Role of G protein–coupled receptors (GPCR), matrix metalloproteinases 2 and 9 (MMP2 and MMP9), heparin-binding epidermal growth factor–like growth factor (hbEGF), epidermal growth factor receptor (EGFR), erbB2, and insulin-like growth factor 1 receptor (IGF-1R) in trenbolone acetate–stimulated bovine satellite cell proliferation[1]

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ABSTRACT: Implanting cattle with steroids significantly enhances feed efficiency, rate of gain, and muscle growth. However, the mechanisms responsible for these improvements in muscle growth have not been fully elucidated. Trenbolone acetate (TBA), a testosterone analog, has been shown to increase proliferation rate in bovine satellite cell (BSC) cultures. The classical genomic actions of testosterone have been well characterized; however, our results indicate that TBA may also initiate a quicker, nongenomic response that involves activation of G protein–coupled receptors (GPCR) resulting in activation of matrix metalloproteinases 2 and 9 (MMP2 and MMP9) that release membrane-bound heparin-binding epidermal growth factor–like growth factor (hbEGF), which then binds to and activates the epidermal growth factor receptor (EGFR) and/or erbB2. Furthermore, the EGFR has been shown to regulate expression of the IGF-1 receptor (IGF-1R), which is well known for its role in modulating muscle growth. To determine whether this nongenomic pathway is potentially involved in TBA-stimulated BSC proliferation, we analyzed the effects of treating BSC with guanosine 5′-O-2-thiodiphosphate (GDPβS), an inhibitor of all GPCR; a MMP2 and MMP9 inhibitor (MMPI); CRM19, a specific inhibitor of hbEGF; AG1478, a specific EGFR tyrosine kinase inhibitor; AG879, a specific erbB2 kinase inhibitor; and AG1024, an IGF-1R tyrosine kinase inhibitor on TBA-stimulated proliferation rate (^{3}H-thymidine incorporation). Assays were replicated at least 9 times for each inhibitor experiment using BSC cultures obtained from at least 3 different animals. Bovine satellite cell cultures were obtained from yearling steers that had no previous exposure to androgenic or estrogenic compounds. As expected, BSC cultures treated with 10 n*M* TBA showed ($P < 0.05$) increased proliferation rate when compared with control cultures. Additionally, treatment with 5 ng hbEGF/mL stimulated proliferation in BSC cultures ($P < 0.05$). Treatment with GDPβS, MMPI, CRM197, AG1024, AG1478, and/or AG879 all suppressed ($P < 0.05$) TBA-induced increases in proliferation. These data indicate that TBA likely initiates a nongenomic response involving GPCR, MMP2 and MMP9, hbEGF, EGFR, erbB2, and IGF-1R, which may play a role in TBA-mediated increases in BSC proliferation.

Key words: bovine, epidermal growth factor receptor, G protein–coupled receptors, satellite cell, trenbolone acetate

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INRODUCTION

Combined estradiol 17β (**E2**) and trenbolone acetate (**TBA**) implants result in significant increases in muscle satellite cell (**SC**) number in feedlot steers (Johnson et al., 1998). Because SC play an integral role in skeletal muscle growth by providing additional nuclei for hypertrophy of existing myofibers, this steroid-induced increase in SC number likely plays a role in steroid-enhanced muscle growth (Campion, 1984; Moss and Leblond, 1971). Although TBA treatment results in increased proliferation of cultured bovine SC (**BSC**), there is no consensus regarding the cellular mechanism or mechanisms by which TBA stimulates BSC proliferation (Kamanga-Sollo et al., 2008). The classical genomic actions of testosterone through the androgen receptor (**AR**) are well characterized (Dehm and Tindall, 2007; Simental et al., 1991); however, recent literature indicates that testosterone likely also initiates a quicker, nongenomic response through the epidermal growth factor receptor (**EGFR**; also known as erbB1) and erbB2, 2 members of the erbB family (Rahman and Christian, 2007; Pignon et al., 2009). Furthermore, recent studies demonstrate that testosterone stimulates proliferation in muscle cells through G protein–coupled receptors (**GPCR**) and proteolytic release of heparin-binding epidermal growth factor–like growth factor (**hbEGF**), resulting in activation of EGFR (Estrada et al., 2003; Filardo and Thomas, 2005; Fu et al., 2012). Furthermore, matrix metalloproteinases (**MMP**) are involved in many cellular functions including cell proliferation and cleavage of proteins from the cell membrane (Montarras et al., 2013). Additionally, the IGF-1 receptor (**IGF-1R**) has been found to have a role in TBA-mediated increases in muscle growth (Kamanga-Sollo et al., 2008). To determine whether GPCR, MMP2 and MMP9, hbEGF, EGFR, erbB2, and IGF-1R are involved in the mechanism of TBA-stimulated BSC proliferation, we have used inhibitors to individually inhibit the activity of each of these molecules.

