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The Effects of Pig Age, Muscularity, the Beta-Adrenergic Agonist Ractopamine, and Cyclic Adenosine Monophosphate on Porcine Muscle Satellite Cell Proliferation In Vitro

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THE EFFECTS OF PIG AGE, MUSCULARITY, THE BETA-ADRENERGIC AGONIST RACTOPAMINE, AND CYCLIC ADENOSINE MONOPHOSPHATE ON PORCINE MUSCLE SATELLITE CELL PROLIFERATION IN VITRO

By

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ABSTRACT

THE EFFECTS OF PIG AGE, MUSCULARITY, THE BETA-ADRENERGIC AGONIST RACTOPAMINE, AND CYCLIC ADENOSINE MONOPHOSPHATE ON PORCINE MUSCLE SATELLITE CELL PROLIFERATION IN VITRO

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Serum-mitogenic activities and satellite cell proliferative capacities of heavy and light muscled pigs were evaluated in porcine muscle satellite cell bioassays. The effects of ractopamine and cAMP on peptide growth factor-induced DNA synthesis were also examined. Blood was collected, and serum harvested at 40 and 90 kg body weights, and satellite cells were isolated at slaughter from 16 crossbred pigs. Carcass muscling traits were measured to assess carcass composition. Clonally-derived satellite cells from 8-week old pigs were used to evaluate serum-mitogenic activities, and ractopamine and cAMP effects. Serum mitogenic activity at 90 kg was correlated with lean gain per day ($R=.82;P<.03$) and semitendinosus weight ($R=.72;P<.05$). Differentiation of satellite cells from heavy and light muscled pigs tended to correlate with lean gain per day and loin eye area. Ractopamine and forskolin had no effect on DNA synthesis in basal serum-free medium. Both compounds enhanced growth factor-induced DNA synthesis. The effects of forskolin were found to be mediated through cAMP-dependent protein kinase.

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INTRODUCTION

Meat is an important source of protein for humans. Concern over dietary fat intake has created greater demand for lean meat products. The meat animal industry has responded with strategies that increase lean tissue accretion and reduce fat deposition in livestock species. Genetic selection for lean, heavily muscled animals is effective but slow. More immediate strategies, such as limit feeding, reduce fat accretion but also slow average daily gain which is an economically important trait for livestock producers. Strategies are needed which have an immediate effect on fat deposition and muscle accretion, and that provide economic benefit for livestock producers. Although not yet approved by the Food and Drug Administration, feeding beta-adrenergic agonists to livestock represents a strategy that would have an immediate impact on muscle and fat deposition. Furthermore, it would provide economic benefit to livestock producers.

Beta agonists have been shown to increase muscle accretion and reduce fat deposition when fed to sheep (Baker et al., 1984; Beermann et al., 1987; Kim et al., 1987; Claeys et al., 1989) and swine (Cromwell et al., 1988; Adeola et al., 1990; Watkins et al., 1990; Yen et al., 1990; Gu et al., 1991a, 1991b). In addition, beta agonists increase average

daily gain and feed efficiency thus making them economically attractive to livestock producers. The beta agonist ractopamine increases protein synthesis causing muscle hypertrophy in pigs (Johnson, 1987; Bergen et al., 1989). Beta agonist-induced protein turnover has been extensively studied (reviewed by Yang and McElligott, 1989). However, effects of beta agonists on skeletal muscle DNA accretion are not well understood. Proliferation and subsequent fusion of satellite cells postnatally accounts for at least 60% of the total DNA found in skeletal muscle of market weight pigs (Allen et al., 1979), and therefore represents a critical aspect of muscle growth. Satellite cell proliferation is controlled by many factors. One important class of these is the polypeptide growth factors which constitute a large portion of the mitogenic activity of serum. This research was conducted to 1) determine whether serum mitogenic activity is correlated with carcass muscling and/or lean growth rate, 2) characterize differences in proliferation and fusion of satellite cells derived from heavy and light muscled pigs, and 3) determine the effect of ractopamine on satellite cell proliferation induced by polypeptide growth factors. A thorough knowledge of factors affecting growth and differentiation of satellite cells, and understanding how beta agonists interact with these factors will provide the tools to greatly increase DNA accretion and muscle growth in pigs.

LITERATURE REVIEW

History

Skeletal muscle has a unique method of postnatal growth. Muscle fiber number is largely determined prenatally and increases only slightly after birth (Allen et al., 1979). Most of the increase in muscle mass therefore is due to hypertrophy. Skeletal muscle is different from other muscle tissues in that it is multinucleated. In early embryonic stages, mononucleated myoblasts fuse to form multinucleated myotubes which eventually become muscle fibers. Once a myoblast has differentiated and becomes part of the multinucleate structure, it has lost its ability to proliferate (Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966; Shafiq et al., 1968; Allbrook et al., 1971; Moss and Leblond, 1971; Cardasis and Cooper, 1975). Therefore, DNA contained within the plasma membrane of muscle fibers cannot replicate. However, as muscle undergoes hypertrophy, DNA content also increases (Enesco and Puddy, 1964; Moss et al., 1964; Winick and Noble., 1965; Moss, 1968a; Cardasis and Cooper, 1975). The source of DNA accumulated postnatally is widely accepted to be muscle satellite cells (Allen et al., 1979) which proliferate and subsequently fuse into existing muscle fibers thereby contributing to the DNA pool.

Role of Satellite Cells in Growth

Satellite cells were first described by Mauro (1961) as mononucleated cells wedged between the plasma membrane and basement membrane of skeletal muscle. Their role in normal muscle growth was not established until Moss and Leblond (1971) showed that satellite cells proliferate and fuse into existing muscle fibers thereby increasing the DNA content of muscle. These findings were supported by the work of Bischoff (1974) who was the first to isolate satellite cells from rat skeletal muscle, and grow these cells in culture. These cells displayed characteristics similar to embryonic myoblasts grown in culture in that they fused to form multinucleated myotubes which could spontaneously contract. In addition, Young et al. (1979) showed that cultured satellite cell-derived myotubes were capable of synthesizing myofibrillar proteins and assembling functional myofibrils. In 1986, Bischoff showed that satellite cells residing on cultured intact myofibers would proliferate in response to mitogens, and subsequently differentiate. Collectively, these studies suggest that muscle satellite cells play a role in normal postnatal muscle growth and development.

To further explore the role of DNA accumulation in muscle growth, studies were undertaken to investigate the effect of nutrient restriction on muscle growth and DNA accumulation. Swatland (1977) restricted feed intake to pigs at a level that would sustain body weight but arrest growth. In these animals, accumulation of myofiber nuclei was greatly reduced

compared to control animals. Winick and Noble (1966) used rats to study cellular responses during malnutrition at three different ages. Experimental animals were fed one half the calculated caloric requirement of a normal rat for 21 days following birth, weaning, or 65 days of age. To achieve caloric restriction during nursing, rat pups were cross-fostered so that 18 nursed from a single dam. Control litters had 9 to 12 rat pups per dam. After each treatment period, rats were given ad libitum access to a commercial rat chow diet. Caloric restriction reduced muscle weight and DNA content in the younger two groups compared to control rats. Refeeding to 133 days did not restore DNA content or muscle weight to control values. Rats placed on the restricted diet at 65 days of age lost muscle weight but did not lose DNA. Refeeding of the latter animals resulted in attainment of normal muscle weights. Moss (1968b) showed that a 2 day starvation period, from 7 to 9 days of age, severely retarded both muscle growth and DNA accumulation in breast muscle of chickens. Subsequent refeeding did not restore these values to those of control animals by 27 days of age. However, the ratio of muscle weight to nuclei number was the same between control animals and starved-refed animals. These data suggest that DNA accumulation may be a prerequisite for normal muscle growth since 1) only those animals whose muscle DNA content was not restricted were able to attain normal muscle weight after refeeding, and 2) weight of muscle per nucleus after refeeding was the same for both groups. Since satellite cells

are the source of DNA accumulated postnatally in skeletal muscle, collectively, these data demonstrate the crucial role that satellite cells play in muscle growth and development.

Satellite cells are not only essential for normal growth, but also play a critical role in the repair process after tissue injury. Snow (1977a,b) showed that satellite cells were the source of regenerating myoblasts in skeletal muscle following muscle mincing and transplantation in the hindlimb of young rats. In 1978, Snow confirmed that satellite cells from young rats could differentiate into multinucleated myotubes following muscle mincing and transplantation into siblings. Bischoff (1975) demonstrated that cultured degenerating muscle fibers retained a population of viable satellite cells which would proliferate and differentiate to form myotubes. Therefore, in addition to the role they play in normal growth and development, satellite cells are critical to the process of muscle regeneration following injury.

Retaining a population of mononucleated cells with the capability of proliferating and fusing into existing muscle fibers, endows skeletal muscle with the capacity to grow by increasing the amount of cellular machinery (DNA and RNA) available for protein synthesis, and theoretically, increase the absolute amount of protein synthesized per unit time. Examination of data shows that the percentage of muscle DNA accumulated postnatally is substantial. Postnatal muscle DNA increases ranges from 50-63% in market weight pigs, to 99% in chickens (Allen et al., 1979). The figure for pigs is from 23

to 118 kg body weight, while the data for chickens are from birth to 266 days of age. Therefore, postnatal DNA accumulation in pigs would certainly be greater than 60% if it was measured from birth to maturity as it was for chickens.

DNA accretion appears to be a prerequisite for normal muscle growth. Therefore, heavy muscled animals should have more DNA per muscle than light muscled animals. Robinson and Bradford (1969) studied skeletal muscle cellularity in mice that had been selected for rapid postweaning growth rate over 14 generations. Total weight and DNA content of the gastrocnemius muscle were greater for the select line of animals than for the control line. In addition, protein to DNA ratio was lower in the select line. Aberle and Doolittle (1976) obtained similar results in mice selected for large body size compared to unselected controls. Powell and Aberle (1975) found that total DNA, and DNA concentration were higher in heavy muscled swine compared to light muscled controls. In addition, protein:DNA was consistently higher in the light muscled group. Harbison et al. (1976) obtained the same results with two genetically different lines of swine, except, DNA concentration did not differ between the lines. Collectively, these four studies demonstrate that selection for increased growth rate, body size, or muscle mass, does not increase protein per nucleus, but increases total DNA content of muscle leading to a larger muscle. Therefore, it appears that these selection schemes have increased satellite cell proliferative activity.

Purchas et al. (1985) showed that satellite cell proliferative activity was greater in a lean strain of mice than in an obese strain. The lean strain also was much heavier muscled as three hindlimb muscles weighed nearly 100% more than those in the obese strain at 8 weeks of age. Allen et al. (1982) demonstrated that serum from a lean strain of pigs stimulated myogenic cell proliferation in culture to a greater extent than serum from an obese strain. These two strains also differed in muscle growth potential as evidenced by an average adjusted loin eye area of 42.1 cm^2 for the lean strain compared to 30.9 cm^2 for the obese strain. These studies support the hypothesis that heavy muscled animals have greater satellite cell proliferative activity than light muscled animals.

Beta-Adrenergic Agonists

Consumer acceptance of meat products has recently become highly dependent on their lean content. As a result, the animal industry has concentrated its efforts on developing growth promotants that increase lean tissue growth while reducing fat deposition. One class of compounds that has been very successful in accomplishing this objective is the beta-adrenergic agonists, usually referred to as beta agonists.

Beta adrenergic agonists are structural analogues of catecholamines, which bind and act through beta-adrenergic receptors. Two subtypes of beta receptors (β_1 and β_2) have been isolated and cloned (Frielle et al., 1987; Kobilka et al., 1987) to facilitate further understanding of the

underlying mechanisms in their regulation. The predominant subtype in skeletal muscle appears to be β_2 (Blocken et al., 1986; Watson-Wright and Wilkinson, 1986; Rothwell et al., 1987). Both subtypes are membrane-spanning proteins with seven alpha helices, 3 intra- and three extracellular loops connecting the membrane-spanning helices (Lefkowitz and Caron, 1988). Ligand binding occurs on three intra-membrane domains (Dohlman et al., 1987). These receptors are coupled to guanine nucleotide regulatory proteins (G-proteins) which mediate metabolic events and cellular responses. (Gilman, 1987; Simon et al., 1991). Beta receptor-associated G-proteins are heterotrimers consisting of α, β and γ subunits. Upon beta agonist binding, the α ($G_{s\alpha}$) subunit dissociates (Simon et al., 1991) and activates the enzyme, adenylate cyclase, which catalyzes production of the second messenger cyclic adenosine monophosphate (cAMP) (Lefkowitz and Caron, 1988). This is the best characterized and most widely accepted pathway by which beta agonists exert their effects. However, beta receptor-associated $G_{s\alpha}$ also stimulates dihydropyridine-sensitive skeletal muscle calcium channels, also known as L-channels (Yatani et al., 1988). This calcium-dependent pathway has been shown to stimulate protein synthesis in cultured cardiac myocytes independent of cAMP (Bishopric et al., 1992). However, this pathway is not likely to be involved with myogenic cell proliferation since the L-channel is apparently not present in undifferentiated myoblasts and satellite cells (Schmid et al., 1984).

It is well established that beta agonists markedly increase muscle accretion in several mammalian species (Yang and McElligott, 1989). McElligott et al. (1989) showed that clenbuterol increased muscle growth 14 to 22% in rats treated for 14 days. Brown et al. (1992) reported that cimaterol increased muscle growth in male rats treated for 21 days. Baker et al. (1984) reported that clenbuterol-fed lambs had 33 to 44% larger longissimus muscle cross-sectional areas compared to controls, and increased hindquarter protein percentages after 8 weeks of treatment. Beermann et al. (1987) showed that cimaterol increased longissimus muscle cross-sectional area in lambs 26 and 32% at 7 and 12 weeks of treatment, respectively. Additionally, weights of semitendinosus, semimembranosus, and biceps femoris muscles were 27, 33, and 31.5%, greater than controls, respectively, at 7 weeks. Total protein content of the semitendinosus muscle also was greater in treated versus control animals at both treatment periods. Kim et al. (1987) found that cimaterol increased longissimus muscle cross-sectional area 38% in lambs, and increased the yield of the four major primal cuts 28%. Claeys et al. (1989) reported that 28 days of clenbuterol treatment increased semitendinosus weight in lambs 21.5%, and increased total protein content 28.5%. Cromwell et al. (1988) reported that cimaterol increased longissimus muscle cross-sectional area in swine 12% during a 6 week treatment period. Bergen et al. (1989) fed ractopamine to pigs for 4 weeks and reported that semitendinosus weights were

25% greater in treated pigs than controls. Feeding ractopamine to pigs for 28 days increased longissimus, psoas major, semitendinosus, biceps femoris and quadriceps femoris weights 8 to 22% (Adeola et al., 1990). Watkins et al. (1990) showed that ractopamine fed to pigs for 45 to 50 days increased longissimus muscle cross-sectional area 14 to 17%. These data clearly indicate that beta agonists markedly increase muscle mass.

The lean tissue growth response to beta agonists has been investigated with pigs that differ in lean tissue growth capacity. Yen et al. (1990b) found that ractopamine increased muscle growth equally in lean and obese pigs. Additionally, no differential response was observed between lean and obese pigs fed cimaterol (Yen et al., 1990a). However, Gu et al. (1991a,b) reported that pigs from leaner genotypes tended to have greater muscle growth responses to ractopamine. Yen et al. (1991) observed greater lean tissue growth responses to ractopamine in U.S. contemporary crossbred pigs than in lighter muscled Chinese pigs. Furthermore, Bark et al. (1992) found that ractopamine increased lean gain per day much more in lean pigs than in obese pigs. Further study is needed to determine the mechanism whereby ractopamine induces differential lean growth responses in pigs divergent in lean growth capacity.

Although it is clear that beta agonists increase muscle mass, the mechanism by which this occurs remains controversial. Studies indicate that beta agonists alter the

rate of protein turnover, but disagree whether this effect is mediated strictly through altered rates of synthesis, degradation, or both. Studies with isolated avian muscles have shown that protein degradation is decreased (Klasing et al., 1985; Rogers et al., 1988). In vivo, Reeds et al. (1986) could not detect an effect of clenbuterol on protein synthesis in rats, and concluded that protein degradation was decreased. Babij and Booth (1988) reported that clenbuterol inhibited loss of α-actin mRNA in atrophying rat hindlimb muscles. However, Bates and Pell (1991) found that clenbuterol increased skeletal muscle protein synthesis in dwarf mice, and had no effect on protein breakdown. Roeder et al. (1987) observed no protein turnover response to beta agonists in cultured rat myogenic cells. However, Harper et al. (1990) reported that cimaterol increased protein synthesis rates in cultured L6 myoblasts while it had no effect on protein degradation. Anderson et al. (1990) found that ractopamine increased protein synthesis in cultured rat myotubes. In addition, beta agonists have been shown to stimulate protein synthesis in cultured avian myogenic cells (Kagen and Freedman, 1974; Young et al., 1988; Shaoquan and Orcutt, 1991). In vivo studies with avian (Morgan et al., 1988), cattle (Williams et al., 1987), and lambs (Bohorov et al., 1987) have all shown beta agonist-induced decreases in protein degradation. Data with swine, however, agree that beta agonists stimulate protein synthesis. Pigs fed ractopamine for 21 and 35 days, had increased fractional protein synthesis

and degradation rates resulting in a net increase in protein accretion (Bergen et al., 1989). Helferich et al. (1990) showed that pigs fed ractopamine for 21 days had increased fractional rates of skeletal muscle α-actin synthesis. Additionally, Johnson (1987) demonstrated an increased fractional myofibrillar protein synthesis rate in the semitendinosus muscle of pigs fed ractopamine for one week. Collectively, these studies indicate there may be species based differences in beta agonist-induced responses which preclude generalizations. Furthermore, the wide variety of methods and beta agonists employed to study protein turnover may contribute to the observed discrepancies. For a review of beta agonist-induced effects, see Yang and McElligott (1989).

