($P<.05$) appearance of dpm in chase media (Fig 2) when added at a concentration of 10^{-4} mM although lower concentrations were not effective. In addition, radioactivity remaining in total protein, the 43 kd band and MHC was greater in leupeptin treated cultures after the 24 h chase period (Table 5). Since no treatments were present in the pre-label incubation period, greater radioactivity/well at the end of the experiment is further evidence of inhibition of protein degradation by leupeptin.

To show an increase in protein degradation, calcium (5 mM) was added to cultures in the chase medium along with 10^{4} ng/ml A23187, a Ca^{++} ionophore, to increase intracellular Ca^{++}. Calcium, with A23187 increased appearance of dpm in the media (Fig 3), indicating increased protein degradation. This was the expected result based on the data of Kameyama and Etlinger, (1979), Lewis et al., (1982) and Rodemann et al., (1982).

Discussion

The dramatic effects of BAA on improving growth and lean/fat ratio of food producing and laboratory animals has generated considerable interest in potential for BAA use as growth and carcass modifiers for livestock or for use as

anti-obesity or atrophy preventative agents in humans. Although much recent research has been focused on determining the mechanism(s) by which BAA increase muscle protein deposition (reviewed by Yang and McElligot, 1989), this area remains confusing (Maltin et al., 1989). Since muscle protein is dynamic in nature, increased protein accumulation could be a result of increased synthesis, decreased degradation or both. While some researchers have concluded that BAA influence protein metabolism entirely through reduction in the rate of protein degradation (eg Scanes et al., 1988), arguments for increased protein synthesis in response to BAA treatment are gaining support.

In work reported here and in previous experiments (Bergen et al., 1989; Anderson et al., 1989), we have demonstrated increased muscle protein synthesis in response to RAC treatment. Based on these results, observed in two diverse biological systems (market weight pigs and cultured myotubes from a myogenic cell line of embryonic rat origin), we conclude that increased muscle protein synthesis is responsible for at least part of the RAC-induced muscle hypertrophy observed in growing animals. This conclusion is strengthened by the observation of Helferich et al. (1988) that abundance of mRNA coding for skeletal muscle alpha actin, a major myofibrillar protein, is increased in skeletal muscle of RAC-treated pigs.

The influence of BAA other than RAC on protein metabolism has been more widely studied than RAC but reports are

often conflicting. For example, Li and Jefferson (1974), Garber et al. (1976), Reeds et al. (1986) Forsberg and Merrill (1986) and Roeder et al. (1987) all reported no effect of various BAA on protein synthesis in various experimental systems. In contrast, Deshaies et al. (1981), Emery et al. (1984), Young et al. (1987), Eadara et al. (1988) and Harper and Buttery (1989) have observed BAA-induced increases in protein synthesis. BAA have also increased protein synthesis in hypophysectomized (Nutting et al., 1982) and denervated (Maltin et al., 1989) rats. Differences among reported data could result from differences in treatment compound, timing of experiments or experimental conditions.

The present work is the first to compare RAC to one of the more widely studied BAA, in this case CLEN. The increased protein synthesis in this muscle cell culture system in response to RAC exceeded the increase in response to CLEN. In this study, CLEN treatment increased protein synthesis 15.0%, a response comparable to the 12% increase reported by Harper and Buttery (1989) in L6 cells treated with CIM. This may partly explain the confusion surrounding reported data. Indeed, if CLEN increases protein synthesis in other experimental systems, but to a lesser extent than RAC, a CLEN-induced increase might escape observation, whereas the larger RAC response would have been observed if the experimenters had chosen to include RAC as a treatment.

While BAA effects on lipid metabolism are known to involve BAA binding to beta receptors (Fain and Garcia-Sainz,

1983; Coutinho et al., 1989), involvement of beta receptors in protein metabolism has not been well described. In work reported here, PROP, a non-selective beta antagonist, included at 10x BAA concentration, partially inhibited effects of both RAC and CLEN. In the work of Garber et al., (1976), BAA effects on protein metabolism were inhibited by PROP and Harper and Buttery (1989) observed complete inhibition of CIM effect when PROP was included at 100 x the concentration of CIM. In the work reported here, it is possible that inclusion of 100-fold greater concentrations of PROP than BAA (instead of 10-fold greater) would have completely abolished the effects of RAC and CLEN. In contrast, PROP did not inhibit CLEN-stimulated protein accretion (Maltin et al., 1987) and Reeds et al. (1988) reported that BAA effects on protein accumulation of rats were not diminished by beta antagonists PROP or atenolol. Hence, it remains possible that RAC and/or CLEN affect muscle cells through some non-beta receptor mediated pathway as well, this area should receive further investigation.

In work reported here neither BAA nor PROP had an effect on degradation of total protein or specific myofibrillar proteins. Young et al. (1987), reported decreased myofibrillar protein degradation in chicken primary muscle cell cultures treated with CIM for 6 d, but no effect was noted in cells treated for 2 h. Observance of chronic, but not acute effects led Young et al. (1987) to conclude that CIM does not directly inhibit activity of proteolytic enzymes. Forsberg and Merrill

(1986), have also reported that CIM (1 μm) decreased protein degradation in rat myotubes. This effect was not observed in mouse myotubes or at higher doses of CIM in rat myotubes. No explanation is apparent for the lack of BAA effect on protein degradation in ELC5 myotubes however we did not evaluate the effects of CIM. Furthermore, we utilized an original subclone of the cell line used by Forsberg and Merrill (1986) and Young et al. (1987) used primary cells.

