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Mechanism of Action of Beta Adrenergic Agonists and Potential Residue Issues

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I.β-Adrenergic Agonist-Stimulated Muscle Hypertrophy

One of the most pronounced effects of feeding a β_2-adrenergic agonist to ruminants is the preferential increase in skeletal muscle mass and/or cross-sectional area of individual muscles. Examples of these skeletal muscle-enhancing characteristics are summarized in Table 1. Generally, addition of either clenbuterol or cimaterol, both presumed β_2-adrenergic agonists, to ruminants resulted in increased longissimus muscle cross-sectional area between 11 and 39 %. In addition, the mass of selected muscles were increased between 8 and 40 %. These data suggested that addition of these β_2-adrenergic agonists to ruminants resulted in dramatic increased muscle mass.

Muscle Fiber Hypertrophy and Fiber Types. Previous studies have investigated the effect of β_2-adrenergic agonists at the individual muscle fiber level. Many of these studies assessed relative changes in both number as well as diameter of type I vs. type II muscle fibers. Miller and colleagues (1988) reported an increase in diameter of type II muscle fibers and a numerical decrease in diameter of type I fibers in clenbuterol-fed (10 mg/hd/d) heifers. Similarly, Smith et al., (1995) observed that mean diameters of type II A muscle fibers were greater in clenbuterol-fed steers as compared to controls. In addition, similar results have been reported in lambs fed cimaterol. Kim et al., (1987) published that the proportion of type I to type II fibers in the longissimus and semitendinosus muscle were unaffected. However, the type II fibers had 50 % greater cross-sectional area in the longissimus and semitendinosus muscle of cimaterol-fed lambs compared to untreated lambs. Cimaterol had no effect on the cross-sectional area of type I fibers (Kim et al., 1987). These data from both cattle and sheep suggested that β_2-adrenergic agonist can increase individual muscle fiber cross-sectional area and the type II fiber apparently were the most responsive to the β_2-adrenergic agonist administration. However, another cimaterol-feeding study in lambs resulted in contradictory data. Beermann et al., (1987) reported that dramatic increases in hind-limb muscle mass of lambs fed cimaterol was a result of radial growth of both type I and type II fibers. These authors also found a reduction in proportion of type I fibers compared to type II fibers due to cimaterol administration. The authors concluded that hypertrophy of type II fibers contributed more to the cimaterol-induced increase in skeletal muscle mass compared to type I hypertrophy. However, the cross-sectional area of type I fibers were increased but cimaterol administration resulted in preferential increase in the number of type II fibers compared to type I fibers.

DNA Accumulation. Due to the dramatic increase in skeletal muscle hypertrophy following β_2-adrenergic agonist administration to ruminants, one would expect satellite cell proliferation and subsequent fusion of the daughter satellite cells, to provide a source of DNA to support the rapid changes in muscle mass. However, the majority of previous work suggested during the 3 to 5 weeks of β_2-adrenergic agonist stimulated muscle hypertrophy, no change in number of nuclei occurred. A constant DNA amount (nuclei number) coupled with rapid changes in muscle mass and consequently, protein accumulation results in the DNA concentration of individual muscles to be lower in β_2-adrenergic agonist-fed animals compared to untreated controls. In lambs fed cimaterol for 56 days, the DNA concentration in the longissimus and semitendinosus muscle was 50 and 35% less, respectively, as compared to control lambs (Kim et al., 1987). These data suggested that the approximately 40 % increased muscle hypertrophy during the 56 day feeding period occurred without a subsequent increase in DNA accumulation, thus suggesting little effect on proliferation and fusion of satellite cells throughout this 56 day period. Likewise, Beermann et al., (1987) reported DNA concentrations were 22 % lower in cimaterol-fed lambs after 49 days of supplementation. However, after 84 days of cimaterol exposure there was no significant differences in DNA concentrations of selected hind-limb muscles. In another study (O'Connor et al, 1991), cimaterol reduced DNA concentration in the semitendinosus muscle of lambs 42 % after 21 days of feeding. The reduction in DNA concentration was somewhat attenuated after 42 days, but was still 25 % lower in treated muscle compared to control.