Another mechanism that may contribute to increased muscle mass in beta agonist-treated animals is increased satellite cell proliferation and fusion into muscle fibers. This would give existing muscle fibers more cellular machinery to synthesize protein. Kim et al. (1987) reported that lambs fed the beta agonist cimaterol for 8 weeks had decreased longissimus and semitendinosus muscle DNA concentrations compared to controls. Beermann et al. (1987) reported similar finding in lambs treated with cimaterol for 7 weeks. However, after 12 weeks of treatment, beta agonist-fed lambs had semitendinosus muscle DNA concentrations equal to controls, but treated lambs had greater semitendinosus weights. This indicates that beta agonist-treated lambs had more total DNA in their muscles. Bergen et al. (1989) reported that

ractopamine-fed pigs had nonsignificant increases in DNA per muscle after 28 days of treatment compared to control pigs. Johnson (1987) observed that pigs fed ractopamine for 1 week had lower longissimus and semitendinosus muscle DNA concentrations than control pigs. Skjaerlund et al. (1993) reported that pigs fed ractopamine for 4 weeks had significantly more DNA per muscle than control pigs. Grant et al. (1990) found a proliferation response of chick myoblasts in culture to ractopamine. Taken together, these data indicate that DNA accretion is not a prerequisite for beta agonist-induced muscle growth during short term treatments. However, longer term in vivo beta agonist treatments may induce DNA accretion. Based on the these studies, length of treatment required to stimulate DNA accretion may vary between species.

Myogenic Cell Isolation and Serum-Free Media

Bischoff (1974) was the first to describe the isolation of myogenic cells from muscle tissue. Since that time, procedures have been published for the isolation of ovine (Dodson et al., 1986), bovine (Dodson et al., 1987), chicken (Matsuda et al., 1983; Yablonka-Reuveni et al., 1987), turkey (McFarland et al., 1988), human (Blau and Webster, 1981) and porcine (Doumit and Merkel, 1992) myogenic cells. Conditions optimizing the environment for proliferation and differentiation of bovine, (Dodson et al., 1987), ovine (Dodson et al., 1990), porcine (Doumit and Merkel, 1992), and turkey (McFarland et al., 1988) satellite cells have also been

described. Many systems have used serum as a source of mitogens. To avoid confounding effects of unknown serum components in serum-containing cell culture media, serum-free media have been developed for primary chicken myoblasts (Dollenmeier et al., 1981), clonally-derived turkey satellite cells (McFarland et al., 1991), human muscle satellite cells (Ham et al., 1988), primary rat satellite cells (Allen et al., 1985) and clonally-derived porcine satellite cells (Doumit et al., manuscript in preparation). These media facilitate studying factors which regulate cell growth and differentiation.

Peptide Growth Factors

Peptide growth factors play an important role in regulating cell growth and differentiation. Basic fibroblast growth factor (bFGF) is a potent mitogen for mesoderm-derived cells including myoblasts and fibroblasts (Gospodarowicz et al., 1987). Basic FGF stimulates proliferation of primary rat satellite cells (Allen et al., 1984), myoblasts from the RMo rat myoblast cell line (Johnson and Allen, 1990), primary and clonally-derived bovine satellite cells (Greene and Allen, 1991) and clonally-derived porcine satellite cells (Doumit et al., 1993). Moreover, FGF is required for proliferation of turkey satellite cells (McFarland et al., 1993). Additionally, acidic FGF (aFGF) increases DNA synthesis of MM14 cells, a permanent mouse skeletal muscle cell line dependent on FGF for proliferation (Seed et al., 1988a). Myogenic cells are not only responsive to exogenous FGF, but

also produce it. Alterio et al. (1990) showed that cultured rat satellite cells express mRNA for both acidic and bFGF. Moreover, extracts from whole chick limb buds will compete with aFGF for binding to Swiss 3T3 cells and stimulate proliferation of MM14 cells (Seed et al., 1988b). While FGF stimulates growth of myogenic cells, it is a potent inhibitor of differentiation. Creatine phosphokinase activity was depressed by FGF in the BC_3H1 muscle cell line (Lathrop et al., 1985). Additionally, FGF depressed differentiation of MM14 cells (Linkhart et al., 1981), primary bovine satellite cells (Greene and Allen, 1991), RMo cells (Johnson and Allen, 1990) and muscle colony-forming cells from developing chick wing buds (Seed and Hauschka, 1988a).

Insulin-like growth factors-I and -II (IGF-I and -II) are unique because they stimulate both proliferation and differentiation of myogenic cells. Insulin, at supraphysiological concentrations, has been reported to act through the type-I IGF receptor (Straus, 1984) and therefore is sometimes used to study the actions of IGF-I and IGF-II. Ewton and Florini (1981) suggested that at supraphysiological concentrations, insulin acts as an analogue of the somatomedins (IGF's) stimulating differentiation of L6 myoblasts. In addition, amino acid uptake, cell proliferation and differentiation are stimulated, and protein degradation is decreased, in response to IGF-I, IGF-II and insulin in L6 myoblasts. All of these responses are mediated through the type-I IGF receptor (Ewton et al., 1987). Duclos et al.

(1991) also showed that both IGF-I and -II stimulate DNA synthesis in chicken muscle satellite cells through the type-I IGF receptor. Insulin and IGF-II promote proliferation of primary rat satellite cells (Dodson et al., 1985). Others have shown that IGF-I stimulates proliferation of L6 cells (Pampusch et al., 1990), RMo cells (Johnson and Allen, 1990), primary bovine satellite cells (Greene and Allen, 1991) and clonally-derived turkey (McFarland et al., 1993) and porcine (Doumit et al., 1993a) satellite cells. Studies measuring primarily myogenic cell differentiation have shown that insulin promotes this process in primary rat satellite cells (Allen et al., 1985) and clonally-derived porcine satellite cells, but not primary porcine satellite cells (Doumit et al., 1993b). Florini et al. (1986a) also demonstrated that IGF's give a biphasic response in L6 cells, stimulating differentiation at low concentrations but inhibiting it at high concentrations. Furthermore, expression of IGF-II mRNA and the IGF-II receptor are greatly increased in C2 myoblasts upon differentiation (Tollefsen et al., 1989a,b; Kou and Rotwein, 1993). Finally, Florini et al. (1991) showed that IGF-I stimulates myogenic differentiation by induction of the myogenic regulatory factor, myogenin, in L6A1 cells.

Platelet-derived growth factor (PDGF) is a hetero- and homodimer consisting of combinations of A and B chains (Ross et al., 1986). Porcine PDGF is primarily the BB isoform (Stroobant and Waterfield, 1984). There are two different PDGF receptors, α and β (Heldin et al., 1988), which dimerize

upon ligand binding (Bishayee et al., 1989) to induce signal transduction. Isoform-specific binding of PDGF (AA, AB, and BB) to PDGF receptors ($\alpha\alpha$, $\alpha\beta$, and $\beta\beta$) has been extensively studied (Heldin et al., 1988; Bishayee et al., 1989; Heldin et al., 1989; Seifert et al., 1989; Kanakaraj et al., 1991; Fretto et al., 1993). The PDGF-B chain binds to both α- and β-receptors and is capable of inducing dimerization of all receptors combinations, while the PDGF-A chain only binds and induces dimerization of α-receptors. PDGF-BB is mitogenic for L6J1 myoblasts (Jin et al., 1990; 1991), C2 myoblasts (Yablonka-Reuveni et al., 1990), chick myoblasts (Yablonka-Reuveni and Seifert, 1993), turkey satellite cells (McFarland et al., 1993), and porcine satellite cells (Doumit et al., 1993a), while PDGF-AA is not. However, proliferating L6J1 and secondary rat myoblasts (Sejersen et al., 1986) and differentiating L6J1 myoblasts (Jin et al., 1990) produce substantial amounts of PDGF-AA, indicating that it may be an important autocrine regulator of myogenic cell function (for PDGF reviews see Heldin et al., 1985; Ross et al., 1986). Although not well characterized, the effects of PDGF on myogenic cell differentiation appear to be inhibitory. Jin et al. (1991) reported that PDGF-BB inhibited myotube formation and CPK activity in L6J1 myoblasts, in a concentration-dependent manner.

TGF-β is a potent inhibitor of myogenic cell differentiation in L6 (Florini et al., 1986b), L6E9, rat, chicken (Massague et al., 1986), C2, BC_3H1 (Olson et al.,

1986) and RMo (Johnson and Allen, 1990) myoblasts. TGF-β also potently inhibits differentiation in rat (Allen and Boxhorn, 1987; 1989), ovine (Hathaway et al., 1991) and bovine (Greene and Allen, 1991) satellite cells. However, conflicting reports exist regarding the effect of TGF-β on myogenic cell proliferation. Factors such as cell source, media type and the presence of other growth factors play an important role in determining the response to TGF-β. Allen and Boxhorn (1987) showed that proliferation of primary satellite cells from mature and neonatal rats was inhibited by TGF-β in serum-containing media. In serum-free medium, proliferation of rat satellite cells stimulated by FGF, IGF-I and the combination of IGF-I and FGF, was inhibited by TGF-β (Allen and Boxhorn, 1989). Greene and Allen (1991) reported that proliferation of primary bovine satellite cells in basal serum-free medium was depressed by TGF-β, while proliferation stimulated by FGF in serum-free medium was increased by TGF-β. However, the same study revealed that clonally-derived bovine satellite cells stimulated by FGF were not sensitive to TGF-β. Pampusch et al. (1990) reported that TGF-β depressed growth of L6 myoblasts in serum-containing medium, as well as IGF-I-stimulated proliferation in serum-free medium. In the same study, proliferation of porcine embryonic myoblasts was inhibited by TGF-β in serum-containing media. Hathaway et al. (1991) reported that proliferation of primary bovine and porcine satellite cells grown in serum-containing medium was depressed by TGF-β, while proliferation of ovine satellite

cells grown in either serum-containing or in serum-free media was not affected by TGF-β. Johnson and Allen (1990) found that proliferation of the RMo muscle cell line was not affected by TGF-β. Proliferation of clonally-derived porcine satellite cells was inhibited by TGF-β in serum-containing medium but stimulated in basal serum-free medium (Cook et al., 1993). Finally, in studying the growth of fibroblasts, Roberts et al. (1985) concluded that the effects of TGF-β on cell growth are dependent on other growth factors and receptors operating in the cell.

Epidermal growth factor (EGF) is mitogenic for human satellite cells (Ham et al., 1988). Clonally-derived porcine satellite cells are not responsive to EGF in serum-free media, but are responsive in serum-containing media. Moreover, EGF synergizes with IGF-I and PDGF-BB in serum-free media to increase porcine satellite cell proliferation (Doumit et al., 1993a). However, EGF has no proliferative effect on chicken (Dollenmeier et al., 1981) myoblasts or cultured rat (Allen et al., 1985) and turkey (McFarland et al., 1993) satellite cells. In ovine satellite cell-derived myotubes, EGF stimulates protein synthesis (Roe et al., 1989). EGF also acts synergistically with FGF and insulin to increase creatine kinase activity in human satellite cells (Askanas and Gallez-Hawkins, 1985).

Cyclic AMP

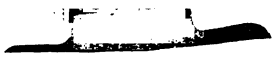
The effects of cAMP on myogenic cell proliferation have not been studied extensively. The 3T3 fibroblast cell line is

mitogenically responsive to cAMP and a brief discussion of this literature is relevant. Rozengurt et al. (1981) reported that cholera toxin-induced increases in 3T3 cell cAMP concentration increased cell sensitivity to the mitogenic action of serum. In serum-free medium, cholera toxin alone had a small effect on DNA synthesis, but synergized with EGF and insulin to greatly increase proliferation. Addition of an adenosine receptor antagonist to quiescent 3T3 cultures increased cAMP concentrations and stimulated reinitiation of DNA synthesis (Rozengurt, 1982). Simultaneous addition of a phosphodiesterase inhibitor markedly potentiated mitogenic responsiveness to the adenosine analog. Shier and Durkin (1982) reported that PDGF induced DNA synthesis, and release of arachidonic acid and E-type prostaglandin (PGE) from 3T3 cells. Similar results were obtained by Rozengurt et al. (1983a,b) who also showed that PDGF and PGE induced similar cAMP concentrations in 3T3 cells. Additionally, PGE-induced DNA synthesis was markedly potentiated by a phosphodiesterase inhibitor. Rozengurt (1986) also reported that cAMP analogues synergize with growth-promoting agents such as fibroblast-derived growth factor, bombesin, vasopressin, phorbol esters, diacylglycerol, and insulin, but not with others known to increase cAMP such as cholera toxin, PGE, and adenosine analogues. Collectively, these studies establish a role for cAMP in the initiation of DNA synthesis in 3T3 fibroblasts. A role for cAMP in myogenic cell proliferation has not been established. Young et al. (1988) reported that cimaterol had

no effect on myotube nuclei in embryonic chicken muscle cell cultures. However, the beta agonist ractopamine has been shown to increase growth of cultured chick embryo myoblasts (Grant et al., 1990). This isolated observation has not been confirmed.

CHAPTER ONE

THE EFFECTS OF PIG AGE AND MUSCULARITY ON SATELLITE CELL PROLIFERATION AND DIFFERENTIATION.



ABSTRACT

The objectives of this study were to 1) determine the effect of pig age and muscularity on serum mitogenic activity (SMA), and 2) determine whether a relationship exists between pig muscularity and satellite cell proliferation and differentiation. Sixteen pigs were catheterized at 40 kg and again at 90 kg body weight. Blood was collected every two hours over an eight hour period, allowed to clot 24 hours at 4°C, and serum harvested by centrifugation. After the 90 kg bleeding, pigs were slaughtered, and the left semitendinosus muscle was excised immediately following exsanguination, placed in cold phosphate-buffered saline, and quickly transferred to the cell culture laboratory for satellite cell isolation. Backfat and muscling traits were measured 24 hours after slaughter to determine carcass composition. To assess SMA, clonally-derived porcine satellite cells from an eight week-old pig were plated at 8,000 cells per 16 mm diameter gelatin-coated culture well in Minimum Essential Medium (MEM) containing 10% fetal bovine serum. After a 24 hour attachment period, cultures were washed with MEM, and treatments of MEM containing 6% catheter-collected serum were applied. MEM-6% commercial porcine serum served as control. Treatments were changed once after 48 hours, and cultures were frozen after 76

hour exposure to treatment media. Proliferation was measured using a fluorometric DNA assay. To determine differentiation potentials of primary satellite cells isolated from pigs in this study, cells were plated at .05 gram equivalents onto 10 cm dishes in MEM-10% FBS and allowed to grow for 7 to 10 days. MEM-2% FBS was then applied for 2 to 4 days to induce fusion, and myogenic colony size was assessed microscopically. Alternatively, cells were plated onto 24-well plates, grown to confluence and fusion induced to determine fusion percentage. The SMA of nine pigs at 40 kg body weight (SMA-40) was consistently greater than at 90 kg. ($P<.001$). SMA-40 was not correlated with percent muscle (PM), lean gain/day (LGD), semitendinosus weight (STW), or loin eye area (LEA) at market weight. SMA-90 was more highly correlated with LGD, PM, STW, and LEA. SMA-90 increased with increasing LGD ($R=.82$; $P<.02$) and STW ($R=.72$; $P<.04$). In addition, the difference between SMA-40 and SMA-90 was negatively correlated ($R=-.65$) with LGD. Myogenic colony size and myotube nuclei from primary cultures were not correlated with carcass muscling. However, fusion percentage tended to increase ($R=.53$; $P<.12$) with increasing lean gain per day.

These data indicate that, serum mitogenic activity was consistently greater in younger pigs while SMA in older pigs was more highly correlated with carcass muscling traits. Satellite cells from pigs with a high rate of lean tissue accretion tended to display higher fusion percentages.

INTRODUCTION

Skeletal muscle satellite cells are the source of DNA accumulated in muscle tissue during postnatal growth (Allen et al., 1979). Postnatal DNA accretion accounts for 99% of total muscle DNA in some meat animal species, and DNA per muscle is indicative of the total cellular machinery available for protein synthesis (Allen et al., 1979). Heavy muscled animals have greater total DNA per muscle than light muscled animals (Robinson and Bradford, 1969; Powell and Aberle, 1975; Aberle and Doolittle, 1976; Harbison et al., 1976). Therefore, factors which affect satellite cell proliferation play a critical role in determining DNA content, and ultimately, skeletal muscle mass. Purchas et al. (1985) found that satellite cell proliferative activity was greater in lean mice than in lighter muscled, obese mice. Inherent proliferative capacity differences and variations in serum mitogenic activity may have contributed to these differences. The contribution of serum-borne factors to satellite cell proliferation can be quantified by collecting serum and determining its mitogenic activity on myogenic cells in vitro. Allen et al. (1982) showed that serum from lean pigs stimulated greater primary myoblast proliferation in vitro than serum from obese pigs. However, that study used

embryonic chicken muscle cells grown in medium containing horse serum, chick embryo extract, and porcine serum. These conditions necessitated assumptions that fibroblast contamination was insignificant, and that synergistic effects were absent when chick embryo extract, horse serum and pig serum were combined in the assay. In addition, Allen et al. (1982) concluded that pure cultures of pig skeletal muscle satellite cells used in a culture system that required only porcine serum would be ideal.

Circulating growth factor concentrations could account for some of the differences in muscling and lean tissue growth of pigs by differentially affecting proliferation and subsequent fusion of satellite cells. Inherent differences in the proliferation and differentiation capacity of satellite cells may also help to create varying degrees of muscularity. Thus, this study was conducted to 1) determine whether mitogenic activity of serum from growing and market weight pigs is correlated with carcass muscling characteristics and/or lean tissue growth rate, and 2) determine whether satellite cells derived from pigs differing in muscularity possess inherent differences in their ability to proliferate and differentiate in culture.

MATERIALS AND METHODS

Materials

Minimum Essential Medium (MEM), antibiotic/antimycotic, gentamicin, and porcine serum (PS) were purchased from Gibco (Grand Island, NY). Pig skin gelatin, bovine insulin, Hoechst 33258 reagent, calf thymus DNA, were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Hazleton (Lenexa, KS), and twenty-four well culture plates were from Corning Glass Works, (Corning, NY). Polyvinyl catheters were obtained from Bolab (Lake Havasu City, AZ).

Live Animal Handling and Blood Collection

Sixteen crossbred pigs weighing approximately 40 kg were selected from the MSU swine barn using genetic records and visual appraisal based on degree of muscling (i.e., heavy or light). Pigs were weighed individually, transported to the metabolism unit, placed in metabolism crates and given ad libitum access to feed and water. Thirty-six hours later, pigs were anesthetized by intramuscular injection of 1 mg Ketamine/kg body weight and 1 mg Telazol/kg body weight. Pigs were then fitted with indwelling ear vein catheters which were sutured to the ear to prevent removal. The catheters were kept patent with sodium citrate, and flushed every 4 to 6

hours with fresh sodium citrate. Pigs were allowed to recover for 24 hours before initiation of blood collection. However, pigs typically recovered full consciousness within 1 hour after catheterization and began consuming feed and water immediately thereafter.