It could be that timing of BAA administration in relation to measurement of protein metabolism affects results. This appears to be true in vivo as Eisemann et al. (1988) reported that acute (1 d) CLEN treatment reduces protein degradation in steers, whereas 9 d of CLEN treatment induced increased protein synthesis. In rats, Emery et al. (1984) and Maltin et al. (1989) observed increased protein synthesis as an immediate effect of BAA treatment while protein synthesis was unaltered after 1 wk of CLEN treatment (Reeds et al., 1986). In the rat, BAA effects on protein accumulation are diminished by 1 wk of treatment (Eadara et al., 1988), an observation that led Maltin et al. (1989) to speculate that Reeds et al. (1986) might have observed a detectable increase in protein synthesis earlier in the treatment period.

In general, conclusions from in vivo experiments with BAA other than RAC have been that BAA decrease protein degradation. However, direct measurement of protein degradation is virtually impossible in vivo, so assumptions must be used. For example, Reeds et al. (1986) measured protein

synthesis and accretion and calculated protein degradation by difference (eg degradation = synthesis - accretion). Validity of this method is contingent upon protein accretion proceeding in linear fashion since the size of the protein pool (used to calculate fractional degradation rate) is calculated as the mean of initial and final protein content. Maltin et al. (1989) have pointed out that this assumption may be invalid in BAA treated rats since the majority of the BAA effect on protein accretion are observed in the first few days of treatment, thus the protein pool would be underestimated if calculated as by Reeds et al. (1986).

Use of muscle cell culture allows accurate measurement of direct BAA effects on protein degradation in the muscle cell. Physiological significance of these cell culture findings is unknown, however, and the entire area of BAA influence on protein degradation remains confusing. Indeed, Bergen et al. (1989) have reported that RAC-treated pigs had increased protein degradation (protein synthesis was increased to a greater extent than degradation, resulting in net increase in protein deposition in treated pigs) but these degradation values were calculated by difference, not measured directly. Maltin et al. (1989) have also observed occasions where synthesis of protein was increased to an extent greater than accretion indicating that degradation was increased.

Figure 1. The effect of BAA on appearance of TCA-soluble dpm in media.

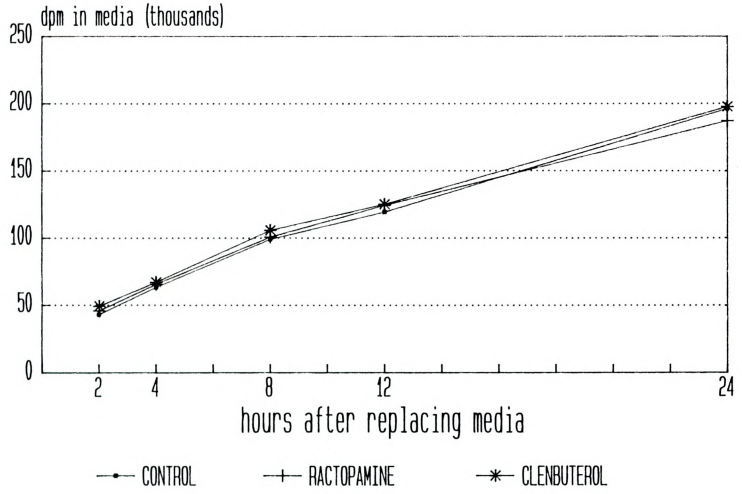

dpm in media (thousands)
250
200
150
100
50
0
2
4
8
12
24
hours after replacing media
CONTROL
RACTOPAMINE
CLENBUTEROL

Figure 2. The effect of leupeptin on appearance of dpm in media.

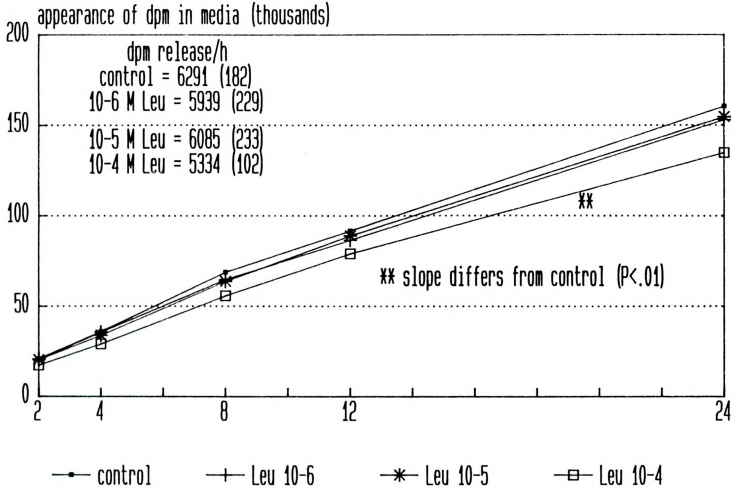

appearance of dpm in media (thousands)
200
150
100
50
0
dpm release/h
control = 6291 (182)
10-6 M Leu = 5939 (229)
10-5 M Leu = 6085 (233)
10-4 M Leu = 5334 (102)
** slope differs from control (P<.01)
**
2
4
8
12
24
control
Leu 10-6
Leu 10-5
Leu 10-4

Figure 3. The effect of 5mM calcium with 10^{4} ng/ml A23187 on appearance of dpm in media.