Taken together, the static DNA level in skeletal muscles experiencing rapid increased muscle hypertrophy would result in reduced DNA concentrations of those muscles. In addition, rapid

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Table 1. Relative changes in individual muscle size and/or mass due to administration of a β2-adrenergic agonist to ruminants.

Species	β2-agonist	Dose	Muscle	% Change	Source
Cattle, steers	Clenbuterol	10 mg/hd/d	Longissimus area	+11	Ricks et al., 1984
Cattle, steers	Clenbuterol	500 mg/hd/d	Longissimus area	+16	Ricks et al., 1984
Cattle, steers	Clenbuterol	7 mg/hd/d	Longissimus area	+28	Shiavetta et al., 1990
Cattle, steers	Clenbuterol	7 mg/hd/d	Longissimus weight	+25	Shiavetta et al., 1990
Cattle, steers	Clenbuterol	10 mg/hd/d	Longissimus area	+16	Miller et al., 1988
Cattle, steers	Clenbuterol	10 mg/hd/d	Longissimus weight	+8	Miller et al., 1988
Sheep	Cimaterol	10 ppm	Longissimus area	+39	Kim et al., 1987
Sheep	Cimaterol	10 ppm	Gastrocnemius weight	+40	Kim et al., 1987
Sheep	Cimaterol	10 ppm	Biceps femoris weight	+33	Beermann et al., 1987
Sheep	Cimaterol	10 ppm	Semimembranosus weight	+27	Beermann et al., 1987
Sheep	Cimaterol	10 ppm	Semitendinosus weight	+32	Beermann et al., 1987
Sheep	Cimaterol	10 ppm	Biceps femoris weight	+27	O′ Connor et al., 1991
Sheep	Cimaterol	10 ppm	Semimembranosus weight	+30	O′ Connor et al., 1991
Sheep	Cimaterol	10 ppm	Semitendinosus weight	+34	O′ Connor et al., 1991

changes in muscle hypertrophy were not a result of accelerated DNA accretion from satellite cells during the first 35 days. Prolonged exposure (> 84 days) to the β_2-adrenergic agonist and the resulting muscle hypertrophy may have stimulated some satellite cell proliferation as indicated by mild increases in DNA levels. In light of previous work, during a typical 28 to 42 day β-adrenergic agonist exposure in cattle, satellite cell proliferation and subsequent fusion in the existing fiber most likely does not account for nor contribute to the rapid increased muscle hypertrophy.

Research has been conducted to evaluate the direct effects of β-adrenergic agonists on cultured muscle cell proliferation and differentiation. Ractopamine was shown to increase the number myotube nuclei in primary cultures of chicken satellite cells at the end of the culture period, suggesting rate of proliferation of the satellite was enhanced (Grant et al., 1990). However, the dose required to elicit this response in muscle satellite cell proliferation would be considered greater than normal physiological levels expected after feeding ractopamine. Likewise, pharmacological doses of ractopamine (10 μ*M*) stimulated rate of C_2C_{12} myoblast proliferation approximately 30% (Shappell et al., 2000). Our preliminary data has shown no effect of zilpaterol on cell proliferation of cultured muscle satellite cells as indicated by [^{3}H]-thymidine incorporation. These equivocal cell culture results coupled with the lack of significant increased DNA content in muscles from β-adrenergic agonist -treated animals, suggest muscle satellite proliferation and subsequent fusion may not be a prerequisite for β-adrenergic agonist-induced muscle hypertrophy. Thus, other contributing factors that lead to muscle hypertrophy may be altered with β-adrenergic agonist administration. For example, an increase in muscle protein synthesis, a reduction in muscle protein degradation, or a combination of both could be responsible for the β-adrenergic agonist-induced increase in muscle mass.