MATERIALS AND METHODS

Bovine Satellite Cell Isolation

Satellite cell isolation was performed as previously described (Hathaway et al., 1991; Hathaway et al., 1994; Frey et al., 1995). Steers were euthanized using procedures approved by the University of Minnesota Institutional Animal Care and Use Committee. Using sterile techniques, approximately 500 g of the semimembranosus muscle was dissected out and transported to the cell culture laboratory. Subsequent procedures were conducted in a sterile field. After removal of connective tissue, the muscle was passed through a sterile meat grinder. The ground muscle was incubated with 0.1% pronase in Earle's balanced salt solution for 1 h at 37°C with frequent mixing. Following incubation, the mixture was centrifuged at 1,500 × *g* for 4 min at room temperature, the pellet was suspended in PBS (140 m*M* NaCl, 1 m*M* KH_2PO_4, 3 m*M* KCl, and 8 m*M* Na_2HPO_4, pH 7.4), and the suspension was centrifuged at 500 × *g* for 10 min at room temperature. The resultant supernatant was centrifuged at 1,500 × *g* for 10 min at room temperature to pellet the mononucleated cells. The PBS wash and differential centrifugation were repeated 2 more times. The resulting mononucleated cell preparation was suspended in cold (4°C) Dulbecco's modified Eagle medium (**DMEM**) containing 10% fetal bovine serum (**FBS**) and 10% (vol/vol) dimethylsulfoxide (**DMSO**) and then frozen. Cells were stored in liquid nitrogen for subsequent use. Clonal analysis of SC cultures established from these preparations showed that between 80 and 90% of the cells were able to establish colonies that contained fusion, indicating that between 80 and 90% of the cells in the preparations were myogenic. Satellite cells used in the experiments described below were isolated from yearling steers that had never been implanted with either estrogenic or androgenic compounds.

Bovine Satellite Cell Culture

Bovine SC preparations were plated on 2-cm^2 culture dishes precoated with reduced growth factor basement membrane Matrigel (Corning, Tewksbury, MA) diluted 1:50 (vol/vol) with DMEM as described previously (Johnson et al., 1998). Media used in this study did not contain phenol red unless otherwise indicated. Plating density was empirically established so that all cultures were approximately 70% confluent after 72 h in culture. Cells were plated in DMEM containing 10% FBS and incubated at 37°C 5% CO_2 in a water-saturated environment. Cultures were rinsed once with DMEM and treated at 72 h with test media consisting of DMEM plus 1% IGF binding protein 3 (IGFBP-3)–free swine serum (**SSS**; prepared by passing sera obtained from 6-wk-old male pigs castrated within 1 wk of birth through an IGFBP-3 immunoaffinity column; Kamanga-Sollo et al., 2004).

Six different inhibitors were used to determine the effect of inhibiting GPCR, MMP2 and MMP9, hbEGF, erbB2, EGFR, and IGF1-R on TBA-mediated increases in BSC proliferation. Inhibition of GPCR was performed using guanosine 5′-O-2-thiodiphosphate (**GDPβS**; Calbiochem, Billerica, MA) as previously described (Estrada et al., 2003; Fu et al., 2012).