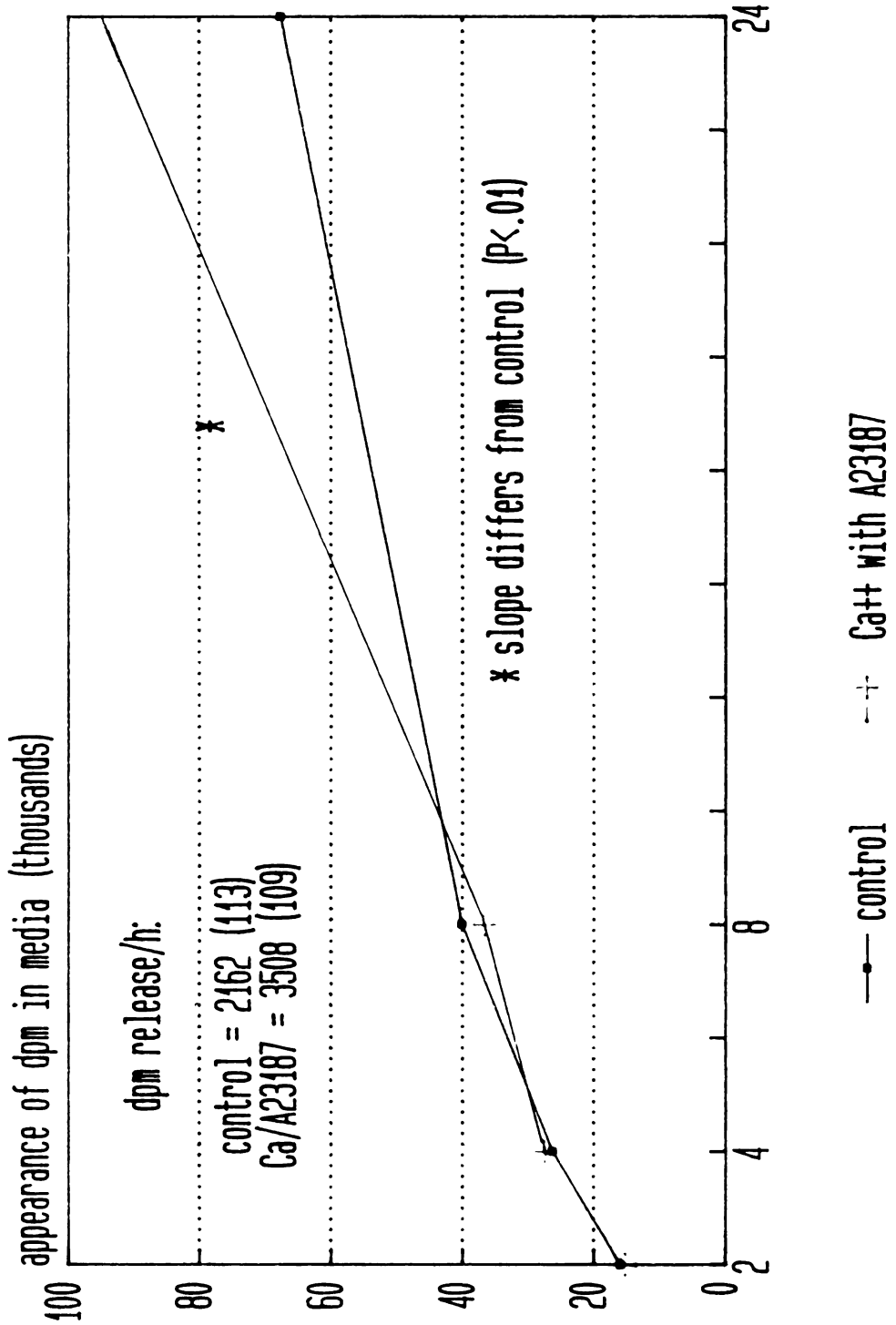

appearance of dpm in media (thousands)
100
80
60
40
20
0
2
4
8
24
dpm release/h:
control = 2162 (113)
Ca/A23187 = 3508 (109)
* slope differs from control (P<.01)
control
Ca++ with A23187

Table 1. The effect of BAA and PROP on ^{35}S methionine incorporation into total protein, 43 kd protein and MHC in ELC5 myotubes

Treatment	Item		
	Total protein	43 kd protein	MHC
	dpm/μg total protein/6h		
Control	2825.2	424.9	359.5
Ractopamine	3559.8[b]	536.3[b]	436.8[b]
Clenbuterol	3232.2[b]	465.9	405.6[a]
Propranolol	2703.2	407.2	353.2
RAC + PROP	3108.6[d]	453.6[c]	397.8[c]
CLEN + PROP	2982.2[c]	461.9[c]	399.6[c]
Pooled SE	124.0	21.4	20.8
df	235	136	136

