Altered mRNA abundance of ASB15 and four other genes in skeletal muscle following administration of β-adrenergic receptor agonists

Tara G. McDaneld,1 Deana L. Hancock,2 and Diane E. Moody1
1Department of Animal Sciences, Purdue University, West Lafayette 47907-2054; and 2Elanco Animal Health, Greenfield, Indiana 46140

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McDaneld, Tara G., Deana L. Hancock, and Diane E. Moody. Altered mRNA abundance of ASB15 and four other genes in skeletal muscle following administration of β-adrenergic receptor agonists. Physiol Genomics 16: 275–283, 2004. First published November 25, 2003; 10.1152/physiolgenomics.00127.2003.—β-Adrenergic receptor agonists (BA) stimulate skeletal muscle growth. However, downstream signaling pathways that facilitate this effect remain poorly defined. Objectives of this study were to identify genes differentially expressed after administration of a novel BA and to evaluate the expression of one of those genes in additional models of skeletal muscle growth. Differentially expressed gene fragments were identified through differential display of skeletal muscle biopsies from five steers 24 h after administration of the BA. Five gene fragments designated DD53, DD143, DD163, DD209, and DD214 were identified. Tissue distribution of these genes was evaluated by RT-PCR. While DD53, DD163, DD209, and DD214 were expressed across tissues, DD143 mRNA expression was most abundant in skeletal muscle. DD143, later identified as bovine ASB15, was evaluated in rats following administration of anabolic compounds. Thirteen 7-wk-old female rats were randomly assigned to each of four treatment groups including: control, clenbuterol, trenbolone acetate (TBA), and growth hormone (GH). Changes in rat Asb-15 mRNA were measured at 30 min, 12 h, and 24 h following intraperitoneal injections of each compound. Clenbuterol treatment decreased Asb-15 mRNA in skeletal muscle at 12 and 24 h (P < 0.01) and also decreased mRNA in lung at 12 h (P < 0.05). TBA and GH treatments did not alter Asb-15 mRNA in any of the tissues evaluated (P > 0.10). These results are the first to associate an Asb gene family member with muscle growth or BA administration and suggest a potential role for ASB15 in β-agonist-induced skeletal muscle hypertrophy.

β-agonist; clenbuterol; hypertrophy

Increasing efficiency of muscle accretion is an important goal in the production of meat animals, as well as the prevention of muscle atrophy associated with a number of human health concerns. The regulation of muscle accretion is a complex process, and many specific mechanisms involved are not yet fully understood. It is known that efficiency of muscle growth is enhanced through administration of β-adrenergic receptor agonists (BA; 2, 30). These compounds influence lipid metabolism through cAMP signaling and phosphorylation of key enzymes in lipid metabolism (see Ref. 28). However, specific mechanisms by which BA stimulate anabolic activity and muscle accretion are not well understood (28, 31). To date, investigations of differential gene expression in skeletal muscle following administration of BA have focused on changes in the expression of specific candidate genes. Increased mRNA expression of myosin light chain (35) and α-actin (10, 14, 22) support a model that BA enhance muscle accretion through increased expression of myofibrillar proteins. Increased mRNA and activity of calpastatin in response to BA administration have also been reported (3, 15, 20), supporting a hypothesis that BA inhibit the calpain protein degradation pathway through regulation of calpastatin. Genes expressed in human skeletal muscle have been characterized through computational analysis of sequence databases (5) and serial analysis of gene expression (SAGE; 37). Results of these studies indicate that functions of less than one-third of all genes expressed in human skeletal muscle are known. Thus we hypothesize that additional genes with unknown functions are differentially expressed in skeletal muscle in response to BA administration, and that these genes participate in physiological pathways contributing to increased muscle accretion. Experiments describing differential mRNA expression of specific candidate genes in response to BA administration have focused on gene expression changes occurring after administration of BA for several weeks. We expect that gene expression changes at these time points reflect the downstream effects of altered physiological pathways.