Briefly, cultures were treated with either 1% SSS or 1% SSS plus 100 n*M* GDPβS for 30 min. Following this 30-min incubation period, media were removed and BSC cultures were treated with DMEM/1% SSS or DMEM/1% SSS containing appropriate additions of 25 ng LR3-IGF-1/mL (GroPep Bioreagents Pty Ltd, Thebarton, SA, Australia; an IGF-1 analog that binds normally to the IGFR-1 but has little or no affinity for IGF binding proteins; Francis et al., 1992), hbEGF (R&D Systems, Minneapolis, MN), or TBA (Steraloids, Inc., Newport, RI) ± 100 n*M* GDPβS.

Matrix metalloproteinases 2 and 9 inhibition was performed using a MMP2 and MMP9 inhibitor (**MMPI**; MMP-2/MMP-9 Inhibitor II; Calbiochem) following previously described methods (Kamanga-Sollo et al., 2014). Briefly, plating media were removed at 72 h, cells were washed once with warm DMEM, and cultures were incubated with DMEM/1% SSS or DMEM/1% SSS plus 10 μ*M* MMPI for 30 min. Following the 30-min incubation period, media were removed and cultures were treated with DMEM/1% SSS or DMEM/1% SSS containing 25 ng LR3-IGF-1/mL or 10 n*M* TBA ± 10 μ*M* MMPI.

Analysis of the role of hbEGF was performed using CRM197 (Sigma Aldrich, St. Louis, MO), a potent antagonist of hbEGF using previously described methods (Kamanga-Sollo et al., 2014). Briefly, plating media were removed at 72 h, cells were washed once with warm DMEM, and cultures were incubated with DMEM/1% SSS or DMEM/1% SSS plus 1 μg CRM197/mL for 30 min. Following the 30-min incubation period, media were removed and cultures were treated with DMEM/1% SSS or DMEM/1% SSS containing 25 ng LR3-IGF-1/mL, 5 ng hbEGF/mL, or 10 n*M* TBA ± 1 μg CRM197/mL.

Inhibition of the EGFR and erbB2 was completed using AG1478 (Calbiochem), an EGFR tyrosine kinase inhibitor, and AG879 (Calbiochem), an erbB2 tyrosine kinase inhibitor, as previously described (Zhou and Brattain, 2005; Reiter et al., 2014). Briefly, plating media were removed at 72 h, cells were washed once with warm DMEM, and cultures were incubated with DMEM/1% SSS or DMEM/1% SSS plus 1 μ*M* AG1478 and/or 1 μ*M* AG879 for 30 min. Following the 30-min incubation period, media were removed and cultures were treated with DMEM/1% SSS or DMEM/1% SSS containing 5 ng hbEGF/mL or 10 n*M* TBA ± 1 μ*M* AG1478 and/or 1 μ*M* AG879.

Inhibition of the IGF-1R was performed using an IGF-1R tyrosine kinase inhibitor (AG1024; Calbiochem) as preciously described (Kamanga-Sollo et al., 2013). Briefly, plating media were removed at 72 h, cells were washed once with warm DMEM, and cultures were incubated with DMEM/1% SSS or DMEM/1% SSS plus 1 μ*M* AG1024 for 30 min. Following the 30-min incubation period, media were removed and cultures were treated with DMEM/1% SSS or DMEM/1% SSS containing 25 ng LR3-IGF-1/mL, 5 ng hbEGF/mL, or 10 n*M* TBA ± 1 μ*M* AG1024.