Ten mL of blood were drawn every 2 hours for 10 hours. Blood was placed in borosillicate glass tubes and allowed to clot at room temperature for 2 hours, then maintained at 4° C overnight. Serum was harvested by centrifugation at 2000 x g for 15 minutes, and stored at -20° C.

After blood collection at 40 kg body weight, catheters were removed, pigs were returned to the finishing barn and randomly allotted to 2 pens. Pigs were provided ad libitum access to a vitamin and mineral-fortified corn-soybean meal based diet containing 16% crude protein. When pigs reached 90 kg, body weight was recorded and the handling and blood collection procedure was repeated.

After the second blood collection, pigs were kept in the metabolism crates until slaughtered. Four pigs were slaughtered every 2 days at the MSU meat laboratory. Pigs were electrically stunned, and immediately exsanguinated. Blood was collected at slaughter and sera harvested as previously described. Immediately following exsanguination, the left semitendinosus muscle was excised, weighed, trimmed free of visible fat and extraneous tissue, placed in cold phosphate-buffered saline (PBS) and transferred to the cell culture laboratory where satellite cells were isolated as

described by Doumit and Merkel (1992). Additionally, perirenal fat was removed after evisceration, weighed and recorded along with hot carcass weight. Because the semitendinosus muscle was removed, pigs were skinned rather than scalded and dehaired. Therefore, 3 mm were added to each backfat measurement recorded.

The following carcass data were collected from each carcass 24 hours after chilling: loin eye area, carcass length, fat depth at the first rib, last rib, last lumbar vertebra and 10th rib. Percent muscle was calculated according to the National Pork Producers Council guidelines (1983). Lean gain per day was calculated using the following assumptions (Mulvaney, 1984): 1) pigs weighing approximately 40 kg would have dressing percentage of 68, and 2) their carcasses would contain approximately 60% muscle. Lean gain per day was calculated by subtracting estimated weight of carcass muscle at the first bleeding from estimated weight of muscle at the second bleeding, and dividing the difference by the number of days between bleedings (NPPC formula, 1983). Loin eye area and semitendinosus weight were adjusted to a common carcass weight of 105 kg.

Satellite Cell Culture

To assess mitogenic activity of serum from each pig at each bleeding weight, clonally-derived porcine satellite cells were plated at approximately 8000 cells per 16 mm diameter gelatin-coated culture well in Minimum Essential Medium containing 10% fetal bovine serum and antibiotics (MEM-10%

FBS). Cells were allowed to attach for 24 hours, then washed with MEM to remove residual FBS, and placed in MEM containing 6% porcine serum obtained by catheter. Commercial porcine serum served as the control. Cells were given fresh treatment media every 48 hours, and cultures were terminated after 76 hours of treatment. Cells were washed once with PBS and stored at -20° C. Proliferation was assessed using a fluorometric DNA assay as described by West et al. (1985). Serum mitogenic activity (SMA) of each pig was determined in three independent experiments for each bleeding weight, expressed relative to the commercial serum control, and ranked relative to all other pigs, for each experiment. Relative rank was evaluated for consistency across experiments, and pigs that did not maintain a consistent SMA rank across at least two experiments were not evaluated further. One cause for inconsistency was serum clotting upon thawing. This phenomenon was associated with a dramatic loss in mitogenic activity.

Correlations were calculated between SMA and lean gain per day, loin eye area, semitendinosus weight, and percentage muscle, and tested against the null hypothesis $\rho=0$.

Primary Satellite Cells

Primary satellite cells isolated from semitendinosus muscles, as described by Doumit and Merkel (1992), were plated at .5 gram equivalents per 16 mm diameter gelatin-coated well in MEM-10% FBS and grown for 96 hours with one media change after 48 hours. Cultures were terminated after 96 hours of

treatment by washing them once in PBS and fixation in 1.5 ml absolute methanol. Cells were stained with Giemsa and counted microscopically in ten random fields per well. Parallel cultures were grown to confluence and placed in MEM-2% FBS for 4 days to induce fusion. Fusion was also assessed microscopically. Alternatively, cells were plated in 10 cm diameter gelatin-coated dishes at clonal density (.05 grams per dish) in MEM-10% FBS. Cells were grown for 12 days, then placed in fusion medium (MEM-2% FBS) for 4 days. Cultures were then washed once with PBS, fixed in absolute methanol and stained with Giemsa. Colonies evaluated microscopically were not counted if they displayed obvious cell overgrowth which is characteristic of fibroblasts. Colonies were grouped by nuclei number (<50, 50-100, 100-200, and >200 nuclei per colony). The number of colonies within a size range was divided by the total number of colonies to obtain the percentage of colonies representing each range. These percentages were used to calculate correlations with lean gain per day, loin eye area and semitendinosus weight, and percentage muscle.

Statistical Analysis

Data in figures 1 and 2 were analyzed with the general linear models program of the statistical analysis system (SAS), and means were separated by a Bonferonni t-test. Correlations were calculated using the CORR statement of SAS and tested against the null hypothesis $\rho=0$.

RESULTS

A summary of carcass muscling indicators and SMA for each pig slaughtered is given in Table 1. Preliminary experiments with catheter serum (data not shown) indicated that differences in SMA between pigs could be maximized by using a serum concentration higher than half maximal (MEM-3%). Therefore, MEM-6% serum was used. The half maximal concentration was established using commercial porcine serum (Figure 1), which stimulated a dose-dependent increase in proliferation. MEM alone did not maintain cell viability. SMA is expressed as a percentage of proliferation induced by MEM-6% commercial porcine serum. SMA of nine pigs was greater ($P<.001$) at 40 kg than at 90 kg (Figure 2). Correlation coefficients were calculated between carcass muscling indicators and several in vitro proliferative indices. Correlations were tested against the null hypothesis $\rho=0$. Since sample size may have been a limiting factor in obtaining statistically significant correlations, P values of .20 or less are presented. Table 2 shows correlations between SMA at both bleedings, and carcass muscling indicators. LGD ($R=.82$; $P<.02$) and STW ($R=.72$; $P<.05$), were positively correlated with SMA-90. Figures 3 and 4 show the regression of LGD and STW, respectively, on SMA-90. The magnitude of SMA reduction

TABLE 1. MUSCLING INDICATORS AND SERUM MITOGENIC ACTIVITIES

PIG #	LGD GRAMS	ADJ. LEA CM^2	ADJ. STW GRAMS	PERCENT MUSCLE	SMA-40[a]	SMA-90[a]
1	275	40.0	409	57.7	NA	NA
2	205	31.0	274	51.8	84.1	56.7
3	266	29.0	381	53.9	NA	70
4	227	29.0	411	51.3	68.0	NA
5	234	29.0	404	52.2	63.5	70.7
6	133	26.5	317	48.5	60.4	NA
7	164	40.6	394	57.1	81.8	71.5
8	302	40.6	438	58.1	NA	72.2
9	201	32.3	370	52.5	66.4	56.5
10	213	28.4	349	55.2	64.8	50.4
11	167	22.6	414	51.3	81.1	55.2
12	189	35.5	346	54.9	74.6	57
13	234	34.8	387	52.6	50.4	NA
14	194	32.3	373	55.2	60.9	NA
15	238	31.6	382	52.7	70.7	64.6
16	179	26.5	306	51.9	51.9	63.1

[a]serum mitogenic activity is expressed as a percentage of commercial porcine serum mitogenic activity
NA=serum not available

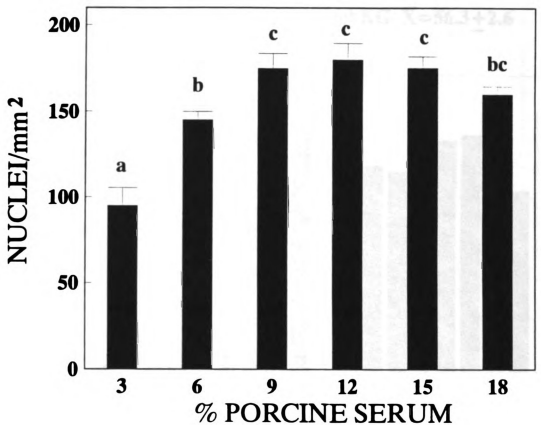


Figure 1. Commercial porcine serum dose response. Clonally-derived porcine satellite cells were plated in MEM-10% FBS and allowed to attach for 24 hours. Cells were then washed in MEM and treated with MEM containing increasing concentrations of porcine serum. Treatment media was changed once after 48 hours, and cells were fixed after 96 hours in treatment. Cells were fixed in absolute methanol and stained with Giemsa. Means are expressed as nuclei per mm^2 ± S.E.M. Means lacking a common letter differ ($P<.05$).

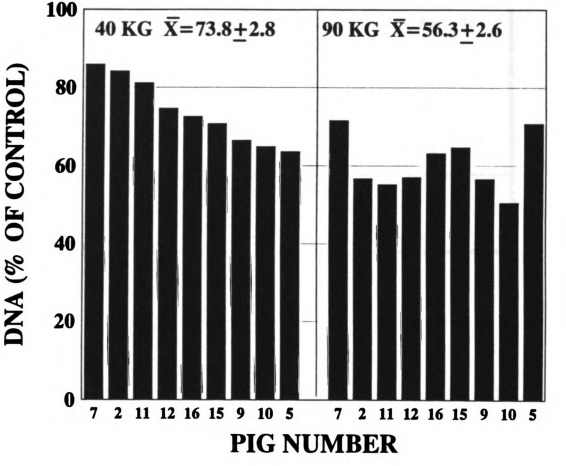


Figure 2. Serum mitogenic activities of nine pigs at 40 and 90 kg body weight. Clonally-derived porcine satellite cells were plated in MEM-10% FBS and allowed to attach for 24 hours. Cells were then washed in MEM and treatments containing MEM-6% catheter serum from each pig were applied. Media was replaced once after 48 hours and cultures were terminated after 96 hours of treatment. DNA was assayed and is expressed as a percentage of proliferation induced by MEM-6% commercial porcine serum.

TABLE 2. CORRELATION COEFFICIENTS BETWEEN CARCASS MUSCLING AND CATHETER SERUM-STIMULATED SATELLITE CELL PROLIFERATION

	PROLIFERATIVE INDEX		
MUSCLING INDEX	40 kg SERUM MITOGENIC ACTIVITY[a]	90 kg SERUM MITOGENIC ACTIVITY[b]	90 kg MINUS 40 kg[c]
LEAN GAIN PER DAY	-.06	.82(.01)	-.65(.08)
ADJ. LOIN EYE AREA	-.13	.28	-.15
ADJ. ST WEIGHT	-.05	.72(.04)	-.41
PERCENTAGE MUSCLE	.01	.20	-.03

() indicates probability R = 0
[a]n=12 (pigs 2,4-6,9-16)
[b]n=8 (pigs 2,3,5,8-10,12,15)
[c]n=8 (pigs 2,5,9-12,15,16)

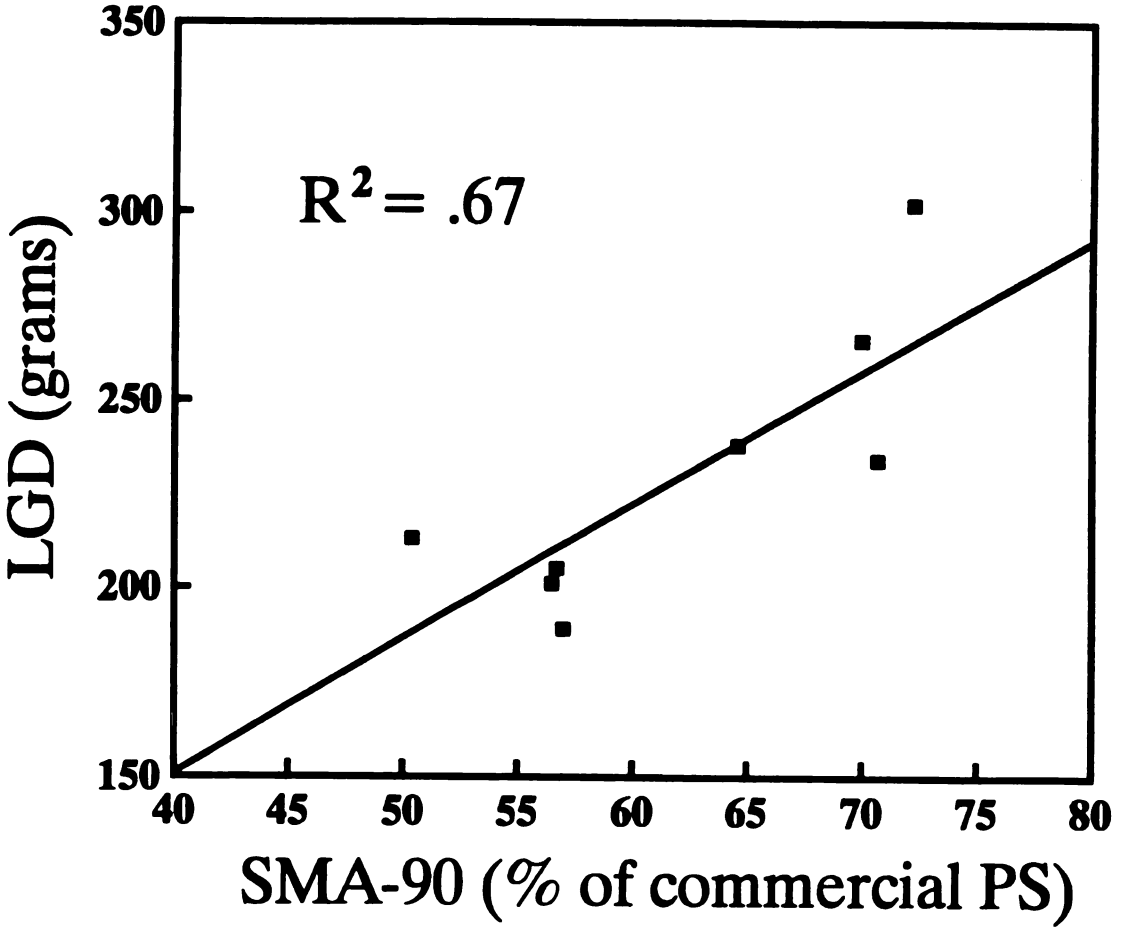


Figure 3. Regression of LGD on SMA-90. SMA-90 of eight pigs was determined as described in materials and methods, and plotted against LGD.

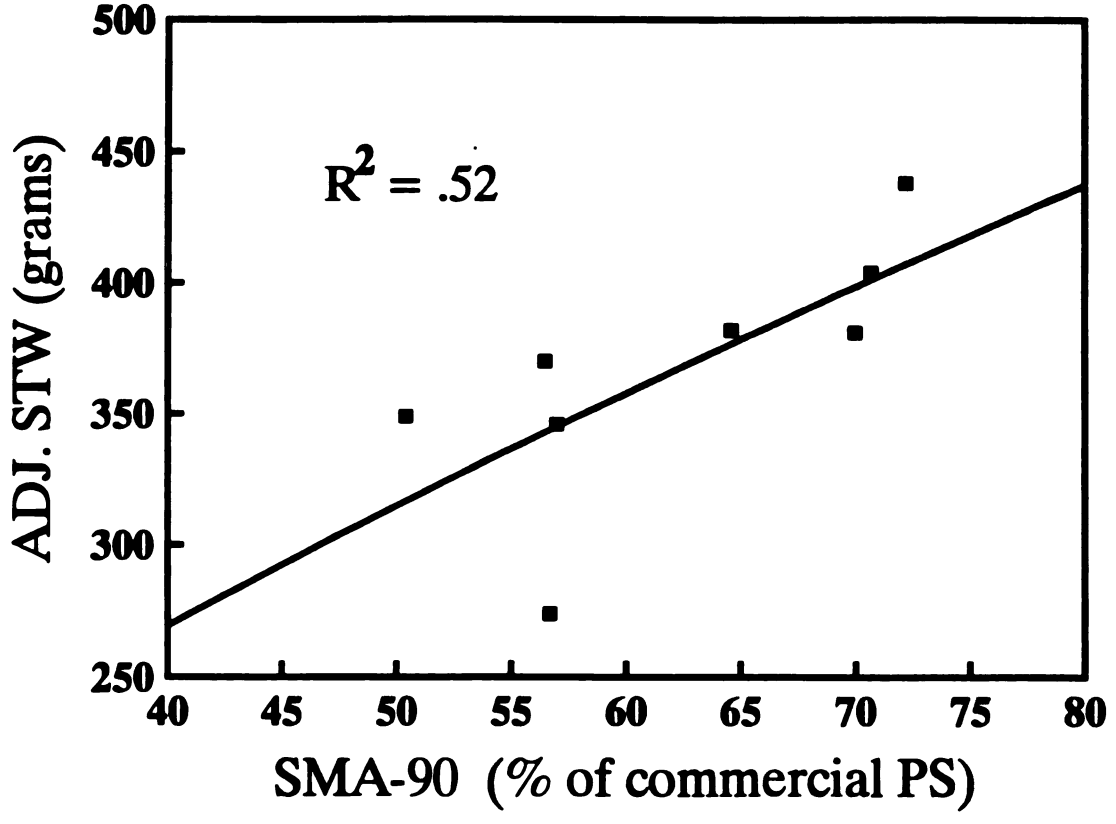


Figure 4. Regression of STW on SMA-90. SMA-90 of eight pigs was determined as described in materials and methods, and plotted against STW.

between 40 kg and 90 kg body weight was negatively correlated (R=-.65; P<.09) with lean gain per day. To determine whether inherent proliferative capacity differences existed between satellite cells from different pigs, primary cultures were established from 10 pigs which were divergent in muscling. Table 3 presents correlations of percentage fusion and myotube nuclei per mm^2 in mass culture, and carcass muscling. Fusion percentages tended to be higher in cultures derived from pigs with high LGD (R=.53; P<.12), and large LEA, (R=.44; P<.20). However, correlations between myotube nuclei and carcass muscling were lower. Figures 5 and 6 show regressions of LGD and LEA on fusion percentage.

