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EFFECT OF THE PHENETHANOLAMINE, RACTOPAMINE, ON THE ADIPOGENIC CELL LINE, TA1

By

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ABSTRACT

EFFECT OF THE PHENETHANOLAMINE, RACTOPAMINE, ON THE ADIPOGENIC CELL LINE, TA1

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The phenethanolamine, ractopamine, reduces total fat accretion in meat-producing animals. This study was undertaken to determine the effect of ractopamine on lipid metabolism in adipocytes. For this study a stable cell line (TA1) was used since these undifferentiated preadipocyte acquires adipocyte functions and morphology after growth to confluence in culture. Prior to the use of this system inital experiments were conducted to establish the experimental conditions necessary to obtain a highly differentiated culture of adipocytes and to establish the response of the adipocytes to the beta-adrenergic agonists, isoproterenol and ractopamine. By monitoring the activity of glycerol-3-phosphate (GPD), malic enzyme (ME) and fatty acid synthase (FAS) during differentiation, dexamethasone, a synthetic glucocorticoid, in combination with the antiinflammatory drug, indomethacin, was observed to trigger a rapid and complete differentiation of TA1 cells 4 days after confluence. Also, TA1 adipocytes were observed to respond

to isoproterenol and ractopamine in a dose-dependent manner as indicated by the agonists stimulation of lipolysis and inhibition of FAS activity. These studies indicate that TA1 adipocytes are a suitable model to investigate the effect of ractopamine on lipid metabloism.

Next, studies were conducted to investigate the effect of ractopamine on TA1 adipocytes at a cellular and molecular level. Changes in activity of GPD, ME, FAS and mRNA abundance for these enzymes and acetyl-CoA carboxylase (ACC) were monitored. Ractopamine at 10^{-6} M maximally inhibited the activity of GPD and ME. Ractopamine (10^{-6} M) was also observed to have an immediate and sustained inhibitory effect on GPD, ME and FAS activity. Studies conducted with the beta-antagonist, propranolol, indicated that these effects were mediated via the beta-receptor. Finally the inhibitory effect of ractopamine on the activity of these enzymes was accompanied by a reduction in GPD, ME, FAS and ACC mRNA abundance. In summary, these studies indicate ractopamine mediates its effect by interacting with the beta-adrenergic receptor present on the adipocyte and altering enzyme activity through a pretranslational event. These studies also suggest that the reduced fat deposition observed in animals fed ractopamine may also be regulated in this manner.

To Lynn

with love

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Introduction

The continued increases in the growth rates of meat-producing animals have been a result of decades of genetic selection. Although these rigorous selection pressures, have improved muscle protein deposition by selecting for larger animals, little improvement has been made in reducing the rate of fat deposition. With the current heightened health awareness, high fat content in red meat is no longer acceptable to the consumer. In order to address the over-production of animal fat, a group of compounds which reduce fat accretion have been developed as potential feed additives for meat-producing animals. These compounds, synthetic beta- adrenergic agonists, are structural analogs of the naturally occurring catecholamines, epinephrine and norepinephrine, and are believed to act specifically at the beta receptor and exert their actions primarily by elevating intracellular concentration of cyclic AMP (Fain and Garcia-Sainz, 1983; Lefkowitz et al., 1976).

Adrenergic agonists were first recognized as potential manipulators of body composition through studies involving administration of the naturally occurring catecholamines,

epinephrine and norepinephrine. In swine, daily injections of epinephrine (0.15 mg/kg body weight) increased nitrogen retention with minimal change in body fat deposition (Cunningham et al., 1963). Based on this limited report it appeared that catecholamines had the potential to alter body composition in meat-producing animals. One difficultly facing further development of the natural catecholamines as growth promoters was that epinephrine and norepinephrine have a very short half life in the body (Christensen et al., 1984). Consequently, a single administration of the catecholamine which would be ideal in a production setting may not be suitable for maximum response. Another potential problem with catecholamines was their interaction with both alpha-and beta-adrenergic receptors which could lead to undesirable side effects in the animal. Therefore, analogues of these catecholamines, specific beta-adrenergic agonists have been developed, which alter body composition in animals, produce minimal side effects and easily adapt into general farm-management practices. Beta-agonists currently under investigation include clenbuterol, cimaterol, ractopamine, L-640,033, LY 104119, BRL 26830 and BRL 35135.

Among the various beta-agonists currently being developed BRL 26830 and LY 104110 reduce fat deposition with negligible influence on muscle protein accretion. In genetically obese mice (ob/ob and db/db) and rats (fa/fa),

BRL 26830 reduced body lipid content with only minor effects on lean body mass (Arch and Ainsworth, 1983; Arch et al., 1984). Similarly, LY 104119 decreased body fat in obese (A^{vy}/a) mice (Yen et al., 1984). In normal rats and mice, however, BRL 26830 and LY 104119 only slightly reduced fat gain while failing to influence protein accretion (Arch et al., 1984; Yen et al., 1984). These studies suggest that BRL 26830 and LY 104119 are effective in reducing body fat accretion in genetically obese animals but have little efficacy in normal animals. Moreover, these data indicate that both have little practical value to the meat-animal industry, however, BRL 26830 and LY 104119 may have an impact as potential anti-obesity drugs.

Clenbuterol, cimaterol, L-640,033, ractopamine, unlike BRL 26830 and LY 104119, increase lean body mass concomitantly with reducing adipose tissue mass. Finishing lambs fed 2 ppm clenbuterol for 8 weeks had 37% less 12th rib fat, 42% larger longissimus areas and 23% greater semitendinosus muscle weights than control lambs (Baker et al., 1984). Similar enhancement of lean body mass and reduced fat accretion have been observed in steers (Ricks et al., 1984), swine (Dalrymple et al., 1984a), broilers (Dalrymple et al., 1984b) and rats (Reeds et al., 1986) fed clenbuterol. Lambs fed cimaterol for approximately two months showed a 25 to 30% increase in the weight of several muscles compared to lambs fed a control diet (Beermann et al., 1986; Kim et al., 1987). L-640,033 altered growth

and carcass characteristics when added to the diets of broiler chickens and rats (Muir et al., 1985; Rickes et al., 1985). In preliminary reports, ractopamine increased muscle hypertrophy and reduced adipose tissue accretion when fed to finishing pigs at 20 ppm (Anderson et al., 1987ab; Hancock et al., 1987; Merkel et al., 1987; Prince et al., 1987).

Although it is well accepted that many beta-adrenergic agonists produce a dramatic increase in skeletal muscle mass as well as a large reduction in body fat, the biochemical aspect of these effects on either tissue is still under investigation. In muscle tissue the effects of beta-agonists appear to be mediated through an increase in the synthesis and/or decreased degradation of muscle protein while in adipose tissue a decrease in lipogenesis and/or an increase in lipolysis may cause the reduced fat accretion (Reeds et al., 1986; Bergen et al., 1989; Claeys et al., 1989; Yang and McElligott, 1989). Since the following research investigated beta-adrenergic agoinsts effects on lipid metabolism the remainder of this review will be devoted to the actions of beta-agonists on lipid metabolism.

Beta-Adrenergic Agonist Regulation of Lipid Deposition

Reduction of body fat is a pronounced physiological effect of chronic beta-adrenergic agonist treatment. Decreased body fat may be a consequence of increased energy expenditure or an alteration in lipid synthesis and/or mobilization. Rothwell et al. (1983) and Reeds et al. (1987) reported that clenbuterol treatment increased energy expenditure 8 and 16%, respectively, in laboratory animals. Similarly, increased energy expenditure has been observed in lambs and calves fed clenbuterol (MacRae et al., 1986; Williams et al., 1987). Cimaterol treatment increased the apparent fasting heat production and energy required for maintenance in lambs (Kim et al., 1989) In rodents, this enhanced energy expenditure may be a result of beta- adrenergic agonist direct stimulation of brown adipose tissue (Arch and Ainsworth, 1983; Yen et al., 1984), however, the existence or importance of brown adipose tissue in meat-producing animals remains questionable (Alexander, 1979). Kim et al. (1989) implied that the enhanced skeletal protein accretion and turnover observed with beta-agonists may account for a sizable portion of the increased heat production while Eisemann et al. (1988) attributed the increased maintenance requirement to increased blood flow and heart rate observed in beta-agonist treated animals. Whatever the mechanism, these studies imply that energy once available for fat deposition

may be diverted towards a heat generating activity, thus limiting overall fat accretion. Eadara et al. (1988), however, reported that chronic cimaterol treatment reduced fat accretion in rats but failed to alter energy balance. This latter study raises doubt that beta-adrenergic agoinsts simply reduce body fat by increasing energy expenditure.

Enhanced lipolysis and/or depressed lipogenesis have also been implicated as possible modes of action of beta-adrenergic agonists. In isolated ovine adipocytes clenbuterol reduced lipogenesis 18% and enhanced lipolysis 28% (Thorton et al., 1984). Duquette and Muir (1985) also showed that clenbuterol was a potent inhibitor of lipogenesis as well as a stimulator of lipolysis in vitro. In the presence of adenosine receptor antagonist, adenosine deaminase or the phosphodiesterase inhibitor, theophylline, isoproterenol, epinephrine, cimaterol and ractopamine depressed fatty acid biosynthesis and stimulated lipolytic activity in porcine adipose tissue cells, in vitro (Coutinho et al., 1989; Lui et al., 1989; Lui and Mills, 1989; Peterla et al., 1990). Other studies examining adipose tissue and hepatic tissue slices and isolated cells from different species corroborate the lipolytic actions of these agonists (Cramb et al., 1982; Campbell and Scanes, 1985; Saggerson, 1985; Hausman et al., 1989).

In contrast to the in vitro data which support both lipogenic and lipolytic control by beta-adrenergic agonists, at least in the rat, in vivo data seem to support only a lipolytic mode of action. Eadara et al. (1986) observed reduced body fat in rats fed cimaterol for 4 weeks. Under these conditions cimaterol supplementation altered fatty acid mobilization in the liver and white adipose tissue without any apparent change in fatty acid biosynthesis. Feeding L-640,033 for 1 week resulted in a reduction of epididymal fat pad weight without altering the activity of the lipogenic enzymes (see Yang and McElligott, 1989). These data imply that chronic administration of beta-agonists enhances fat mobilization without affecting overall fatty acid biosynthesis in rat adipose tissue, implying that these compounds act primarily as lipolytic agents in rodents.

Merkel and coworkers (1987), however, reported that in adipose tissue from ractopamine fed pigs, both lipogenesis and lipolysis were altered and for lipogenesis the decrease was related to a reduction in the activity of enzymes involved in fatty acid biosynthesis. In cattle supplemented with clenbuterol for 50 days, the activities of lipogenic enzymes were reduced in subcutaneous adipose tissue but not in either the intramuscular or perirenal fat depots (Miller et al., 1986). Cimaterol, L-644,969 and ractopamine have been shown to have lipolytic capabilities in porcine adipose tissue in vivo (Hanrahan et al., 1986;

Merkel et al., 1987; Wallace et al., 1987; Peterla et al., 1990). These data clearly indicate that in livestock species beta-adrenergic agonists may effect both fatty acid biosynthesis and triacylglycerol hydrolysis unlike the case in rodents.

Beta-adrenergic receptors have been localized on plasma membrane of adipocytes (Williams et al., 1976). Recent data presented by Hausman et al. (1989) illustrated that ractopamine stimulated lipolysis and inhibited lipogensis by direct interaction with beta-adrenergic receptors. Mersmann et al. (1974), however, claimed that clenbuterol failed to directly influence lipolysis in porcine adipose tissue, in vitro, indicating that clenbuterol may have an indirect effect on lipid metabolism in swine. Timmermann (1987) stated that lipophilic beta-adrenergic agonists, such as clenbuterol, may pass the blood brain barrier resulting in modification of metabolism via altered sympathetic outflow or endocrine system function. Unfortunately, this remains to be substantiated since evidence on circulating concentrations of hormones in beta-adrenergic agonist treated animal have not been shown to differ from controls (Emery et al., 1984; Beermann et al., 1987). It appears then that the action of beta-agonists are mediated through the hormone-receptor

coupling system which ultimately leads to the activation of the cyclic AMP/protein kinase A cascade (Krebs and Beavo, 1979).

Regulation of Fatty Acid Biosynthesis by Cyclic AMP

Binding of beta-agonists to adipocyte beta-adrenergic receptor, activates the adenylate cyclase system which in turn increases cyclic AMP production and ultimately leads to a modification and coordination of the activity of key enzymes. This modulation and coordination can be subdivided into an immediate or short-term mechanism of regulation and a long-term mechanism. Short-term regulation operates on a minute to minute basis and allows for immediate response to environmental changes while long-term regulation involves adaptive changes in enzymatic activity due to fluctations in absolute amount of enzymes and requires hours to take effect. Both of these cyclic AMP-induced regulatory mechanisms are involved in regulation of long-chain fatty acid biosynthesis.

Biogenesis of long-chain fatty acids involves two enzymes, acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthase (EC 2.3.1.85). Acetyl-CoA carboxylase catalyzes the ATP-dependent carboxylation of acetyl CoA in the formation of malonyl CoA. Malonyl CoA and acetyl CoA are then condensed to form palmitate by the multi-functional

enzyme, fatty acid synthase. Cyclic AMP, a second messenger, has been implicated in acute regulation of fatty acid biosynthesis with acetyl-CoA carboxylase representing the favored site for short-term regulation (Geelen et al., 1980). The classical theory of short-term regulation of acetyl-CoA carboxylase suggests that citrate, the precursor of cytosolic acetyl-CoA, functions as a positive feed forward activator of fatty acid synthesis by inducing polymerization of acetyl CoA carboxylase to its active form (Numa et al., 1967; Moss and Lane, 1972; Lane et al., 1974); the pace at which fatty acid synthesis occurs reflects the citrate concentration of the cytosol. Under conditions of elevated cyclic AMP production, citrate production is decreased due to the inhibition of glycolysis which leads to the dissaggregation of acetyl-CoA carboxylase and lowered fatty acid production. However, several investigators (Harris and Yount, 1975; Cook et al., 1977; Watkins et al., 1977; McGarry et al., 1978; Clarke et al., 1979) reported that dibutyryl cyclic AMP inhibition of fatty acid synthesis did not correlate with the level of citrate. These data question the importance of the physiological role of citrate in the short-term regulation of fatty acid synthesis. In a recent review, Kim et al. (1989) suggested that metabolite levels may serve to fine-tune the synthesis of long-chain fatty acids under different physiological conditions.

Long-chain acyl-CoA esters have been implicated as allosteric regulators of acetyl-CoA carboxylase. More specifically, increases in free fatty acids concentration, in vitro, result in a depression of fatty acid synthesis which is caused by an increase in intracellular fatty acyl-CoA esters levels (Goodridge, 1973; McGee and Spector, 1975). Goodridge (1973) suggested that this elevation may inhibit acetyl-CoA carboxylase directly. In vitro, long-chain acyl-CoA esters have been shown to inhibit purified acetyl-CoA carboxylase (Botz and Lynen, 1963; Ogiwara et al., 1978; Nikawa et al., 1979). In fact, Nikawa et al. (1979) indicated that a specific binding site for long-chain acyl-CoA esters may be involved in this attenuation of acetyl-CoA carboxylase. Although long-chain acyl-CoA esters may alter acetyl-CoA carboxylase, in vitro, and although beta-adrenergic agonists are known to promote increased levels of long-chain acyl-CoA esters, compartmentalization of these esters in the cells make it unlikely that in vivo long-chain esters alter fatty acid biosynthesis. Moreover, glucagon-induced increase of acyl-CoA was observed to be confined to the mitochondria (Goodridge, 1973; Christiansen, 1977, 1979), therefore, the likelihood that the cyclic AMP-induced inhibition of lipogenesis was caused by an increase in long-chain acyl-CoA esters is remote since acetyl-CoA carboxylase activity is localized in the cytoplasm (Geelen et al., 1980).