Trenbolone acetate was initially dissolved in 95% ethanol, GDPβS and CRM197 were dissolved in cell culture grade water, and MMPI, AG1478, and AG1024 were dissolved in DMSO. All cultures including the control contained a final concentration of 0.1% ethanol (vol/vol) and 0.1% DMSO (vol/vol) in assays containing MMPI, AG1478, and AG1024. The concentrations of ethanol and DMSO added to the cultures did not affect proliferation rate (data not shown). All assays contained triplicate measurements for each inhibitor assayed. Cells isolated from a minimum of 3 separate steers were used and cells from each steer were used in 3 separate assays to assess the effects of each inhibitor. This means that each of the assays was performed at least 9 times for each different inhibitor used.

Measurement of ^{3}H-thymidine Incorporation in Bovine Satellite Cell Culture

Incorporation of ^{3}H-thymidine was measured as previously described (Johnson et al., 1998; Kamanga-Sollo et al., 2004, 2008). In brief, after 93 h in culture, ^{3}H-thymidine was added to the media on the cells to give a final concentration of 1 μCi/mL and incubated for 3 h. At 96 h, test media were removed and cells were washed 3 times with cold DMEM. Cells were fixed with 1 mL/well of cold 5% trichloroacetic acid (**TCA**) overnight at 4°C. Unincorporated ^{3}H-thymidine was removed when 5% TCA was removed and cells were washed twice with cold 5% TCA. Incorporation of ^{3}H-thymidine into cellular DNA was determined by dissolving the cell material in 0.5 *M* NaOH for 30 min. Cell material was then placed in scintillation vials and counted in a scintillation counter. All experiments contained triplicate measurements.

Statistical Analysis

Statistical analysis was completed using the MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC). All data are presented as the least squares means ± SE of the mean. Data from multiple assays performed on cells isolated from different animals were combined. Preliminary analyses indicated that there were no effects ($P > 0.05$) observed for either the assay number or the different animals; as such, these 2 factors were included as random variables in the model. The model included treatment as a fixed effect and assay number and the animal the BSC were

isolated from as random effects. When treatment differences were found to be significant ($P < 0.05$), least squares means were separated using Tukey–Kramer adjustments ($P < 0.05$).

RESULTS

Effect of Inhibiting GPCR Activity on Trenbolone Acetate–Stimulated Bovine Satellite Cell Proliferation

Addition of both TBA and LR3-IGF-1 increased ($P < 0.001$) BSC proliferation rates as compared with SSS controls (Fig. 1, 2, and 3). To ensure that GDPβS was not indiscriminately inhibiting proliferation, we assessed the effect of GPCR inhibition in the presence of LR3-IGF-1 (Fig. 1) and found that GDPβS did not inhibit LR3-IGF-1–stimulated BSC proliferation (Fig. 1). Furthermore, treatment with GDPβS suppressed ($P < 0.001$) the stimulatory effect of TBA on proliferation (Fig. 1), indicating that GPCR are involved in TBA-mediated increases in BSC proliferation.

Effect of Inhibiting MMP2 and MMP9 Activity on Trenbolone Acetate–Stimulated Bovine Satellite Cell Proliferation

Treatment of BSC cultures with 5 ng hbEGF/mL increased ($P < 0.001$) proliferation rate, establishing that BSC respond to hbEGF treatment (Fig. 2, 3, and 4). Inhibition of MMP2 and MMP9 activity by treatment with 10 μ*M* MMPI suppressed ($P < 0.001$) TBA-, hbEGF-, and LR3-IGF-1–mediated increases in BSC proliferation (Fig. 2). Although TBA-mediated increases in proliferation are significantly reduced by treatment with MMPI, it does not appear to be specific to TBA, as the same results are seen with treatment of both hbEGF and LR3-IGF-1 (Fig. 2).

Effect of Inhibiting hbEGF Activity on Trenbolone Acetate–Stimulated Bovine Satellite Cell Proliferation

As expected, treatment of BSC with 1 μg CRM197/mL inhibited ($P < 0.001$) hbEGF-stimulated BSC proliferation (Fig. 5). Additionally, treatment with 1 μg CRM197/mL also inhibited ($P < 0.001$) TBA-stimulated proliferation in BSC cultures (Fig. 5). In contrast, treatment with CRM197 had no effect on the ability of LR3-IGF-1 to stimulate BSC proliferation (Fig. 5), indicating that inhibiting hbEGF activity did not have a general inhibitory effect on the ability of BSC to respond to mitogenic stimuli.