Myogenic cell proliferation also was assessed by a second method. Cells derived from pigs divergent in muscling were plated at clonal density onto 10 cm dishes and grown for 12 days. Fusion was then induced for four days. Table 4 shows correlation coefficients between the percentage of colonies in a given size range and carcass muscling indicators. No significant relationships were observed.

TABLE 3. CORRELATION COEFFICIENTS BETWEEN CARCASS MUSCLING AND PRIMARY SATELLITE CELL PROLIFERATION AND DIFFERENTIATION IN MASS CULTURE

	PROLIFERATIVE INDEX	
MUSCLING INDEX	FUSION PERCENTAGE[a]	MYOTUBE NUCLEI PER mm^{2a}
LEAN GAIN PER DAY	.53(.12)	.30
ADJ. LOIN EYE AREA	.44(.20)	.30
ADJ. ST WEIGHT	.33	.15
PERCENTAGE MUSCLE	.29	.10

() indicates probability R = 0
[a]n=10 (pigs 1,3,6,8,11-16)

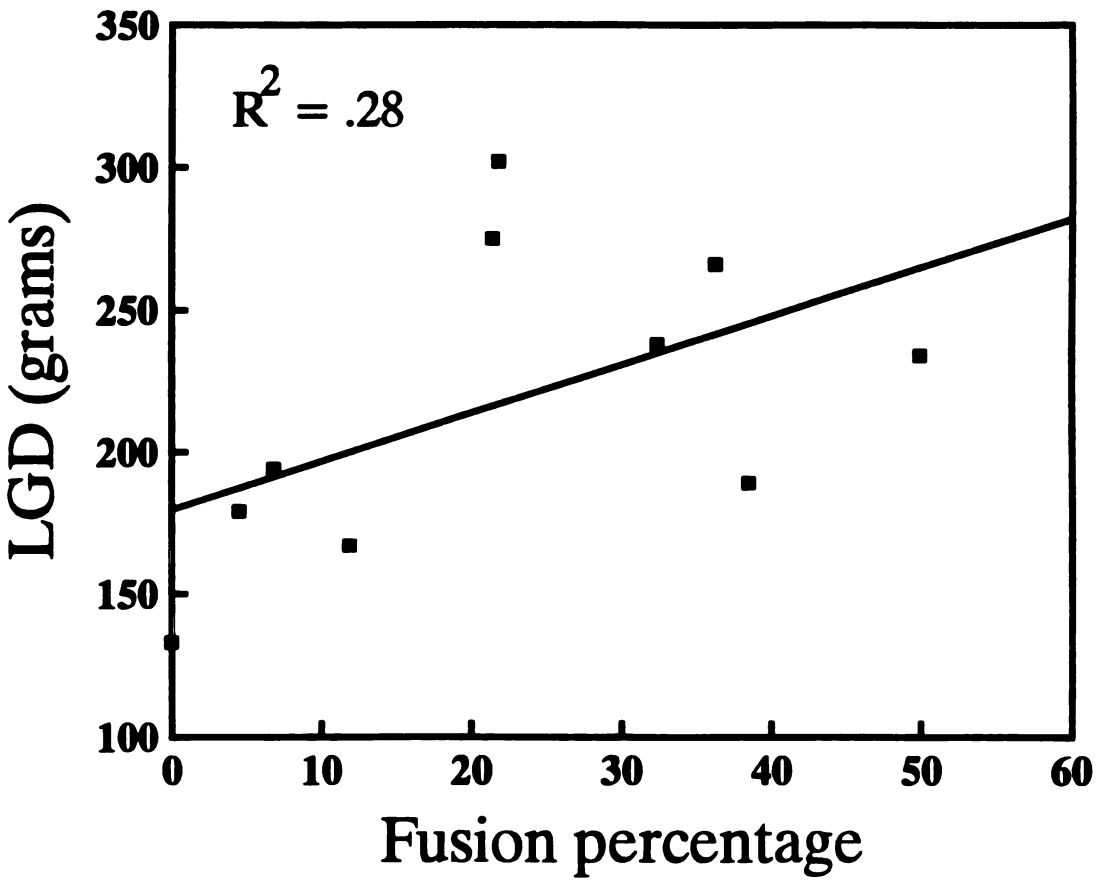


Figure 5. Regression of LGD on fusion percentage. Fusion percentages of primary cultures derived from 10 pigs divergent in muscling were determined as described in materials and methods, and plotted against LGD.

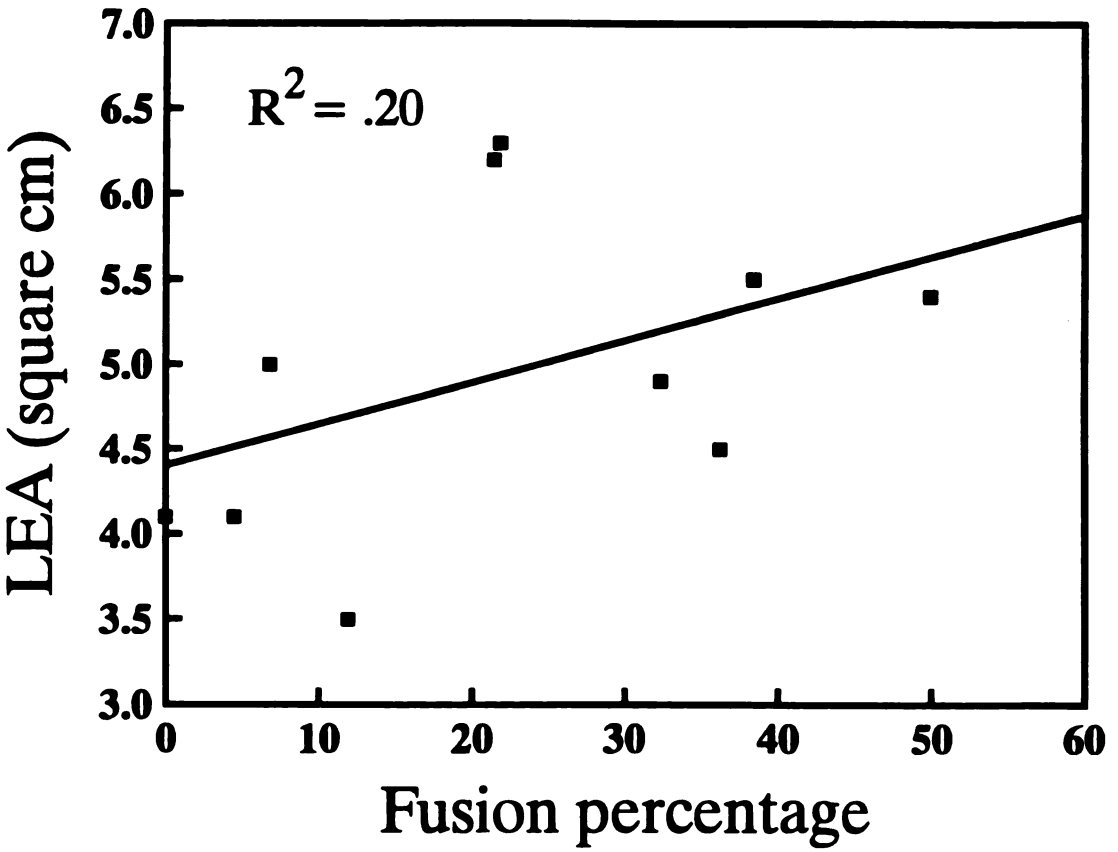


Figure 6. Regression of LEA on fusion percentage. Fusion percentages of primary cultures derived from 10 pigs divergent in muscling, were determined as described in materials and methods, and plotted against LEA.

TABLE 4. CORRELATION COEFFICIENTS BETWEEN CARCASS MUSCLING AND SATELLITE CELL PROLIFERATION AT CLONAL DENSITY

	COLONY SIZE			
MUSCLING INDEX	<50[a]	50-100[a]	100-200[a]	>200[a]
LEAN GAIN PER DAY	.17	-.04	-.18	-.17
ADJ. LOIN EYE AREA	-.12	.14	.13	.05
ADJ. ST WEIGHT	-.02	.30	-.08	-.08
PERCENTAGE MUSCLE	.11	.10	-.13	-.19

[a]n=10 (pigs 1,3,6,8,11-16)

DISCUSSION

Muscularity in swine is directly related to nuclear content of muscle fibers (Ezekwe and Martin, 1975; Powell and Aberle, 1975; Harbison et al., 1976; Buhlinger et al., 1978;). This relationship also has been documented in mice (Robinson and Bradford, 1969; Aberle and Doolittle, 1976; Martin et al., 1979) indicating it is a highly conserved mechanism of muscle growth. Taken together, these studies demonstrate that selection for rapid growth, large body size, or total muscle mass produces animals which possess more DNA per muscle than unselected, slower growing, or lighter muscled controls. Satellite cells are the source of postnatal DNA accumulation in skeletal muscle tissue (Allen et al., 1979). Therefore, differences in satellite cell proliferative activity and fusion properties are likely responsible for divergent muscle growth.

To obtain a representative serum sample over time from each pig, catheters were surgically implanted into each pig and blood was collected every two hours. This also alleviated problems associated with snaring pigs which causes extreme excitation, and likely alters hormonal status. Therefore, samples which more accurately reflect a natural physiologic state were obtained. However, inherent difficulties with this

method, such as blood clotting in the catheters, and pigs pulling out catheters, prevented sample collection from all pigs. In preliminary experiments, mitogenic activity of serum collected at slaughter was compared to catheter SMA from four pigs. Serum collected at slaughter promoted greater satellite cell proliferation than catheter serum (data not shown). This is consistent with the finding that commercial porcine serum stimulated greater proliferation than catheter serum. A possible reason for this is that excitation at slaughter may alter concentrations of hormonal factors important for satellite cell proliferation.

Allen et al. (1982) demonstrated that serum mitogenic activity decreased with increasing age in heavy and light muscled pigs. Our results agree with these data as shown in Figure 2. Average SMA of nine pigs decreased 24% ($P<.001$) from 40 kg to 90 kg body weight. These data also are consistent with findings of Purchas et al. (1985) who reported that satellite cell proliferative activity was greater in younger mice than in older mice. In addition, Swatland (1977) observed an age-dependent decrease in nuclear accumulation of pig skeletal muscle.

Allen et al. (1982) found that serum from heavily muscled pigs stimulated greater myogenic cell proliferation in vitro than serum from lighter muscled pigs. Results of the current study agree, showing that SMA-90 was positively correlated with LGD ($R=.82$; $P<.02$) and STW ($R=.72$; $P<.05$) (Table 2). These data support the hypothesis that sera from heavy muscled

pigs stimulates greater satellite cell proliferation than sera from light muscled pigs, and thus provides heavy muscled pigs more cellular machinery to synthesize protein.

Allen et al. (1982) detected consistently greater SMA of heavy muscled pigs than light muscled pigs, before 100 days of age. Beyond 100 days of age, no consistent differences in SMA were detected. However, data from our study indicate that SMA-40 was not correlated with any measure of muscularity examined. Possible reasons for this discrepancy include the following: Selection over more than one generation for muscle growth may inadvertently produce animals which have a higher serum mitogen concentration than unselected controls at a young age, but not at older ages. The current study, however, did not utilize two distinct lines of pigs. Instead, pigs from the MSU swine herd representing a broad range of muscularity were obtained. In this scenario, young pigs may be growing at similar rates and possess similar concentrations of serum mitogens. Lighter muscled pigs may have an earlier drop in SMA while heavier muscled pigs maintain SMA longer. This theory is supported by the observation that LGD was negatively correlated ($R=-.65$; $P<.09$) with a loss in SMA between 40 and 90 kg.

Purchas et al. (1985) demonstrated that proliferative activity of satellite cells is greater in heavy muscled mice than in lighter muscled mice. However, whether differences in proliferation were due to inherent characteristics of satellite cells, or differences in circulating mitogens was

not investigated. In addition to calculating correlation coefficients between SMA and carcass muscling, satellite cells isolated from pigs in the current study, were used to determine whether inherent differences existed in proliferative capacities, and whether these differences were correlated with carcass muscling. In mass primary cultures, myotube nuclei per mm^2 were used an indicator of myogenic cell proliferation. No significant relationships were found between carcass muscling and myotube nuclei. This lack of significant correlations should be interpreted with caution, however, since fusion percentages ranged from 0 to 50% between cultures of 10 pigs studied. Low fusion percentages could be interpreted as a lack of myogenic cells. However, fibroblasts typically formed colonies which were easily identified after staining due to their characteristic overgrowth. Cultures lacking myotube formation contained no more of these colonies than cultures which readily formed myotubes. Therefore, cultures which lacked myotube formation probably did not lack myogenic cells, but rather lacked the ability to fuse. Therefore, determination of myogenic cell proliferation between cultures with different fusion percentages is difficult, and should not be over interpreted.

In addition to using primary satellite cells grown in mass culture, proliferation potential was assessed using primary cultures plated at clonal density. Cells were plated in 10 cm dishes, grown for 12 days and induced to fuse. The wide range of fusion percentages observed in mass culture was

also evident at clonal density. Nearly 100% of colonies from some pigs formed myotubes, while no fusion was observed in any of the colonies from other pigs. Again, fibroblast overgrowth was not symptomatic of non-fusing cultures. Colonies which displayed typical fibroblast characteristics were not evaluated. Colonies were grouped by size and correlation coefficients were calculated between carcass muscling and the percentage of colonies in a given size range. Using this method, no significant correlations were found. Taken together, these proliferation data, obtained with two independent methods, suggest that inherent differences in satellite cell proliferation potentials do not play a major role in determining pig muscularity. Rather, SMA appears to be a more likely source of variation responsible for divergent muscling in pigs. It should be noted that both methods used to evaluate myogenic cell proliferation have limitations. Myotube nuclei per mm^2 underestimates the total number of myogenic cells due to a lack of fusion in some cultures. In cultures with the greatest fusion percentage observed, (50%) myogenic cell number is probably still underestimated because 100% of colonies from that same pig gave rise to myotubes at clonal density. The second method (evaluating colony size at clonal density) may overestimate satellite cell number. The lack of fusion and absence of fibroblastic overgrowth in some cultures suggests that many cells were myogenic but lacked critical factors necessary for fusion. However, the possibility that fibroblast colonies were included with

myogenic colony evaluation cannot be ruled out.

To determine whether differentiation was related to carcass muscling, correlation coefficients were calculated between fusion percentage and carcass muscling traits. LGD and LEA were weakly correlated with fusion percentage, $R=.53$ ($P<.12$) and $R=.44$ ($P<.20$), respectively. These data suggest that satellite cells from heavier muscled pigs possess a greater propensity to differentiate than cells from lighter muscled pigs. Schultz (1974) suggested that the percent incorporation of available satellite cells decreases with age in mice. Satellite cells derived from lighter muscled pigs may have lacked the ability to fuse because these pigs were earlier maturing and thus more physiologically mature. Chronological pig age had a range of 10 days, which is not likely to explain these differences.

CONCLUSIONS

1. SMA-90 was positively correlated with LGD and STW, while SMA-40 was not correlated with carcass muscling in this study. The magnitude of reduction in SMA between 40 and 90 kg was negatively correlated with carcass muscling indicating that a loss in SMA, may in part be responsible for early maturity and light muscling in pigs.

2. Differences in myogenic cell proliferation in this study were not correlated with carcass muscling. However, wide variation in apparent fusion capabilities between pigs makes interpretation of these data difficult.

3. Fusion percentage tended to be correlated with LGD. This may reflect inherent differences between satellite cells from pigs divergent in lean growth rate. Differences in physiological pig maturity could also explain these findings.

CHAPTER TWO

EFFECTS OF THE BETA-ADRENERGIC AGONIST RACTOPAMINE, THE ADENYLATE CYCLASE ACTIVATOR FORSKOLIN, AND THE PKA INHIBITOR HA1004, ON THE PROLIFERATION OF CLONALLY-DERIVED PORCINE SATELLITE CELLS

ABSTRACT

The objective of this study was to characterize the effect of ractopamine [1-(4-hydroxyphenyl-2-(1-methyl-3-(4-hydroxyphenyl)propylamino)ethanol], a beta-adrenergic agonist, on proliferation of clonally-derived porcine satellite cells grown in serum-free and serum-containing media. Satellite cells were isolated from the semimembranosus muscle of an 8-wk-old pig, cloned using a cloning-ring technique, and expanded to the fourth passage. Cells were plated at 5000 cells per 16 mm gelatin-coated well in Minimum Essential Medium containing 10% fetal bovine serum (MEM-10%FBS) and allowed to attach for 24 hours. Cells were then washed once in MEM, and porcine serum (PS) or serum-free media treatments were applied every 24 hours for 72 hours. Proliferation was assessed using a fluorometric DNA assay. Ractopamine (10^{-5} to 10^{-10}M) had no effect ($P>.05$) on proliferation of cells grown in MEM-2% commercial PS. Ractopamine also had no effect ($P>.05$) on proliferation of satellite cells grown in media containing porcine serum collected from pigs divergent in muscling. In basal serum-free medium, ractopamine increased ($P<.01$) proliferation in a dose-dependent manner. However, refinements to the serum-free medium abolished this response. In serum-free medium, ractopamine enhanced ($P<.01$) the

individual mitogenic actions of platelet-derived growth factor-BB (PDGF), giving a maximal response at 10^{-8}M. Ractopamine also enhanced IGF-I and FGF-induced DNA synthesis. Thirty minute pretreatment of satellite cells with ractopamine had no effect on PDGF-induced proliferation. During a 72 hour treatment period, continuous exposure to ractopamine enhanced PDGF-induced proliferation more than 24 hour exposure, and more than exposure during the first and last 24 hours only. The effect of ractopamine on PDGF-induced proliferation was blocked ($P<.01$) by the beta-antagonist propranolol (10^{-7}M). Dibutyrl cAMP had no effect ($P>.05$) on PDGF-induced proliferation, or proliferation in basal serum-free medium. Forskolin (10^{-5}M), a direct activator of adenylate cyclase, had no effect ($P>.05$) on satellite cell proliferation in basal serum-free medium, but mimicked the effect of ractopamine by stimulating ($P<.01$) proliferation induced by PDGF. The cAMP-dependent protein kinase (PKA) inhibitor HA1004, (10^{-4}M to 10^{-7}M) slightly depressed PDGF-induced proliferation, and blocked ($P<.01$) the forskolin effect on PDGF-induced proliferation. Using a new lot of PS, 10^{-8}M ractopamine, and 10 ng/ml PDGF, individually stimulated ($P<.01$) satellite cell proliferation. In combination, their effect was greater than additive. These results demonstrate that the beta-adrenergic agonist ractopamine enhances PDGF-induced DNA synthesis in cultured porcine satellite cells, and suggest a role for cAMP in myogenic cell proliferation. The effect of ractopamine on DNA synthesis in MEM-2% PS appears to be PS lot-specific.