In contrast, we are interested in early changes in gene expression in response to BA administration. The identification of these genes may reveal pathways not previously known to be associated with BA-stimulated muscle growth. The objective of this study was twofold. First, we wanted to identify genes differentially expressed in bovine skeletal muscle following administration of a novel BA. The BA used in this study was an aryloxypropanolamine previously shown to be a specific agonist of the β3-adrenergic receptor (16) and known to elicit anabolic activity in beef cattle (unpublished data). Differential gene expression was investigated using the differential display technique to compare gene expression before and 24 h after administration of the BA compound.

Second, we wanted to confirm the regulation of a differential display product, subsequently identified and called ankyrin and SOCS box protein 15 (bovine or human ASB15, rodent Asb15; 27), in additional animal models of muscle accretion. We propose that regulation of genes essential to the physiological response in skeletal muscle to BA compounds will be conserved across species. Additionally, genes regulated by anabolic compounds that utilize different mechanisms to stimulate muscle accretion may play a central role in the regulation of muscle growth. The rat was chosen as a model in which to
validate the regulation of Asb-15, and the anabolic compounds investigated included trenbolone acetate (TBA), growth hormone (GH), and clenbuterol. TBA, GH, and clenbuterol have all been shown to increase muscle mass and growth rate. TBA is a steroid component in anabolic implants used to increase total muscle mass in cattle (8, 11). Administration of GH results in hypertrophy of muscle fibers and overall increased muscle mass (7, 36). Finally, clenbuterol is a β2-adrenergic receptor agonist that functions as a repartitioning agent to stimulate skeletal muscle growth through hypertrophy of muscle fibers (29, 30, 38).

MATERIALS AND METHODS

Differential Display Experiment

Tissue collection and experimental treatments. Five steers (~318 kg) of similar genetic background (primarily Angus) were used. All experimental protocols were approved through the Institutional Animal Care and Use Committee, Elanco Animal Health. Steers were housed in individual tie stalls and acclimated to the experimental environment, handling, and feeding conditions for a period of 7 days. On days when tissue biopsies were obtained, steers were provided a standardized amount of feed such that tissues were biopsied ~1 h after feed consumption. Tissue biopsies (~5 g) were taken from the longissimus dorsi muscle while the steers were restrained and under the effects of local anesthesia (lidocaine). Tissues were rinsed in sterile phosphate-buffered saline, trimmed of fat and connective tissue, and frozen in liquid nitrogen. Tissues were stored at ~80°C pending RNA extraction.

Two tissue biopsies were taken from each steer and were excised from the same relative location of contralateral longissimus dorsi muscles. The first tissue biopsy (control) was taken prior to administration of the experimental compound. The BA compound was administered by two intravenous injections (12 h apart) of 0.05 mg/kg body wt. A second tissue biopsy (treated) was taken 24 h following the initial injection of the BA compound. This experimental design was used so that steers would serve as their own control, avoiding potential differences due to genetic background.

Blood area nitrogen (BUN) analysis. Blood samples were collected immediately prior to obtaining the control and treated tissue biopsies for measurement of BUN. Decreased BUN in response to BA administration has been reported in steers (6). Therefore, altered BUN was investigated included trenbolone acetate (TBA), growth hormone (GH), and clenbuterol. TBA, GH, and clenbuterol have all been shown to increase muscle mass and growth rate. TBA is a steroid component in anabolic implants used to increase total muscle mass in cattle (8, 11). Administration of GH results in hypertrophy of muscle fibers and overall increased muscle mass (7, 36). Finally, clenbuterol is a β2-adrenergic receptor agonist that functions as a repartitioning agent to stimulate skeletal muscle growth through hypertrophy of muscle fibers (29, 30, 38).