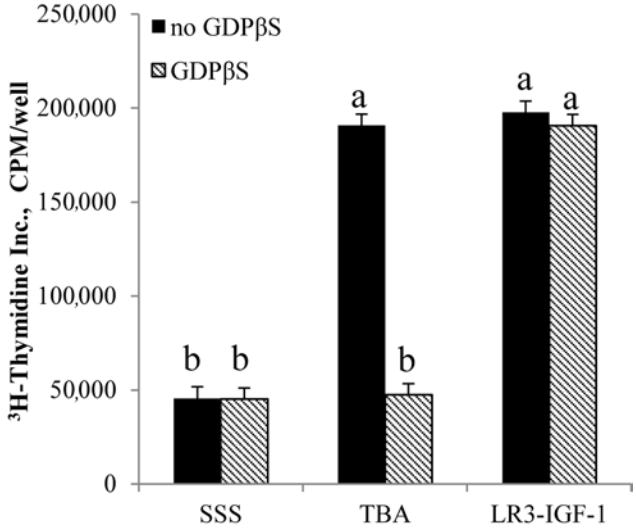

Figure 1. Effect of inhibition of G protein coupled receptors using 100 n*M* guanosine 5′-O-2-thiodiphosphate (GDPβS) on proliferation (^{3}H-thymidine incorporation [Inc.]) in bovine satellite cell (BSC) cultures treated with Dulbecco's modified Eagle medium (DMEM)/1% IGF binding protein-3–free swine serum (SSS) plus 25 ng LR3-IGF-1/mL or 10 n*M* trenbolone acetate (TBA). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed 3 times with DMEM, and cultures were fed with test media consisting of DMEM/1% SSS plus the indicated additions of LR3-IGF-1/mL, TBA, and/or GDPβS. Cultures treated with GDPβS were preincubated for 30 min with GDPβS before addition of LR3-IGF-1 or TBA to the culture. Incorporation of ^{3}H-thymidine was measured at 96 h as described in the Materials and Methods. ^{a,b}Bars with different letter designations are significantly different from each other ($P < 0.001$). Data represent least squares means ± SEM from 9 separate assays using BSC isolated from at least 3 different animals. Each assay contained triplicate determinations. CPM = counts per minute.

Effect of Inhibiting EGFR and erbB2 Activity on Trenbolone Acetate–Stimulated Bovine Satellite Cell Proliferation

Treatment of BSC cultures with AG1478 and/or AG879 suppressed ($P < 0.001$) TBA- and hbEGF-mediated increases in BSC proliferation (Fig. 4), indicating that both the EGFR and erbB2 have a role in the mechanism through which TBA increases BSC proliferation. Therefore, it appears that the 2 receptors may function independently of one another, because they both decrease TBA-mediated increases in BSC proliferation both alone and when they are applied to cultures together.

Effect of Inhibiting IGF-1R Activity on Trenbolone Acetate–Stimulated Bovine Satellite Cell Proliferation

Treatment of BSC cultures with AG1024 suppressed ($P < 0.001$) TBA- and LR3-IGF-1–mediated increased in BSC proliferation (Fig. 4), indicating that the IGF-1R has a role in the mechanism through which TBA increases BSC proliferation. Treatment of