Covalent modulation of acetyl-CoA carboxylase has also been implicated as a possible mode of action of adrenergic agonists short-term control on lipogenesis. Lee and Kim (1978) studied in vivo control of acetyl-CoA carboxylase in adipose tissue following intraperitoneal administration of epinephrine and reported that ^{32}P-labeling of acetyl-CoA carboxylase coincided with appropriate alterations in the activity of this enzyme. Holland et al. (1985) provided evidence that the mechanism of inhibition of acetyl-CoA carboxylase in adipocytes treated with glucagon involved increased phosphorylation. In fact, both glucagon and epinephrine changed the kinetic parameters and phosphorylation state of the carboxylase. Further support for the covalent-modulation of acetyl-CoA carboxylase in both adipocytes and hepatocytes was presented by Witter et al. (1979). In a series of experiment these investigators demonstrated that a ^{32}P-labeled cytosolic protein from adipocytes and hepatocytes could be specifically and completely precipitated with chicken and rat liver acetyl-CoA carboxylase antisera. Furthermore, complete amino acid sequences of acetyl-CoA carboxylase from chicken and rat sources deduced from cDNA sequence data (Takai et al., 1988; Lopez-Casillas et al., 1988) have allowed the identification and localization of phosphorylation sites. Cyclic AMP dependent protein kinase has been shown to participate in the phosphorylation of several of these sites (Lent and Kim, 1982; Munday et al., 1988). The

activity of acetyl-CoA carboxylase, independent of citrate, was depressed by phosphorylation and enhanced by dephosphorylation; thus confirming short-term regulation of acetyl-CoA carboxylase by a phosphorylation-dephosphorylation mechanism (Lent and Kim, 1982; Munday et al., 1988).

Fatty acid synthase (EC 2.3.1.85), the other enzyme required for long-chain fatty acid synthesis, has also been implicated as a site for short-term control of fatty acid biosynthesis. In vitro, the existence of two forms of fatty acid synthase have been observed. Holo-a, a high specific activity-low phosphorus content form of fatty acid synthase and holo-b, a low specific activity-high phosphorous content form of fatty acid synthase, have been demonstrated to arise from each other (Qureshi et al., 1975). Also, the existence of fatty acid synthase as an inactive form in liver of fasted rats and the increase in fatty acid synthase activity upon refeeding in the absence of protein synthesis, suggests that the inactive form was converted to an active form (Liou and Donaldson, 1977). Tweto et al. (1971) demonstrated that the prosthetic group of fatty acid synthase turned over at a much faster rate than the rest of the molecule and suggested that removal and replacement of this group may be a means of control of the overall activity of fatty acid synthase. Although these alternative forms of fatty acid synthase may be

important in regulation of lipogenesis in the short-term under some conditions, the link between these altered forms with cyclic AMP remains to be established. In fact, with regard to the phosphorylated state of fatty acid synthase, Witters et al. (1979) demonstrated in isolated hepatocytes that within 15 minutes of the addition of glucagon, acetyl-CoA carboxylase activity was decreased while fatty acid synthase activity was unaffected. Similar results were observed in vivo (Klain and Weiser, 1973). More specifically, within 15 to 30 minutes after intravenous injection of glucagon, a decrease in hepatic fatty acid synthesis and acetyl-CoA carboxylase activity was apparent, while the activity of fatty acid synthase, citrate cleavage enzyme, NADP-malic, glucose-6-phosphate- and isocitrate dehydrogenase were not affected. Brownsey et al. (1977) reported that incubation of intact rat epididymal adipocytes with epinephrine resulted in the phosphorylation of acetyl-CoA carboxylase but not fatty acid synthase. These data imply that fatty acid synthase is not involved in the short-term control of lipogenesis by cyclic AMP elevating agents.

Although ample evidence indicates that fatty acid biosynthesis is under short-term control by cyclic AMP generating agents, in regard to continual feeding of beta-adrenergic agonists to animals, this mechanism is probably not the predominate regulatory mechanism. Reduction in fat deposition is probably a result of long-term regulation of

lipid metabolism. Long-term control of fatty acid biosynthesis by cyclic AMP, unlike short-term regulation, appears to be a coordinated control system which involves the regulation of lipogenic capacity through an adaptive change in the absolute amount of key enzymes (Gibson et al., 1972; Goodridge et al., 1974; Lane et al., 1974; Numa and Yamashita. 1974; Volpe and Vagelos, 1976; Porter, 1978). Treatment of cultured hepatocytes with glucagon for 3 days resulted in a decrease in malic enzyme activity primarily because the relative synthesis rate of malic enzyme declined (Goodridge and Alderman, 1976). This effect of glucagon on malic enzyme synthesis was mediated by cyclic AMP (Goodridge et al., 1974; Goodridge and Alderman, 1976). Similarly, Rudack et al. (1971) reported cyclic AMP/glucagon attentuated the synthesis of the lipogenic enzyme glucose-6-phosphate dehydrogenase. The synthesis of fatty acid synthase in rat liver was also regulated by both glucagon and cyclic AMP with the effects of glucagon being mediated through cyclic AMP (Lakshmanan et al., 1972). In 3T3-L1 adipocytes, preincubation for 15 minutes with isoproterenol or 40 minutes with dibutyryl cyclic AMP produced a 4-fold reduction in the relative synthesis rate of the enzyme (Weiss et al., 1980). Similarly, Spiegelman and Green (1981) reported that the treatment of 3T3-F442A adipocytes for 30 hours with dibutyryl cyclic AMP or epinephrine resulted in the

reduction of the synthesis of fatty acid synthase and glycerol-3-phosphate dehydrogenase. The effect of dibutyryl cyclic AMP alone was slightly less than epinephrine while cyclic AMP with theophylline resulted in a sligher greater reduction in the synthesis of these enzymes. These effects were reversed after the removal of the agents; within 24 hours an increase in synthesis was observed and by 72 hours the rate of synthesis was equal to that of the controls.

The effect of cyclic AMP on synthesis of enzymes involved in fatty acid biosynthesis resides at a pretranslational level. Goodridge (1986) demonstrated that treatment of avian hepatocytes with glucagon resulted in a reduction in the synthesis of fatty acid synthase as well as the relative abundance of fatty acid synthase mRNA. In vivo adminstration of glucagon also resulted in a reduction in the synthesis of fatty acid synthase pattern accompanied by a comparable change in the relative abundance of fatty acid synthase mRNA abundance (Wilson et al., 1986). In 3T3 adipocytes dibutyryl cyclic AMP caused a 60% decrease in fatty acid synthase mRNA and in normal fasted/refed mice, dibutyryl cyclic AMP inhibited induction of fatty acid synthase mRNA (Paulauskis and Sul, 1988). The down regulation of fatty acid synthase mRNA abundance resides at the trancriptional level as indicated by Back et al. (1986a) and Paulauskis and Sul (1989).

Malic enzyme is also regulated at the pretranslational level. Glucagon treatment of hepatocytes previously treated with insulin plus triiodothyronine abolished the stimulatory effect insulin combination had on the synthesis of malic enzyme protein. Under these conditions the abundance of full length malic enzyme mRNA was correlated positively with the decrease in synthesis of enzyme (Winberry et al., 1983). Back et al. (1986b) demonstrated that glucagon altered the stability of malic enzyme mRNA in avian hepatocytes.

The studies described above indicate that although the synthesis of lipogenic enzymes is reduced by cyclic AMP, the mechanisms responsible for the reduced synthesis of these enzymes are different. Fatty acid synthase concentration appears to be mediated at a transcriptional level while malic enzyme concentration is regulated at a posttranscriptional level. Therefore, although cyclic AMP long-term regulation of fatty acid biosynthesis results in a coordinated decrease in the activities and amounts of lipogenic enzymes, several independent mechanisms are involved in the process.

Long-term control of lipogenesis by cyclic AMP has also been implicated as an indirect effect mediated by the increase in long-chain acyl-CoA esters. Long-chain acyl-CoA esters may signal repression of the synthesis of acetyl-CoA carboxylase resulting in the reduction of the

overall rate of fatty acid biosynthesis (Kamiryo et al., 1979). It has also been demonstrated that palmityl-CoA increases the rate of degradation of purified acetyl-CoA carboxylase by lysosomal extracts (Tanabe et al., 1977). Thus, it is conceivable that long-chain acyl-CoA esters are the factors resulting in the augmentation of lipogenesis. However, Spiegelman and Green (1981) investigated the link between inhibition of lipogenic enzymes with cyclic AMP-elevating agents via lipolysis by using cells allowed to differentiated in the absence of biotin. Under these conditions the cells synthesize no lipid but show characteristic lipogenic enzyme activities (Kuri-Harcuch et al., 1978). In cells whose triacylglyceride accumulation was prevented by this means; dibutyryl cyclic AMP with theophylline reduced the synthesis of fatty acid synthase, glycerol-3-phosphate dehydrogenase and malic enzyme to the same extent as cells possessing lipids. These agents therefore, affect synthesis of lipogenic enzymes even though no products of lipolysis are formed. This implies that the regulation of cyclic AMP is mediated directly on lipogenic enzymes rather than through the products of lipolysis (free fatty acids).

In summary long-term control of lipogenesis by cyclic AMP is a result of the alteration of mRNA abundance at a trancriptional and post-transcriptional level. Available data suggest that many of the enzymes involved in fatty acids biosynthesis are regulated on a long-term basis

unlike the short-term regulation which involves the regulation of only acetyl-CoA carboxylase. The enzymes regulated by cyclic AMP on a long-term basis result in a coordinated regulation in the amount of enzyme protein and thus, a coordinated decrease in the lipogenic capacity of the tissue (liver or adipose). This type of regulation implies that administration of agents which elevate cellular cyclic AMP concentrations may result in the reduction in fat accretion, in part, by reducing lipogenic capacity of the liver and/or adipose tissue at a pre-translational level.

Regulation of Gene Expression by Cyclic AMP

It is clear that cyclic AMP modulates the transcription and stability of messenger RNA for lipogenic enzymes, however, the mode of action by which this modulation occurs remains unresolved. In fact the negative regulation of gene expression by cyclic AMP is poorly understood, while the positive regulation of gene expression by cyclic AMP has been extensively investigated. Both the regulatory and catalytic subunits of protein kinase have been implicated in the cyclic AMP stimulation of gene expression. The regulatory subunit of protein kinase bears significant sequence homology to the prokaryotic cyclic AMP catabolite gene activator protein of

E. coli (Weber et al., 1982), suggesting that the regulatory unit may act in a similar manner to this prokaryotic protein in regulating gene expression. In a report by Constantinou et al. (1985) evidence was presented that the regulatory subunit can act directly on DNA to alter its conformation. The phosphorylation form of this subunit, after activation by cyclic AMP, possessed an intrinsic topisomerase activity toward several DNA substrates and can relax both positive and negative superhelical turns in plasmid DNA. These findings suggest that the regulatory subunit of protein kinase changes the conformational state of the chromatin, thus allowing the transcription of genes previously unavailable for interaction with RNA polymerase. Recently, Shabb and Granner (1988), however, reported that the regulatory subunit of cyclic AMP-dependent protein kinase does not contain intrinsic topisomerase activity, thus, refuting the role of the regulatory subunit as a mediator of cyclic AMP regulation of gene expression. Furthermore, even if the regulatory subunit was associated with topoisomerase activity, currently known topoisomerases show no DNA sequence specificity (Wang, 1985), therefore, it seems unlikely that the interaction of the regulatory subunit of protein kinase with specific gene sequences would occur.

The catalytic subunit of protein kinase has been proposed as the mediator of cyclic AMP positive regulation of gene expression. Basically, it has been reported that the catalytic subunit of protein kinase phosphorylates a nuclear DNA binding protein which in turn binds with high affinity to a conserved sequence 5′ to the trancriptional start site of cyclic AMP responsive genes (Roesler et al., 1988). This cyclic AMP responsive element (CRE) has been identified and shown to confer cyclic AMP regulation in several genes (Hod et al., 1984; Nagamine et al., 1984; Tsukada et al., 1985; Montminy et al., 1986; Short et al., 1986; Wyanshaw-Boris et al., 1986; Montminy and Bilezikjian, 1987). Unfortunately, some cyclic AMP responsive genes do not contain the CRE (see Montminy et al., 1986; Milsted et al., 1987) suggesting that the catalytic subunit cascade may not directly regulate all genes influenced by cyclic AMP.

In a review, Roesler et al. (1988) proposed a third mechanism for cyclic AMP regulation of gene expression in which ongoing protein synthesis is required for cyclic AMP regulation of gene expression. More specifically, cyclic AMP may stimulate synthesis of a particular protein which in turns stimulates the transcription of the target gene(s). To date, there is no direct evidence supporting this mechanism and therefore, further research is needed to verify this model.

In a recent review, Gaskin et al. (1989) postulated a negative mechanism of cyclic AMP regulation of gene expression similar to the indirect mechanism proposed by Roesler et al. (1988). Basically, Gaskin et al. (1989) proposed that cyclic AMP stimulates the expression of the cfos gene, and then cfos protein inhibits the expression of adipocyte specific genes. This hypothesis was based upon two lines of evidence. First, cfos gene expression has been shown to be under the regulation of cyclic AMP through the CRE/CREB system (Greenberg et al., 1985; Kruijer et al., 1985; Bravo et al., 1987) and second, cfos protein has been shown to suppress the expression of adipocyte P2 gene by binding to the fat-specific element II of this gene (Distel et al., 1987). Unfortunately there is no direct evidence to date to support this mechanism, however, if the model were validated it would not only provide support for an indirct mechanism of gene regulation by cAMP but it may also provide insight into the negetive regulation of gene expression by cyclic AMP.

Summary of Enzymes and cDNA Probes

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyzes the ATP-dependent carboxylation of acetyl CoA to malonyl CoA in the rate limiting step of long-chain fatty acid biosynthesis (Volpe and Vagelos, 1973; Lane et al., 1974; Kim, 1983;

Numa and Tanabe, 1984). Acetyl-CoA carboxylase is a relatively large enzyme in which the protomer consists of two identical subunits with a molecular weight of approximately 260,000 d (Ahmad et al., 1978; Song and Kim, 1981). The corresponding mRNA is also unusually large, approximately 10 kb and is present at low levels in cells (Lopez-Casillas et al., 1988). Recently, several cDNA clones of acetyl-CoA carboxylase have been isolated and the entire acetyl-CoA carboxylase coding region has been sequenced (Bai et al., 1986; Lopez-Casillas et al., 1988).

Fatty acid synthase (EC 2.3.1.85) catalyzes the conversion of acetyl CoA and malonyl CoA to palmitate. This dimeric enzyme with a subunit molecular weight of 250,000 d contains distinctive catalytic domains of acetyl transacylase, malonyl transacylase, B-ketoacyl synthase, B-ketoacyl reductase, B-hydroxyacyl dehydrase, enoyl reductase, acyl carrier protein and thioesterase (Wakil et al., 1983). The size and number of mRNAs encoding the mammalian fatty acid synthase appear to be species specific. In rat liver two mRNA bands, corresponding to either 9.2 and 8.4 kb (Nepokroeff et al., 1984) or 8.8 and 8.1 kd (Yan et al., 1985; Paulauskis and Sul, 1988) have been reported. Duck and goose fatty acid synthase are encoded by two mRNAs of 12.2 and 10.8 kb (Back et al., 1986a). Rat mammary gland also contains two fatty acid synthase mRNAs (Braddock and Hardie, 1988). In mice and 3T3-L1 adipocytes, however, only a single 8.2 kb mRNA

encodes for fatty acid synthase (Paulauskis and Sul, 1988). Southern blots of genomic DNA suggest that the mRNA(s) arise from a single gene (Yan et al., 1985; Back et al., 1986a) with the two mRNA species resulting from alternative polyadenylation sites (Back et al., 1986a). Presently, it remains unclear as to why the mouse has only one fatty acid synthase transcript, however, during the evolutionary process the mouse gene may have lost a polyadenylation site.

Malic enzyme (EC 1.1.40) catalyzes the NADP-dependent oxidative decarboxylation of malate to pyruvate. NADPH generated by malic enzyme is a major source of the reducing equivalents required for de novo biosynthesis of palmitate and other long chain fatty acids (Frenkel, 1975). The enzyme is a tetramer of four structurally identical subunits (Pry and Hsu, 1980). Each subunit is 64,000 d. Avian liver contains a unique 2.1 kb malic enzyme mRNA (Winberry et al., 1983) while mouse and rat cells express two mRNAs of markedly different sizes (2.0 and 3.1 kb) (Sul et al.,1984; Dozin et al., 1985). Both mRNAs have been found on polysomes and appear to code for the same malic enzyme subunit polypeptide (Magnuson et al., 1983; Sul et al., 1984; Dozin et al., 1985). The co-expression of the 2.0 and 3.1 kb mRNAs results from a post-transcriptional processing step. More specifically, the 2.0 kb mRNA results from the utilization of a poly A addition signal

that truncates the 3′ noncoding sequence by approximately 1 kb (Bagchi et al., 1987).

NAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) is an anabolic enzyme that converts a glycolytic intermediate metabolite, dihydroxyacteone phosphate, to a precursor of the glycerol backbone of triacylglycerides, glycerol-3-phosphate (Pollak et al., 1976; Schlossman and Bell, 1976; Wise and Green, 1978). The molecular weight of glycerol-3-phosphate is 37,600 d which is encoded by a 3.6 kb mRNA in the mouse (Kozak and Birkenmeier, 1983; Spiegelman et al., 1983). Rat glycerol-3-phosphate dehydrogenase mRNA is 4.6 kb (Kumar et al., 1985). The difference between these transcripts appears to be the length of their 3′ untranslated region (Kumar et al., 1985; Dobson et al., 1987).

Cell Line Jusification

Chapman et al. (1984) described the isolation and characterization of a stable adipogenic cell line, TA1, derived from 5-azacytidine treated 10T1/2 mouse embryo fibroblasts. At subconfluent densities, these cells resemble a fibroblast, however, several days after reaching confluence, TA1 cells loose their fibroblastic characteristics and begin to express both morphogical and phenotypic attributes of adipocytes (i.e. these cells

accumulate lipid droplets and express enzymes involved in fatty acid and triacylglycerol synthesis) (Chapman et al., 1984). Therefore, the TA1 adipocytes appear to be a suitable model to study lipid metabolism.

3T3 cell (Green and Kehinde, 1974, 1975, 1976) have been extensively characterized as compared to TA1 cells, therefore, any basic characterization of TA1 cells would be of relative importance. Preliminary studies on the differentiation and maturation of either cell line would have been conducted prior to the main studies, therefore, it seemed logical to use the TA1 cell since any information obtained would significantly add to the information on this cell line rather than duplicating information already available on the 3T3 cells.

Summary and Disseration Objectives

Beta-adrenergic agonists alter lipid deposition in animals. Ractopamine and other beta-adrenergic agonists appear to reduce lipid deposition, in part, by decreasing fatty acid biogenesis and enhancing lipid mobilization. This effect also appears to be mediated through the beta-adrenergic receptor. Chronic elevation of cyclic AMP reduces lipogenic capacity of the adipocyte at a pretranslational level. Presently, however, this has not been confirmed after long-term exposure of adipocytes to ractopamine. Therefore, the present research was conducted

to evaluate the long-term control of ractopamine on fatty acid biosynthesis. To best address this question a highly differentiated adipocyte system was established. Then, the in vitro system was utilized to examine the direct effect of ractopamine on lipid metabolism both at the enzymatic and pretranscriptional levels.

CHARACTERIZATION OF THE STABLE ADIPOGENIC CELL LINE, TA1

Introduction

Chapman et al. (1984) described the isolation and characterization of the stable adipogenic cell line, TA1, derived from 5-azacytidine treated 10T1/2 mouse embryo fibroblasts. When subconfluent, these preadipocytes are indistinguishable from their parent cell type, whereas after reaching a growth arrested state, TA1 cells exhibit the morphology characteristic of mature adipocytes, which is apparent by the appearence of lipid droplets. Chapman et al. (1984, 1985) also reported that the synthetic glucocorticoid, dexamethasone, accelerates the conversion of TA1 preadipocytes to adipocytes. Similarly, Knight et al. (1987) illustrated that the antiinflammatory drug, indomethacin, as well as dexamethasone, stimulates differentiation of TA1 cells. Within 3 days of indomethacin treatment, virtually all cells contain lipid droplets, whereas dexamethasone treated cells have only begun to differentiate. These data indicate indomethacin promotes a more rapid and complete conversion of preadipocytes to adipocytes than dexamethasone.

Torti et al. (1985) reported that mature adipocyte cultures of TA1 cells are capable of lipid mobilization and down regulation of enzymes involved in triacylglycerol synthesis upon insult by cachectin (tumor necrosis factor). These workers also noted the striking similarity of response of these cells to physiological events which occur in vivo, indicating TA1 cells as a plausible in vitro model for studying regulation of lipid metabolism by exogenous agents. My ultimate objective is to utilize TA1 adipocytes to investigate the direct effect of ractopamine, a beta-adrenergic agonist, on lipid metabolism at a cellular and molecular level. Prior to further use of this system, however, it seems imperative to establish: 1) the experimental conditions necessary to obtain a highly differentiated culture, 2) the time required for the cells to become a fully mature adipocyte population, and 3) the responsiveness of the adipocytes to beta-adrenergic agonists. The present studies, therefore, were designed to address these three objectives.

Experimental Protocols and Procedures

Drug Induced Adipogenesis: The conversion of preadipocytes to adipocytes is characterized by the induction of enzymes involved in fatty acid and triacylglycerol biosynthesis as well as the appearance of cytosolic lipid droplets. Fatty

acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (GDP) are enzymes induced during the conversion process (Mackall et al., 1976; Kuri-Harcuch and Green, 1977) and, thus can be used as an indicator of differentiation (Mackall et al., 1976; Kuri-Harcuch and Green, 1977; Coleman et al., 1978; Grimaldi et al., 1978; Wise and Green, 1979; Morikawa et al., 1982). Therefore, the activities of FAS and GPD were measured in the following experiments to evaluate dexamethasone and indomethacin as inducers of differentiation.

In a preliminary study confluent cells (day 0) were triggered to differentiate by the addition of 1.0 uM dexamethasone (DEX), 125 uM indomethacin (INDO) or the combination of both dexamethasone and indomethacin (DEX/INDO) to the medium for 48 hours or allowed to differentiate spontaneously. On day 2 inducer containing medium was replaced with standard medium, and cells were allowed to differentiate until day 6 at which time cells were harvested for GPD activity determination. Similarly, in the second experiment, on day 0 cells were treated with DEX, INDO, and DEX/INDO for 48 hours. Inducer-containing medium was replaced with standard medium on day 2 and cells were allowed to mature until being harvested for FAS activity determination on day 6 and day 12.

Time Course Study: Two phases of development exist during adipogenesis; an initial phase which is recognized by an exponential increase in the activity of enzymes involved in lipid synthesis and mobilization and a second phase characterized by a steady-state level of enzyme activity (Mackall et al., 1976; Kuri-Harcuch and Green, 1977; Coleman et al., 1978; Grimaldi et al., 1978; Wise and Green, 1979). The initial phase represents the induction or conversion of preadipocytes to adipocytes while the latter phase represents a climax population of adipocytes. Since the overall objective is to study regulation of lipid metabolism and not differentiation the following experiments were designed to establish the maturation curve of TA1 cells.

In the first set of experiments GPD and malic enzyme (ME) activity was measured during adipogenesis while FAS activity was focused upon in the second set of experiments. More specifically, confluent cells (day 0) were treated with the combination of 1.0 uM dexamthasone and 125 uM indomethacin for 48 hours as described above and cells were harvested on day 0, 1, 2, 3, 4, 5, 6, 8, 10, 12 post-confluence for GPD and ME activity determination while in the FAS experiments cells were harvested on day 0, 2, 4, 6, 8.

Responsiveness of Adipocytes to Beta Adrenergic Agonists: The overall objective of these experiments was to establish that TA1 adipocytes are responsive to specific beta-adrenergic agonists. The beta-agonists, isoproterenol and ractopamine, have been shown to stimulate triacylglycerol mobilization and depress de novo fatty acid biosynthesis in isolated adipocytes (Hausman et al., 1989; Saggerson et al., 1985). Triacylglycerol mobilization can be quantitated by measuring the amount of glycerol released from the cells since glycerol is an end-product of the degradation process which is not reutilized by the adipocytes. The activity of FAS is correlated to the rate of fatty acid biosynthesis (Volpe and Vagelos, 1976), therefore, the lipogenic capacity of adipocytes can be assessed by measuring the activity of FAS. Two independent studies were conducted to compare the efficacy and potency of isoproterenol and ractopamine to stimulate glycerol release and depress fatty acid synthase activity in TA1 adipocytes. In the lipolytic studies fully differentiated TA1 cells were treated with either isoproterenol or ractopamine at concentration of 10^{-10} to 10^{-4} M for 2 hours and lipolytic response was assessed by collection of the medium for glycerol concentration determination. In the lipogenic studies treatments were imposed at concentration of 10^{-9} to 10^{-4} M for 24 hours. Following the treatment period cells were harvested for FAS activity determination. Dose-response curves were

generated for the comparison of the effectiveness of the agonists.

Cell Culture Conditions: TA1 cells were generously provided by Dr. Gordon M. Ringold (Institute of Biological Science, Syntex Research Laboratories, Palo Alto, CA). Culture dishes (35 mm) were inoculated with 50,000 TA1 cells and grown to confluence (3 to 4 days) in 2.0 ml of Dulbecco's modified Eagle medium (4500 units glucose) (Gibco Laboratories, Grand Island, NY) containing 10% heat inactivated fetal bovine serum (Gibco Laboratories, Grand Island, NY) and 10,000 units of gentamicin (Gibco Laboratories, Grand Island, NY) in a humidified atmosphere of 5% CO_2, 95% air at 37 C. Unless otherwise indicated medium was replaced every three days during the experiments.

Enzyme assays: For the determination of GPD and ME activity, cells were rinsed with Dulbecco's phosphate buffered saline (pH 7.4) and removed from plates by scraping with a rubber policeman into 0.15 M KCl containing 5 mM 2-mercaptoethanol (Allee et al., 1971). Cell suspensions were homogenized with two 30 second pulses at setting no. 4 by the Polytron (Brinkman Corporation, Westbury, NY). Cell lysates were then centrifuged at 20,000 x g for 15 minutes at 4 C. The resulting

supernatants were immediately assayed for both enzymes and aliquots were frozen (-20 C) for later protein determination (Lowry et al., 1951). Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) activity was determined as outlined by Kozak and Jensen (1974) with the modifications specified by Wise and Green (1979). The assay system consisted of 100 mM triethanolamine/HCl buffer (pH 7.5), 2.5 mM EDTA, 0.12 mM NADH, 0.2 mM dihydroxyacetone phosphate, 0.1 mM 2-mercaptoethanol and 1 to 100 ug of cytosolic protein (based upon expected activity) with the reaction initiated by the addition of dihydroxyacetone phosphate in a final reaction volume of 1.0 ml. The change in absorbance at 340 nm was followed with a Gilford model no. 252 recording spectrophotometer (Gilford Laboratories, Inc., Oberlin, OH) at 25 C for 5 minutes. Units of enzyme activity equal 1 nmole of NADH oxidized per minute and activity was standardized on a per milligram protein basis.

Malic enzyme (EC 1.1.1.40) activity was assayed according to the method of Yeh et al., (1970) which was a modification of the Ochoa method (1955). The standard reaction mixture contained 125 mM glycylglycine buffer (pH 7.4), 0.25 mM $NADP^+$, 25 mM $MnCL_2$, 1.1 mM malate and enzyme (1 to 100 ug protein) in a final volume of 1.0 ml. The reaction was conducted at 25 C for 5 minutes after initiation by the addition of malic acid. Units of enzyme activity are equivalent to nmoles of $NADP^+$ reduced per minute and standardized by protein content.

Digitonin solution containing 0.25 M sucrose, 17 mM MOPS (pH 7.4), 2.5 mM EDTA and digitonin at 0.8 mg per ml was used to release cytosolic proteins from the monolayer of mature adipocytes for fatty acid synthase activity determination. Plates were rinsed with Dulbecco's phosphate buffered saline (pH 7.4) and then treated with 1.5 ml of digitonin solution for 10 minutes at room temperature (Student et al., 1980). Typically, 4 to 6 35 mm plates were combined to yield sufficient activity throughout the differentiation process. The resulting cytosolic protein suspensions were made to 200 mM potassium phosphate by the addition of 1 volume of 0.4 M potassium phosphate to stabilize FAS activity and was then, clarified by centrifugation at 20,000 x g for 15 minutes at 4 C. Supernatants were immediately assayed for FAS activity in 1.0 ml of reaction mixture which contained 200 mM potassium phosphate buffer, pH 6.8, 1 mM 2-mercaptoethanol, 1 mM EDTA, 100 uM NADPH, 33 uM acetyl CoA, 100 uM malonyl CoA and 1 to 100 ug of cytosolic protein (Muesing and Porter, 1975). Reactions were started by the addition of malonyl CoA and the change in absorbance at 340 nm was followed for 10 minutes at 30 C on a Cary 2200 spectrophotometer (Varian, Sugarland, TX). Data are expressed in units per milligram protein in which units represent nmoles NADPH oxidized per minute.

Lipolysis Assay: Lipolysis assay was conducted to evaluate the lipolytic response of TA1 adipocytes. The assay medium consisted of basal medium (Gibco Laboratories, Grand Island, NY) supplemented with 3% bovine albumin and 0.56 mM ascorbic acid (Coutinho et al.,1989). Prior to the onset of the experiment assay medium was equilibrated at 37 C in 5% CO_2, 95% air for 4 hours. At commencement of the experiment cells were washed twice with 3 ml of basal medium followed by the addition of the assay medium plus the effectors. Incubations were performed at 37 C in 5% CO_2, 95% air, for 2 hours, over which time release of glycerol was linear. After the incubation period, assay medium was removed and stored at -20 C for later analysis of glycerol content. After removal of assay medium cells were trypsinized and counted using a hemocytometer.

Glycerol concentration in the assay medium was determined enzymatically by utilizing the Glycerol Enzymatic Food Analysis Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). To maximize the number of samples per assay kit, the solutions in the kit were diluted 1:3 with water. Sample volume of 0.3 ml was used in the reaction mixture. Glycerol concentrations are expressed as nmoles of glycerol released per hour per 10^6 cells.

Compounds: Dexamethasone, indomethacin and isoproterenol were obtained from Sigma Chemical Co. (St. Louis, MO). Ractopamine was kindly donated by Lilly Research Laboratories (Eli Lilly and Company, Greenfield, IN).

Statistical Design: For the induction experiment, means for fatty acid synthase were analyzed using a two-way analysis of variance and comparisons were conducted using all-wise comparison (Gill, 1978). Time course experiments were designed as a randomized complete block design and means were analyzed using one-way analysis of variance (Gill, 1978). Responsiveness data were analyzed using one-way analysis of variance and comparisons were conducted using Bonferroni t statistic (Miller, 1966; Gill, 1978).

Results

Comparison of drug induced adipogenesis

To establish a rapid induction system for the conversion of TA1 cells a series of studies were conducted to compare dexamethasone and indomethacin and their combination as potential adipogenic inducers. As previously described by Chapman et al. (1984, 1985) and Knight et al. (1987) treatment of confluent TA1 cells with dexamethasone or indomethacin accelerates the morphological adipose conversion. In a preliminary study GPD activities

in spontaneously differentiated cells and DEX-, INDO- and DEX/INDO-treated cells were measured. In spontaneously differentiated cells relatively low levels of GPD activity were observed 6 days post confluence (Table 1) and lipid droplets were not apparent in these cells (Figure 1A), indicating little conversion had occurred at this time. DEX approximately doubled GPD activity while INDO treatment caused a 12-fold rise in activity over the spontaneously differentiated cells. Figure 1 (B,C) show DEX- and INDO-treated cells prior to harvesting. Only isolated lipid containing cells were observed in the DEX-treated cells while fat droplets were apparent in the INDO-triggered cells. DEX/INDO combination resulted in the highest GPD activity which was 26-fold higher in the spontaneously differentiated cells. Treatment of cells with DEX/INDO produced a uniformly differentiated cultures in which at least 90% of the cells contained visible lipid droplets (Figure 1D).