Skeletal Muscle Protein Synthesis. Ractopamine has been shown to increase fractional protein synthesis rate in pigs (Bergen et al., 1989). This research was further supported by muscle cell culture work, in which ractopamine increased both total and myosin heavy-chain protein synthesis rate in L_6 cells (Anderson et al., 1990). These data confirmed that the ractopamine-induced increase in muscle cell protein accretion was due in part, to the increased total and myofibrillar protein synthesis rate. Interestingly, ractopamine, a β_1-adrenergic agonist, did not alter protein degradation rates in cultured L_6 muscle cells (Anderson et al., 1990). Thus, the authors concluded that ractopamine-enhanced muscle protein accretion was due to increased protein synthesis rates with no detectable effect on protein degradation rates. Additionally, ractopamine increased myosin light-chain mRNA abundance in the longissimus muscle of beef cattle approximately fivefold as compared to untreated cattle (Smith et al., 1989). Likewise, clenbuterol increased myosin light-chain mRNA levels after 50 d of treatment as compared to control steers (Smith et al., 1995). These increased myofibrillar protein mRNA species could be a result of β-adrenergic agonist-induced gene transcription and/or increased stability of the mRNA. Regardless of the mechanism, increased mRNA abundance of muscle-specific proteins was related to the marked increase in muscle hypertrophy, potentially through alterations in protein synthesis. These conclusions are further supported with muscle cell culture results in which clenbuterol stimulated both fractional and absolute rates of protein synthesis in primary cultures of neonatal rat muscle cells (McMillan et al., 1992).

Skeletal Muscle Protein Degradation. Rate of protein degradation can also impact net protein accretion in skeletal muscle. Often, protein degradation rate is calculated by differences between measured protein accretion and fractional synthesis rates (Beermann, 2002). Alternatively, activities of muscle proteases (calcium-dependent and lysosomal) and their inhibitors have been determined following β-adrenergic agonist administration. Generally, results of various studies suggest that muscle protein degradation may be reduced or not affected by β-adrenergic agonist administration. In cattle, the majority of work in the area of protein degradation has been conducted with the β-adrenergic agonist, L-644,969. This β_2-adrenergic agonist caused a 27 % reduction in fractional protein degradation rate in steers com-

pared to untreated controls (Wheeler and Koohmaraie, 1992). Additionally, the activity of a specific inhibitor to the calpains, calpastatin, was elevated in bovine muscle samples obtained from steers fed L-644,969 (Wheeler and Koohmaraie, 1992: Killefer and Koohmaraie, 1994). Often, these reductions in protein degradation are the basis for observed decreases in meat tenderness from animals treated with β-adrenergic agonists. Specifically, the muscle proteases and inhibitor activities that are affected *in vivo* often follow parallel responses in the postmortem meat. Ractopamine fed to steers and heifers at the 200 $mg \cdot hd^{-1} \cdot d^{-1}$ level had no effect on tenderness (Schroeder et al., 2003). However, as dose increased to 300 $mg \cdot hd^{-1} \cdot d^{-1}$, shear-force values also increased compared to controls (Schroeder et al., 2003). This reduction in tenderness with ractopamine at 300 $mg \cdot hd^{-1} \cdot d^{-1}$ was somewhat surprising due to the preponderance of data suggesting ractopamine had no effect on rate of protein degradation in skeletal muscle (as previously discussed). However, it may indicate that at higher doses ractopamine, a presumed βadrenergic agonist, can bind to β_2-adrenergic receptors and elicit a biological response consistent with that type of receptor.

Insulin-like growth factor-I (IGF-I) as a potential mediator of β-adrenergic agonist action in skeletal muscle. Insulin-like growth factor-I (IGF-I) is a potent stimulator of skeletal muscle growth and differentiation. It is thought to stimulate skeletal muscle protein synthesis and reduce skeletal muscle protein degradation. Other skeletal muscle-enhancing agents, like anabolic steroids, have been shown to induce postnatal skeletal muscle hypertrophy in part through increased circulating and locally-produced (skeletal muscle) IGF-I. Specifically, trenbolone acetate and estradiol-17β (TBA/E_2) implant administration to cattle has been reported to increase circulating IGF-I and skeletal muscle IGF-I mRNA levels compared to nonimplanted, control steers (Johnson et al., 1996; Johnson et al., 1998; Dunn et al., 2003; Pampusch et al., 2003; White et al., 2003). In addition, TBA/E_2 administration to steers resulted in an increase in the number of actively proliferating, satellite cells within 35 days of implantation (Johnson et al., 1998). The rapid elevation of muscle IGF-I mRNA levels after implantation is very significant in view of a recent report that virally induced over-expression of IGF-I in the muscle tissue of mice resulted in a 15 % increase in muscle mass in young adult mice and maintenance of muscle mass in old mice that would otherwise be in a muscle atrophy state (Barton-Davis et al., 1998). Furthermore, another report found that over-expression of IGF-I in muscle tissue increased the proliferative capacity of satellite cells (Chakravarthy et al., 2000). These results were consistent with the above data showing that the number of actively, proliferating-satellite cells that can be isolated from the semimembranosus muscle was greater in steroid-implanted steers than in nonimplanted controls (Johnson et al., 1998). These findings strongly supported a mechanism for anabolic steroid-induced muscle growth in beef cattle that involved increases in the local production of muscle IGF that caused enhanced satellite cell activity and consequently increased skeletal muscle hypertrophy.