[a]Tends to differ from control (P<.1)
[b]Differs from control (P<.01)
[c]Tends to differ from propranolol (P<.1)
[d]Differs from propranolol (P<.01)

Table 2. The effect of BAA on incorporation of ^{35}S methionine during 6 h incubations in ELC5 myotube cultures in the presence of 1 mM added methionine.[a]

	control	ractopamine	clenbuterol
dpm/μg	126.5	147.7[b]	139.4[c]

[a]Pooled SE = 5.43, 24 observations/treatment
[b]Differs from control (P<.01)
[c]Differs from control (P<.05)

Table 3. Effects of BAA on protein metabolism in ELC5 myotubes[a]

	Slope	Intercept
	specific activity (dpm/μg)	
Control	-7.53 (.83)	710.5 (30.2)
Ractopamine	-10.40 (.94)[c]	805.2 (33.9)[b]
Clenbuterol	-9.10 (.69)[b]	755.1 (25.3)
	dpm released from bound pool per well	
Control	-1208.3 (197.3)	114701 (7171)
Ractopamine	-1709.6 (240.6)[c]	128846 (8741)
Clenbuterol	-1587.2 (139.3)[c]	123554 (5061)
	protein degradation (%/h)	$t_{1/2}$ (h)
Control	1.00 (.16)	69.3
Ractopamine	1.05 (.15)	65.9
Clenbuterol	1.13 (.10)	61.1

[a]Means calculated by regression, $t_{1/2}$ calculated as .693/rate of degradation, n = 6 observations/treatment at each of 6 time points
[b]Tends to differ from control (P<.1)
[c]Differs from control (P<.05)

Table 4. Effects of BAA and PROP on protein degradation in ELC5 myotubes[a,b]

	Protein degradation (%/h)	$t_{1/2}$ (h)
Control	1.18 (.10)	58.5
Ractopamine	1.21 (.12)	57.2
Clenbuterol	1.31 (.11)	52.9
Propranolol	1.19 (.11)	58.3
Rac + Prop	1.23 (.14)	56.0
Clen + Prop	1.27 (.13)	54.7

[a]Means calculated by regression, 5 observations/treatment at each of 4 time points
[b]No significant differences

Table 5. Effects of BAA and PROP on protein degradation in ELC5 myotubes[a,b]

	43 kd protein degradation (%/h)	$t_{1/2}$ (h)
Control	.73 (.09)	94.9
Propranolol	.77 (.11)	89.6
	MHC degradation (%/h)	$t_{1/2}$ (h)
Control	1.15 (.09)	60.5
Propranolol	1.19 (.13)	58.2

[a]Means calculated by regression, 5 observations/treatment at each of 4 time points
[b]No significant differences

Table 6. Effects of leupeptin on protein degradation in ELC5 myotubes[a]

	Concentration of leupeptin			
	0	1μM	10μM	100μM
	1000 dpm/well			
Appearance in media[b]	6.29	5.94	6.09	5.33[d]
SE	.18	.23	.23	.10
Remaining total protein[c]	94.6	100.3	104.5[d]	108.2[d]
SE	2.3	2.3	2.3	1.6
Remaining 43 kd protein[c]	21.7	25.6	27.4[d]	26.5[d]
SE	1.2	1.2	1.2	.8
Remaining MHC[c]	11.5	12.0	11.7	11.8
SE	.7	.7	.7	.5

[a]n = 40
[b]Appearance/h of TCA-soluble radioactivity in media, means calculated by regression.
[c]Radioactivity remaining in total protein, 43 kd protein or MHC after 24 h degradation period.
[d]Differs from control (P<.05).

Table 7. Effect of increased calcium on protein degradation in ELC5 myotubes[a]

	control	treated
	1000 dpm/well/h	
Appearance in media (SE)	2.16 (.1)	3.51[b] (.1)

[a]Treated cultures received 10^4 ng/ml of the calcium ionophore A23187 and 5 mM additional calcium.
[b]Differs from control ($P<.01$).

Chapter 3

A PRELIMINARY INVESTIGATION INTO THE MOLECULAR BIOLOGY OF RACTOPAMINE-INDUCED INCREASES IN PROTEIN SYNTHESIS IN ELC5 MYOTUBES IN CULTURE

Introduction

This work was undertaken based on two key findings. First, ractopamine (RAC) increases muscle protein synthesis in vivo (Bergen et al., 1989) and in cell culture experiments using myotubes from ELC5, a myogenic cell line (Anderson et al., 1989). Second, the in vivo protein synthesis response to RAC is accompanied by (and perhaps a result of) pretranslational changes that result in a 2 to 3-fold increase in the relative abundance of mRNA coding for skeletal muscle alpha actin (SKMAA; Helferich et al., 1988).

With these results in mind, the objective of this investigation was to determine if RAC administration to ELC5 myotubes increased the relative abundance of mRNA coding for SKMAA in these cells.