Since treatment of the adipogenic cell line, TA1, with dexamethasone and/or indomethacin increased GPD activity as compared to the untreated controls, a further study was conducted to compare the ability of these agents to accelerate differentiation. Confluent cultures of TA1 preadipocytes were treated with DEX, INDO and DEX/INDO for 48 hours following confluence (day 0) and were subsequently harvested for FAS activity determination on day 6 and day

TABLE 1: Comparison of Dexamethasone, Indomethacin and Dexamethasone-Indomethacin Combination as Accelerators of Adipogenesis in TA1 cells

Tretmeant	Glycerol-3-phosphate Dehydrogenase (% of maximum of response)
CONTROL	3.6
DEX	7.0
INDO	49.0
DEX/INDO	100.0

At confluence TA1 cells were treated with 1 uM dexamethasone, 125 uM indomethacin or both for 48 hours. Cells were harvested 6 days after confluence for GPD activity determination. Values represent one observation. 100.0% equals 101.8 units per mg protein.

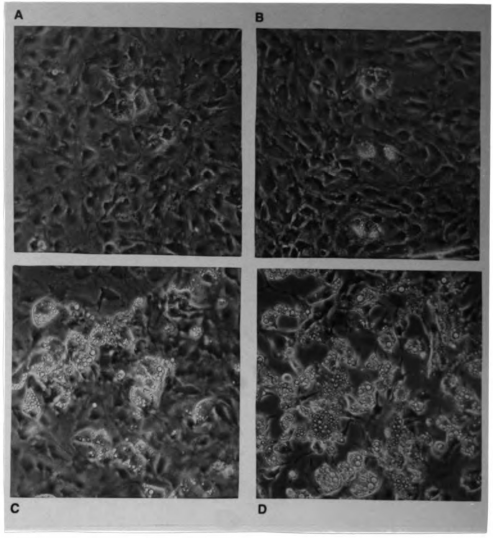

Figure 1. Induction of Adipogenesis by Dexamethasone and Indomethacin in TA1 Cells. Confluent TA1 cells were exposed to A) vehicle, B) 1 uM Dexamethasone, C) 125 uM Indomethacin or D) Dexamethasone and Indomethacin combination for 48 hours. Pictures were taken on Day 6.

12. The effect of these compounds on FAS activity are shown in Figure 2. A significant treatment by day interaction was observed in this study, therefore, comparisons were confined within day. On day 6, INDO- and DEX/INDO-treated cells had higher ($P<0.05$) FAS activity than the control and DEX-treated cells. Activity of the DEX/INDO group also was greater ($P<0.05$) than the INDO group. These results indicate that DEX is a poor inducer of differentiation while INDO is an effective inducer of differentiation. Furthermore it appears that the combination (DEX/INDO) is a more effective accelerator of adipogenesis than either DEX or INDO alone. The combined FAS activities of the DEX-and INDO-groups cannot account for the activity observed in the DEX/INDO group; thus indicating that the more pronounced conversion observed with DEX/INDO is a result of a synergistic effect between dexamethasone and indomethacin. On day 12, FAS activity in INDO- and DEX/INDO-treated cells was still elevated ($P<0.05$) over the spontaneously differentiated cells. DEX/INDO treated cells possessed greater ($P<0.05$) FAS activity than either the DEX or INDO groups. The FAS activity of the INDO group, however, was not different ($P>0.05$) from the DEX group, unlike on day 6. Also, the FAS activity observed in the dual-treated cells could be accounted for by the individual treatments. These results indicate that DEX, INDO and DEX/INDO induce differentiation of TA1 cell at different rates (DEX < INDO < DEX/INDO) but

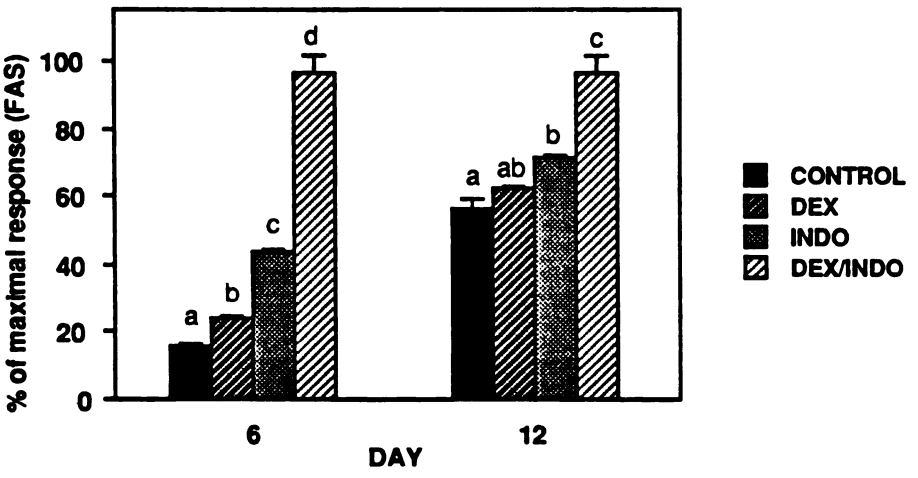

Figure 2. Acceleration of Differentiation of TA1 cells. TA1 cells were grown to confluence (Day 0) and treated with 1 uM dexamethasone (DEX), 125 uM indomethacin (INDO), both dexamethasone and indomethacin (DEX/INDO) or no treatment (control) for 48 hours. Cells were harvested on Day 6 and Day 12 for fatty acid synthease (FAS) activity determination. Values are mean +SE (n=2). Different letters indicate differences ($P<0.05$) within each day. 100% equals 19.3 units/mg protein.

suggest that the ultimate plateaus are similar.

Adipogenesis in Dexamethasone-Indomethacin-Induced TA1 cells

The kinetics of the increase in GPD, ME and FAS activity were determined in dexamethasone-indomethacin-induced TA1 cells. As shown in Figure 3 (A,B) GPD, ME, and FAS activity increased gradually between day 1 and day 4, approaching a plateau at day 4. Stable activity for the three enzymes persisted through the remainder of the time points except ME activity decreased slightly between day 8 and 10. Cytoplasmic triacylglycerol droplets were apparent in the majority of the cells by day 3, however, the droplets were relatively small and occupied only a small fraction of the cytoplasm (Figure 3C). By day 6 more than 90% of the cells exhibited lipid accumulation and the lipid droplets occuppied the majority of the cytoplasmic space (Figure 3D). These data are consistent with a cause and effect relationship between increased enzyme activity and triacylglycerol formation since activity of key enzymes involved in fatty acid and triacylglycerol synthesis preceded the appearence of lipid droplets. The maximal inductions for GPD, ME, and FAS were 84-, 6.5- and 4.0-fold respectively, indicating that a major reorganization of celluar metabolism occurred during differentiation.

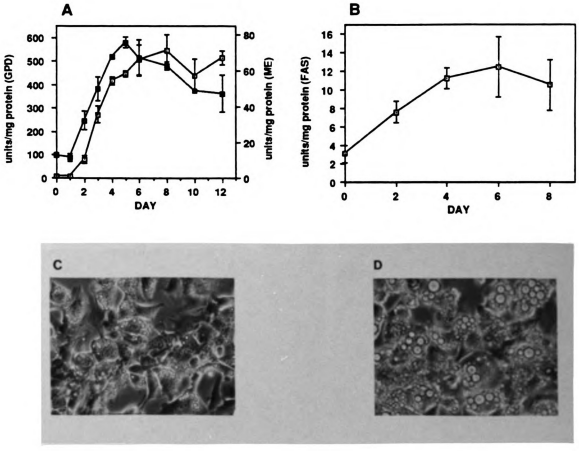

Figure 3. Changes in Glycerol-3-phosphate Dehydrogenase Activity, Malic Enzyme Activity and Fatty Acid Synthase Activity in TA1 Cells During Adipogenesis. Differentiation was induced in TA1 cells by exposure to 1 uM dexamethasone and 125 uM indomethacin for 48 hours. Cells were harvested at indicated times for enzyme activity determination. A) Glycerol-3-phosphate dehydrogenase activity (open squares) and malic enzyme activity (closed diamonds) (n=2). B) Fatty acid synthase activity (open squares) (n=4). C) TA1 cells on day 3; small lipid droplets are apparent in the majority of the cells. D) TA1 cells on day 3: large lipid droplets are apparent in the majority of the cells.

Furthermore, these data illustrate that a stable adipocyte population is established by day 4; therefore, experiments addressing regulation of lipid metabolism may be conducted in TA1 cells 4 days after dexamethasone-indomethacin treatment.

Responsiveness of Adipocytes to Adrenergic Agonists

Isoproterenol interacts with both subtypes of the beta receptor (Mersmann et al., 1974, Mersmann, 1984ab) whereas the receptor preference of ractopamine has not been fully investigated but from limited data it appears to react with the beta subtypes (Hausman et al., 1989). These compounds have also been shown to stimulate lipolysis and inhibit fatty acid synthesis in isolated adipocytes (Hausman et al., 1989; Countinho et al., 1989). The responsiveness of TA1 adipocytes to beta- adrenergic challenges has not been previously investigated, therefore, the lipolytic and antilipogenic actions of isoproterenol and ractopamine on TA1 adipocytes were investigated. Figure 4 (A,B) illustrates the experimental results. The minimum concentration of isoproterenol and ractopamine required to inhibit fatty acid biosynthesis, as indicated by FAS activity was 10^{-7} M ($P<0.05$) (Figure 4A). No additional inhibition of FAS activity was observed with

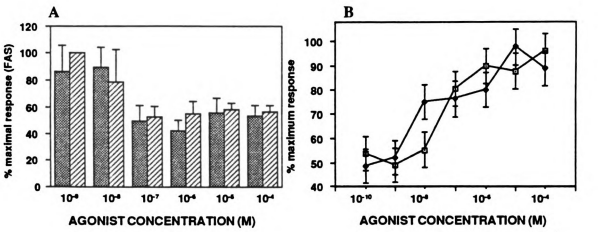

Figure 4. Effect of Isoproterenol and Ractopamine on Fatty Acid Synthase Activity and Lipolytic Activity in TA1 Adipocytes. A) Fatty acid synthase activity in isoproterenol (stippled bars) and ractopamine (diagonial lined bars) treated TA1 adipocytes. 100% equals 8.2 units/mg protein. B) Lipoylsis activity in isoproterenol (open squares) and ractopamine (closed diamonds) treated TA1 adipocytes. Values represent mean + SE from 2 independent studies (n=4). 100% equals 440.0 nmols glycerol/10^6 cells/hr. Basal rate equals 239.6 nmols glycerol/10^6 cells/hr.

higher concentrations of either beta adrenergic agonist, indicating maximal inhibition of FAS was achieved at 10^{-7} M.

Triacylglycerol mobilization, as measured by glycerol release, was enhanced by both isoproterenol and ractopamine in a dose response manner (Figure 4B). Basal lipolytic activity of TA1 cells was observed at 50% of maximal response. Isoproterenol stimulated ($P<0.05$) triacylglycerol mobilization at 10^{-7} M while ractopamine induced ($P<0.05$) mobilization at a concentration 10-fold lower (10^{-8} M). These results indicate that ractopamine is more effective than isoproterenol in stimulating triacylglycerol mobilization in TA1 adipocytes. Concentrations above 10^{-7} M and 10^{-8} M of isoproterenol and ractopamine, respectively, also stimulated ($P<0.05$) glycerol release as compared to the basal level, however, extent of stimulation was similar between the compounds at similar concentration. Therefore, the maximal stimulation of lipolysis by these compounds is similar.

The dose-response curves for the stimulation of lipolysis and inhibition of lipogenesis, as measured by FAS activity, by isoproterenol are comparable. At concentrations of 10^{-7} M and above isoproterenol stimulated triacylglycerol mobilization and inhibited FAS activity. These results indicate that isoproterenol is equipotent and equally effective in stimulating lipolysis and inhibiting

FAS activity in TA1 adipocytes. Ractopamine, however, appears more effective in stimulating lipolysis than inhibiting lipogenesis since a 10-fold higher concentration of ractopamine was required to inhibit ($P<0.05$) FAS activity than to stimulate lipolysis.

Discussion

Chapman and coworkers (1984) described the isolation and characterization of the stable adipogenic cell line, TA1. At subconfluent densities these cells resemble their fibroblastic counterparts, 10T1/2 cells, however, upon reaching a growth arrested state, these cells loose their fibroblastic nature and begin to display an adipocyte phenotype apparent by lipid accumulation. Although the conversion to mature adipocytes can occur spontaneously in TA1 cells, differentiation can be accelerated by treatment of confluent cultures with either dexamethasone or indomethacin with indomethacin acting as a much more potent initiator (Chapman et al., 1984, 1985; Knight et al., 1987). In the present investigation adipogensis was accelerated by treatment of confluent TA1 cells with dexamethasone and indomethacin; however, a more pronounced acceleration was observed when the two drugs were combined. It appears that dexamethasone and indomethacin act in a synergistic fashion when combined, since the sum of

activities expressed in cells supplemented with either inducer alone could not account for the total activity observed in the dual treated cultures. Similarly, Rubin et al., (1978) reported that the mixture of dexamethasone and the cyclic nucleotide phosphodiesterase inhibitor, methylisobutylxanthine, proved to be highly effective in promoting differentiation of confluent 3T3-L1 cultures. More specifically, dexamethasone or methylisobutylxanthine alone triggered approximately 20 to 40% of the cells to differentiate while in combination these drugs resulted in an 80 to 90% conversion rate. Therefore, adipogenic cell lines can be differentiated more rapidly and more completely when specific agents are used in combination.

Dexamethasone and indomethacin are recognized as potent inhibitors of prostaglandin synthesis (Hong and Levine, 1976ab; Miyamoto et al., 1976). Moreover, it has been proposed that the inhibition of prostaglandin synthesis may play a role in triggering the activation of the differentiation program (Russell and Ho, 1976; Williams and Polakis, 1977; Chen and London, 1981). In TA1 cells, indomethacin clearly inhibits prostaglandin production, however, it does so at concentrations far lower than those required to stimulate cellular differentiation (Knight et al., 1987), thus, implying that prostaglandin levels are not critical or responsible for adipocyte differentiation. Knight et al. (1987) proposed that an activating substance is responsible for initiating conversion. Once cells

reach confluence, the substance begins to accumulate and upon reaching a critical level it promotes the cells to differentiate. Inducers of differentiation (i.e. dexamethasone and indomethacin) probably alter this system by either reducing the threshold or level of this substance required for conversion or by increasing the synthesis rate of the substance (Knight et al., 1987). The acceleration of adipogenesis observed in the present investigation can be explained by this hypothetical activating substance since the rate of induction between each treatment was different but the final level of differentiation was comparable. Unfortunately, until the activating substance has been identified this entire concept will remain pure conjecture.

The sequential changes in the activity of three enzymes involved in lipid synthesis (GPD, ME, FAS) were determined to follow the maturation of TA1 cells differentiated by addition of the combination of dexamethasone and indomethacin. The present investigation showed that addition of dexamethasone-indomethacin results in a coordinated induction of fatty acid synthase, malic enzyme and glycerol-3-phosphate dehydrogenase activity. The overall kinetics in the increase of these three enzymes were similar. Basically, enzyme activity began to increase on day 2 and approached a plateau on day 4. Differentiation, therefore, was complete within 5 days of

confluency and the cells continued to exhibit a stable adipocyte phenotype for approximately 5 more days. The maximal FAS activity reported under these experimental conditions was similar to the activity reported in spontaneously differentiated 3T3-L1 cells (Mackall et al., 1977), 3T3-L1 cells differentiated by methylisobutylxanthine, dexamethasone and insulin (Student et al., 1980) and by methylisobutylxanthine, and dexamethasone (Weiss et al., 1980). Similarly, ME activity was comparable to that recorded by Kuri-Harcuch and Green (1977). Chapman et al. (1984) stated that GPD activity increased more than 100-fold during differentiation of the adipogenic cell line, TA1. In these experiments only an 84-fold increase GPD activity was apparent in cells triggered by dexamethasone-indomethacin, however, the maximum activity obtained was similar to that reported by Chapman et al. (1984). Collectively these reports suggest that both TA1 cells and 3T3 subclones have similar capacities to differentiate into adipocytes.

The differentiation curve also indicated a two phase development process in TA1 cells; an initial exponential phase and a second steady state phase. The first phase indicates recruitment and maturation of the cells while the second phase represents a stable adipocyte population. Experiments conducted during phase 1 would best address regulation of differentiation while experiments conducted in phase 2 would address regulation of lipid metabolism,

Since the overall objective is to utilize this cell line as an in vitro model to study the regulation of lipid metabolism it is imperative to have fully operational adipocytes and, therefore, all experiments must be conducted during phase 2 of development.