With rapid increases in skeletal muscle hypertrophy due to increased protein synthesis and decreased protein degradation with β-adrenergic agonist, one might propose IGF-I could be mediating this response. In lambs, cimaterol-feeding (10 ppm) has been shown to reduce circulating IGF-I levels 46.5 % at 42 days and 21.5 % at 84 days compared to sera from untreated lambs (Beermann et al., 1987). Similarly, in recent studies by Walker et al. (submitted), ractopamine-feeding to Holstein steers attenuated the TBA/E_2-induced increase in circulating IGF-I. Additionally, ractopamine,-feeding reduced the longissimus muscle IGF-I mRNA levels compared to levels obtained prior to ractopamine feeding.

In support of the local IGF-I mRNA data above, clenbuterol administration to rats reduced local production of IGF-I in the plantaris muscle after 14 days of exposure but had no effect on the tibialis anterior muscle (Yimlamai et al., 2005). Awede et al. (2002) reported that clenbuterol administration resulted in a transient change in soleus muscle IGF-I. The authors noted an increase in muscle IGF-I protein in the soleus muscle after 3 days, but there was no difference between clenbuterol and untreated muscle at 9 days. Taken together these data suggest IGF-I may not be mediating the effects of β-adrenergic agonist in skeletal muscle hypertrophy. Furthermore, IGF-I is known to stimulate the proliferative capacity of muscle satellite cells which in turn supports postnatal muscle hypertrophy. The lack

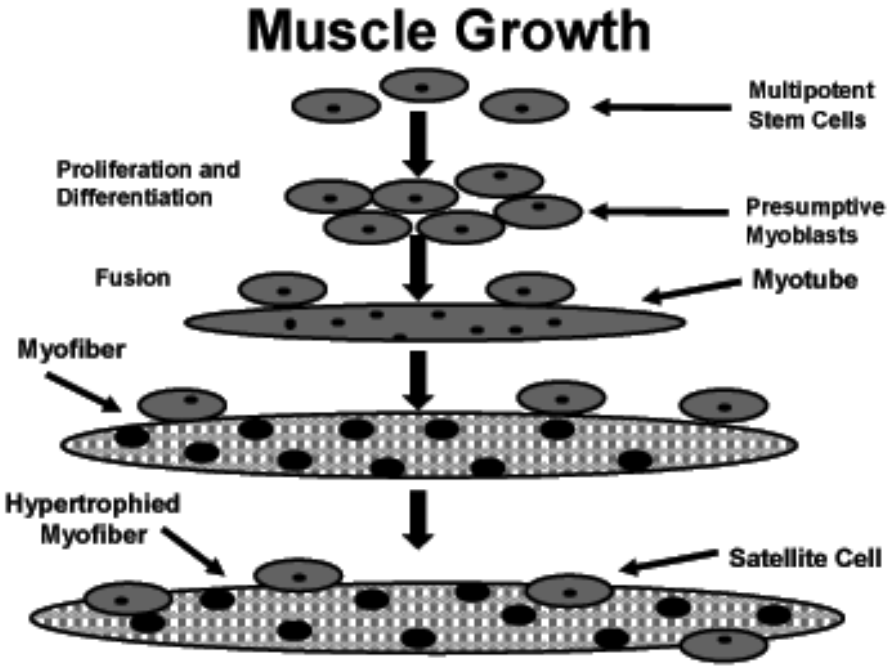

Figure 1. Overview of muscle cell proliferation and differentiation during embryonic and postnatal development.

of DNA accumulation in β-adrenergic agonist stimulated skeletal muscle growth may be in part due to the reduced local and circulating IGF-I levels caused by β-adrenergic agonist feeding. More research is needed to confirm the reduction in local and/or circulating IGF-I is related to the disconnect in satellite cell proliferation and fusion during β-adrenergic agonist-stimulated skeletal muscle hypertrophy. The ability to circumvent these relationships could potentially sustain greater degrees of muscle hypertrophy following administration of β-adrenergic agonists.