Materials and Methods

Cell culture. ELC5 myoblasts were multiplied, plated in 6 well dishes and allowed to fuse as described in chapters 1 and 2 and treated as if to compare protein synthesis between RAC and control as in chapter 2.

RNA extraction. RNA was extracted from two 6 well dishes per treatment after 48 h of RAC treatment using an adaptation of the procedure of Chirgwin et al. (1979) as described by Helferich et al. (1988). Briefly, media were removed and the cell monolayer washed 3 times with Dulbecco's phosphate buffered saline. Proteins were hydrolyzed and myotubes removed from culture wells by scraping with a sterile rubber policeman after addition of 4 M guanidinium isothiocyanate (GIT). This solubilized virtually all of the cellular proteins and nucleic acids. To ensure complete solubilization, the GIT mixture from each treatment was pooled into a single sample which was was homogenized for 30 sec with a Brinkmann polytron with a .7 cm generator probe.

After homogenization, the cell/GIT solution was centrifuged at 11,000 x g for 15 min at 4 C to remove non-solubilized material and debris. The supernatant (4 ml) was layered over a 1 ml cesium chloride (5.7 M) cushion and centrifuged for approximately 16 h at 100,000 x g in a Beckman ultracentrifuge using an SW 50.1 rotor. The RNA pellet was resuspended in 7 M guanidine-HCl, 20 mM Na acetate, 1 mM

dithiothreitol, 10 mM iodoacetic acid and 1 mM Na_2EDTA and transferred to a 1.5 ml microcentrifuge tube. RNA was precipitated with 2 volumes of absolute ethanol (-20 C) and one-tenth volume of .3 M Na acetate. RNA precipitates were washed once with 3 M Na acetate, 10 mM iodoacetate (pH 5.0, 4 C), then with 66% ethanol, 33 mM Na acetate (pH 5.0) and finally with absolute ethanol (-20 C). The final RNAs were resuspended in 10 mM Tris, 1 mM EDTA (pH 8.0) and stored at -80 C. Quality and purity of the RNA solutions was checked spectrophotometrically by scanning from 220-320 nm. The A260/A280 ratio was determined and RNA concentration determined from the A260.

Northern blot analysis. Duplicate RNA samples (5 ug/lane) were size separated by electrophoresis through a 1.2% agarose, 2.2 M formaldehyde, .4M MOPS, 100 mM NaAc and 10 mM EDTA (1 x MAE) denaturing gel for 16 h at 40 volts (Maniatis et al., 1982). RNA was then transferred using the capillary method (Southern, 1975) onto nitrocellulose paper with 25 mM NaPO4, pH 6.5 (Maniatis et al., 1982). Lanes containing size standards and samples were sliced from the gels, stained with ethidium bromide, destained to remove background flourescence and photographed under UV light.

Blots were briefly washed with 200 ml 2x-SSC to remove any adherent agarose, placed on dry 3MM paper and dried under a heat lamp. Blots were then baked in a vacuum oven for 2 h at 80 C. Blots were prehybridized in "seal-a-meal" bags

containing 50% formamide, 1.0 M NaPO4, 5x Denhardt's, .2 M EDTA, 10 mg/ml tRNA for 3 h at 42 C in a shaking water bath. Following prehybridization, prehybridization solution was replaced with hybridization solution (identical to prehybridization solution except Denhardt's was reduced to 1 x) which contained 2 million cpm of appropriate ^{32}P labeled cDNA insert. The cDNA inserts were random primed with ^{32}P (Feinberg and Vogelstein, 1983; 1984) using a commercially prepared kit (Boehringer Mannheim). One blot was allowed to hybridize overnight at 42 C to a SKMAA cDNA probe (# 248) obtained from Dr. L. Kedes at Stanford University. The probe was random primed 700, 800 bp Pst/PvuII inserts excised from pHM A-1 plasmid. An identical blot was allowed to hybridize in similar fashion to a rat beta actin cDNA probe (# 280) also obtained from Dr. Kedes. The beta actin cDNA insert was excised from the recombinant plasmid with PvuII and SmaI resulting in 2 fragments - 600 and 1200 bp and a 2400 bp remaining piece of plasmid. The 600 and 1200 bp inserts were purified together and the combination used in random priming as above. Following hybridization, blots were washed with 2 x SSC, 0.1% SDS at room temperature for 10 min, then washed 3 times for 45 min each, in .1 x SSC and 0.1 % SDS at 55 C. Washed blots were partially dried under a heat lamp and wrapped in plastic. Autoradiography was conducted using an X-ray cassette equipped with intensifying screens, blots were exposed to X-ray film (Kodak X-OMAT AR) for 48 h at -80 C.