In the present study the response of TA1 adipocytes to beta-adrenergic agonists was investigated. Isoproterenol and ractopamine enhanced triacylglycerol mobilization and inhibited fatty acid synthase activity in a dose dependent manner. At low concentrations of the agonists little to no change in either of these parameters occurred, however, as concentrations were increased lipolytic and antilipogenic response became apparent which eventually plateaued. Isoproterenol appears to be equipotent in stimulating lipolysis and inhibiting lipogenesis in TA1 cells while ractopamine appears to be a more effective stimulator of lipolysis than inhibitor of lipogenesis. Ractopamine at 10^{-8} M concentration stimulated lipolysis but was ineffective in inhibiting fatty acid synthase, an indicator of lipogenesis. Ractopamine also appears to be slightly a more effective lipolytic agent than isoproterenol since a lower concentration of ractopamine was required to stimulate lipid mobilization. The maximal lipolytic response of both beta-adrenergic agonists, however, were comparable, indicating that the overall potency of the two agents is similar in TA1 adipocytes. In isolated rat

adipocytes, isoproterenol and ractopamine have been shown to be equally effective as well as equipotent in stimulating lipolysis and inhibiting fatty acid synthesis (Hausman et al.,1989). In isolated porcine adipocytes, however, isoproterenol appears to be more effective and more potent effector than ractopamine in stimulating lipolysis (Coutinho et al., 1989). These studies suggest that isoproterenol and ractopamine stimulate lipolysis and inhibit lipogenesis in adipocytes but the agents overall effectiveness and potency appear to be species specific. It may be inferred that these differences between species may be a result of different receptor number and/or receptor subclass present on the adipocyte.

In summary treatment of confluent TA1 cells with dexamethasone and indomethacin in combination leads to a rapid lipid accumulation and a concomitant increase in enzymes involved in lipid synthesis in TA1 cells. The present studies also illustrate that TA1 cells under these experimental conditions become a stable adipocyte population within 4 days and indicate that this in vitro system is a valuable tool to study the regulation of lipid metabolism by beta-adrenergic agonists.

INFLUENCE OF THE PHENETHANOLAMINE, RACTOPAMINE, ON THE ADIPOGENIC CELL LINE, TA1

Introduction

The phenethanolamine, ractopamine, (Figure 5), when included in the diet of finishing swine at 20 ppm, reduces total fat deposition (Anderson et al., 1987ab, 1988; Hancock et al., 1987; Prince et al., 1987). The reduction in fat accretion appears to be a result of enhanced lipid mobilization and depressed fatty acid biosynthesis (Merkel et al., 1987). Also the lowered lipogenic capacity observed in adipose tissue isolated from ractopamine fed pigs is caused by the reduction in the activity of fatty acid synthase and malic enzyme, key lipogenic enzymes (Merkel et al., 1987). In isolated rat adipocytes, the lipolytic and antilipogenic action of ractopamine is mediated, in part, through the beta receptor (Hausman et al., 1989) thus, implicating a direct mode of action on adipose tissue by ractopamine as well as the involvement of the adenylate cyclase-cyclic AMP system in the regulation of fat deposition by ractopamine.

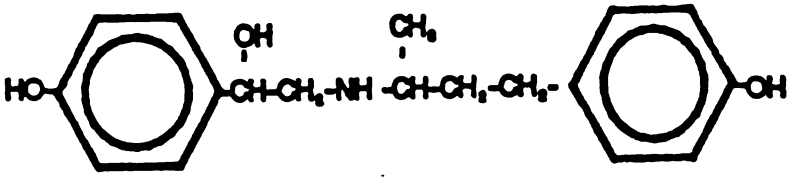

Figure 5. Structure of the Phenethanolamine, Ractopamine.

The classical theory of cyclic AMP on lipid metabolism implies both an immediate and chronic form of regulation. Initially, cyclic AMP reduces fatty acid biogenesis by inactivating acetyl-CoA carboxylase and stimulates triacylglycerol mobilization by activating triacylglycerol lipase through covalent modification (Steinberg, 1972, 1976; Fredrikson et al., 1981; Fain and Garcia-Sainz, 1983; Kim et al., 1989). Chronic elevation of cyclic AMP depresses the synthesis of enzymes involved in lipid metabolism, such as acetyl-CoA carboxylase, fatty acid synthase, malic enzyme and glycerol-3-phosphate dehydrogenase (Lakshmanan et al., 1972; Goodridge et al., 1973; Goodridge and Adelman, 1976; Volpe and Vagelos, 1976; Spiegelman and Green, 1981; Paulauskis and Sul, 1988) Lowered enzyme synthesis results in lowered enzyme content which translates to a lower capacity to synthesize lipids. Therefore, long-term reduction in enzyme activity observed during continual cyclic AMP production is a result of depressed enzyme content rather than a depressed catalytic efficiency. The depressed synthesis of fatty acid synthase, malic enzyme and glycerol-3-phosphate dehydrogenase has been shown to be associated with a reduced abundance of mRNA for each enzyme (Dobson et al., 1987; Paulauskis and Sul, 1988). This indicates that the long-term regulation of fatty acid and triacylglycerol synthesis is mediated at the pretranslational level. Since the actions of ractopamine

on lipid metabolism appear to be mediated through cyclic AMP, it seems likely that the down regulation of lipogenic enzymes observed in adipose tissue from pigs fed ractopamine (Merkel et al., 1987) may also be a result of a pretranslational regulation. Unfortunately, these effects of ractopamine on lipid metabolism have not been fully investigated.

The adipogenic cell line, TA1 (Chapman et al., 1984) rapidly and uniformly differentiates into adipocytes when cultured in medium containing both dexamethasone and indomethacin. This conversion is characterized by the induction of enzymes involved in fatty acid and triacylglycerol synthesis (Chapman et al., 1984, previous chapter). TA1 adipocytes are responsive to beta-adrenergic agonists (previous chapter) which indicates that when fully differentiated TA1 cell are a suitable culture model to study the effect of beta-adrenergic agonists on lipid metabolism. Therefore, a comprehensive study, employing TA1 adipocytes was conducted to investigate the direct influence of ractopamine on lipid metabolism at a cellular and molecular level.

Experimental Protocols and Procedures

Dose-Response Experiment: In order to determine the concentration of ractopamine required to maximally inhibit fatty acid biosynthesis and triacylglycerol synthesis, TA1 adipocyte were used in a dose-response experiment. Fully differentiated TA1 adipocytes were treated with 10^{-8} to 10^{-4} M ractopamine (Eli Lilly and Co., Greenfield, IN) for 3 consecutive days. Twenty-four hours following the final exposure to ractopamine, cells were harvested and analyzed for glycerol-3-phosphate dehydrogenase (GPD) and malic enzyme (ME) activity.

Mean values reported for GPD activity represent the average of duplicate samples from three independent experiments while ME means were compiled from four experiments. Studies were designed as a randomized complete block design with each experiment representing a block and analyzed by two-way analysis of variance. Mean comparisons were made between each treatment concentration and the control using Dunnett's t test (Dunnett, 1955, 1964; Gill, 1978).

Time Course Experiment: The optimum concentration of ractopamine determined by the dose-response experiments was utilized to investigate the time-dependent changes in the activity of GPD, ME, and FAS. Fully differentiated TA1 cells were treated with 0 or 10^{-6} M ractopamine for 24

hours. In the initial experiments, cell were harvested at 0, 0.25, 0.5, 2, 4, 8, 12, and 24 hours after ractopamine treatment, for the determination of GPD and ME activity while in the later studies examing FAS activity, cells were harvested at 0, 0.5, 4 and 24 hours post treatment. Means reported for GPD and ME were compiled from three independent studies, while FAS means were established from two separate experiments.

Mean values from these studies were blocked by experiment and analyzed as a three-way analysis of variance (Gill, 1978) with the factors being experiment, treatment, and time. For each time point mean comparisons between control and ractopamine treatment were conducted utilizing the Bonferroni t statistic (Miller, 1966; Gill, 1978).

Antagonist Experiment: To establish that the effect of ractopamine on the fatty acid synthase was mediated through the beta receptor, a series of experiments utilizing propranolol, a known beta antagonist (Pessin et al.,1983; Lager et al., 1986) were conducted. In the initial study the optimum concentration of propranolol required to reverse the inhibitory action of ractopamine (10^{-6} M) on FAS activity was investigated. In a factorial design experiment TA1 cells were treated simultaneously with ractopamine (10^{-6} M) and 0, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M propranolol (Sigma Chemical Co., St. Louis, MO) for 24 hours. Concentrations above 10^{-5} M propranolol were toxic

to TA1 cells. Following the treatment period, cells were harvested for determination of FAS activity. The resulting data were analyzed as a two-factor analysis of variance (Gill, 1978) and specific mean comparisons were conducted between control and ractopamine at each concentration of propranolol using Bonferroni t statistic (Miller, 1966; Gill, 1978).

Next a series of four 2 x 2 factorial experiments were conducted to determine whether the action of ractopamine was completely mediated via the beta-adrenergic receptor. Adipocytes were treated simultaneously with either 0 or 10^{-6} M ractopamine and 0 or 10^{-5} M propranolol for 24 hours prior to harvesting for the determination of FAS activity. Bonferroni t statistic (Miller, 1966; Gill, 1978) was used to compare the means within and across treatments.

Cyclic AMP Experiments: A series of independent studies were designed to compare the effect of ractopamine and cyclic AMP on FAS activity. In the initial studies N^6-2'O-dibutyryl adenosine 3',5'cylic monophosphate (dibutyryl cyclic AMP) (Sigma Chemical Co., St. Louis, MO) was used while in the latter studies a more potent cyclic AMP analogue, 8-(4-chlorophenylthio)-adenosine 3',5'cyclic monophosphate (8CPT-cyclic AMP) (Sigma Chemical Co., St. Louis, MO) (Braumann et al., 1986) was used. In the

dibutyryl cyclic AMP studies fully differentiated TA1 cells were treated with 10^{-6} M ractopamine and 0.5 mM dibutyryl cyclic AMP for 24 hours. Following the treatment period, cells were harvested and analyzed for FAS activity. Treatment effects were analyzed as a two-way analysis of variance and comparisons between treatments means were made by utilizing the Bonferroni t statistic (Miller, 1966; Gill, 1978).

In the 8CPT-cyclic AMP study TA1 adipocytes were treated with ractopamine (10^{-6} M) and 8CPT-cyclic AMP (0.05 and 0.5 mM) for 24 hours. Cell were harvested and extracts prepared for FAS activity determination. Treatment effects were analyzed as a one-way analysis of variance and comparisons between treatment means were made utilizing the Bonferroni t statistic (Miller, 1966; Gill, 1978).

mRNA Abundance Experiment: To determine whether the effect of ractopamine was regulated at the pretranslational level the mRNA abundance of acetyl CoA carboxylase (ACC), FAS, ME, GPD and B-actin were measured by dot blot analysis. Two studies were conducted to investigate the effect of ractopamine after 24 hour exposure. Cells were treated with ractopamine (10^{-6} M) and harvested at 24 hours for total RNA isolation. An addition study was conducted to investigate the time related change in the mRNA abundance for ACC, FAS, ME, GPD and B-actin. Cells were treated with ractopamine (10^{-6} M) and harvested at 0, 4, 8 and 24 hours

for total RNA isolation. Following RNA extractions, mRNA abundance for each enzyme was determined by blotting samples and hybridizing the filters with the respective probe. Prior to use of any probe northern blots were conducted to confirm that the probes bind to the appropriate mRNA band(s). Each probe was found to bind to their respective band(s) with minimal background.

Dot blots were scanned by TLC scanner for determination of the relative amounts of mRNA present. Means from the 24 hour study were analyzed by two-way analysis of variance while the time course experiment was analyzed by one-way analysis of variance (Gill, 1978). Means comparisons were made between control and treated samples within time using Bonferroni t statistic (Miller, 1966; Gill, 1978).

Cell Culture Conditions: Culture dishes (35 mm) were inoculated with 50,000 TA1 cell (Dr. Gordon M. Ringold, Institute of Biological Science, Syntex Research Laboratories, Palo Alto, CA) and cells were grown to confluence in 2.0 ml of Dulbecco's modified Eagles medium (Gibco Laboratories, Grand Island, NY) containing 10% heat inactivated fetal bovine serum (Gibco Laboratories, Grand Island, NY) and 10,000 units of gentemicin (Gibco Laboratories, Grand Island, NY) in a humidified atmosphere of 5% CO_2, 95 % air at 37 C. To accelerate differentiation

confluent cells (day 0) were treated with 1 uM dexamethasone (Sigma Chemical Co., St Louis, MO) and 125 uM indomethacin (Sigma Chemical Co., St. Louis, MO) in combination for 48 hours. After the 48 hours, medium was replaced with inducer-free medium and cells were allowed to maturate until commencement of the experiment on day 4. Unless otherwise indicated medium was replaced every 3 days throughout all studies.

Enzyme Assays: For the determination of GPD and ME, cells were rinsed with 2.0 ml of Dulbecco's phosphate buffered saline (pH 7.4) and then harvested in 0.15 M KCl containing 5.0 mM 2-mercaptoethanol (Allee et al., 1971). Cells were homogenized with two 30-second pulses at setting 4 on the Polytron (Brinkmann, Westbury, NY). Cell lysates were clarified by centrifugation at 20,000 x g for 15 minutes at 4 C. The resulting supernatants were immediately analyzed for GPD and ME activity and aliquots of the supernatants were frozen at -20 C for later determination of protein content (Lowry et al., 1951). Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) activity was determined as outlined by Kozak and Jensen (1974) with the modifications specified by Wise and Green (1979). The assay system consisted of 100 mM triethanolamine/HCl buffer (pH 7.5), 2.5 mM EDTA, 0.12 mM NADH, 0.2 mM dihydroxyacetone phosphate, 0.1 mM 2-mercaptoethanol and 1 to 100 ug of cytosolic protein (based upon expected activity) in a final

volume equaling 1.0 ml. Reaction was initiated by the addition of dihydroxyacetone phosphate. The change in absorbance was followed with a Gilford model no. 252 recording spectrophotometer (Gilford Laboratories Inc., Oberlin, OH) at 25 C for 5 minutes. Units of enzyme activity equal 1 nmole of NADH oxidized per minute. Enzyme activity was standardized on a per milligram protein basis.

Malic enzyme (EC 1.1.1.40) was assayed according to the method of Yeh et al. (1970) which is a modification of the method by Ochoa (1955). The standard reaction mixture consisted of 125 mM glycylglycine buffer (pH 7.4), 0.25 mM $NADP^+$, 25 mM $MnCl_2$, 1.1 mM malic acid and cellular extract (1 to 100 ug protein) in a final volume of 1.0 ml. Enzymatic activity was measured at 25 C for 5 minutes after initiation by the addition of malic acid. Units of enzyme activity are equivalent to nmoles of NAD^+ reduced per minute. Enzymatic activity was standardized on a per milligram protein basis.

Digitonin solution containg 0.25 M sucrose, 17 mM MOPS (pH 7.4), 2.5 mM EDTA and digitonin at 0.8 mg per ml was used to release cytosolic proteins from the monolayer of mature adipocytes for FAS activity determinations. Plates were rinsed with Dulbecco's phosphate buffered saline (pH 7.4) and then treated with 1.0 ml of digitonin solution for 10 minutes at room temperature (Student et al., 1980). Typically, three 35 mm plates were combined in

order to yield sufficient assayable activity. The resulting digitonin suspensions were brought to 200 mM potassium phosphate to stablize the FAS enzyme by the addition of an equal volume of 0.4 M potassium phosphate. Cellular extracts were centrifuged at 20,000 x g for 15 minutes at 4 C to remove any cellular debris and insoluble digitonin. Supernatants were immediately analyzed for FAS activity in 1.0 ml of the following reaction mixture: 200 mM potassium phosphate buffer, pH 6.8, 1 mM 2-mercaptoethanol, 1mM EDTA, 100 uM NADPH, 33 uM acetyl CoA, 100 uM malonyl CoA and 1 to 100 ug of cytosolic protein (Muesing and Porter, 1975). The change in absorbance was monitored on a Cary 2200 spectrophotometer (Varian, Sugarland, TX) for 10 minutes at 30 C. Data are expressed in units per milligram protein in which units are equal to nmoles of NADPH oxidized per minute.