Differential display PCR. Total RNA was extracted from each tissue sample using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer’s protocols. Equal quantities of total RNA from each steer were pooled within treatments, and all analyses were done using pooled RNA. All RNA samples were treated with DNase (Ambion, Austin, TX). Total RNA was reverse transcribed to cDNA using the SuperScript Preamplification System (Invitrogen) and three different anchor primers (T12A, T12G, or T12C). Differential display PCR (DD-PCR; 24) was done using 80 unique arbitrary primers in combination with the three anchor primers, for a total of 240 unique primer pairs. All reactions were performed in duplicate with a total volume of 20 μl consisting of 1× PCR buffer, 2 μM dNTPs, 15 mM (α32P)-dATP, 1 μM anchored primer, 1 μM arbitrary primer, 1 U AmpliTaq Gold (PerkinElmer, Wellesley, MA), and 20 ng of cDNA. Cycling conditions were as follows: initial denaturation at 92°C for 2 min, 40 cycles of denaturation at 92°C for 15 s, 2 min annealing beginning at 40°C and increasing 0.5°C per second until 72°C, extension at 72°C for 1 min; final extension at 72°C for 5 min.

The DD-PCR products were separated on 6% polyacrylamide sequencing gels. Following electrophoresis, gels were transferred to paper, dried, and exposed to autoradiographic film (24 h). Films were visually examined to identify DD-PCR products that were subjectively scored as either up- or downregulated in treated relative to control in each duplicate reaction.

Cloning and sequencing of potentially differentially expressed fragments. A total of 72 potentially differentially expressed PCR products were excised from dried polyacrylamide gels and amplified by PCR using the same reaction conditions and primers as in the DD-PCR amplification. Amplification products were cloned using the pCR-2.1 TOPO TA cloning system (Invitrogen). Plasmid DNA from 10 colonies representing each amplified gene fragment was isolated, and inserts were sequenced using an ABI 377 instrument. Similarity between all unique sequences and sequences in the GenBank database was determined using BLAST (1).

Confirmation of differential expression. Confirmation of differential expression was achieved by duplex semi-quantitative RT-PCR using primers for β-actin as an internal control to illustrate consistent amounts of cDNA in the reaction. The cDNA for semi-quantitative PCR was reverse transcribed from the same pooled total RNA samples used in DD-PCR using an oligo-dT primer and diluted to final concentrations of 5 and 25 ng/μl based on the initial amount of RNA that was reverse transcribed. The concentration of β-actin primers was optimized such that clear differences in amplification products from 5 and 25 ng of cDNA were evident. Differentially expressed PCR products were selected for validation based on sequence homology and relative differences in expression as subjectively determined from DD-PCR results. Primers specific to 35 sequences of potentially differentially expressed gene fragments were designed. Duplex PCR including β-actin and experimental primers was carried out using Advantage cDNA Polymerase Mix (Clontech, Palo Alto, CA) according to standard protocols. Final concentrations of β-actin and experimental primers were 20.8 nM and 300 nM, respectively. Sequences of primers that confirmed differential expression of five genes, as well as the β-actin primers, are provided in Table 1.

The PCR products that confirmed differential gene expression by semi-quantitative RT-PCR were used as probes on Northern blots. mRNA was purified from total RNA using a NucleoTrap mRNA Purification kit (Clontech). Pooled mRNA (3 μg) representing control

Table 1. Sequences of PCR primers used for semi-quantitative and quantitative reverse transcription and PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD53</td>
<td>AGGTGGATTGGGAAGAAGGCAA</td>
<td>GCAATGGTGGTTTCAAGAAGTA</td>
</tr>
<tr>
<td>DD143</td>
<td>GAGACAAGATGGTGTAGAG</td>
<td>TGAAGCATTCTGCAAGTCT</td>
</tr>
<tr>
<td>DD163</td>
<td>GATCGAAGCTTCTGCTAG</td>
<td>TGGCTCCTGAGAAGAAGAAA</td>
</tr>
<tr>
<td>DD209</td>
<td>CTTAGGGAAAGCAGAAGAAGA</td>
<td>TGGCCATTGTGAGATACCAT</td>
</tr>
<tr>
<td>DD214</td>
<td>CTAGGATTGGAAGATGAGAT</td>
<td>TGGCTCCTGAGAAGAAGAAA</td>
</tr>
<tr>
<td>Bovine β-actin</td>
<td>AACTGGGAGAGCAGAATGAGAA</td>
<td>TGGCCATTGTGAGATACCAT</td>
</tr>
<tr>
<td>Rat Asb-15</td>
<td>ATGCTGCCGCTTCTCTTTCT</td>
<td>GACGCTGAGCATGATCTCA</td>
</tr>
<tr>
<td>Rat GAPDH</td>
<td>GAACATCATCCCTGATCAGCA</td>
<td>CCACTGAGCTTCCCTGCTTA</td>
</tr>
</tbody>
</table>
and treated tissue and an RNA ladder (Invitrogen) were used to make Northern blots using a NorthernMax kit (Ambion). Radioactively labeled probes were made by random priming of purified PCR products. Probes were hybridized to Northern blots for 12 to 16 h using UltraHyb hybridization buffer (Ambion) and washed according to standard protocols. Northern blots were exposed to autoradiographic film (2 to 10 days) at −80°C with intensifying screens. The β-actin PCR product was labeled and used as a control to illustrate consistent amounts of mRNA on the blot.