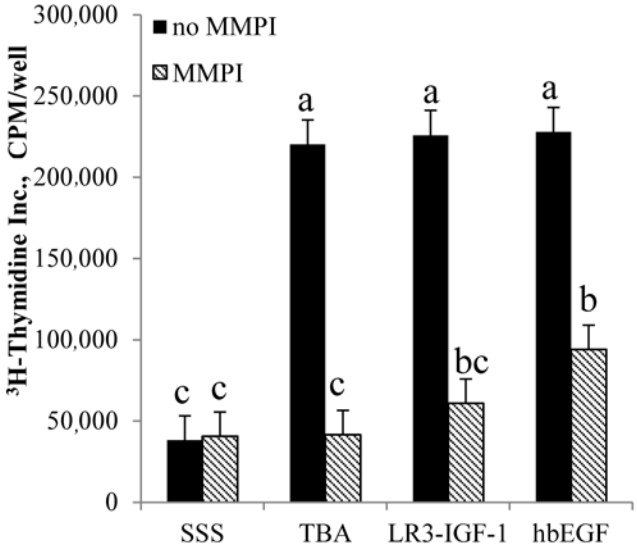

Figure 2. Effect of inhibition of matrix metalloproteinases 2 and 9 (MMP2 and MMP9) using 20 μ*M* of MMPI on proliferation (^{3}H-thymidine incorporation [Inc.]) in bovine satellite cell (BSC) cultures treated with Dulbecco's modified Eagle medium (DMEM)/1% IGF binding protein-3–free swine serum (SSS) plus 25 ng LR3-IGF-1/mL, 5 ng heparin-binding epidermal growth factor–like growth factor (hbEGF)/mL, or 10 n*M* trenbolone acetate (TBA). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed 3 times with DMEM, and cultures were fed with test media consisting of DMEM/1% SSS plus the indicated additions of LR3-IGF-1/mL, hbEGF, TBA, and/or MMPI. Cultures treated with MMPI were preincubated for 30 min with MMPI before addition of LR3-IGF-1, hbEGF, or TBA to the culture. Incorporation of ^{3}H-thymidine was measured at 96 h as described in the Materials and Methods. $^{a–c}$Bars with different letter designations are significantly different from each other ($P < 0.001$). Data represent least squares means ± SEM from 9 separate assays using BSC isolated from at least 3 different animals. Each assay contained triplicate determinations. CPM = counts per minute.

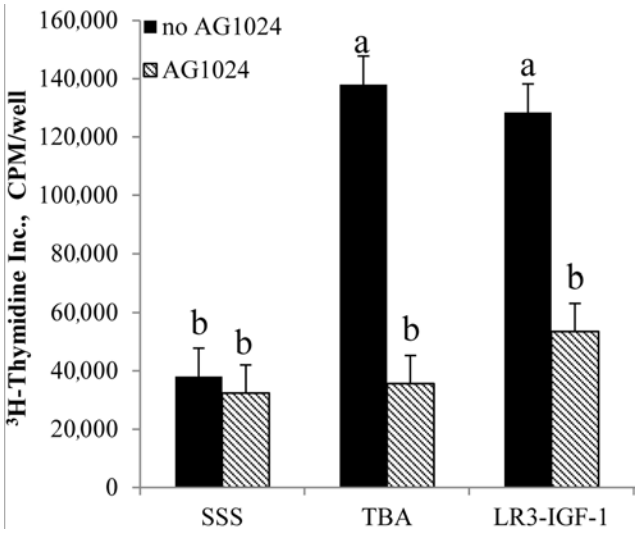

Figure 3. Effect of inhibition of the IGF-1 receptor (IGF-1R) using 1 μ*M* AG1024, an IGF-1R tyrosine kinase inhibitor, on proliferation (^{3}H-thymidine incorporation [Inc.]) in bovine satellite cell (BSC) cultures treated with Dulbecco's modified Eagle medium (DMEM)/1% IGF binding protein-3–free swine serum (SSS) plus 25 ng LR3-IGF-1/mL or 10 n*M* trenbolone acetate (TBA). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed 3 times with DMEM, and cultures were fed with test media consisting of DMEM/1% SSS plus the indicated additions of LR3-IGF-1, TBA, and/or AG1024. Cultures treated with AG1024 were preincubated for 30 min with AG1024 before addition of LR3-IGF-1 or TBA to the culture. Incorporation of ^{3}H-thymidine was measured at 96 h as described in the Materials and Methods. ^{a,b}Bars with different letter designations are significantly different from each other ($P < 0.001$). Data represent least squares means ± SEM from 9 separate assays using BSC isolated from at least 3 different animals. Each assay contained triplicate determinations. CPM = counts per minute.