Crosshybridization was observed between the SKMAA probe

containing 50% formamide, 1.0 M NaPO4, 5x Denhardt's, .2 M EDTA, 10 mg/ml tRNA for 3 h at 42 C in a shaking water bath. Following prehybridization, prehybridization solution was replaced with hybridization solution (identical to prehybridization solution except Denhardt's was reduced to 1 x) which contained 2 million cpm of appropriate ^{32}P labeled cDNA insert. The cDNA inserts were random primed with ^{32}P (Feinberg and Vogelstein, 1983; 1984) using a commercially prepared kit (Boehringer Mannheim). One blot was allowed to hybridize overnight at 42 C to a SKMAA cDNA probe (# 248) obtained from Dr. L. Kedes at Stanford University. The probe was random primed 700, 800 bp Pst/PvuII inserts excised from pHM A-1 plasmid. An identical blot was allowed to hybridize in similar fashion to a rat beta actin cDNA probe (# 280) also obtained from Dr. Kedes. The beta actin cDNA insert was excised from the recombinant plasmid with PvuII and SmaI resulting in 2 fragments - 600 and 1200 bp and a 2400 bp remaining piece of plasmid. The 600 and 1200 bp inserts were purified together and the combination used in random priming as above. Following hybridization, blots were washed with 2 x SSC, 0.1% SDS at room temperature for 10 min, then washed 3 times for 45 min each, in .1 x SSC and 0.1 % SDS at 55 C. Washed blots were partially dried under a heat lamp and wrapped in plastic. Autoradiography was conducted using an X-ray cassette equipped with intensifying screens, blots were exposed to X-ray film (Kodak X-OMAT AR) for 48 h at -80 C.

Crosshybridization was observed between the SKMAA probe

and beta actin mRNA (ELC5 cells) and between the beta actin probe and alpha actin mRNA (pig). Consequently, blots were rewashed using higher stringency conditions, 0.1 x SSC and 0.1 % SDS at 65 C and re-exposed to X-ray film as above. Crosshybridization was not detected using these this more stringent wash.

Results

The A260/A280 ratio of RNA was between 1.8 and 2.0 for each sample. Quality of RNA was excellent as determined by ethidium bromide staining of agarose-formaldehyde gels, as very little degradation was evident and the 28S:18S intensity appeared to be near 2:1.

A photograph of the final autoradiogram is shown in Fig 1. In this preliminary observation, with only one RNA sample/treatment, ELC5 RNA showed virtually no hybridization to the SKMAA 248 probe and substantial hybridization to the beta actin 280 probe. The intensity of the beta actin band in the lane containing RNA from RAC-treated cells compared to RNA from control cells, suggests increased relative abundance of the beta actin message in the RAC-treated cells compared to control.

RNA (3 ug/lane) from 4 wk old and market weight pig longissmus dorsi muscle which contain SKMAA was used as a standard. The pig RNA hybridized to the SKMAA probe, but not the beta actin probe.

Discussion

Failure of ELC5 RNA to hybridize to the SKMAA 248 probe, as observed in RNA from pig skeletal muscle, is perplexing but may be explained by one of the following theories. It is likely that these cells, at the stage of development that they were utilized in this experiment, synthesize predominately beta, but not alpha actin. Perhaps the cells, considered to be early myotubes because of their multinucleation, characteristic of myotubes, would produce alpha actin later after differentiation. If so, a similar experiment, conducted with cells later in development, might give different results. These cells can be kept viable for approximately 4-8 days longer than those used in this experiment, but cell death increases at 7-8 days after differentiation.

Perhaps the beta actin mRNA observed resulted from contaminant cells. In this experiment, measures were not taken to rid cultures of myoblasts, mononucleated precursor cells which do not produce alpha actin. These cultures were morphologically similar to others which routinely exhibit fusion of approximately 50%. If 50% of the RNA extracted from these mixed cultures originated from myoblasts, abundance of beta actin mRNA could exceed that of SKMAA mRNA which could readily explain the presence of the beta actin mRNA in this experiment. This would not, however, preclude presence of SKMAA mRNA. Indeed, even if only 30% of the nuclei originated

from myotubes and these cells had only half as much mRNA/nucleus as the myoblasts, myotubes would still have contributed approximately 18% of the RNA obtained which would correspond to about 1 ug/lane on the northern blots. If SKMAA mRNA is an abundant message in the myotube mRNA pool, 1 ug of total RNA/lane should have been adequate to detect it using the methods described above.