Tissue collection and experimental treatments. Seven-week-old female Fischer 344 rats in the weight range of 100–120 g were obtained from Harlan Teklad (Madison, WI). All animals were maintained in individual cages at 25°C with a 12:12-h light-dark cycle for 5–7 days prior to the experiment and had ad libitum access to water and feed (Rodent Laboratory Chow; Ralston Purina, St. Louis, MO) throughout the experiment. All animals were handled in accordance with the protocol approved by the Purdue Animal Care and Use Committee.

Thirteen animals were randomly assigned to control and three treatment groups, including TBA, clenbuterol, and porcine GH. Tissues were harvested 30 min, 12 h, and 24 h after treatment administration, with three, five, and five rats, respectively, representing each time point for each treatment and control group. Experimental compounds (Sigma, St. Louis, MO) were diluted to a final concentration of 0.25 mg/ml. Clenbuterol and TBA were prepared by dissolving 2.5 mg of each compound in 1 ml of ethyl alcohol, then diluting to 0.25 mg/ml with 1:1 PEG-200:phosphate-buffered saline. GH was prepared in a similar manner, except that it was initially dissolved in ammonium bicarbonate. The control treatment was prepared the same as clenbuterol and TBA treatments, except that no compound was added. All solutions were sterilized by filtration. Compounds were administered via intraperitoneal injections at a dosage of 1 mg/kg of body wt. Rats were euthanized by CO2 asphyxiation at the appropriate time such that all tissues were collected between 6 and 10 PM. For the 24 h time point, two injections were given at time 0 and 12 h.

Tissues were collected from each animal, immediately frozen in liquid nitrogen, and stored at −80°C pending RNA extraction. Tissues collected included brain (including pituitary), heart, lung, kidney, liver, spleen, white adipose tissue, gracilis muscle, and reproductive tissues (ovaries and uterus). Truncal blood was collected, and serum was isolated and stored at −20°C. BUN was measured via methods of Kerscher and Ziegenhorn (18). Data for BUN concentration were obtained by analysis of variance using the GLM procedure of SAS. Treatment means for each time point were compared with the control treatment using contrasts to determine whether there was a significant change in BUN concentration.

RNA extraction and cDNA synthesis. Total RNA from skeletal and heart muscle was extracted using the Qiagen RNAeasy Mini kit following the manufacturer’s recommended protocol, including an additional step of protein kinase digestion for muscle tissue (Qiagen, Valencia, CA). Extraction of RNA from the remaining tissues was done with TRIZol reagent following the manufacturer’s recommended protocol (Invitrogen). Contaminating DNA was removed by digestion with DNase (RNA-free DNase, Qiagen; or DNA-Free, Ambion). Concentration of RNA was determined by measuring absorbance at 260 nm, and RNA quality was evaluated by gel electrophoresis. One microgram of total RNA was reverse transcribed to cDNA using the Superscript Preamplification System (Invitrogen).