INTRODUCTION

Muscle satellite cells proliferate and subsequently fuse with existing muscle fibers thereby increasing DNA content of skeletal muscle postnatally (Allen et al., 1979). DNA content of muscle is directly related to muscle mass (Robinson and Bradford, 1969; Ezewke and Martin, 1975; Powell and Aberle, 1975; Aberle and Doolittle, 1976; Harbison et al., 1976; Buhlinger et al., 1978; Martin et al., 1979). Heavy muscled animals have greater satellite cell proliferative activity than light muscled animals (Purchas et al., 1985), and serum from heavy muscled animals promotes greater myogenic cell proliferation in vitro than serum from light muscled animals (Allen et al., 1982). Myogenic cell proliferation is controlled by many factors, including polypeptide growth factors. Clonally-derived porcine satellite cells respond mitogenically to insulin-like growth factors-I and -II (IGF-I and -II), basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF-BB), and epidermal growth factor (EGF) (Doumit et al., 1993a). These mitogens are likely physiologic regulators of porcine satellite cell proliferation in vivo. Enhancing peptide growth factor activity represents a potential means of increasing satellite cell proliferation to greatly increase muscle growth in pigs.

Beta-adrenergic agonists stimulate muscle accretion when fed to rats, mice, and several livestock species (reviewed by Yang and McElligott, 1989). Muscle DNA accretion does not appear to be a prerequisite for beta agonist-induced muscle growth. However, some studies have demonstrated increased muscle DNA in beta agonist-treated animals compared to controls (Beermann et al., 1987; Skjaerlund et al., 1993). This suggests that beta agonists may directly, or indirectly affect satellite cell proliferation in vivo. Young et al. (1988) reported that cimaterol had no effect on myotube nuclei in chicken muscle cell cultures. However, Grant et al. (1990) found that ractopamine increased chick embryo myoblast proliferation in serum-containing medium. These conflicting reports have not been resolved.

Beta-adrenergic receptor stimulation leads to increased intracellular cAMP concentrations (Gilman, 1987). Cyclic AMP is important for DNA synthesis in 3T3 cells (Rozengurt, 1986). However, a definitive role for cAMP in myogenic cell proliferation has not been established. The purpose of this study was to characterize the response of clonally-derived porcine satellite cells, grown in serum-free and serum-containing media, to the beta agonist ractopamine. The effects of ractopamine, and the direct adenylate cyclase activator forskolin, on peptide growth factor mitogenicity in serum-free medium were studied. A possible second messenger pathway also was examined.

MATERIALS AND METHODS

Materials

Minimum Essential Medium (MEM), antibiotic/antimycotic, gentamicin, Deutsch fetuin, porcine serum (PS) and human recombinant IGF-I, bFGF, PDGF-BB and EGF were purchased from Gibco (Grand Island, NY). Additional porcine sera was collected at the MSU Swine farm from six pigs divergent in lean tissue growth rate. Pure crystalline ractopamine and the peptide growth factors mentioned above were a generous gift from Lilly Research Laboratories (Greenfield, IN). Pig skin gelatin, MCDB-110, bovine serum albumin (BSA), bovine transferrin, bovine insulin, dexamethasone, Hoechst 33258 reagent, calf thymus DNA, Forskolin, Propranolol and HA1004 were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Hazleton (Lenexa, KS), and twenty-four well culture plates were from Corning Glass Works, (Corning, NY).

Satellite cell culture

Satellite cells were isolated from the semimembranosus muscles of 8-week-old pigs as described by Doumit and Merkel (1992). Pure myogenic clones were established using a cloning ring technique, as described by Doumit et al. (1993), and one highly myogenic clone (designated M1) was used in all

experiments. Cells were plated at approximately 5000 cells per 16 mm diameter gelatin coated culture well in MEM-10% FBS and antibiotics (50 units penicillin, 50 μg streptomycin, .13 μg fungizone, and 10 μg gentamicin/ml). Cells were allowed to attach for 24 hours before treatments were imposed, and fresh media were supplied every 24 hours thereafter for 72 hours, unless otherwise specified. After the attachment period, cells were washed in MEM to remove residual FBS before treatments were applied. Cultures were maintained in a humidified, CO_2 incubator (95% air and 5% CO_2) at 37°C. Basal serum-free medium initially consisted of MEM:MCDB-110 in a 4:1 ratio, .5 mg/ml BSA, 100 μg/ml transferrin, 62.5 μg/ml Deutsch fetuin, 10^{-7} M dexamethasone, 10^{-10} M insulin, and antibiotics. Subsequent experiments were conducted without fetuin but with water-soluble linoleic acid (.5 μg/ml). A complete discussion of this serum-free medium will appear in a forthcoming manuscript (Doumit et al., manuscript in preparation).

Stock solutions of ractopamine, propranolol, dibutyrl cAMP forskolin, and HA1004 were made in the following manner: ractopamine was dissolved in MEM (.1 or 1.0 mg/ml); propranolol was also dissolved in MEM (1.0 mg/ml); dibutyrl cAMP was dissolved in absolute ethanol (10 mg/ml); forskolin was dissolved in dimethylsulfoxide (DMSO) (10mg/ml); and HA1004 was dissolved in 95% ethanol, 5% water (10mg/ml). Ethanol and DMSO were tested in culture at the highest concentrations used and had no effect on proliferation in basal media, PDGF-containing media, and media containing PDGF

and forskolin.

Proliferation was assessed using a fluorometric DNA assay as described by West et al. (1985). Fluorescence was measured using a Varian CARY 2200 spectrophotometer with fluorescence attachment. Calf thymus DNA served as standard. Data were analyzed using general linear model procedures of the Statistical Analysis System (SAS, 1985). Data were blocked by experiment, and least-squares means for each treatment were separated based on least significant differences.

RESULTS

Ractopamine (10^{-5} to 10^{-9}M) had no effect (P>.05) on porcine satellite cell proliferation in MEM-2% PS (Figure 1). Ractopamine also did not affect (P>.05) proliferation of satellite cells grown in media containing 6% porcine serum obtained from heavy and light muscled pigs (Figure 2). In basal serum-free medium, ractopamine (10^{-6} to 10^{-10}M) stimulated (P<.05) proliferation with a maximal effect at 10^{-8}M (Figure 3). However, after removal of fetuin from the serum-free medium, ractopamine had no effect on proliferation in basal serum-free medium. Proliferation induced by PDGF (Figure 4), IGF-I (Figure 5), or FGF (Figure 6) was enhanced (P<.03) by ractopamine. The concentrations of growth factors used in these experiments induced maximal proliferation (Doumit et al., 1993). Of the three growth factors studied, the magnitude of ractopamine response was consistently greatest with PDGF. The numerically maximal response was observed at 10^{-8}M ractopamine. This is the approximate concentration found in the bloodstream of pigs consuming 20 ppm ractopamine (personal communication with Dr. D.B. Anderson, Lilly Research Laboratories). Therefore, 10^{-8}M ractopamine was used in subsequent experiments. To determine whether ractopamine increases the sensitivity of satellite cells to PDGF, PDGF was

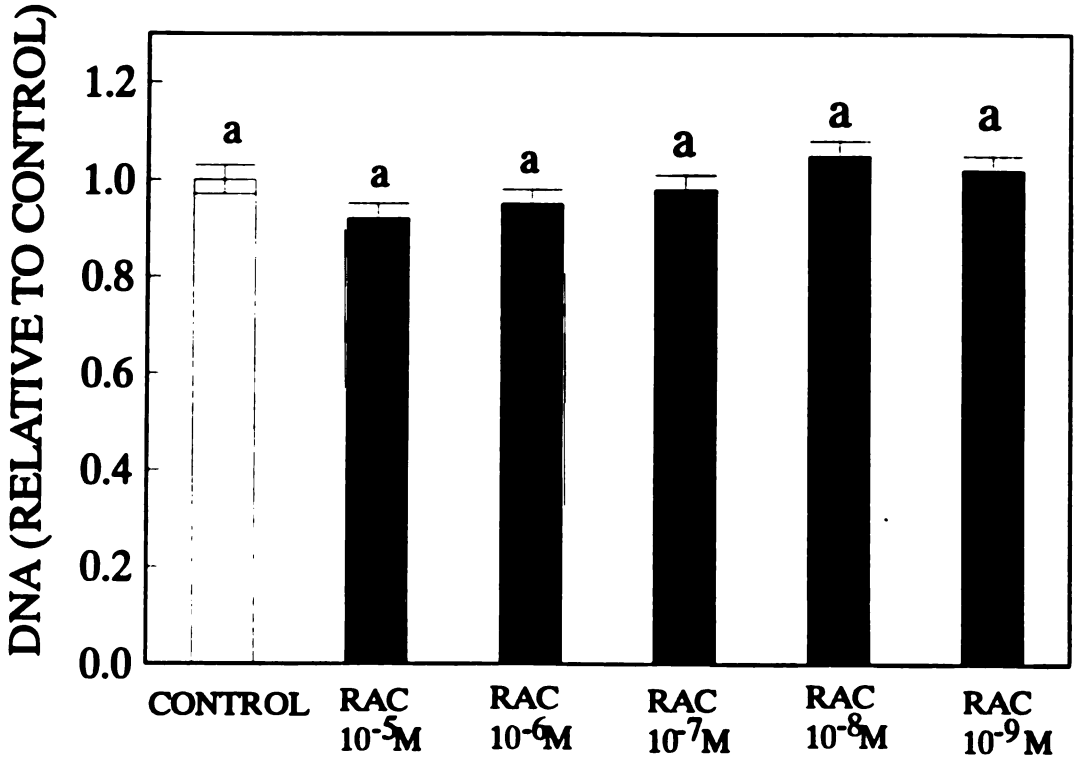


Figure 1. Ractopamine dose response in MEM-2% PS. Satellite cells were treated with MEM-2% PS and increasing concentrations of ractopamine. Treatments were supplied fresh every 24 hours for 72 hours, and DNA was assayed as described in materials and methods. Bar represent means ± S.E.M. of eight replicate wells expressed relative to MEM-2%PS. Means with a common letter do not differ (P>.05).

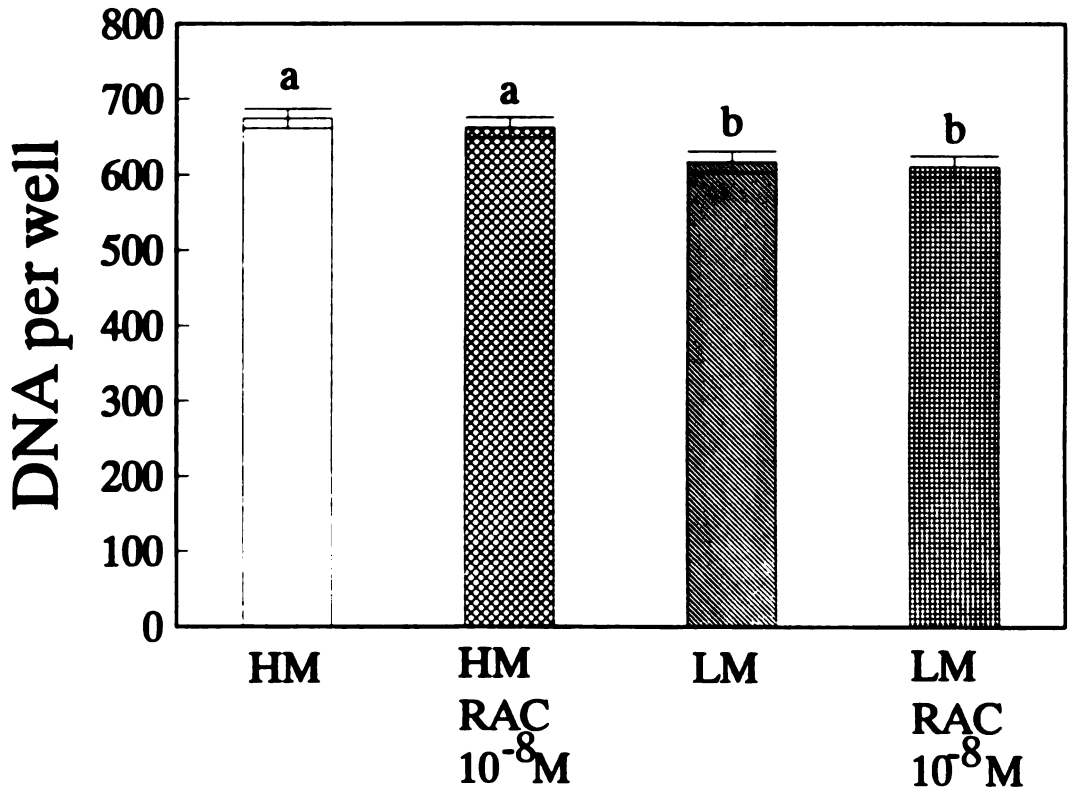


Figure 2. The effect of 10^{-8}M ractopamine on proliferation induced by serum obtained from heavy and light muscled pigs. Two sera pools were formed representing three pigs with a high lean tissue accretion rate, and three pigs with a low lean tissue accretion rate. Cells were grown in MEM-6% serum with and without ractopamine. Cultures were fed every 24 hours for 48 hours and DNA was assayed as described in materials and methods. Bars represent means of four replicate wells ± S.E.M. Means lacking a common letter differ ($P<.05$).

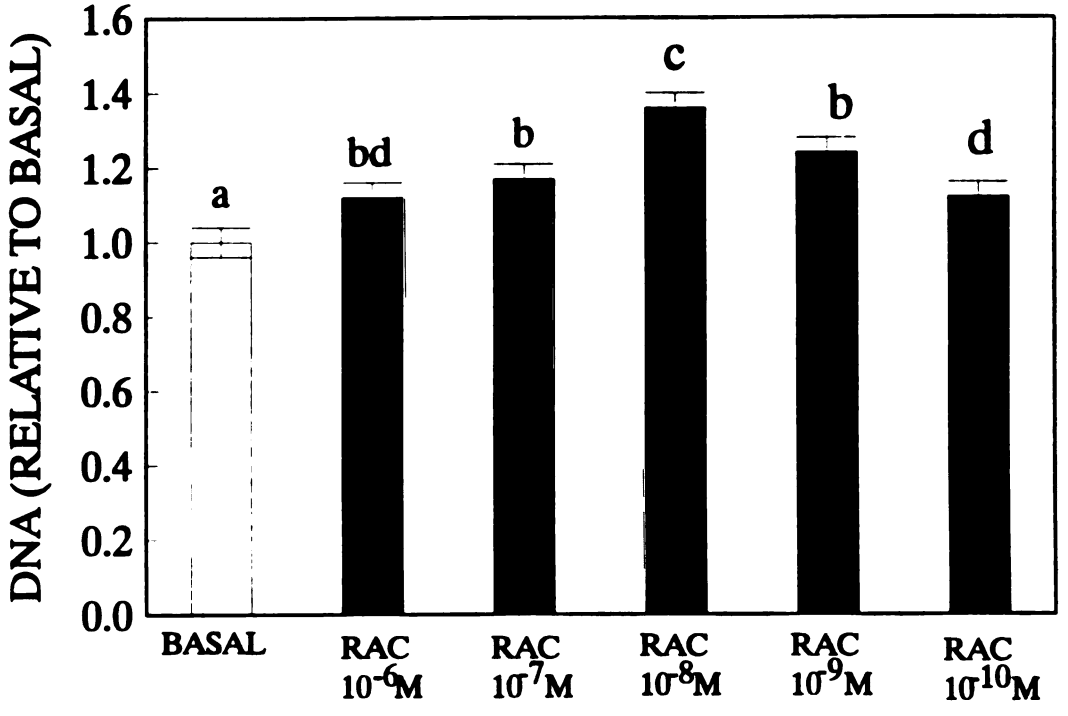


Figure 3. Ractopamine dose-response in basal serum-free medium containing fetuin. Cells were fed basal serum-free medium with increasing concentrations of ractopamine every 24 hours for 72 hours. DNA was assayed as described in materials and methods. Bars represent means of eight replicate wells ± S.E.M. are expressed relative to the basal serum-free medium control. Means lacking a common letter differ ($P<.05$).

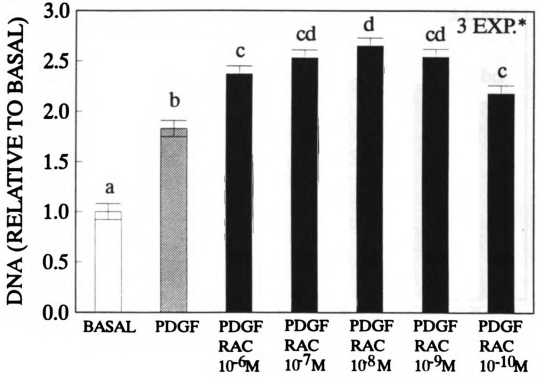


Figure 4. Ractopamine dose-response on PDGF-induced DNA synthesis. Ractopamine (10^{-6} to 10^{-10}M) was added to serum-free medium containing 10 ng/ml PDGF. Treatments were applied every 24 hours for 72 hours and DNA was assayed as described in materials and methods. Bars represent means of 12 replicate wells ± S.E.M. expressed relative to basal serum-free medium. Means lacking a common letter differ ($P<.03$). Asterisk indicates treatment by experiment interaction.