II. Practical Considerations

Dose and Duration. The dose (acute) and duration of that sustained dose (chronic) of β-adrenergic agonists has been shown to affect the maximal response. It has been well documented that theβAR-mediated increase in cAMP is transient. Continuous, receptor activation by the β-adrenergic agonist ligand is necessary to maintain the cAMP levels and to potentiate the response. Exposure of a constant dose of β-adrenergic agonist to the receptor will eventually cause acute desensitization or inactivation of receptor-mediated signaling. This phenomenon occurs due to phosphorylation of the βAR by both protein kinase A and βARKs (Hausdorff et al., 1990). Acute desensitization can be circumvented to some degree by increasing the dose and potentiating the signal. Long-term exposure (chronic) at elevated doses of β-adrenergic agonist leads to internalization or loss of the receptor from the cell surface and down-regulation in βAR mRNA abundance (Hausdorff et al., 1990). These alterations appear to be irreversible at least in the short-term feeding period.

Sustained skeletal muscle hypertrophy. Another practical issue in regards to duration of feeding of β-adrenergic agonist is skeletal muscle's ability to sustain the dramatic increase in hypertrophy without the concomitant increase in DNA to aid in maintaining the extra protein mass. Anabolic steroid (TBA/E_2) administration to beef cattle has been shown to stimulate muscle satellite cell proliferation (Johnson et al., 1998). Proper timing of implant administration prior to β-adrenergic agonist exposure may be crucial to stimulate muscle satellite cell proliferation and subsequent fusion into the existing fiber (Figure 2). The added DNA to the fiber will become beneficial during the rapid muscle hypertrophy phase caused by β-adrenergic agonist administration. The increased DNA accumulation due to previous anabolic steroid treatment could result in more sustained muscle hypertrophy during β-adrenergic agonist administration.

Comparative Efficacy of Steroid Implants and β*AA.* With the recent approvals of β-adrenergic agonists for beef cattle in the United States, producers will now be able to administer two distinct types of growth promotants to beef cattle during the finishing phase: steroid implants and β-adrenergic agonists. Naturally, questions have been raised as to the comparative efficacy of these two classes of growth promotants used together in beef cattle production. In theory, steroid implants and β-adrenergic agonist should be nearly additive at stimulating muscle growth in beef cattle due to presumably different mechanisms of action. Earlier research evaluating the effects of bovine somatotropin (bST) and steroids revealed additive effects of these two growth promotants on weight gain and protein deposition in beef cattle (Preston et al., 1995). The conclusion that each had distinct mechanisms of action in stimulating growth was surprising since E_2 had been shown to increase circulating ST levels. In fact research suggests that E_2 and bST were not additive in the effects on circulating ST. Taken together these data suggested that growth-promoting effects of estrogens were not fully mediated by the increase in ST secretion (Enright et al., 1990).

Steroid implants stimulate skeletal muscle growth through both increases in muscle hypertrophy as well as activation of muscle satellite cells to provide the critical DNA to sustain muscle hypertrophy (Johnson et al., 1996; 1998). It appears locally-produced IGF-I can mediate many of these effects in skeletal muscle (Dunn et al., 2003; Pampusch et al., 2003; White et al., 2003). As stated earlier, β-adrenergic agonists had no effect at increasing DNA content of skeletal muscle. Therefore administration of a steroid implant 60 to 90 days prior to feeding β-adrenergic agonists should have additive effects on lean tissue deposition in beef cattle due to different mechanisms of action. In fact, research with ractopamine and anabolic steroids in steers has shown opposite effects of these to growth enhancers on circulating and local, muscle IGF-I mRNA concentrations. Again, the above data would suggest the two classes of compounds are working independently of each other which should then result in additive effects. Surprisingly, ractopamine did not increase