Quantitative analysis of Asb-15 mRNA. Primers specific to bovine ASB15 sequence were used to amplify a 650-bp region of rat Asb-15 cDNA. The rat Asb-15 PCR product was sequenced (GenBank accession no. AY339371), revealing 91% and 86% identity to mouse Asb-15 and bovine ASB15, respectively. The rat sequence was used to design PCR primers for a quantitative real-time PCR assay (QRT-PCR) that amplified 120 bp of rat Asb-15 (Table 1). The QRT-PCR assay was carried out in the Bio-Rad iCycler (Bio-Rad, Hercules, CA) in a 25-μl final reaction volume. Quantitation of PCR products was achieved using SYBR Green (PerkinElmer, Wellesley, MA) reagents following the manufacturer’s recommended protocol with the following thermal cycling conditions: 95°C, 10 min (1 cycle); 95°C, 1 min, 60°C, 30 sec (35 cycles); 4°C hold. The PCR products were visualized on an ethidium bromide-stained agarose gel to ensure there was no nonspecific PCR amplification. All assays were done in duplicate in a 96-well plate format. Control samples were also run in duplicate on each 96-well plate to establish a standard curve for determining the log starting copy number (LSCN) of Asb-15 template in each cDNA sample. Controls were log dilutions of the Asb-15-specific target (108 copies to 102 copies) constructed from purified plasmid DNA (TOPO pcR2.1 vector, Invitrogen) containing the Asb-15 PCR product as an insert. Expression of GAPDH was measured in separate QRT-PCR assays for normalization of cDNA starting quantities. Primers specific to rat GAPDH (GenBank accession number NM_017008) were designed to amplify a 77-bp product region of the gene (Table 1). Controls were run for GAPDH as described for Asb-15, except using a vector containing the GAPDH PCR product.

The regression of LSCN on cycle threshold was calculated for the control samples to establish a standard curve for predicting LSCN for each experimental cDNA. The average GAPDH LSCN for the duplicate reactions from each cDNA sample was calculated. Normalized Asb-15 was calculated as the difference between each Asb-15 LSCN and the average GAPDH LSCN for the cDNA sample. The LSCN of Asb-15, GAPDH, and normalized Asb-15 were analyzed by analysis of variance using the mixed model procedures of SAS (33). Significant time by treatment interactions (P < 0.01) were observed for Asb-15 and normalized Asb-15 data, so these were analyzed separately for each time point. The final model included treatment as a
fixed effect and rat within treatment as a random effect. When significant differences among treatment means were observed ($P < 0.05$), differences between treatment and control means were defined using contrasts.

RESULTS

**Differential Display Experiment**

BUN levels significantly decreased 24 h after administration of the aryloxypropanolamine BA compound ($P < 0.01$). This indicates that a significant metabolic response to the BA compound had occurred at the time of treated tissue biopsy (Fig. 1). A total of 118 potentially differentially expressed gene fragments were identified by visual inspection of autoradiographic films (Fig. 2). Differential expression of five gene fragments, designated DD53, DD143, DD163, DD209, and DD214, was confirmed by semi-quantitative RT-PCR (Fig. 3) and Northern blots (Fig. 4). Results from semi-quantitative RT-PCR and Northern blots were consistent, except that the DD209 Northern hybridization resulted in very weak signal that appeared as two bands, both greater than 5 kb. The consensus sequence of three to seven clones for each gene fragment can be found in the dbEST database of GenBank (see Table 2 for accession numbers). Three of these gene fragments, DD163, DD209, and DD214, have high similarity (>90% identical over >100 bp) to full-length human genes with unknown functions; DD53 is 99% identical to *Bos taurus* seryl tRNA synthetase; and DD143 was weakly similar (86% identical over 61 bp, and 100% identical over 23 bp) to regions of a human genomic sequence (see Table 2). The DD143 sequence has since been extended to reveal its identity as ankyrin and SOCS box containing protein 15 (ASB15; 27). The estimated lengths of mRNA transcripts detected on Northern blots...
are given in Table 2. The sizes of DD53, DD209, and DD214 are consistent with the reported lengths of sequences to which they showed greatest similarity, while the observed length of the DD163 transcript (1.7 kb) is smaller than the sequence to which it showed greatest similarity (2.3 kb). The differentially expressed genes were detected in multiple tissues by RT-PCR (Fig. 5). Three genes (DD53, DD209, and DD214) were expressed across all tissues evaluated, and DD209 was more abundant in muscle than other tissues. The DD163 PCR product was detectable in all tissues, but at very low levels in all tissues except skeletal muscle and pituitary. Transcripts of DD143 were most abundant in skeletal muscle, heart, and pituitary (Fig. 5).