BSC cultures with AG1024 had no effect ($P > 0.05$) on the ability of hbEGF to increase proliferation of BSC cultures, indicating that AG1024 is not a nonspecific inhibitor of cell proliferation in the presence of mitogenic stimuli (data not shown).

DISCUSSION

Anabolic steroid implantation of beef cattle has been shown to increase growth (10–30%), feed efficiency (5–15%), and carcass leanness (5–8%) as well as result in an economic benefit to cattle producers of US$20 to $75 per animal beyond the initial cost of implanting (Beermann, 1994; Duckett et al., 1996; Preston, 1999). Implants containing both estrogens and androgens are most effective in promoting growth of beef cattle as compared with implants containing either steroid alone (Hayden et al., 1993; Johnson et al., 1996; Dayton and White, 2014). Currently, there is no consensus regarding the cellular mechanism responsible for improved growth for either steroid compound. As such, it is important that the mechanisms responsible for both TBA- and E2-enhanced postnatal muscle growth are determined to aid in the development of future strategies to further improve beef production practices.

Trenbolone acetate has a greater relative androgenic and anabolic activity of 3 to 5 and 5 to 8 times higher, respectively, when compared with testosterone (Bouffault and Willemart, 1983). Steers implanted with a combined E2 and TBA implant produce BSC cultures with significantly higher proliferation rates compared with BSC cultures from nonimplanted steers (Johnson et al., 1996). Furthermore, treatment of BSC cultures with TBA results in significant increases in proliferation rate compared with control cells (Kamanga-Sollo et al., 2008). Additionally, studies completed in both humans and animals have demonstrated that testosterone increases muscle fiber diameter, the number of myonuclei present in muscle fibers, the absolute number of SC, and the percentage of SC relative to myofiber nuclei present in muscle (Bhasin et al., 2001;