RNA Isolation: Total cellular RNA was prepared by the method of Chirgwin et al. (1979). Cells were suspended in 4 M guanidium thiocynate and homogenized using a Polytron (Brinkmann, Westbury, NY). The resulting cell lysate was layered on a 5.7 M CsCl cushion and centrifuged at 80,000 x g for 20 hour. The RNA pellet was re-extracted in 7 M guanidine hydrochloride, 20 mM Na acetate, 1 mM dithiothreitol, 10 mM iodoacetate, 1.0 mM Na_2EDTA, pH 7.0, and precipitated with ethanol (Jump et al., 1984). RNA was washed in 3 M Na acetate, 10 mM iodoacetate, pH 5.0 (4 C),

then in 3 mM Na acetate, pH 5.0 containing 66% ethanol and finally with absolute ethanol (Jump et al., 1984). RNAs were resuspended in 10 mM Tris, pH 7.6, 0.1 mM EDTA, quantitated (A_{260}) and stored at -80 C for later uses.

Analysis of RNA: One to 10 ug of total RNA isolated from untreated control and ractopamine treated TA1 adipocytes were denatured with 2.2 M formaldehyde in 50% formamide, 10mM NaH_2PO_4 (pH 7) at 65 C for 15 minutes (Maniatis et al., 1982). Samples were blotted onto a damp nitrocellulose paper (Schleicher and Shuell, BA85) equilibrated with 6X standard saline citrate solution (SSC) (1X SSC: 0.15 M NAcl, 0.015 M Na citrate, pH 7.0) (White and Bancroft, 1982). After blotting the nitrocellulose paper was dried and baked at 80 C for 2 hours under vacuum.

Hybridization conditions: Dot blots were prehybridized for at least 2 hours at 42 C in 50% formamide, 5X SSC, 4X Denhardt's solution (1X Denhardt's solution: 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone (Denhardt, 1966)), 0.05 M $NaPO_4$, 0.01% sodium dodecyl sulfate (SDS), 2 uM EDTA and 0.1 mg per ml heat denatured yeast tRNA (Maniatis et al., 1982). Following prehybridization, prehybridization solution was replaced with the hybridization (50% formamide, 5X SSC, 1X

Denhardt's solution, 0.05 M $NaPO_4$, 0.01% SDS, 2 uM EDTA and .1 mg per ml heat denatured yeast tRNA) which contained 2 x 10^6 cpm per ml of the probe. Probes were labeled with ^{32}P using a random prime kit (Boeringer Mannheim Biochemicals, Indianapolis, IN). Hybridizations were conducted at 42 C for 24 hours for all probes except malic enzyme in which a 48 hour hybridization time was used. Following hybridization blots were washed in 2X SSC, 0.1% SDS at room temperture for 15 minutes followed by a a series of three washes with 0.1X SSC, 0.1% SDS at 65 C for 45 minutes (Jump et al., 1984). Blots were dried, wrapped in saran wrap and were allowed to exposed Kodak XAR-5 film at -80 C utilizing an intensifying screen. The resulting autoradiograms were quantitated by scanning with an integrating Shimadzu chromatography scanner (Shimadzu Corporation, Kyoto, Japan).

Probes: Acetyl-CoA carboxylase cDNA was kindly provided by Dr. Ki-Han Kim (Department of Biochemistry, Purdue University, West Lafayette, IN). Plasmid, p(181-16) contains a 506 bp insert subcloned at the EcoR1 site of pGEM3 (Promega, Madison, WI). The insert cDNA corresponds to the 3' untranslated region of acetyl-CoA carboxylase mRNA (Bai et al., 1986; Lopez-Casillas et al., 1988). Fatty acid synthase cDNA was provided by Dr Stuart Smith (Children's Hospital Oakland Research Institute, Oakland, CA). A 1200 bp insert was subcloned into the EcoR1 sites

of pUC19 forming the plasmid p(FAS1). The cDNA insert codes for the acyl carrier protein and its flanking domains (Witkowski et al., 1987). The malic enzyme cDNA probe was a kind gift from Dr. Vera M. Nikodem (Clinical Endocrinology Branch, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Bethesda, MD). The insert, approximately 1000 bp, was inserted into EcoR1 sites of pUC13. The insert codes for the entire coding region of malic enzyme mRNA (Magnuson et al., 1983, 1986). Glycerol-3-phosphate dehydrogenase cDNA was provided by Dr. Bruce Spiegelman (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA) The 900 bp EcoR1 fragment from pBR322 corresponds to the coding region of glycerol-3-phosphate dehydrogenase mRNA (Dobson et al., 1987). B-actin cDNA was provided by Dr. L. Kedes (Standford University, Palo Alto, CA). A cDNA insert, approximately 1800 kb, was cloned into the orginal Okayama-Berg vector and was excised by PvuII and Pst I digestion (Gunning, unpublished). The plasmid isolations were conducted by SDS-NaOH method described by Birnborn and Doly (1979).

Results

The effect of ractopamine on lipid synthesis in mature adipose cell cultures of TA1

Glycerol-3-phosphate dehydrogenase and malic enzyme were used to investigate the effect of varying concentrations of ractopamine on triacylglycerol synthesis and fatty acid synthesis in the adipogenic cell line TA1 and to establish the minimum concentration of ractopamine required to significantly depress GPD and ME. A dose-dependent response was observed for both enzymes. Figure 6 (A,B) shows the effect of increasing ractopamine concentrations on GPD and ME activity. Ractopamine at concentrations of 10^{-6} M and above, effectively inhibit GPD and ME activity. More specifically at 10^{-6} M ractopamine GPD activity was reduced ($P<0.05$) approximately 49% as compared to the control while concentrations above 10^{-6} M reduced ($P<0.05$) GPD activity by approximately 65% and 74% of control, respectively. Similarly, ME activity was reduced ($P<0.10$) at 10^{-6} M ractopamine by 32% while at 10^{-5} M and 10^{-4} M ractopamine depressed ($P<0.05$) ME activity by approximately 39% and 48%, respectively. Concentrations of ractopamine below 10^{-6} M ractopamine failed to significantly depress GPD or ME activity. Although at 10^{-7}

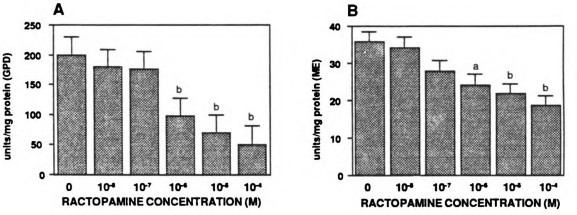

Figure 6: Effect of Ractopamine on Glycerol-3-phosphate Dehydrogenase Activity and Malic Enzyme Activity in TA1 adipocytes. TA1 adipocytes were exposed to varying concentrations of ractopamine for 72 hours, prior to harvesting for GPD and ME activity determination. A) Glycerol-3-phosphate dehydrogenase activity in TA1 adipocytes. B) Malic enzyme activity in TA1 adipocytes. Values are mean + SE of 3 studies for GPD (n=6) and 4 studies for ME (n=8). (a is different from control, $P<0.10$ and b is different from control, $P<0.05$).

M ractopamine, ME activity was slightly reduced (22%); this reduced activity did not prove to be different ($P>0.10$) from control. From these data it appears that the minimal concentration of ractopamine which reduces glycerol-3-phosphate dehydrogenase activity and malic enzyme activity in TA1 adipocytes is 10^{-6} M ractopamine, therefore, in subsequent studies 10^{-6} M ractopamine was utilized as the optimum inhibitory concentration of these enzymes.

In a previous study (previous chapter) the minimal concentration of ractopamine necessary to inhibit FAS activity was reported as 10^{-7} M, a 10-fold lower concentration than the concentration required to reduce GPD activity and ME activity in the present study. Two explanations can account for these difference. One, FAS activity may be more sensitive to ractopamine than GPD or ME activity. Two, there may be a down-regulation of responsiveness in TA1 adipocytes to ractopamine after 72 hour exposure which is not present at 24 hours. With prolong exposures to ractopamine, adipocytes may become refractory to the compound and thus, higher concentration of the beta-agonist may be needed to maintain maximal inhibition.

Figure 7 illustrates the change in cytosolic protein content at the varying concentrations of ractopamine. Protein content per plate was not altered by any ractopamine concentration from 10^{-4} to 10^{-8} M; this illustrates that the reduction in enzyme activity most

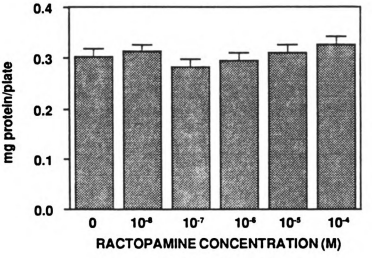

Figure 7. Effect of Ractopamine on Protein Content Per Plate in TA1 Adipocytes. TA1 adipocytes were treated with varying concentrations of ractopamine for 72 hours and then harvested for protein determination. Values are mean of 4 independent studies (n=8). Error bars represent SE.

likely is the result of altered metabolism rather than a general toxic effect. If the effect of ractopamine on TA1 adipocytes was toxic one would expect the protein content per plate to decline as cell death occurred.

Time-course evalution of the effects of ractopamine on lipid metabolism

In order to further evaluate the effect of ractopamine on TA1 cells, time-dependent changes in GPD and ME activity were investigated. Figure 8 (A,B) depicts the changes in GPD and ME activity during the 24 hour treatment period. GPD activity was reduced ($P<0.10$) by approximately 37% 15 minutes following ractopamine treatment and remained significantly depressed through 24 hours with the exception of the 2 hour sample. Similarly, ME activity was depressed ($P<0.05$) after 30 minutes. ME activity remained depressed ($P<0.10$) throughout the remainder of the experiment.

In two separate experiments the change in FAS activity with regard to time after treatment was monitored (Figure 9). Within 30 minutes of ractopamine supplementation, FAS activity was reduced ($P<0.05$) as compared to the untreated controls. FAS activity of ractopamine treatment group remained depressed ($P<0.05$) throughout the 24-hour sampling period. Twenty-four hours of treatment with ractopamine resulted in greater than 50% reduction in activity of FAS.

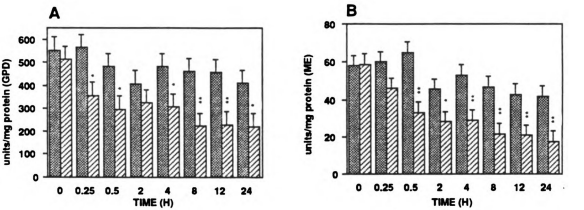

Figure 8.Changes in Glycerol-3-phosphate Dehydrogenase Activity and Malic Enzyme Activity with Ractopamine Treatment . TA1 adipocytes were treated with ractopamine (10^{-6} M) and harvested at the specified times for GPD and ME activity determination. A) Glycerol-3-phosphate dehydrogenase activity across time (untreated control, stippled bars; ractopamine treated, diagonial lined bars). B) Malic enzyme activity across time (untreated controls, stippled bars; ractopamine treated, diagonial lined bars). Values represent mean +SE (n=3). * indicates treatment mean is different ($P<0.10$) from the control and ** indicates treatment mean is different ($P<0.05$) from control.

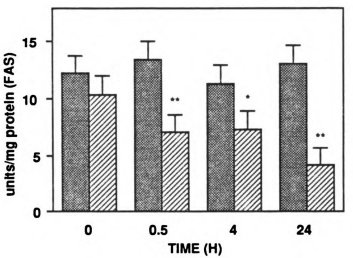

Figure 9. Changes in Fatty Acid Synthase Activity with Ractopamine Treatment in TA1 adipocytes. TA1 adipocyte were treated with ractopamine (10^{-6}) and harvested at the specified times for FAS activity determination. Control are represented in the stippled bars and ractopamine treatment are in the diagonial lined bars. Values are mean of 3 studies. (n=6). Error bars represent SE. * indicates ractopmine treatment is different ($P<0.10$) from control and ** indicates ractopamine treatment is different ($P<0.05$) from control.

In these experiments as observed with GPD and ME activity, FAS activity also was inhibited immediately and continually by ractopamine.

The effect of the beta adrenergic antagonist, propranolol on the inhibition of fatty acid synthase activity by ractopamine

To determine whether the effect of ractopamine was mediated through the beta receptor of TA1 adipocytes, a series of experiments utilizing the beta-adrenergic antagonist, propranolol, were designed. Initially a dose-response experiment was conducted to determine the optimium propranolol concentration. Concentrations from 10^{-8} to 10^{-6} M propranolol failed to significantly reduce the inhibitory actions of 10^{-6} M ractopamine on FAS activity (Figure 10A). However, propranolol (10^{-5} M) blocked the inhibitory action of ractopamine on FAS activity, since the cells treated with 10^{-5} M propranolol and 10^{-6} M ractopamine FAS activity was not different from the untreated control cells.

Following determination of the dose-response curve, four more experiments were conducted to evaluate the relationship between the beta agonist and beta antagonist. Under these experimental conditions, ractopamine (10^{-6} M) depressed ($P<0.05$) FAS activity in the absence of propranolol. With the addition of propranolol (10^{-5} M),

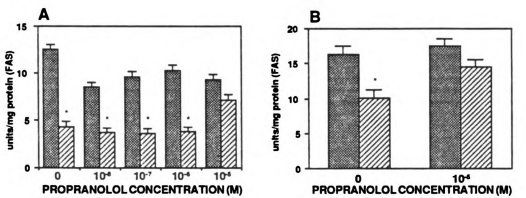

Figure 10. Blockage of Ractopamine's Inhibitory Effect on Fatty Acid Synthase Activity by the Antagonist, Propranolol. A) FAS activity was determined in TA1 adipocytes which had been treated with varying concentrations of propranolol and 0 (stippled bars) or 10^{-6} M ractopamine (diagonial lined bars) for 24 hours. Values represent mean + SE of a single study (n=2). B) TA1 adipocytes were treated with 10^{-5} M propranolol in combination with 0 (stippled bars) or 10^{-6} M ractopamine (diagonial lined bars) for 24 hours. Values represent mean + SE of 4 independent studies (n=8). * indicates ractopamine means were different from controls ($P<0.05$)

however, ractopamine was unable to significantly reduce FAS activity in TA1 cells (Figure 10B). These data indicate that ractopamine elicits its inhibitory effect on FAS via the beta receptor.

Cyclic AMP effect on fatty acid synthase activity in mature cultures of the adipogenic cell line TA1

Since the above results indicate that ractopamine mediates its inhibitory effect on FAS activity via the beta receptor it seems likely that cyclic AMP would have a similar inhibitory action on FAS activity as ractopamine. To test this hypothesis, two experiments were conducted to compare dibutyryl cyclic AMP and ractopamine inhibitory effect on FAS activity. Dibutyryl cyclic AMP has been shown to inhibit FAS activity at 0.5 mM (Weiss et al., 1980) and therefore, this concentration was used in the following experiments. FAS activity (Table 2) of the untreated control cells was 10.66 units/mg protein while in the dibutyryl cyclic AMP and ractopamine treatment groups, FAS activity was 23% and 34% lower, respectively, than the controls. FAS activity in both treated groups was depressed ($P<0.05$).

A further study using a more potent cyclic AMP analogue was conducted to evaluate lower levels of cyclic AMP and ractopamine. 8CPT-cyclic AMP at 0.05 and 0.5 mM

TABLE 2: Comparison of Ractopamine and Dibutyryl cAMP on Fatty Acid Synthase Activity in the Adipogenic cell line, TA1

Tretmeant	Fatty Acid Synthase Activity (units/mg protein)
Control	9.3
Ractopamine	5.8[a]
dibutyryl cAMP (0.5 mM)	6.9[a]
SEM	.5

On Day 4 TA1 adipocytes were treated with ractopamine (10E-6 M) and dibutyryl cyclic AMP (0.5 mM) for 24 hours. Adipocytes were harvested for FAS activity determination. [a] indicates means are different ($P<0.05$) from control. (n=4).

concentrations (Buchler et al., 1988) and ractopamine at 10^{-6} M were used to compare their inhibitory action on FAS activity (Table 3). Both concentrations of 8CPT-cyclic AMP lowered ($P<0.05$) FAS activity. Ractopamine also depressed ($P<0.05$) FAS activity similarly to the 8CPT-cyclic AMP treated cells. The combination treatment of 8CPT-cyclic AMP (0.5 mM) and ractopamine (10^{-6} M) reduced ($P<0.05$) FAS activity as compared to the untreated control, however, there was no additive effect of this combination as compared to either drug alone. These data, as well as the previous studies, clearly illustrate that ractopamine elicits similar antilipogenic actions as does cyclic AMP, providing supportive evidence that the inhibitory action of ractopamine is mediated through cyclic AMP.