**Rat Experiment**

Concentrations of BUN decreased at 12 h for both TBA and clenbuterol and at 24 h for clenbuterol (P < 0.05), but BUN levels were unchanged for GH (Fig. 6). No differences in GAPDH LSCN were observed across treatments (P > 0.05). Similar results were generated from analyses using Asb-15 LSCN and normalized Asb-15 data. A significant decrease in Asb-15 mRNA following clenbuterol administration was observed in the gracilis muscle (P < 0.01; Fig. 7) at the 12 and 24 h time points. The TBA and GH treatments did not alter Asb-15 mRNA relative to the control (P > 0.10). The mRNA of Asb-15 was most abundant in rat heart, gracilis muscle, kidney, and lung but undetectable by RT-PCR in rat brain (including pituitary), liver, reproductive tract, spleen, and white adipose tissue (Fig. 8). A significant decrease (P < 0.05) in Asb-15 mRNA was observed in the lung 12 h after clenbuterol administration (Fig. 8), but not at other time or treatment combinations. No significant changes in Asb-15 mRNA were observed in heart, kidney, or adipose tissues following

<table>
<thead>
<tr>
<th>GenBank Entry with Greatest Similarity*</th>
<th>E value</th>
<th>Identity</th>
<th>Estimated mRNA Transcript Length, kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seryl tRNA synthetase</td>
<td>0.0</td>
<td>99% (580)</td>
<td>1.8</td>
</tr>
<tr>
<td>BAC clone</td>
<td>9 × e-6</td>
<td>86% (61), 100% (23)</td>
<td>5.3</td>
</tr>
<tr>
<td>RP11-390E23 from HSA 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asc-1 complex subunit P50</td>
<td>2 × e-44</td>
<td>92% (134)</td>
<td>1.7</td>
</tr>
<tr>
<td>mRNA for KIAA1824 protein</td>
<td>3 × e-93</td>
<td>90% (300)</td>
<td>6.3</td>
</tr>
<tr>
<td>Immediate early response 5 (IER5)</td>
<td>1 × e-56</td>
<td>91% (190), 86% (182)</td>
<td>2.35</td>
</tr>
</tbody>
</table>

*GenBank accession numbers are for the dbEST database (http://www.ncbi.nlm.nih.gov/entrez). *All are human sequences, except AF297553, which is bovine. “E value” indicates the probability of finding a similar sequence in the database by chance. “Identity” indicates the percentage of identical nucleotides over a region including the number of nucleotides indicated in parentheses. Two regions of identity are indicated when two regions of sequence similarity were interrupted by dissimilar (<70 identity) sequence. The “Estimated mRNA Transcript Length” is the length of mRNA transcript estimated by Northern blot. The DD143 fragment has been extended to generate a complete coding sequence with high homology to ASB15 (27).
clobuterol administration, although a numerical decrease was seen for the heart (Fig. 8).