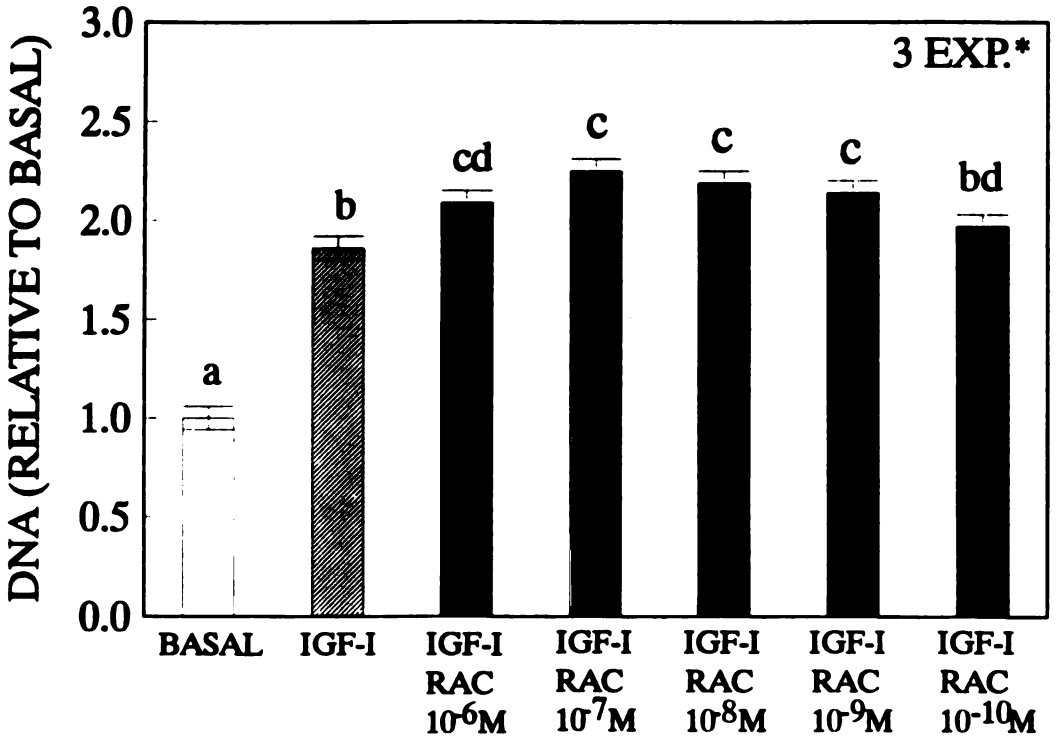


Figure 5. Ractopamine dose-response on IGF-induced DNA synthesis. Ractopamine (10^{-6} to 10^{-10}M) was added to serum-free medium containing 50 ng/ml IGF-I. Treatments were applied every 24 hours for 72 hours and DNA was assayed as described in materials and methods. Bars represent means of 12 replicate wells ± S.E.M. expressed relative to basal serum-free medium. Means lacking a common letter differ ($P<.05$). Asterisk indicates treatment by experiment interaction.

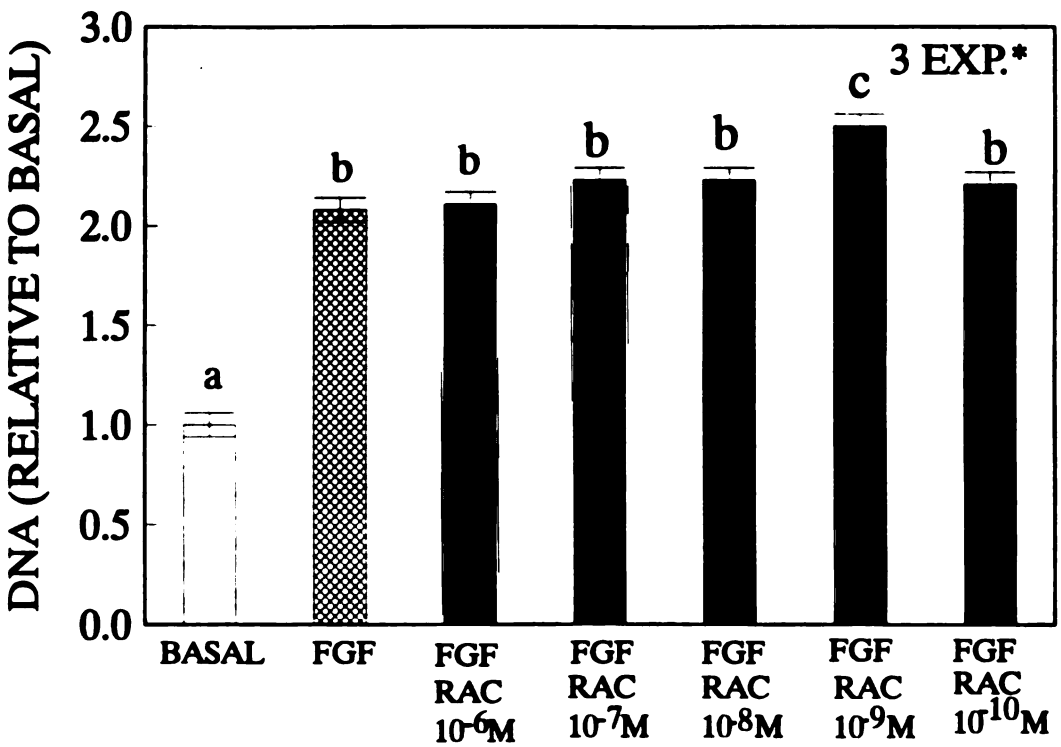


Figure 6. Ractopamine dose-response on FGF-induced DNA synthesis. Ractopamine (10^{-6} to 10^{-10}M) was added to serum-free medium containing 10 ng/ml FGF. Treatments were applied every 24 hours for 72 hours and DNA was assayed as described in materials and methods. Bars represent means of 12 replicate wells ± S.E.M. expressed relative to basal serum-free medium. Means lacking a common letter differ ($P<.01$). Asterisk indicates treatment by experiment interaction.

dosed out with and without ractopamine. Figure 7 shows that ractopamine does not affect the sensitivity of satellite cells to PDGF but does increase the maximal proliferative response. To determine whether brief, preexposure to ractopamine could enhance the mitogenic activity of PDGF, cells were given ractopamine in basal serum-free medium for 30 min prior to addition of serum-free medium containing PDGF. Response to this treatment was not different ($P>.05$) from PDGF alone (Figure 8). Over a 72 hour period, PDGF-induced DNA synthesis was maximized (greater than PDGF alone $P<.05$) in cultures treated with ractopamine for 72 hours, compared to ractopamine treatment for the first 24 hours only, or ractopamine treatment for the first and last 24 hours of growth (Figure 8). To verify that ractopamine acts through beta receptors, the beta antagonist propranolol (10^{-7}M) was added simultaneously with ractopamine, and was found to block ($P<.01$) the mitogenic action of ractopamine on PDGF-induced DNA synthesis (Figure 9). To investigate whether the second messenger, cyclic adenosine monophosphate (cAMP), was involved in porcine satellite cell proliferation, dibutyrl cAMP (10^{-3} to 10^{-7}M) was added to basal serum-free medium, and serum-free medium containing 10 ng/ml PDGF. Direct addition of cAMP had no effect ($P>.05$) on proliferation in neither basal serum-free medium (Figure 10) nor in PDGF-containing medium (Figure 11). However, the direct activator of adenylate cyclase, forskolin (10^{-5} to 10^{-7}M), enhanced ($P<.01$) the mitogenic action of PDGF (Figure 12), but had no effect ($P>.05$) on proliferation in

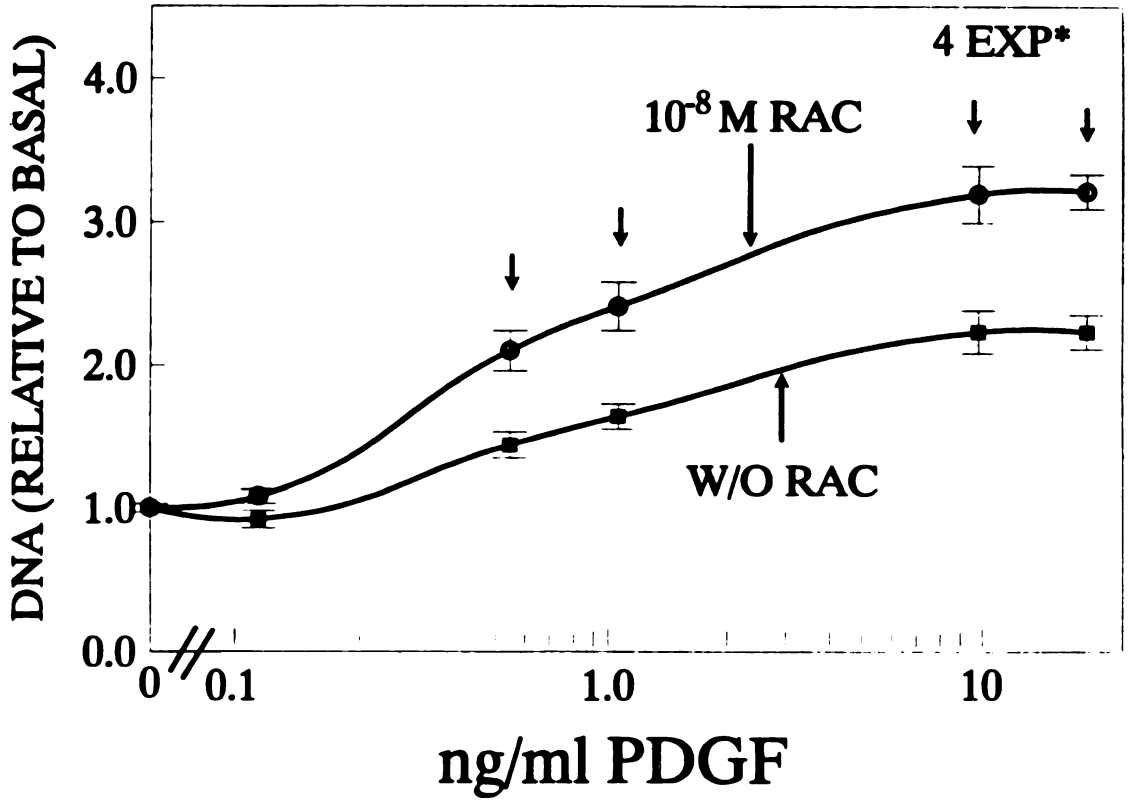


Figure 7. PDGF dose-response in serum-free media with and without ractopamine. PDGF (.1 to 20 ng/ml) was dosed out in serum-free medium alone, and in serum-free medium containing 10^{-8}M ractopamine. Treatments were applied every 24 hours for 72 hours and DNA was assayed as described in materials and methods. Small arrows over open symbols indicate ractopamine effect ($P<.05$). Asterisk indicates treatment by experiment interaction.

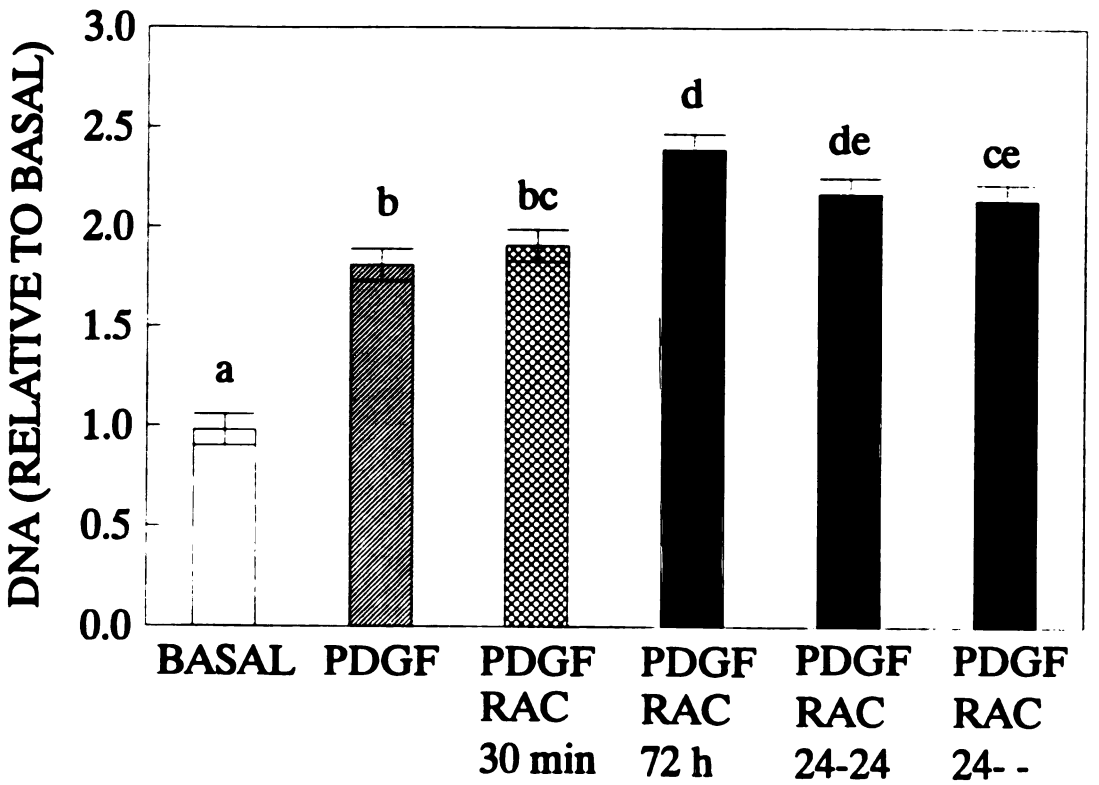


Figure 8. Effects of pre-exposure to ractopamine, and ractopamine exposure time on PDGF-induced DNA synthesis. After cell attachment, cultures were washed in MEM and cells were exposed to 10^{-8}M ractopamine in basal serum-free medium for 30 min. Cells were then washed again, and serum-free medium containing 10 ng/ml PDGF was applied every 24 hours for 72 hours. Alternatively, cultures were fed serum-free medium containing 10 ng/ml PDGF for 72 hours, and 10^{-8}M ractopamine was included for 24 hours, 72 hours, or the first 24 and last 24 hours. DNA was assayed as described in materials and methods, and is expressed relative to basal serum-free medium. Bars represent means ± S.E.M. of 12 replicate wells. Means lacking a common letter differ ($P<.05$).

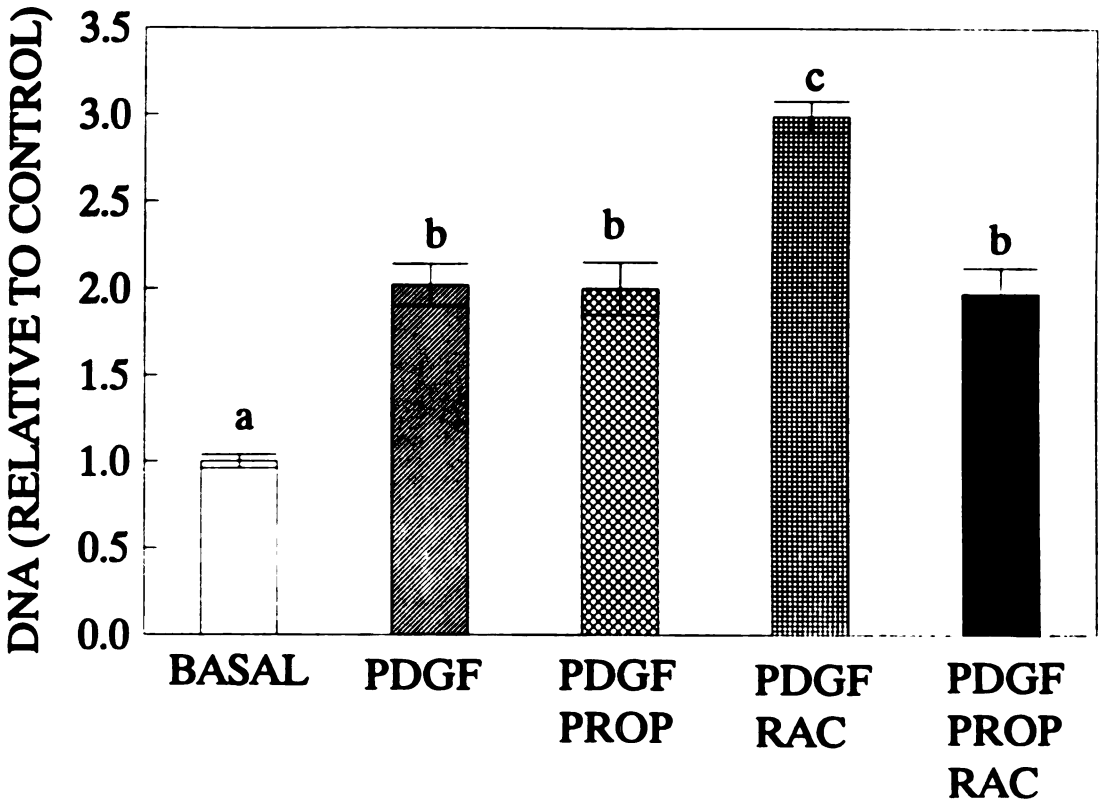


Figure 9. The effect of a beta antagonist, propranolol, on proliferation induced by PDGF and ractopamine. Propranolol (10^{-7}M) was included in serum-free media containing 10 ng/ml PDGF, or PDGF and 10^{-8}M ractopamine. Treatments were applied every 24 hours for 72 hours. DNA was assayed as described in materials and methods and is expressed relative to basal serum-free medium. Bars represent means ± S.E.M. of eight replicate wells. Means lacking a common letter differ ($P<.01$).