Dibutyryl cyclic AMP at 0.5 mM reduced FAS activity by approximatel 30% while 8-CPT cyclic AMP reduced FAS activity by 58%. These results suggest that 8-CPT cyclic AMP is a more powerful inhibitor of FAS activity than dibutyryl cyclic AMP. Braumann et al. (1986) reported that 8-CPT cyclic AMP is superior to other cyclic AMP analogs because it is more lipophilic and more resistant to hydrolysis by phosphodiesterase. These properties, thus, are responsible for the superior performance of 8-CPT cyclic AMP as compared to dibutyryl cyclic AMP in the present study.

TABLE 3: Comparison of Ractopamine and 8CPT-cyclic AMP on Fatty Acid Synthase Activity in the Adipogenic cell line, TA1.

Tretmeant	Fatty Acid Synthase Activity (units/mg protein)
Control	12.0
Ractopamine (10^{-6} M)	8.2[a]
8CPT-cyclic AMP (0.05 mM)	7.6[a]
8CPT-cyclic AMP (0.5 mM)	5.1[a]
8CPT-cyclic AMP (0.5 mM)/Ractopamine	7.4[a]
SEM	.6

On Day 4 TA1 adipocytes were treated with ractopamine (10E-6 M), 8CPT-cyclic AMP (0.05 or 0.5 mM) and ractopamine (10E-6 M) in combination with 8CPT-cyclic AMP (0.5 M) for 24 hours. Adipocytes were harvested for FAS activity determination. [a] indicates means are different ($P<0.05$) from control. (n=2).

Effect of Ractopamine on mRNA Abundance in TA1 Adipocytes

Ractopamine inhibits the activity of fatty acid synthase and malic enzyme as well as glycerol-3-phosphate dehydrogenase, however, the mode of action by which ractopamine inhibits the activity of these enzymes has not been previously investigated. To determine whether the effect of long-term exposure to ractopamine is mediated through a pre- or a post-translational event, a series of studies were conducted to measure the relative abundance of mRNA for acetyl-CoA carboxylase, fatty acid synthase, malic enzyme, and glycerol-3-phosphate dehydrogenase. Initially the abundance of these messages was measured after TA1 adipocytes were exposed to ractopamine for 24 hours. Total RNA was isolated from the treated and control cells for analysis of the message of interest. Figure 11 illustrates the results of these studies. Acetyl-CoA carboxylase, FAS, ME and GPD mRNA abundance was reduced ($P<0.05$) by ractopamine treatment. More specifically, ACC was reduced by approximately 60% while FAS and GPD mRNA abundances were depressed by approximately 50% as compared to the untreated controls. ME mRNA abundance was also reduced by ractopamine treatment but to a much lesser extent than that of the other enzymes. ME mRNA abundance was reduced by approximately 30%.

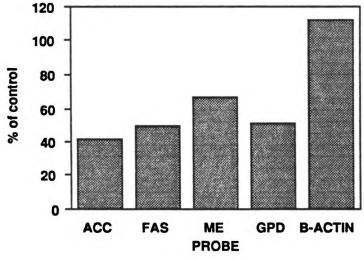

Figure 11. Effect of Ractopamine on the Relative Abundance of Specific mRNAs. TA1 adipcytes were treated with 10^{-6} M ractopamine for 24 hours. Cells were harvested and analyzed for relative abundance of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), malic enzyme (ME), glycerol-3-phosphate dehydrogenase (GPD) and B-actin mRNAs. Values are expressed as % of control and represent mean from 2 independent studies (n=4). Relative abundance of ACC, FAS, ME and GPD was reduced ($P<0.05$) as compared to controls. B-actin was not effected by ractopamine treatment.

B-actin mRNA abundance was also measured to determine whether the effect of ractopamine was specific to the lipid synthesizing enzymes or a generalized effect on all messages. B-actin mRNA levels were not different ($P>0.05$) from the levels observed in the untreated controls. These data suggest that the antilipid synthesis effect of ractopamine may be in part regulated at a pretranslational level.

A further study was conducted to investigate the kinetics of the change in these mRNAs over time. TA1 cells were harvested for RNA analysis at 0, 4, 8 and 24 hours following ractopamine treatment. Figure 12 shows the results from this study. Acetyl-CoA carboxylase and glycerol-3-phosphate mRNA (Figure 12A,C) were reduced ($P<0.05$) at 4, 8, 24 hours following exposure to ractopamine. Fatty acid synthase mRNA abundance, however, only tended to be depressed at 4 hours but was reduced ($P<0.05$) at 8 and 24 hours (Figure 12B).

Discussion

Clenbuterol, cimaterol and ractopamine have been shown to enhance muscle protein deposition and depress fat accretion in laboratory and meat-producing animals (Deshaies et al., 1981; Beermann et al., 1986; Hanrahan et al., 1986; Anderson et al., 1987ab; Bergen et al., 1989). The mode of action of these agonists, are currently under

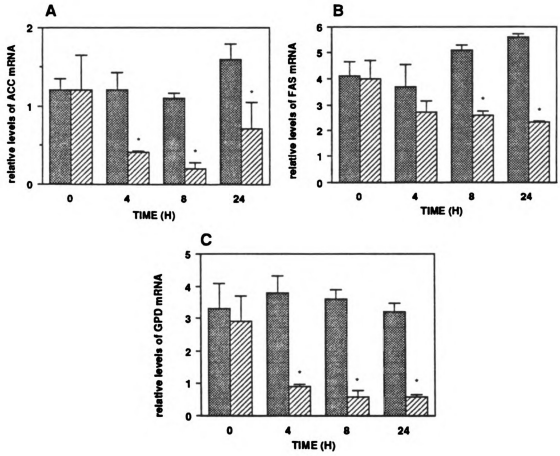

Figure 12. Changes in the Relative Abundance of Specific mRNAs during Ractopamine Treatment. TA1 adipocytes were exposed to 0 (stippled bars) or 10^{-6} M ractopamine (diagonial lined bars). Cells were harvested at the indicated times and the relative abundance of mRNA for: A) acteyl-CoA carboxylase (ACC), B) fatty acid synthase (FAS) and C) glycerol-3-phosphate dehydrogenase (GPD). Values represent mean + SE (n=2). * indicates treatment mean is different ($P<0.05$) from control mean.

investigation. Two possible modes of action are: 1) an indirect effect through endocrine or paracine systems and 2) a direct effect on target tissues through the beta receptor-G-protein-adenylate cyclase system. Although the former presents an interesting and viable option (Grant et al., 1990), the latter, at least with regard to lipid metabolism, seems to be the primary mechanism (Yang and McElligott, 1989). Beta receptors have been identified on adipose cells (Stiles et al., 1984; Williams et al., 1976) and interaction of beta-agonist with these receptors has been shown to enhance lipolysis and inhibits lipogenesis, in vitro (Mersmann et al., 1974; Saggerson, 1985). Incubation of mature TA1 adipocytes with the phenethanolamine ractopamine in the present study markedly decreased GPD, ME and FAS activity, thus further illustrating that ractopamine reduces lipid synthesis via a direct interaction with the adipocyte. These results also suggest that the reduced fat accretion observed in animals fed ractopamine may be, in part, a result of the direct interaction of ractopamine with adipose tissue.

Propranolol, a specific beta-adrenergic antagonist, suppressed the antilipogenic action of ractopamine in TA1 adipocytes. These data indicate that the beta-adrenergic receptor is involved in the inhibition of fatty acid synthase activity by ractopamine since the inhibitory effect of ractopamine was reversed by the addition of the

beta-blocker, propranolol. Other mechanisms, however, may also be involved since complete reversal of fatty acid synthase activity was not observed with the addition of 10^{-5} M propranolol. Propranolol at concentration greater than 10^{-5} M were toxic to TA1 adipocytes (Dickerson, unpublished data). Hausman et al. (1989) reported that propranolol reduced the lipolytic and antilipogenic effects of ractopamine. Hausman et al. (1989) also showed that 10^{-4} M propranolol could not completely abolish the lipolytic and antilipogenic activity at maximal effective ractopamine dose (10^{-6} M). These studies, thus, illustrate that ractopamine inhibits lipogenesis and stimulates lipolysis, in part, through its interaction with the beta receptor. Furthermore, the use of cyclic AMP analogs in the present studies provides indirect evidence that cyclic AMP may be the mechanism by which ractopamine mediates its inhibitory action on fatty acid synthase activity since similar reductions in fatty acid synthase activity by ractopamine can be obtained with dibutyryl cyclic AMP and 8CPT-cyclic AMP treatment.

Incubation of TA1 adipocytes with ractopamine immediately reduced glycerol-3-phosphate dehydrogenase activity, malic enzyme activity and fatty acid synthase activity. This indicates the involvement of short-term regulation of these enzymes. Classical short-term regulation mechanisms of biological pathways by cyclic AMP are a result of altered substrate supply, allosteric

effectors and covalent modification and generally target the rate-limiting enzyme in the pathway. Acetyl-CoA carboxylase is recognized as the rate limiting enzyme in fatty acid biosynthesis and has been shown to be under short-term regulation by cyclic AMP via reversible phosphorylation (Kim et al., 1989). The present findings of an immediate control of fatty acid synthase, malic enzyme, or glycerol-3-phosphate dehydrogenase by ractopamine, therefore, is surprising since these enzymes are not recognized as rate-limiting enzymes. It appears then that in TA1 adipocytes an unknown short-term regulatory mechanism is involved in regulating these enzymes.

In the present study, incubation of TA1 adipocytes with ractopamine also resulted in a continual suppression of the activity of glycerol-3-phosphate dehydrogenase, malic enzyme and fatty acid synthase. The decline in activity was accompanied by the reduction of the mRNA abundance for these enzymes as well as acetyl-CoA carboxylase. Ractopamine was shown to inhibit fatty acid synthase activity by approximately 73%, glycerol-3-phosphate dehydrogenase activity by 46% and malic enzyme activity by 58%. Relative mRNA abundance for fatty acid synthase, glycerol-3-phosphate dehydrogenase and malic enzyme were reduced by approximately 60%, 50% and 30%, respectively. Therefore, it appears that ractopamine lowers

the activity of these enzymes, at least in part, by a pre-translational event. The involvement of other mechanisms to reduce enzyme activity such as degradation of enzyme protein, however, cannot be ruled out. Detailed investigations of lipogenic enzymes have documented that the dramatic changes in enzyme activity observed with the elevation of cyclic AMP levels are a result of lowered synthesis and elevated degradation rates of lipogenic enzymes (Lakshmanan et al., 1972; Volpe et al., 1973; Volpe and Marasa, 1975; Bloch and Vance, 1977; Joshi and Wakil, 1978). Therefore, ractopamine probably reduces the activity of enzymes involved in lipid sythesis by both reducing mRNA levels as well as enhancing the degradation of these enzymes.

The reduced synthesis of lipogenic enzymes has been linked to a lower mRNA abundance of these enzymes (Back et al., 1986a; Dobson et al., 1987; Paulauskis and Sul, 1988). The reduction in the level of these mRNA(s) may be a result of depressed transcription of the gene(s), processing and/or decreased stability of the message. Cyclic AMP has been shown to enhance the degradation of malic enzyme mRNA and lowered the transcription of fatty acid synthase gene (Back et al., 1986ab; Paulauskis and Sul, 1989). Therefore, ractopamine may lower the mRNA abundance of acetyl-CoA carboxylase, fatty acid synthase, malic enzyme and glycerol-3-phosphate dehydrogenase by a transcriptional and/or post-transcriptional event.

In summary, ractopamine inhibits glycerol-3-phosphate dehydrogenase activity, malic enzyme activity as well as fatty acid synthase activity in TA1 adipocytes, indicating a direct interaction of ractopamine with adipose cells, in vivo. Ractopamine appears to have an immediate and persistent inhibitory action on glycerol-3-phosphate dehydrogenase, malic enzyme, and fatty acid synthase activity. These inhibitory actions are, mediated through the beta-adrenergic receptor and thus, the underlying effector of the antilipogenic actions appears to be cyclic AMP. Furthermore, the down regulation of the lipogenic enzymes as well as glycerol-3-phosphate dehydrogenase by ractopamine appears to be modulated at a pretranslational level. Moreover, these studies suggest that the reduced fat deposition observed in animals fed ractopamine may be mediated similarly.

APPENDIX

Growth of Bacteria and Purification of Plasmid DNA

I. Growth of Bacteria:

A. Reagents

1. L-broth

Bacto-tryptone	10 g
Bacto Yeast Extract	5 g
NaCl	10 g

dissolve in 900 ml HOH
adjust pH to 7.5 with 1 N NaOH
bring to 1 liter
autoclave 45 min, 20 p.s.i.
add antibiotics after cooled to < 55 C

Hard Agar Plates

L-broth	1.0 l

(as described above, prior to autoclaving)

Bacto Agar	15.0 g

autoclave 45 min, 20 p.s.i.
add antibiotics after solution cools to 55 C
Pour 25 ml/85 mm plate
let solidify and dry overnight
store at 4 C

3. Antibiotics:

1. Tetracycline: 12.5 mg/ml in absolute ETOH,
   filter and store at -20 C
   Working concentration: 15 ug/ml (1.2 ml/l)
   Note: tetracycline is light sensitive, store
   in dark.

2. Ampicillin: 25 mg/ml in HOH,
   filter, store at -20 C
   Working concentration: 50 ug/ml (2.0 ml/l)
   Note: ampicillin plates are good for only 2
   weeks after preparation.

4. 10X-M9 salts:

Na_2HPO_4-7HOH	113.0 g
KH_2PO_4	30.0 g
NaCl	5.0 g
NH_4Cl	10.0 g

adjust to 1 l
autoclave 45 min, 20 p.s.i.
store at room temperature.

5. 0.25 M $MgSO_4$

$MgSO_4$-7 HOH	6.2 g

adjust to 100 ml
autoclave 45 min, 20 p.s.i.
store at room temperature

6. 10 mM $CaCl_2$

$CaCl_2$ (dihydrate)	0.15 g

adjust to 100 ml
autoclave 45 min, 20 p.s.i.
store at room temperature

7. 15% casamino acids

Bacto casamino acids (vitamin free)	15.0 g

adjust to 100 ml
autoclave 45 min, 20 p.s.i.
store at room temperature

8. 20% glucose (dextrose)

Glucose	20.0 g

adjust to 100 ml
filter
store at room temperature

9. 1 mg/ml thiamine:

Thiamine	10.0 g

adjust to 100 ml
filter
store at 4 C

10. Solution A:

1 M Tris-Cl, pH 7.5	25.0 ml
0.2 M EDTA, pH 8.0	50.0 ml
sucrose (RNase free)	150.0 g

dissolve in 1 l
filter
store at 4 C

11. Solution B:

NaOH	0.8 g
10% SDS	10.0 ml

adjust to 100 ml
store at room temperature

12. 3 M Na-acetate, pH 5.0

Na-acetate (trihydrate)	204.0 g
glacial acetic acid	100.0 ml

dissolve in 400 ml
adjust pH to 5.0 with glacial acetic acid
bring to 500 ml
filter and store at 4 C

13. 13% Polethylene glycol (PEG)

polyethylene glycol	26.0 g

dissolve in 200 ml HOH
filter and store at 4 C

14. TE-8

1.0 M Tris-Cl, pH 8.0	10.0 ml
0.2 M EDTA, pH 8.0	5.0 ml

adjust to 1 l
autoclave, 45 min, 20 p.s.i.
store at room temperature

15. M9 Growth Medium (250 ml)

(assemble medium aseptically)

autoclaved HOH	185.0 ml
L-broth	25.0 ml
10X M9 salts	25.0 ml
10 mM $CaCl_2$	2.5 ml
250 mM Mg SO_4	0.5 ml
15% Casamino acids	8.3 ml
20% glucose	2.5 ml
1 mg/ml thiamine	.25 ml
antibiotic	
tetracycline (12.5 mg/ml)	.3 ml
or ampicillin (25 mg/ml)	.5 ml

16. Chloroform:isoamyl alcohol

chloroform	960.0 ml
isoamyl alcohol	40.0 ml

store at room temperature

17. Phenol:Chloroform:isoamyl alcohol

buffer saturated phenol	200.0 ml
chloroform:isoamyl alcohol	200.0 ml

mix
layer top with TE-8
store in brown bottle, 4 C

B. Growth of Bacteria

1. Day 1: streak a culture from a frozen culture onto a fresh plate containing the appropiate antibiotic. Let grow overnight at 37 C.