**DISCUSSION**

Understanding specific cellular mechanisms that regulate growth and muscle accretion is critical to the development of novel methods to improve the efficiency of muscle accretion. Decreased mRNA abundance following BA administration was confirmed for DD143, subsequently identified as ASB15 (27), in both cattle and rats. This result supports our initial hypothesis that BA administration alters expression of previously uncharacterized genes in skeletal muscle, even at early time points relative to BA administration. We selected ASB15 for further investigation in the rat because the expression of DD143 was limited almost exclusively to skeletal muscle and pituitary in cattle. Additionally, ASB15 is a member of an emerging gene family that has been reported to be involved in a variety of cellular processes including cellular proliferation and differentiation (12, 21, 25). Members of the Asb gene family are characterized by the presence of both ankyrin repeat and suppressors of cytokine signaling (SOCS) box motifs. Ankyrin repeat motifs were first identified in the ankyrin gene family and are generally thought to function in protein-protein interactions (for review, see Ref. 4), while members of the SOCS box gene family play key roles in the negative regulation of signaling pathways (19, 41). Initial data describing functions of Asb gene family members, combined with the role of SOCS proteins in signal transduction pathways (19, 41), have led us to hypothesize that ASB15 may be involved in mediating or regulating initial signal transduction pathways stimulated by BA compounds in skeletal muscle.

A decrease in Asb-15 mRNA abundance was confirmed 12 and 24 h after administration of clobuterol in rat skeletal muscle. However, Asb-15 mRNA was not altered following administration of TBA or GH. This result suggests the regulation of Asb-15 mRNA may be specific to the BA signaling pathway. However, reduced BUN concentrations, indicating increased amino acid utilization and anabolic activity, were

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**Fig. 5.** Tissue distribution of differentially expressed fragments was evaluated by reverse transcription and PCR of RNA extracted from bovine tissues. The amount of cDNA representing each tissue was adjusted to give approximately equal amplification of β-actin. Tissues represented include: 1) longissimus dorsi muscle, 2) semitendinosus muscle, 3) tongue, 4) heart, 5) small intestine, 6) large intestine, 7) backfat, 8) omental fat, 9) kidney fat, 10) brown fat, 11) hypothalamus, 12) pituitary, 13) thyroid, 14) adrenal, 15) lung, 16) liver, 17) spleen, 18) kidney, 19) rumen, 20) reticulum, 21) abomasum, and 22) negative control.

**Fig. 6.** Rat BUN at 30 min, 12 h, and 24 h for control, trenbolone acetate (TBA)-, clobuterol-, and growth hormone (GH)-treated rats. *b* < 0.05, significant difference from the control treatment.
consistently observed only in clenbuterol-treated rats. The lack of significant, consistent effects of TBA and GH on BUN concentrations was unexpected because significant effects on growth rate and nitrogen retention have been documented in rats for these compounds (26, 39, 40). It is particularly unclear why a significant effect of TBA was observed at 12 but not 24 h. However, the fact that no change in Asb-15 mRNA was observed when a significant decrease in BUN concentration occurred 12 h after TBA administration suggests the regulation of Asb-15 is different following TBA vs. clenbuterol administration. In cattle, it has been shown that bovine GH decreases BUN levels as early as 24 h after treatment (13), and we had anticipated a similar response in rats. Nevertheless, the times used in this study were chosen to correspond to early metabolic changes associated with BA administration and may not accurately reflect significant metabolic changes stimulated by TBA or GH. Thus, although the data from this experiment indicate the regulation of Asb-15 is specific to the BA pathway, the possibility that Asb-15 mRNA is altered during times of increased anabolic activity stimulated by other compounds cannot be eliminated.

The expression and regulation of Asb-15 by clenbuterol was investigated in multiple rat tissues. Tissue distribution data from cattle indicated expression of ASB15 was primarily limited to muscle and pituitary. However, Asb-15 mRNA was detected in rat skeletal muscle, heart, lung, and kidney. The differences in tissue distribution between species may be caused by numerous factors, such as the age or physiological state of the animals at the time of tissue collection or unknown environmental effects on Asb-15 expression. Additionally, the different tissue distribution patterns may reflect species-specific differences in Asb-15 expression. Despite the presence of Asb-15 in multiple rat tissues, its downregulation following clenbuterol administration was limited to skeletal muscle (36% and 24% reduction in mRNA after 12 and 24 h, respectively) and lung (12% reduction in mRNA after 12 h), with the magnitude of response much greater in skeletal muscle. The effect of clenbuterol on gene expression in the lung is not surprising given the bronchodilator effects of clenbuterol on lung tissue (32). The expression of Asb-15 in adipose tissue was specifically investigated because of the known effects of BA on adipose tissue and lipid metabolism (17). However, Asb-15 mRNA was not detected in rat white adipose tissue either before or after clenbuterol administration. The tissue-specific regulation of Asb-15 may prove to be an important factor in mediating the effects of BA compounds.