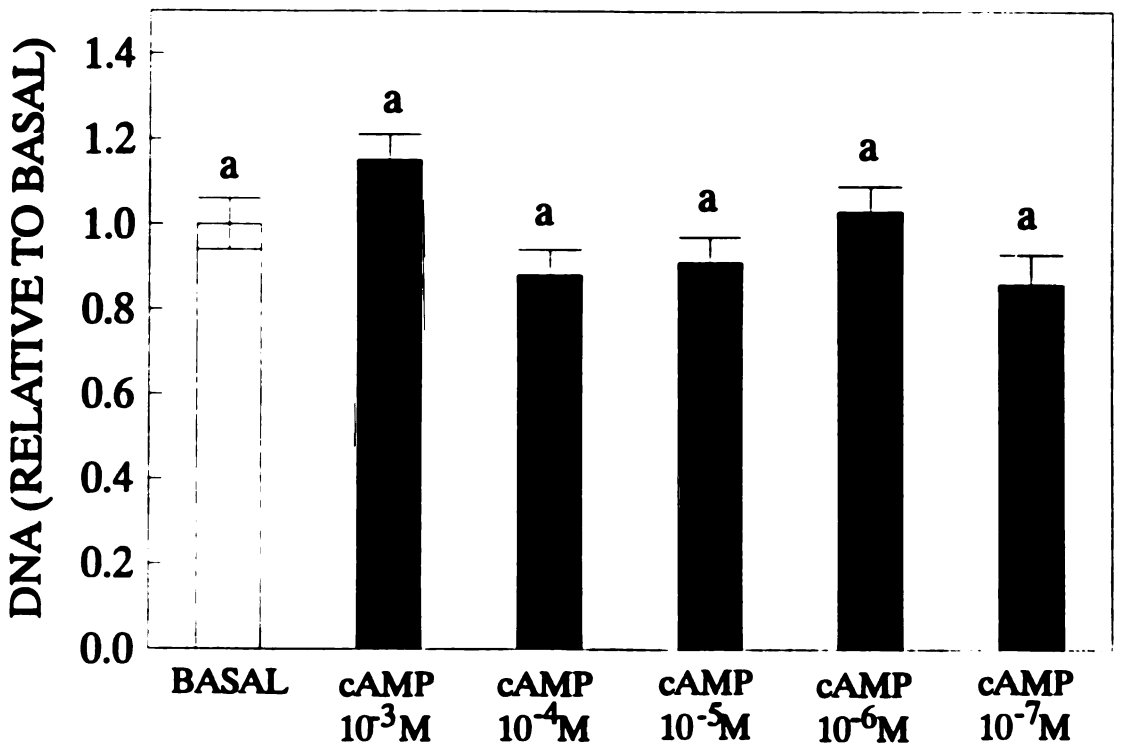


Figure 10. Effect of dibutyrl cAMP on DNA synthesis in basal serum-free medium. Cyclic AMP (10^{-3} to 10^{-7}M) was included in basal serum-free medium. Treatments were applied every 24 hours for 72 hours and DNA was assayed as described in materials and methods. Bars represent means ± S.E.M. of four replicate wells. Means with a common letter do not differ (P>.05).

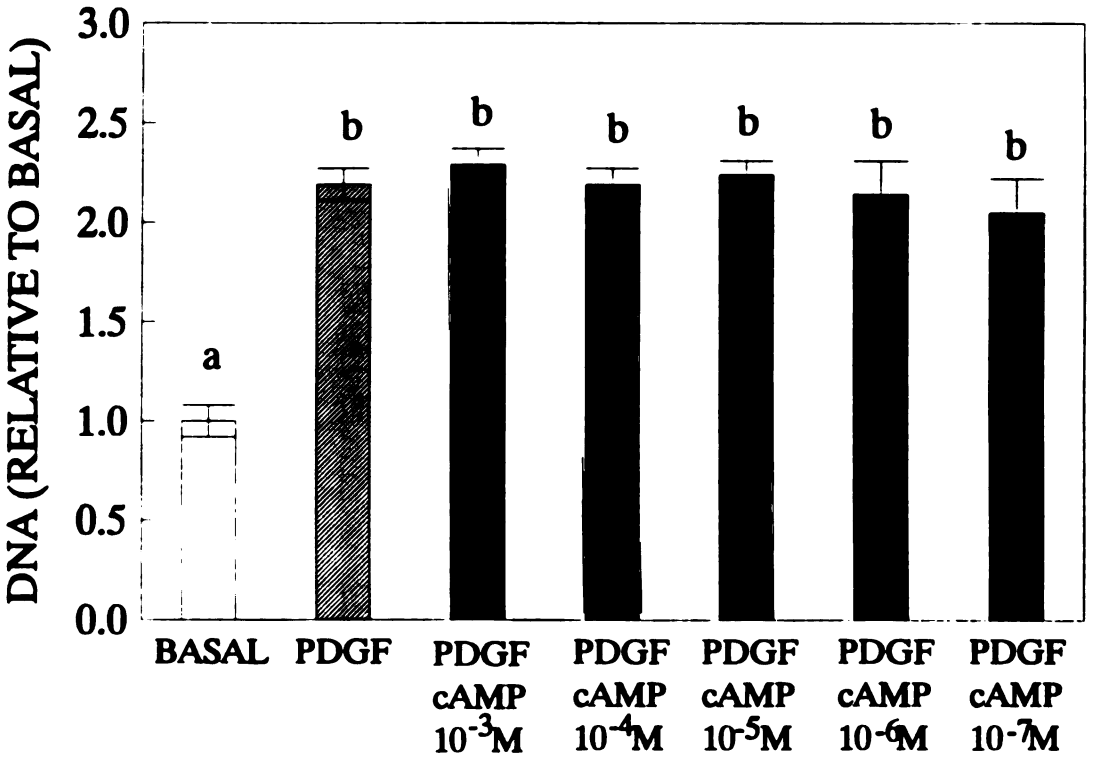


Figure 11. Effect of dibutyrl cAMP on PDGF-induced DNA synthesis. Cyclic AMP (10^{-3} to 10^{-7}M) was included in serum-free medium containing 10 ng/ml PDGF. Treatments were applied every 24 hours for 72 hours and DNA was assayed as described in materials and methods. Bars represent means ± S.E.M. of eight replicate wells. Means with a common letter do not differ (P>.05).

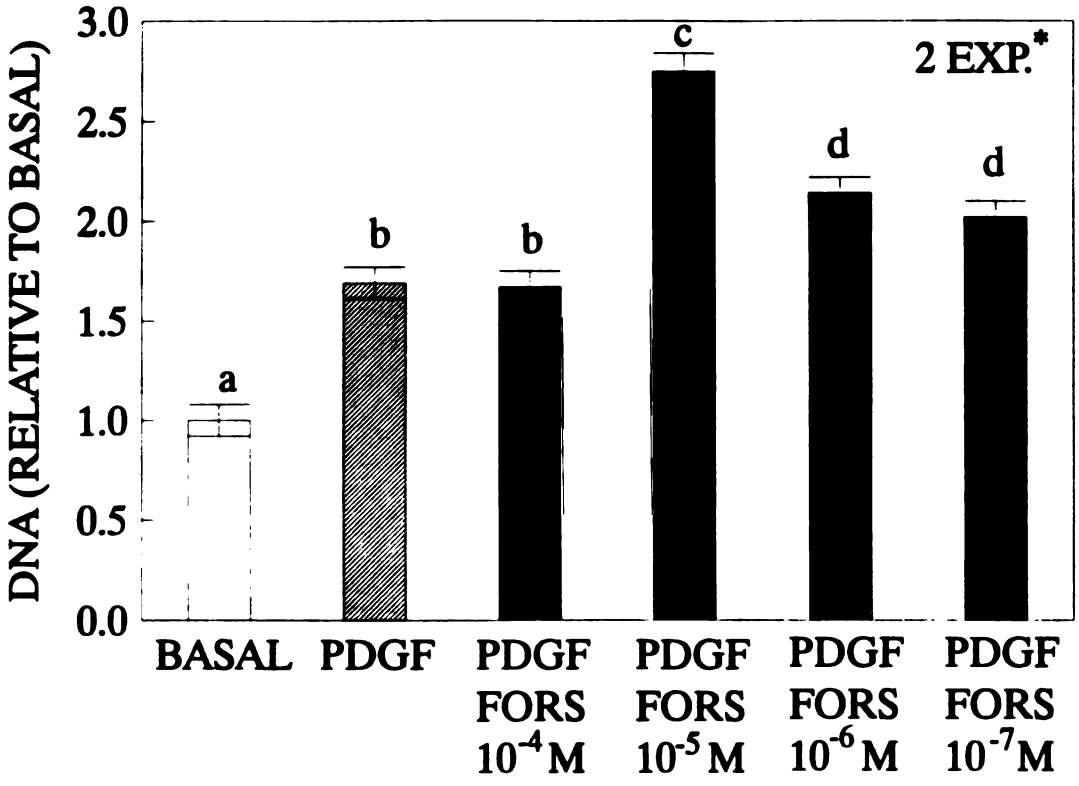


Figure 12. Effect of the adenylate cyclase activator forskolin, on PDGF-induced DNA synthesis. Forskolin (10^{-4} to 10^{-7}M) was included in serum-free medium containing 10 ng/ml PDGF. Treatments were applied every 24 hours for 72 hours and DNA was assayed as described in materials and methods. Bars represent means ± S.E.M. of eight replicate wells. Means lacking a common letter differ ($P<.01$). Asterisk indicates treatment by experiment interaction.

basal serum-free medium (Figure 13). A direct comparison of the magnitude of effect between ractopamine and forskolin is shown if Figure 14. Both ractopamine and forskolin enhanced PDGF-induced proliferation ($P<.01$), but the magnitude of forskolin effect was greater ($P<.01$) than the ractopamine effect. Figure 15 shows that the cAMP-dependent protein kinase (PKA) inhibitor HA1004, slightly depressed PDGF-induced proliferation, and blocked the forskolin effect ($P<.01$) on PDGF-induced proliferation (Figure 16). Using a new lot of PS, 10^{-8}M ractopamine, and 10 ng/ml PDGF, individually stimulated ($P<.01$) satellite cell proliferation (Figure 17). In combination, their effect was greater than additive.

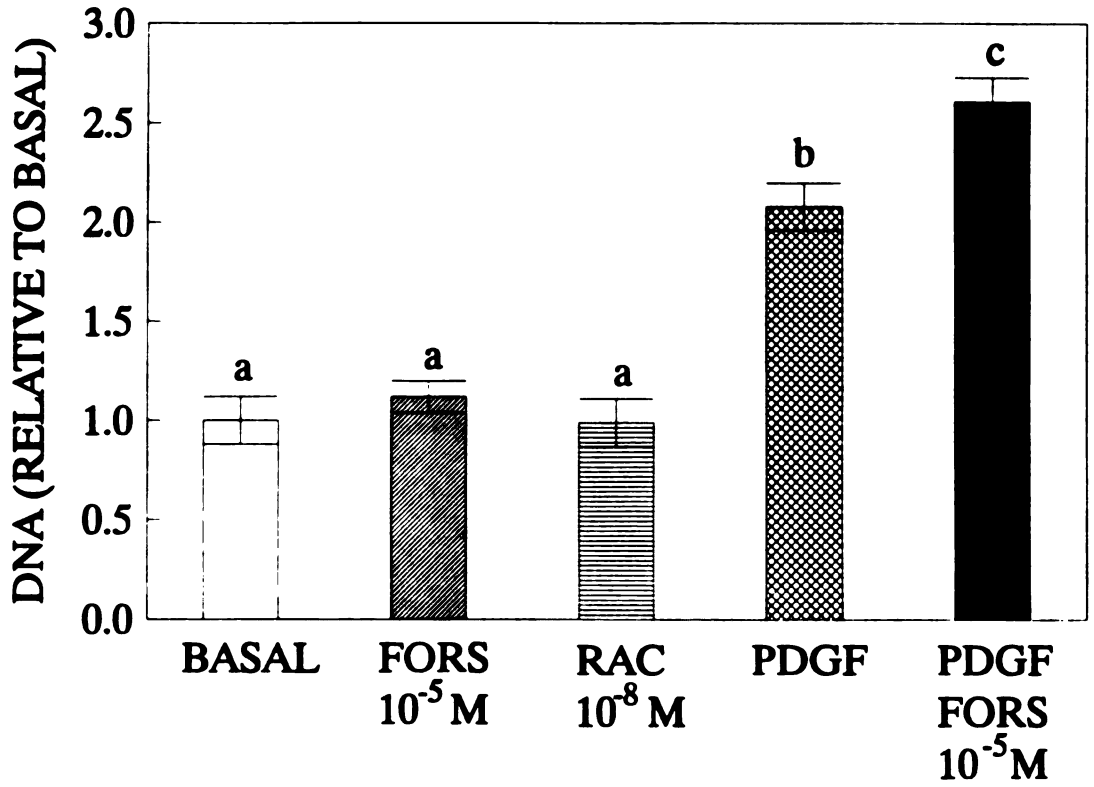


Figure 13. Effects of ractopamine and forskolin on porcine satellite cell proliferation in basal serum-free medium. Ractopamine (10^{-8}M) and forskolin (10^{-5}M) were added to basal serum-free medium and cells were fed every 24 hours for 72 hours. A forskolin positive control is also shown in PDGF-containing media. DNA was assayed as described in materials and methods and is expressed relative to basal media. Bars represent means ± S.E.M. of eight replicate wells.

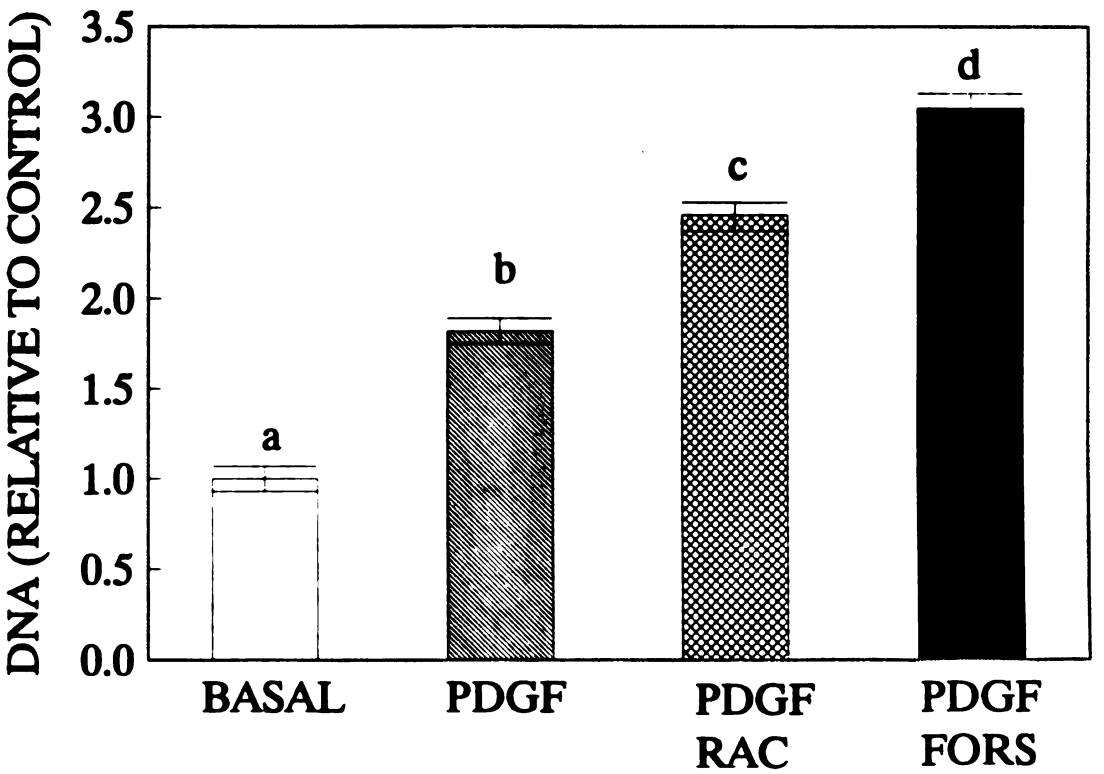


Figure 14. Effects of ractopamine and forskolin on porcine satellite cell proliferation induced by PDGF. Ractopamine (10^{-8}M) and forskolin (10^{-5}M) were added to serum-free medium containing 10 ng/ml PDGF, and cells were fed every 24 hours for 72 hours. DNA was assayed as described in materials and methods and is expressed relative to basal media. Bars represent means ± S.E.M. of eight replicate wells. Means lacking a common letter differ ($P<.01$).

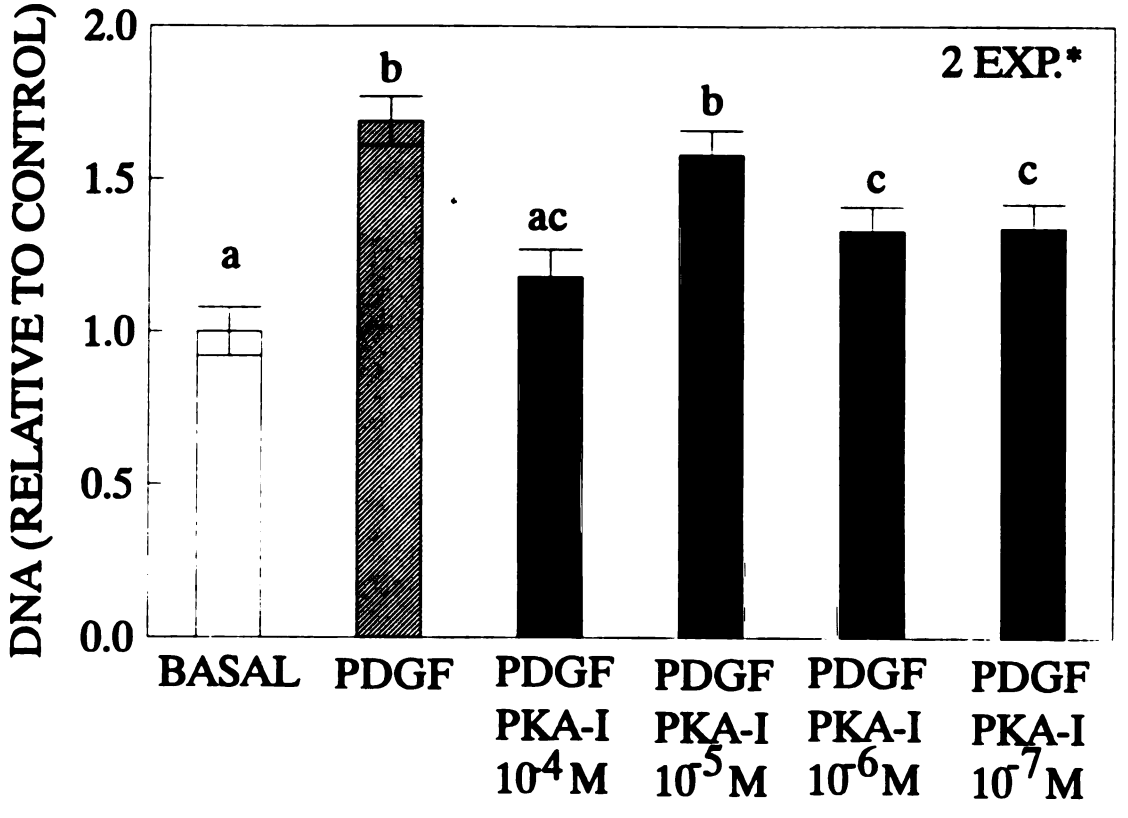


Figure 15. Effects of the protein kinase A inhibitor HA1004, on PDGF-induced proliferation. HA1004, (10^{-4}M to 10^{-7}M), was included in serum-free medium containing 10 ng/ml PDGF. Cells were fed every 24 hours for 72 hours, and DNA was assayed as described in materials and methods. Bars represent means ± S.E.M. of eight replicate wells expressed relative to basal serum-free medium. Means lacking a common letter differ ($P<.05$). Asterisk indicates treatment by experiment interaction.