2. Day 2: use a single colony to inoculate 10 ml L-broth with 0.2% glucose and appropiate antibiotic in 50 ml Erlenmeyer flask. Grow overnight at 37C in shaking water bath.

3. Day 3: add 1 ml of overnight culture to 250 ml M9 medium (approximately 1-2 x10^9 cells), containing antibiotic.
   i. incubate at 37 C in shaking water bath
   ii. let cells grow to a density of 0.6-0.8 A600/ml (approximately 3-4 H)
   iii. add chloramphenicol to 0.2 mg/ml (1.4 ml of 34 mg chloramphenicol/ml in absolute ethanol)
   iv. let cells grow overnight at 37 C in shaking water bath

4. Day 4: Harvest cells (4 C)
   i. transfer contents of flask to 250 ml polypropylene bottle, sediment cells at 5K for 15 min.
   ii. decant supernatant, resuspend pellet in 25 ml cold 10 mM NaCl, transfer to 30 ml corex tube.
   iii. sediment cells at 5 K for 15 mins, decant supernatant, drain pellet.
   iv. transfer cells to -80 C for storage or until ready to prepare plasmid DNA.

II. Isolation of Plasmid DNA:

A. Lysis of Bacteria

1. Recover frozen cell pellets from -80 C freezer and thaw cells.

2. resuspend pellet in 4 ml Solution A

3. once cells are resuspended, add 2 ml of Solution A with 6 mg/ml lysozyme. Let cells lyse at 4 C for 15 min. Suspension should become viscous.

4. add 12 ml Solution B, mix genetle until solution becomes clarified.

5. add 7.5 ml 3 M Na-acetate (pH 5.0) and mix by inversion, keep in ice 10 min, then sediment at 5K for 10 min.

6. Recover supernatant, transfer to 150 ml corex bottle and treat with RNase
   i. add 20 ug/ml RNase A (stock: 1 mg/ml in 25 mM Na-acetate, heated to 80 C for 10 min to inactivate DNase, store at -20.
   ii. add .5 ml RNase stock/ 25 ml supernatant.
   iii. incubate 20 min at 37 C.

B. Organic extraction to remove nucleic acid

1. add 1 volume (25 ml) of phenol:chloroform: isoamyl alcohol, stopper bottle, shake vigorously for 5 min, centrifuge for 10 min at 3 K, recover supernatant and transfer to clean 150 ml corex bottle.

2. repeat above manipulation with 1 vol of chloroform:isoamyl alcohol.

3. ethanol precipitation: add 2 vol of absolute ethanol, mix, store at -20 C for 1 hour to overnight. recover precipitated nucleic acids by centrifugation at 5K for 10 min, rinse pellet with 70% ethanol, sediment, drain and dry pellet.

C. Polyethylene glycol (PEG) precipition

1. dissolve pellet in 1.6 ml sterile HOH

2. transfer to 15 ml corex tube, add 0.4 ml 4 M NaCl and mix

3. add 2 ml 13% PEG. Note: the volume of HOH, NaCl and PEG can be varied: the objective is to resuspend the nucleic acids in HOH, to adjust to 1M NaCl then to add 1 vol of 13% PEG to achieve a final concentration of 6.5% PEG and 0.5 NaCl.

4. incubate on ice for 60 min, sediment DNA at 5K for 10 min. drain and rinse pellet with 70% ethanol, sediment at 5 K for 10 min. drain and dry pellet.

5. respend in 1.0 ml TE-8. Determine DNA concentration by UV absorbance at 260 and 280 (ratio > 1.8 and approximately 0.5 mg DNA/250 ml culture.

6. Store plasmid DNA at 4 C in a flat-bottomed sterile tube. Label with plasmid name and concentration.

III. Restriction Digestion:

1. Mini digest to check plasmid DNA. Consult a known restriction map for selection of appropriate enzyme. Use 1 ug plasmid DNA 1 ul enzyme of each enzyme, 1 ul buffer in a final volume of 10 ul. Digest at 37 C for 2 hours. Add 10.0 ul 2X DNA loading dye. Analyze the DNA on a 1% agarose-TBE gel.

   a. Gel

agarose	2.5 g
HOH	225.0 ml
10 X TBE	25.0 ml

   microwave 4 min on high, cool to 55 C before pouring.

   b. Gel Buffer

HOH	225.0 ml
10X TBE	25.0 ml

   c. run gel at 60 volts for 3-4 hours.

   d. stain gel for 10 min in 100 ml HOH with 25 ul EtBr solution. destain 10 min in HOH.

2. Large scale digestion for insert isolation:

   a. 200 ug DNA
   1 unit restriction enzyme/ug DNA
   1/10 vol digestion buffer
   bring to 200 ul with HOH

   incubate at 37 C overnight

   b. Day 2: add to DNA 1/20 vol 4 M NaCl, vortex add 2 ml absolute ethanol, vortex, precipitate 8 hours to overnight at -20 C.

   c. Pour 1% separating gel (use prep comb), remove DNA from freezer, gently votex, microfuge 4 min, drain and dry pellet. resuspend pellet in 60 ul TE-8, add 60 ul 2X DNA loading dye buffer, load gel, run gel for 13 hours at 10 volts.

   d. stain and destain gel as above. cut out appropiate band and place in dialysis tubing for DNA collection. Add 1 ml TE-8 to tubing, clamp, and place in gel chamber with gel buffer. Run at 90 volts for 3 hours to elute DNA.

e. After dialysis, squeeze out gel. Roll tubing to resuspend DNA. Remove suspension and place in microfuge tube. Centrifuge 1 min and remove 150 ul supernatant and place in clean microfuge tube (Take caution not to get any of the agarose pellet. Repeat above procedure until all supernatant has been removed.

f. Fill microfuge tube to top with butanol, vortex, spin 10 sec, discard top butanol layer and put bottom layer in clean tube. Repeat butanol step until bottom layer is 450 ul.

g. To the 450 ul bottom layer add 450 ul phenol:chloroform:isoamyl alcohol, vortex, spin 10 sec, collect top layer and place in clean microfuge tube. Add 450 ul chloroform:isoamyl alcohol, vortex, spin 10 sec, collect top layer, add 1/20 vol 4 M NaCl, vortex, add 1 vol isopropyl alcohol, votex, and precipitate at -20 C for 20 min to 1 hour.

h. After precipitation vortex, spin 2 min, pour off supernatant, rinse pellet in 100 ul 70% ethanol, vortex, spin for 2 min pour off supernatant, dried pellet and then resuspend in 25 ul TE-8. Quanitate by titrating against known DNA standards, use EtBr to visualize DNA. Label tube with concentration and store at 4 C.

References:

A. Maniatis et al. 1982. Molecular Cloning A Laboratory Manual. Clone Spring Harbor, NY.

B. Birnboim and Doly. 1979. Nucleic Acids Res. 7:1513-1523.

C. Davis et al. 1986. Methods in Molecular Biology. Elsevier Science Publishing Co., Inc. New York. NY.

RNA DOT BLOT PROCEDURE

I. Sample Preparation:

1. add 12 ug sample to microfuge tube (the amount of RNA can be varyied depending upon the relative concentration of the mRNA of interest).

2. bring volume to 150 ul with TE-8

3. add 150 ul denaturing solution, vortex and heat to 65 C for 10 min, cool on ice.

   a. Denaturing solution (for 30 samples):

10X-MAE, pH 7.0	300 ul
deionized formaldehyde	1080 ul
deionized formamide	3120 ul

II. Filter paper preparation and manifold assembly:

1. Nitrocellulose paper (Schleicher and Shuell, BA85) and 3 blotting filter (Schleicher and Shuell, #470) are soaked in HOH for 10 min and then in 6X SSC (1 X SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) for 10 min.

2. Assemble manifold apparatus:

   a. place presoaked #470 paper on manifold
   b. place nitrocellulose on manifold
   c. assemble top and clamp unit
   d. attach vacuum line

III. Application of RNA:

1. wash each well with 500 ul 6X SSC

2. apply 25, 50, 75, and 100 ul of each sample to different wells. Keep track of the order of samples.

3. After all samples are applied, rinse each well with 500 ul 6X SSC.

4. Remove the filter from the manifold, place on dry 3MM paper, dry under heat lamp, label filter and bake for at least 2 hours in a vacuum oven at 80 C -25 mm Hg.

IV. Prehyridization and Hybridization Conditions:

1. Prehybidization solution (20 ml):

formamide	10.0 ml
25X SSC	4.0 ml
100X Denhardt's	1.0 ml
10% SDS	0.2 ml
1 M $NaPO_4$	1.0 ml
0.2 M EDTA	0.1 ml
HOH	2.7 ml

in a boiling water bath heat for 10 min
2.0 ml tRNA, add to above mixute.

(Denhardt's solution (1X): 0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone)

2. Transfer the blot to a seal-a-meal bag

3. Transfer pre-hydridization buffer to seal-a-meal bag with blot. De-bubble, seal the bag and place in shaking water bath at 42 C for at least 2 hours.

4. Hybridization solution (20ml):

formamide	10.0 ml
25X SSC	4.0 ml
100X Denhardt's	0.2 ml
10% SDS	0.2 ml
1 M $NaPO_4$	1.0 ml
0.2 M EDTA	0.1 ml
HOH	3.7 ml

in a boiling water bath heat for 10 min
1.0 ml tRNA, and 2* 10^6 cpm/ml of radioactive cDNA insert then add to above mixute.

5. Remove pre-hybridization solution from bag and replace it with hybridization solution, debubble, seal bag and place in shaking water bath at 42C overnight.

V. Washing Procedure:

1. Carefully remove blot from bag and immediately place blot into 2X-SSC, 0.1% SDS, 500 ml.

HOH	445 ml
20X SSC	50 ml
10% SDS	5 ml

   wash 5-10 min at room temperature.

2. Transfer blot to 0.1X SSC, 0.1% SDS, 500 ml pre-warmed to 65 C

HOH	1970 ml
20X SSC	10 ml
10% SDS	20 ml

   wash 45 min at 65 C- repeat two more times.

3. Place blot onto 3MM paper and dry under heat lamps

4. Wrap blot in saran-wrap and expose to Kodak XAR-5 film at -80 C with intensifying screen.

References:

A. White and Bancroft. 1982. J. Biol. Chem. 257: 8569-8572.

B. Jump et al. 1984. J. Biol. Chem. 259:2789-2797.

C. Maniatis et al. 1982. Molecular Cloning A Laboratory Manual. Clone Spring Harbor, NY.

Random Priming Procedure for cDNA inserts:

The following procedure utilizes the random prime kit from Boeringer Mannheim Biochemical and ^{32}P labeled dCTP from NEN.

1. In an microfuge tube combine 100 ng insert DNA and 2 ul reaction mixture (Solution #6). Place sealed tube in boiling water for 10 min, then cool on ice.

2. Add in order: 3 ul of a 1:1:1 mixture of dATP, dGTP and dTTP (Solution #2,4,and 5 are used to make this mix), 5ul ^{32}P dCTP (approximately 50 uCi), 8 ul sterile water and 1 ul Klenow (Solution # 7). Incubate for 30 min at 37 C.

3. Stop reaction by adding 2 ul 0.2 M EDTA and heat sample to 65 C for 10 min.

4. Purify labeled DNA by ethanol precipitation.

   a. add 10 ul 10 ug/ul tRNA, 10 ul 5 M ammonium acetate and 100 ul isopropanol. Mix well and place at -20 C for 10 min.

   b. microfuge for 10 min, remove supernatant and rinse pellet with 100 ul 70% ethanol.

   c. resuspend pellet in 100 ul TE-8, then add 40 ul 5 M ammonium acetate and 300 ul isopropanol. Mix well and place at -80 C for 10 min.

   d. microfuge for 10 min, remove supernatant and rinse pellet with 100 ul 70% ethanol. Allow pellet to dry.

   e. resuspend pellet in 100 ul TE-8.

   f. to quantify take 1 ul of radioactive solution and place it in a microfuge tube. Add 20 ul of 10 ug/ul tRNA and 1.0 ml of 1 M Na-pyro-phosphate. Vortex and filter mixture through a glass filter. Place filter in 10 ml scintillation fluid and count with scintillation counter.

Electrophoresis of RNA: Northern Blot Procedure

I. Gel Recipe:

1. 0.8% gel is used for large mRNA like acetyl CoA carboylase and fatty acid synthase.

10X MAE, pH 7.0	25.0 ml
LE-agarose	2.0 g
HOH	181.0 ml

dissolve agarose in 10X MAE and HOH by microwaving on high for 4 min, cool to 60 C and add 44.0 ml formaldehyde slowly while mixing, then por gel. The gel should be in the fume hood. Cover gel with pan.

(1 X MAE: 40mM MOPS, pH 7.0; 10 mM Na-acetate; 1 mM EDTA, pH 8.0)

II. Electrode Buffer:

10X MAE, pH 7.0	100.0 ml
HOH	720.0 ml
deionized formaldehyde	180.0 ml

mix just before use, keep in fume hood.

III. Sample preparation:

1. Samples should contain desired amount of total RNA in a total volume of 5.5 ul TE-8.

2. add 14.5 ul of denaturing mix to each RNA sample, cap microfuge tube and heat denature, 10 min at 65 C, cool on ice.

Denaturing Mixture (20 samples):

10X MAE, pH 7.0	20 ul
deionized formaldehyde	70 ul
deionized formamide	200 ul

3. add 5 ul 4X dye mix to each sample

4X dye mixture: 50% glycerol,
0.4% bromophenol blue,
0.4% xylene cyanol, 1 mM EDTA.

samples are ready to be loaded on gel.

4. submerge gel in electode buffer, and apply samples. Cover gel chamber with saran wrap to prevent loss of formaldehyde.

5. Electrophoretically separate RNA for 16 hours at 40 volts.

IV. Transfer of RNA to Nitrocellulose:

1. Rinse gel briefly in distilled water to remove excess formaldehyde. Incubate gel in 50 mM NaOH for 30 min to help facilitate transfer of larger RNA. Incubate in 0.1 M Tris-HCl, pH 7.0 for 30 min to neutralize the gel.

2. Cut Nitrocellulose to exact size of the gel. Wet nitrocellulose in distilled water and the soak in 10X SSC for 15 min.

3. Cut 2 pieces of 3 MM paper slightly larger than the gel and about twice as long as the gel. Also cut 5 pieces of 3 MM paper to the size of the gel as well as paper towel. You will need to count enough paper towels to equal a 3" stack.

4. Wet the 2 largest pieces of 3 MM paper in 10X SSC. Place them on a glass plate with the ends of the paper hanging over the ends of the plate. Place plate on glass tray filled with 10X SSC. Make sure the ends of the paper are submerged in the solution.

5. Carefully place the gel, face down on the paper. Outline the edges of the gel with saran wrap. Then set the nitrocellulose on the gel. Make sure that no air bubbles are trapped between the gel and the membrane.

6. Place the 5 pieces of dry 3MM paper on top of the membrane, and place the paper towels on top of the paper.

7. Place a small weight on top of the paper towels. Allow the transfer to continue for 16 to 24 hours. Change paper towels as needed and add more 10X SSC if needed.

8. After transfer is completed, mark well location on the nitrocellulose membrane and then rinse membrane in 2X SSC to remove residual agarose. Dry membrane under a heat lamp and bake it in a vacuum oven at 80 C for at least 2 hours.

9. **At this point the membrane is ready for pre-hybridization/hybridization procedure listed above.**

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