Our differential display experiment confirmed BA regulation of additional genes in bovine skeletal muscle. This supports our hypothesis that multiple, currently unknown genes may be involved in mediating the effects of BA compounds in skeletal muscle. To date, the function of only one of these genes, seryl tRNA synthetase, is well characterized. Seryl tRNA synthetase catalyzes the specific attachment of serine to its appropriate tRNA (34). Because increased muscle accretion can occur through increased protein synthesis, it is reasonable to expect increased expression of tRNA synthetase mRNA following administration of a BA compound. It is not currently known whether other tRNA synthetases were also regulated but not

Fig. 7. Rat Asb-15 mRNA in gracilis skeletal muscle (SM) was measured by quantitative RT-PCR. Treatments included control, TBA, clenbuterol, and GH. Asb-15 mRNA was measured at 30 min, 12 h, and 24 h. Results are presented as the log of the estimated starting copy number (LSCN) of Asb-15 (n = 3 [30 min] or 5 [12 and 24 h] rats per treatment). *P < 0.01, significant difference from the control group.

Fig. 8. Asb-15 mRNA in rat tissues was measured by quantitative RT-PCR for control and clenbuterol-treated rats. Results are presented as the LSCN of Asb-15 (n = 5 rats per treatment). Data represent gracilis skeletal muscle (SM), heart, lung, kidney, brain, liver, reproductive tract (Repro), spleen, and white adipose tissue (WAT) at 12 h. *P < 0.01 and **P < 0.05, significant differences from the control treatment. Asb-15 was considered not detected (ND) if cDNA amplification was not different from the negative control.
identified in this experiment or whether seryl tRNA synthetase plays a unique function in the response of skeletal muscle to BA.

Altered mRNA abundance following BA administration was confirmed for three genes with high similarity to genes or proteins whose functions have not been well characterized. Highest similarity to DD163 was found with a human gene, Asc-1 complex subunit P50. The Asc-1 complex is an Na+-independent neutral amino acid transporter (9), but the specific function of the P50 subunit within this complex is not well defined. The similarity between DD163 and human Asc-1 complex subunit P50 was limited to a 150-bp region of the PCR product, and the mRNA transcript detected by Northern blotting using the DD163 PCR product was smaller than the Asc-1 complex subunit P50 coding sequence. Thus DD163 may represent a related gene or possibly an alternatively spliced form of the human gene. Differential expression of DD209 was confirmed by RT-PCR, but this gene was not clearly detectable by Northern blot hybridization. The DD209 sequence displayed highest sequence similarity to a human protein, KIAA1824, of unknown function. Highest sequence similarity to DD214 was found with a human mRNA sequence encoding immediate early response factor 5 (IER5). Immediate early genes are rapidly induced by growth factors and other stimuli not affected by protein synthesis inhibitors (23). Genes belonging to a variety of protein families, including transcriptional regulators, zinc-finger proteins, secreted cytokines, and cytoplasmic proteins, have been characterized as immediate early genes. The observation that DD214 mRNA was down-regulated in response to the BA compound was unexpected, given that IER5 and other immediate early response genes are up-regulated in response to anabolic growth factors.

In conclusion, five gene fragments differentially expressed in response to a BA compound that stimulates anabolic activity and muscle accretion in cattle were identified. These genes may be components of the early/acute physiological response to BA. The differential expression of one of these genes, ASB15, was confirmed in rat skeletal muscle in response to the BA clenbuterol, supporting the data in cattle. These results are the first to associate an Asb gene family member with muscle growth or BA administration, and suggest a potential role for ASB15 in β-agonist-induced skeletal muscle hypertrophy. Additional research to characterize further the expression and function of ASB15 and other genes influenced by BA administration may reveal currently unknown mechanisms that regulate complex physiological processes important to muscle growth.

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REFERENCES