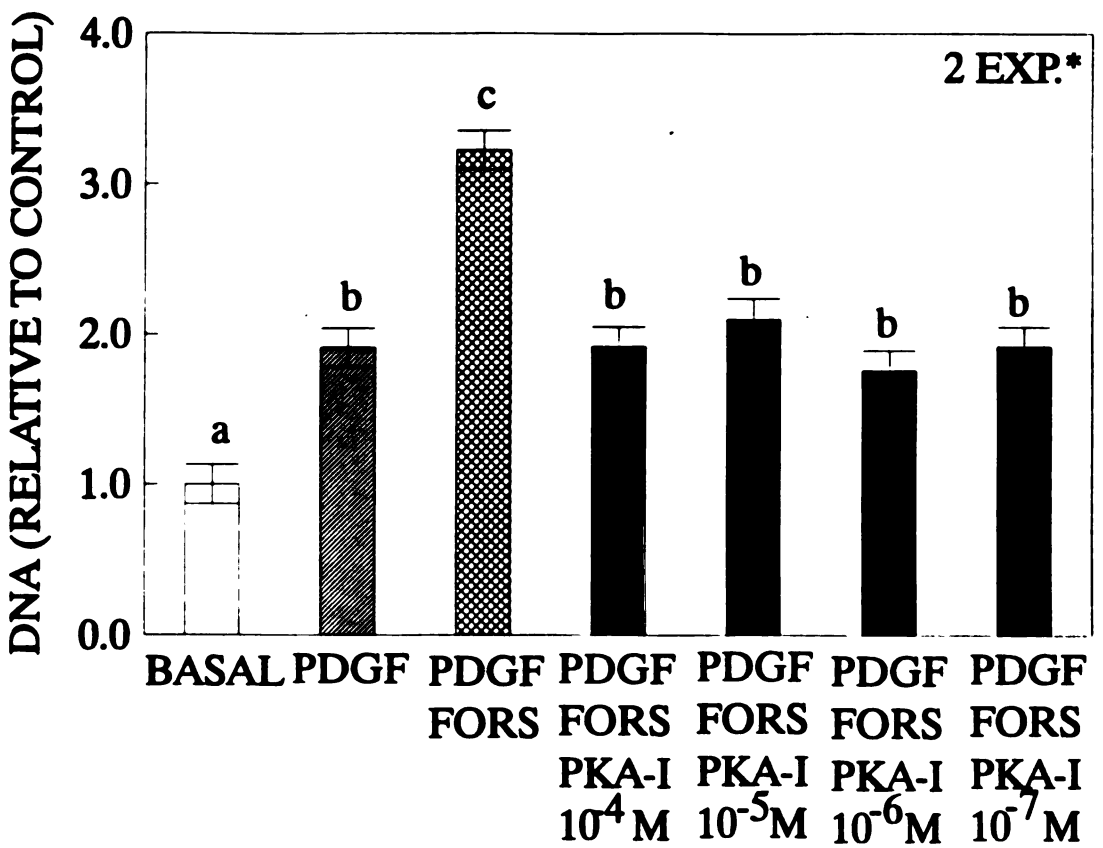


Figure 16. Effects of the protein kinase A inhibitor HA1004, on forskolin-enhanced proliferation. HA1004, (10^{-4}M to 10^{-7}M), was included in serum-free medium containing 10 ng/ml PDGF, and 10^{-5}M forskolin. Cells were fed every 24 hours for 72 hours, and DNA was assayed as described in materials and methods. Bars represent means ± S.E.M. of 12 replicate wells, with the exception of 10^{-4}M HA1004 in which the mean of eight wells is presented. Data are expressed relative to basal serum-free medium. Means lacking a common letter differ ($P<.05$). Asterisk indicates treatment by experiment interaction.

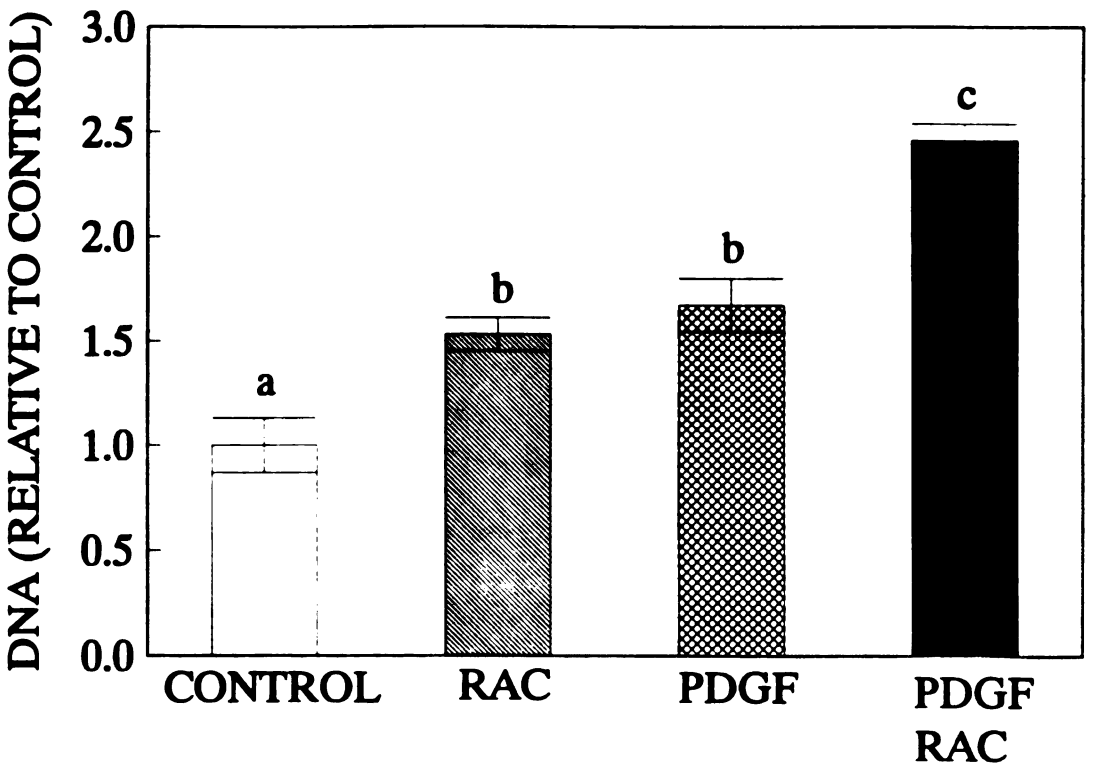


Figure 17. The effects of ractopamine and PDGF on proliferation in MEM-2% PS using a new lot of PS. Satellite cells grown in MEM-2% PS were treated with 10^{-8}M ractopamine and 10 ng/ml PDGF, individually and in combination. Treatments were supplied fresh every 24 hours for 72 hours, and DNA was assayed as described in materials and methods. Bar represent means ± S.E.M. of eight (MEM-2% PS with and without PDGF) or 16 (ractopamine treated) replicate wells expressed relative to MEM-2%PS. Means lacking a common letter differ (P<.01).

DISCUSSION

Skeletal muscle DNA accretion is directly related to muscularity (Allen et al., 1979). Beta agonist-induced muscle growth involves alteration of protein turnover (reviewed by Yang and McElligott, 1989). However, conflicting reports exist regarding beta agonist effects on skeletal muscle DNA accretion. Kim et al. (1987), and Beermann et al. (1987) reported that lambs fed cimaterol for 8 and 7, weeks respectively, had decreased muscle DNA concentrations compared to controls. Johnson (1987) observed similar responses in pigs fed ractopamine for 1 week. In lambs fed cimaterol for 12 weeks, however, Beermann et al. (1987) reported increased muscle DNA content compared to controls. Skjaerlund et al. (1993) reported similar DNA content per muscle findings in pigs fed ractopamine for 4 weeks. In vitro, Young et al. (1988) reported that cimaterol had no effect on myotube nuclei in embryonic chicken muscle cell cultures. However, ractopamine has been shown to increase proliferation of cultured chick embryo myoblasts (Grant et al., 1990). The results of the present study indicate that the proliferative response of porcine satellite cells to ractopamine may depend on the particular lot of PS used. However, no response to ractopamine was observed when satellite cells were grown in

media with two separate sera pools representing pigs with high and low lean tissue growth capacity. Discrepancies between and among the present study and other in vitro studies may represent differences in culture conditions, or species based differences. Furthermore, the vast array of serum components acting on cells grown in vitro makes serum containing media a difficult growth medium in which to study specific mitogenic effects.

In basal serum-free medium containing fetuin, ractopamine stimulated a dose-dependent increase in proliferation of porcine satellite cells. However, removal of fetuin from the serum-free medium abolished this effect. Nie et al. (1991) reported that the growth promoting effects of fetuin could be completely separated from fetuin itself. In the present study, it is likely that ractopamine was interacting with a contaminant of fetuin to increase DNA synthesis.

Ractopamine enhanced the mitogenicity of PDGF, IGF-I, and FGF, although the maximal response occurred at different concentrations for each peptide. The effect on PDGF-induced DNA synthesis was greater than that of IGF-I or FGF, and was therefore investigated further. These initial ractopamine dose-response experiments in serum-free medium containing one growth factor, were performed in media containing fetuin and thus are somewhat confounded. However, all serum-free media experiments subsequent to Figure 6, were performed without fetuin, and demonstrate that ractopamine does have a true effect on PDGF-induced proliferation. Figure 7 shows that

ractopamine enhances the mitogenic activity of .5 ng/ml PDGF at concentrations up to 20 ng/ml PDGF, but does not increase satellite cell sensitivity to PDGF. Epinephrine, acting through beta receptors, has been shown to regulate insulin binding in skeletal muscle. (Webster et al., 1986). Increased PDGF binding would likely shift the PDGF dose response-curve to the left, and therefore does not appear to be a likely mechanism of ractopamine-regulated PDGF mitogenicity. However, direct measurement of PDGF binding in response to ractopamine was not undertaken in this study.

Beta receptors undergo rapid desensitization after ligand binding (Lefkowitz and Caron, 1988). To provide insight as to whether this was occurring in our system, the exposure time to ractopamine was varied over a 72 hour PDGF-treatment. Cells exposed to ractopamine for 72 hours had the greatest proliferation. Exposure for the first 24 hours only, or the first and last 24 hours of the 72-hour treatment period had similar levels of proliferation, but less proliferation than 72 hour exposure ($P<.08$ first and last 24 hours less than 72 hours). These results suggest that the amount of signal (presumably cAMP) generated by beta receptor stimulation is greatest over a 72 hour treatment period. Beta receptor desensitization is a rapid process which occurs within 10 minutes after ligand binding, and reduces adenylate cyclase activity to 60% of maximum (Lohse et al., 1990). Thus, withdrawal of ractopamine for 24 hours would abolish desensitization while the ligand is not present. But, upon

reexposure to the ligand, receptors would elicit a maximal cAMP response for only 10 minutes and then be desensitized. It should be noted that this discussion is speculative as direct measurement of receptor desensitization was not undertaken in this study. Preexposure to ractopamine for 30 min did not alter the magnitude of PDGF-induced proliferation. Collectively, these results suggest that ractopamine is not sensitizing cells to PDGF, but is providing a complementary signal while the agonist is present.

To verify that ractopamine was acting through beta receptors, the mixed $beta_1$ and $beta_2$ antagonist propranolol (Stiles et al., 1984) was added simultaneously with ractopamine in media containing PDGF. Propranolol had no effect on PDGF-induced DNA synthesis, but completely blocked the ractopamine effect, demonstrating that ractopamine was eliciting its effects through beta-adrenergic receptors.

Beta-adrenergic receptor effects are mediated through the $G_{s\alpha}$ protein which couples the receptor to the enzyme adenylate cyclase, which in turn, catalyzes formation of the second messenger cyclic adenosine monophosphate (cAMP) (Gilman, 1987). Degradation of cAMP is catalyzed by cAMP phosphodiesterase (Beavo et al., 1982). Cyclic AMP is involved in 3T3 fibroblast proliferation (reviewed by Rozengurt, 1986) but its role in myogenic cell growth is not clear. Direct addition of 10^{-3} to 10^{-7}M dibutyrl cAMP to basal serum-free media and PDGF-containing media, had no effect on porcine satellite cell proliferation. However, a direct

activator of adenylate cyclase, forskolin (Huang et al., 1982), mimicked the ractopamine effect by enhancing PDGF-induced DNA synthesis. Forskolin had no effect on proliferation in basal serum-free medium. Reasons for the apparent ineffectiveness of dibutyrl cAMP to mimic ractopamine effects are unclear. A possible explanation is that normal cAMP production may be compartmentalized such that overwhelming the entire cell with the addition of dibutyrl cAMP may not provide the signal necessary for enhancing PDGF mitogenicity. However, Rozengurt (1982) reported that inclusion of a phosphodiesterase inhibitor in the culture media of 3T3 cells markedly potentiated the effects of cAMP elevating agents. It is possible that porcine satellite cells possess sufficient quantities of cAMP phosphodiesterase to necessitate simultaneous addition of phosphodiesterase inhibitors with cAMP analogues to prevent immediate cAMP breakdown. This phenomenon might be overcome with forskolin since it continually activates adenylate cyclase, and would provide the cell a constant supply of cAMP. Addition of cAMP every 24 hours, however, would allow substantial cAMP degradation before media replenishment.

In Swiss 3T3 cells, cAMP acts as a mitogen synergizing with serum, insulin, phorbol esters, epidermal growth factor and fibroblast-derived growth factor to stimulate DNA synthesis (Rozengurt et al., 1981). Furthermore, PDGF initiates DNA synthesis in 3T3 cells via a mechanism which involves arachidonic acid metabolism (Shier and Durkin, 1982)

and subsequent production of prostaglandin (Rozengurt et al., 1983a,b). Prostaglandin production, release from the cell, and binding to extracellular receptors results in cAMP accumulation (reviewed by Rozengurt, 1986) and initiation of DNA synthesis in 3T3 cells (Rozengurt et al., 1983a,b). Therefore, cAMP is a known factor involved with PDGF-induced DNA synthesis in 3T3 cells. It is likely that cAMP alone is not mitogenic for porcine satellite cells since 10^{-5}M forskolin had no effect on DNA synthesis. Still, it appears that cAMP enhances PDGF mitogenicity in cultured porcine satellite cells.

A direct comparison of proliferative enhancement by ractopamine and forskolin revealed that forskolin enhanced PDGF mitogenicity more than ractopamine. Beta receptors have three mechanisms of desensitization (Lohse et al., 1990): 1. phosphorylation of receptors by PKA; 2. phosphorylation by the specific agonist-dependent beta-adrenergic receptor kinase; and 3. receptor sequestration away from the cell surface. All three of these mechanisms cause rapid desensitization of the receptor-stimulated adenylate cyclase response. Forskolin, however, directly stimulates adenylate cyclase and therefore would not be subject to these desensitization mechanisms. This may explain why forskolin enhances PDGF-mitogenicity more than ractopamine.

Hidaka et al. (1984) reported that isoquinolinesulfonamides are potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C

(PKC). HA1004 was found to specifically inhibit PKA (Hidaka et al., 1984) and has recently been shown to specifically inhibit PKA activity in cultured rat satellite cells (Lagord et al., 1993). To investigate the next putative step in the cAMP signal transduction pathway, HA1004 was added to media containing PDGF, and to media containing the combination of PDGF and forskolin. The PKA inhibitor partially blocked proliferation induced by PDGF alone, indicating that PKA is involved with the mitogenic signal generated by PDGF. Whether PDGF raises cAMP concentrations in myogenic cells as it does in 3T3 cell remains to be established. It is possible that a basal level of cAMP is sufficient to generate the PKA activity associated with PDGF-induced proliferation. However, it appears likely that increased PKA activity through forskolin-induced cAMP production is responsible for the enhanced proliferative response observed in porcine satellite cells.

CONCLUSIONS

These data demonstrate that the beta-adrenergic agonist ractopamine, the adenylate cyclase activator forskolin, and dibutyrl cAMP do not affect porcine satellite cell proliferation in basal serum-free medium. Ractopamine, acting via beta-adrenergic receptors, enhances the mitogenicity of PDGF, and this effect is mimicked by forskolin, indicating it likely occurs through increases in cAMP concentrations. Furthermore, the PKA inhibitor HA1004, partially blocks PDGF-induced DNA synthesis, and fully blocks the forskolin effect on PDGF-induced DNA synthesis. Therefore, it is likely that cAMP, and PKA are important regulators of the PDGF signal transduction pathway in porcine satellite cells. Further study of these mechanisms will provide the knowledge necessary to manipulate satellite cell proliferation in vivo and increase lean tissue accretion in pigs.

OVERALL SUMMARY AND CONCLUSIONS

The data presented here indicate that the serum-mitogenic activity of 90 kg pigs is positively correlated with lean gain per day and adjusted semitendinosus weight. Serum-mitogenic activity at 40 kg body weight does not appear to be highly correlated with carcass muscling, although more pigs might be needed to detect lower, but important correlations. The inherent ability of satellite cells to proliferate does not appear to be a major factor in pig muscling, although, due to problems associated with myogenic cell identification, this theory remains to be proven unequivocally. However, differences in myogenic cell proliferation do seem to be directly related to the mitogenic activity of serum, which in turn is correlated with lean tissue accretion.

The beta agonist ractopamine, enhances the mitogenicity of platelet-derived growth factor-BB in cultured porcine satellite cells, and this effect is mimicked by the adenylate cyclase activator, forskolin. Furthermore, this response utilizes a cAMP-dependent protein kinase pathway. These findings indicate that carcass muscling is highly related to myogenic cell proliferation, and that use of beta agonists simultaneously with a mitogen, may hold great potential to influence myogenic cell proliferation in vivo.

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