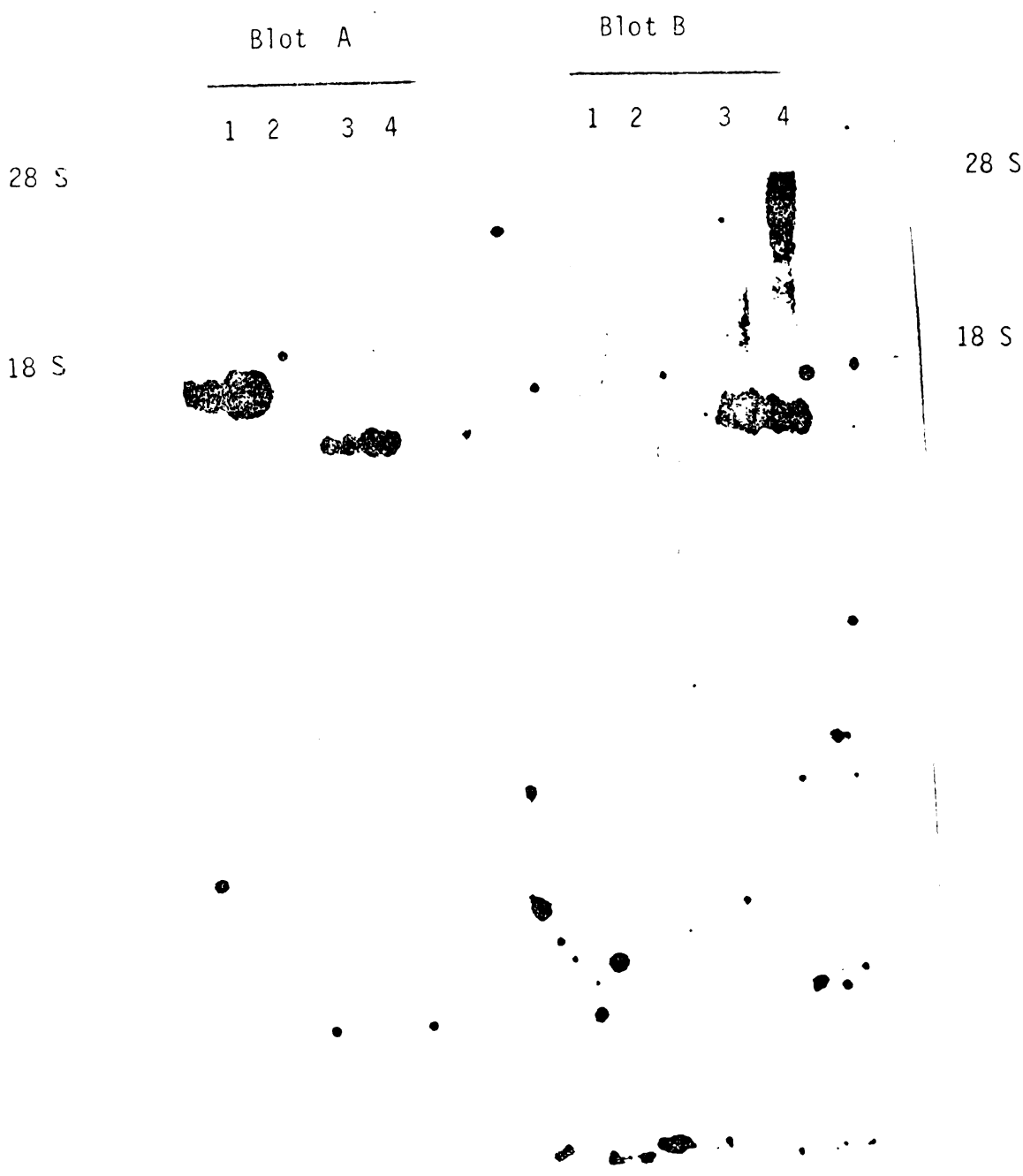
Perhaps the RNA extraction procedure that was utilized selectively extracted RNA from myoblasts or somehow prohibited degradation of RNA in myoblasts but not myotubes. This seems unlikely, since in our laboratory use of this procedure is routinely successful for extraction of RNA from mature muscle, which has very few myoblasts.

Thus, from these preliminary observations a likely conclusion is that these cells do not contain a message that has close homology to the SKMAA message. These cells may contain some isoform of actin that is similar but not identical to beta actin but the relatively high stringency conditions used in washing these blots (65 C, .1 x SSC) suggests that the message observed does code for beta or a similar form of actin. There seems little chance that the beta actin message is not translated efficiently, hence, substantial presence of beta actin in these cells is inferred. Given this, it may be that these cells have ability to assemble myofibrils that include beta actin, an event that has not been demonstrated in other systems. It may also be possible that the myotubes utilized in this experiment were simply not mature enough to

synthesize alpha actin at the stage of differentiation at which these studies studies were conducted.

Before the molecular biology of the response of these cells to RAC or other treatments is investigated further, the contribution of various isoforms of actin to the cellular protein pool in these cells should be clarified. If alpha actin appears at a further stage of development, then its message would likely be present in high enough abundance for detailed investigations.

Fig 1. Northern blot analysis of RNA isolated from ELC5 cells and porcine muscle. Blot A was hybridized to a ^{32}P random primed rat beta actin probe, blot B to a ^{32}P random primed human alpha actin probe. Lane 1 = ELC5 (control), lane 2 = ELC5 (RAC-treated), lanes 3 and 4 = porcine muscle. Blots were washed at 65 C and .1 x SSC and exposed to X-ray film for 72 h at -80 C.


Blot A
Blot B
1 2 3 4
1 2 3 4
28 S
18 S
28 S
18 S

SYNTHESIS EXPERIMENTS

I. Cell Culture

A. Plate cells at density of 10^4/cm2 (10^5/well in 6 well dishes), allow to multiply in DMEM with 10% FBS and .5% Pen-Strep until confluent.

B. When confluent, lower FBS to 2% for 24 h, then return to 10%.

C. Add treatments when FBS is returned to 10%.

D. Measure synthesis 48 h later.

II. Measurement of protein synthesis

A. Incubation medium includes:
1. Methionine deficient (Met-) DMEM
2. 10% FBS
3. 584 mg/l L-Glutamine (Met- DMEM doesn't contain gln).
4. ^{35}S Met (5 μCi/well)

B. Remove proliferation medium and replace with 2 ml incubation medium. If synthesis is to be assessed in more than 2 or 3 6 well dishes, they should be staggered about 10 min apart to provide adequate time to remove cells after incubation. If they aren't staggered some will incubate longer than others, it takes about 10 min to trypsinize, remove and spin 2 or 3 6 wells.