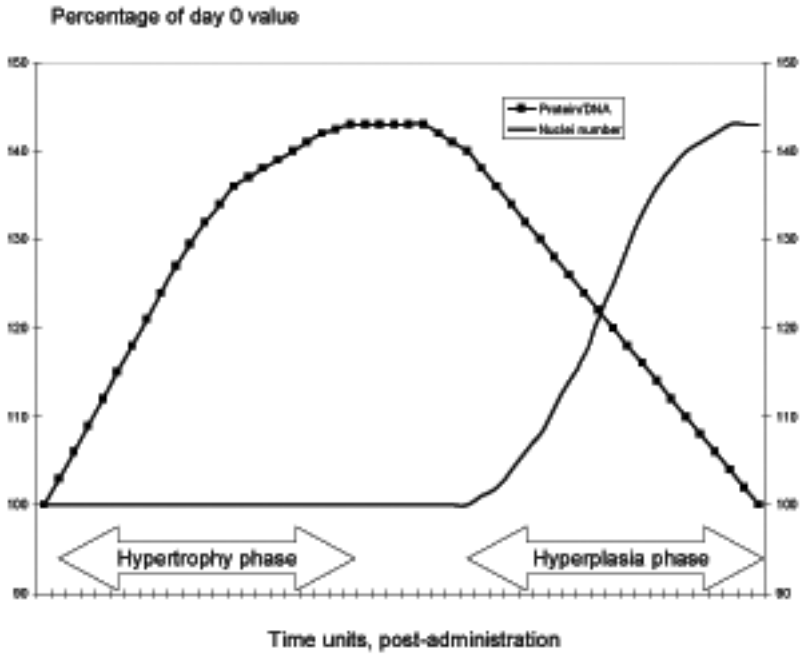

Figure 2. Idealized change in skeletal muscle hypertrophy following anabolic steroid administration in beef cattle. These changes are brought about by the increase in DNA accumulation as a result of increased muscle satellite cell proliferation and subsequent fusion. These changes in satellite cell activity aid in sustaining the increased muscle hypertrophy (adapted from Anderson and Johnson, 2004).

nitrogen retention in steers implanted with TBA/E_2 but did increase nitrogen retained in the nonimplanted steers. These findings suggested a potential interaction in a biological response like nitrogen retention following administration of the two classes of growth promoters (Walker et al., submitted).

Since in commercial application the anabolic steroid treatment will always be administered previous to β-adrenergic agonist feeding, we were interested in the effect of anabolic steroids on expression of β_1AR and β_2AR mRNA levels in total RNA obtained from biopsies of the longissimus muscle of beef cattle. Our preliminary data suggested that TBA/E_2 administration reduced the expression of β_1AR mRNA and had no detectable effect on the abundance of β_2AR mRNA (Figure 3). One might hypothesize that positive changes in number or affinity of the βAR for the ligand, caused by anabolic steroids, could potentially result in a synergistic effect of the two compounds. In contrast, a reduction in number or affinity of the βAR for the ligand, caused by anabolic steroids, could lead to an interaction in some biological response like skeletal muscle growth.

III. Safety Considerations with Unapproved-β-Adrenergic Agonists

As stated previously both clenbuterol and salbutamol are presumed β_2-adrenergic agonists that have been studied as growth promotants in many species including beef cattle. The biological effects of clenbuterol in beef cattle were first described by Ricks et al. (1984). Clenbuterol is chemically described as a benzyl alcohol, 4 amino-alpha-(tbutylamino)methyl-3,5-dichloro. Earlier research studies have indicated that clenbuterol was a very potent β_2-adrenergic agonist in cattle. Very little information is available in the scientific literature in regards to salbutamol.

Neither clenbuterol nor salbutamol are approved for use in meat-animal production in the U.S. The pharmaceutical company, American Cyanamid, abandoned the approval process for clenbuterol in the early 1990s when it appeared there may be a residue toxicity issue with clenbuterol in certain edible tissues of beef. Clenbuterol received much negative attention in the early 1990s when its illegal feeding to cattle resulted to cases of food toxicity in both Spain and France (Martinez-Navarro, 1990; Salleras et al., 1995). Individuals consuming liver from animals illegally-fed clenbuterol possessed symptoms of tachycardia, tremor, headache, and dizziness.