C. After 6h, remove incubation medium, wash cell monolayer twice with 1 ml DMEM, once with .5 ml trypsin, then add 1 ml trypsin to remove them. Place in freezer or refrigerator for 3-5 minutes (cold facilitates removal).

D. When loose, remove cells from wells (I usually combine 2 wells to form 1 experimental unit, this assures adequate protein for a couple of do overs for Lowrys or gels if their are any problems), rinse wells with 1 ml DMEM, add this to trypsin-cell solution to inactivate trypsin.

E. Centrifuge cell/DMEM/trypsin mixture at 3-5000 rpm for 4 min to precipitate cells.

F. Pour off DMEM-trypsin solution. Use a pipette tip to remove any DMEM-trypsin that settles to the bottom

of the tube.

G. Freeze ASAP.

III. Sample processing and assays

A. While frozen, remove frozen pellet from bottom of 15 ml tube with end of a small weigh spatula, and place in a 1.5 ml Eppendorf tube. This step can be avoided and 15 ml tubes boiled instead.

B. Add 150 µl protein denaturing buffer (electrophoresis loading buffer) per well to pellets.

C. Vortex mixture, boil 2 or 3 minutes, vortex and boil another 2 or 3 minutes.

D. Triturated mixture with pipette tip. If not liquid, vortex again and boil until liquid.

E. Remove an aliquot precipitate protein by adding 100% TCA and centrifuging for 5 min at 13000 x g. Pour off supernatant and rinse pellet with water. If pellet is yellow, rinse once with ETOH, if white, ignore. A yellow pellet seems to be due to SDS or bromophenol blue dye, this causes Lowry values to be unusually high. Somehow, ETOH cures this. Care should be taken with water and ETOH washes, it is easy to loosen a portion of the pellet and toss it right down the drain, if protein values are quite variable, this may be an explanation.

F. Resuspend protein in 300 µl 1 N NaOH, vortex, and leave at room temp for 20 min, then heat (40-50 C) for 20 min.

G. Divide NaOH soln.]
1. 50 µl in scint vial, add 4 ml Safety Solve, count.
2. 100 µl in each of 2 tubes for Lowry.

H. After protein content is calculated, electrophorese no more than 35 µg of protein per sample for 12-16 h at 15 milliAmperes per gel. Counting bands isn't very accurate if more than 35 µg of protein is used.

I. Stain (24 h) and destain (48 h) gels. Cut out actin and MHC bands, place in scintillation vials with Safety-Solve and count.

DEGRADATION EXPERIMENTS

I. Cell Culture: same as in synthesis exps.

II. Pre-label

A. Pre-label medium includes:
   1. DMEM
   2. 10% FBS
   3. 5 μCi/well ^{35}S Met
   * I have also used 50% DMEM with 50% Met- DMEM (including gln), cells get hotter, release of label is greater but protein degraded per unit of time is the same. One may choose this method in order to use a shorter pre-label or some thing. Could get by with less isotope, probably much less.

B. Add pre-label medium when FBS is returned to 10%

C. Incubated cells for 48 h.

III. Measurement of degradation

A. Chase medium is DMEM + 10% FBS + 1.7 g/liter of L-Met, this brings medium to 4X normal met content, at room temp this is all I can get to dissolve.

B. After 48 h of pre-label, remove media, replace with chase media and left for 1 h. According to theory intracellular free amino acids are either incorpor ated or released during this time, this is not the case in this system.

C. After 1 h, remove media and replace with fresh chase medium, this is time 0. Six wells of cells per treatment are harvested as described in synthesis section.

D. Remove 50 μl of media, place in TCA, vortex and freeze ASAP. Harvest cells at various times.

IV. Sample processing and assays

A. Pellets - handled as described above. For measurement of radioactivity of the intracellular free pool, count supernatant after TCA precipitation.

B. Media - thaw media samples and centrifuge at 13000 for 6 min, pipette liquid off and count in 3 ml Safety Solve (ppt = dead cells etc).

C. Data - to calculate total counts in media multiply counts in a 50 μl sample by 40. If repeated samples are taken from a well, correct later samples for radioactivity removed in early samples.

Source of materials

Dulbecco's Modified Eagle Medium - Gibco Laboratories, Grand Island, NY.

Fetal bovine serum (lot # 1111759 used for all experiments) - HyClone Laboratories, Logan, UT.

Culture dishes - Costar, Cambridge, MA.

^{35}S L-methionine (specific activity 1151 Ci/mmol) - New England Nuclear, Boston, MA.

Leupeptin - Peptides International, Louisville, KY.

A23187 (calcium ionophore) - Sigma Chemical Co., St. Louis, MO.

Ractopamine (94% purity) - Lilly Research Laboratories, Greenfield, IN.

Clenbuterol - Boeringher Ingelheim

DL-propranolol - Sigma Chemical Co., St. Louis, MO.

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