Indiscriminate use of clenbuterol and other non-approved β_2-adrenergic agonists such as salbutamol continue to be a major issue across the globe. Individuals who illegally feed these agents to cattle often do so at rates much greater than were ever tested in research trials. This can result in residue levels in both the kidneys and liver at levels great enough to cause food poisoning. Research by Smith and Paulson, 1997 revealed that clenbuterol residues were greatest in lung, kidney, and liver in cattle fed 3 mg/kg BW of clenbuterol. These concentrations averaged 8.36 ppm (mg/kg) in lung tissue, 5.9 ppm in kidney, and 5.04 ppm in liver. It has been suggested that the effective oral dose of clenbuterol in humans can be as low as 10 µg per day. Levels above this could begin to cause toxicity. At residue of 0.5 ppm (0.5 µg/g), an individual would only need to consume 20 g to equal the effective dose in humans. This is a very small quantity. Due to these human health concerns, clenbuterol and other potent β_2-adrenergic agonists will most likely never be approved for use in meat-animal production.

The two β-adrenergic agonists that have been approved for use in the U. S. and other countries around the globe, ractopamine and zilpaterol, have been studied extensively from a residue standpoint. When fed at the approved dose and label directions are followed for withdrawal times, negligible to undetectable levels of residues have been reported. For example, zilpaterol feeding at the normal approved dose resulted in liver and kidney residue levels of 0.18 and 0.09 ppm after a 48 h withdrawal (Zilmax FOI Summary, NADA 141-258, August, 2006). These absolute concentrations are much less than those reported for clenbuterol. In addition the effective oral dose of zilpaterol in humans in many fold higher than that reported for clenbutrol. Taken together, these data indicate that there are key differences in the metabolism, excretion and activity of different β-adrenergic agonists.

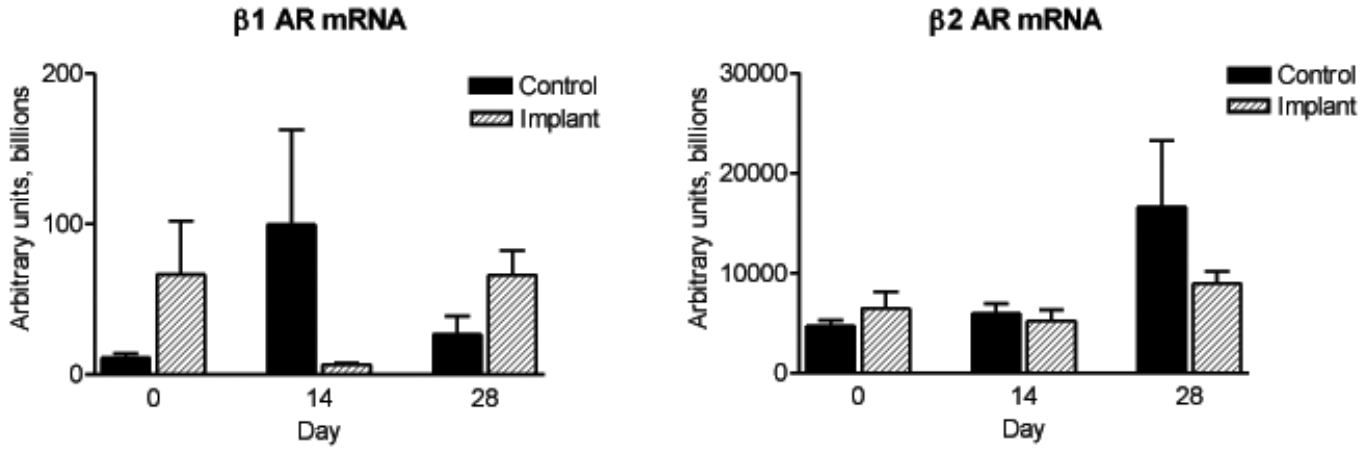

Figure 3. The effect of anabolic steroids on expression of βAR mRNA in longissimus muscle samples of yearling steers.

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