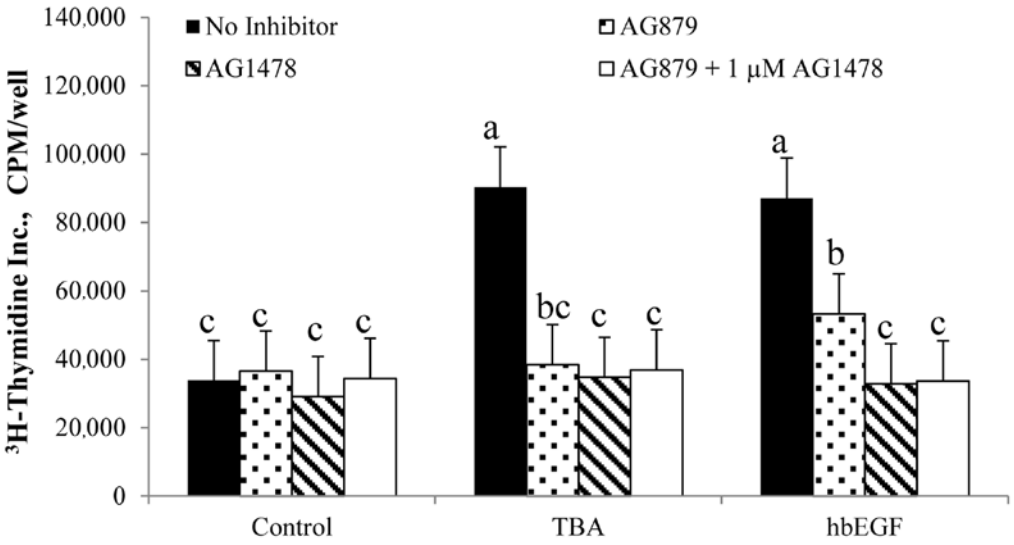

Figure 4. Effect of inhibition of the epidermal growth factor receptor (EGFR; also known as erbB1) using 1 μ*M* AG1478, an EGFR tyrosine kinase inhibitor, and erbB2 using 1 μ*M* AG879, an erbB2 tyrosine kinase inhibitor on proliferation (^{3}H-thymidine incorporation [Inc.]) in bovine satellite cell (BSC) cultures treated with Dulbecco's modified Eagle medium (DMEM)/1% IGF binding protein-3–free swine serum (SSS) plus 5 ng heparin-binding epidermal growth factor–like growth factor (hbEGF)/mL or 10 n*M* trenbolone acetate (TBA). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed 3 times with DMEM, and cultures were fed with test media consisting of DMEM/1% SSS plus the indicated additions of hbEGF, TBA, and/or AG1478 and/or AG879. Cultures treated with AG1478 and/or AG879 were preincubated for 30 min with inhibitor before addition of hbEGF or TBA to the culture. Incorporation of ^{3}H-thymidine was measured at 96 h as described in the Materials and Methods. $^{a–c}$Bars with different letter designations are significantly different from each other ($P < 0.001$). Data represent least squares means ± SEM from 9 separate assays using BSC isolated from at least 3 different animals. Each assay contained triplicate determinations. CPM = counts per minute.

Sinha-Hikim et al., 2002, 2003). Testosterone or TBA treatment increases protein synthesis rate and degradation rate in fused SC cultures (Kamanga-Sollo et al., 2011). These data are consistent with the anabolic actions both testosterone and TBA have on growth properties of skeletal muscle. The genomic actions of testosterone in which the AR acts as a ligand inducible transcription factor modulating target gene transcription have been well characterized (Dehm and Tindall, 2007; Simental et al., 1991). Several studies have also found that the AR and the EGFR and erbB2 may be involved in functionally significant cross-talk in cancerous cell lines, although it is currently unknown whether this occurs in skeletal muscle cells (Naderi and Hughes-Davies, 2008; Pignon et al., 2009). More recently, it has been demonstrated that testosterone or TBA may also initiate a quicker, nongenomic cellular response, which promotes growth and proliferation of muscle cells (Lieberherr and Grosse, 1994; Benten et al., 1999; Estrada et al., 2003).

Previous studies completed in skeletal muscle cells have shown that testosterone has an ability to activate GPCR, resulting in downstream physiological changes within the cell (Estrada et al., 2003; Fu et al., 2012). Studies in nonmuscle cells show that activation of GPCR results in transactivation of the EGFR through a mechanism involving MMP cleavage and release of membrane-bound hbEGF and its subsequent effects on EGFR (Luttrell et al., 1999; Prenzel et al., 1999; Liu and Armant, 2004; Filardo and Thomas, 2005; Chantrain et al., 2006; Almendro et al., 2010). G protein–coupled receptors have also been found to be capable of directly activating both EGFR and erbB2 (Chan et al., 2006; Arora et al., 2008). Previous research from our lab has also indicated that the IGF-1R is involved in TBA-mediated increases in BSC proliferation (Kamanga-Sollo et al., 2008). Additionally, data from our lab as well as from other labs suggests that expression of the EGFR may mediate expression of the IGF-1R (Riedemann et al., 2007; Reiter et al., 2014). Based on these findings, we hypothesized that this mechanism involving GPCR, MMP, hbEGF, and EGFR may also function in primary BSC. To test this hypothesis, we examined the effects of inhibiting GPCR, MMP2 and MMP9, hbEGF, erbB2, EGFR, and IGF-1R on TBA-mediated increases in BSC proliferation rates. A summary of this mechanism as well as the inhibitors used to test it can be seen in Fig. 6.

Our results show that when GPCR activity is inhibited by GDPβS, TBA-stimulated proliferation is completely inhibited, indicating the GPCR are required in TBA-induced BSC proliferation. This result is supported by previous research in muscle cells from other species analyzing the effects of testosterone-induced

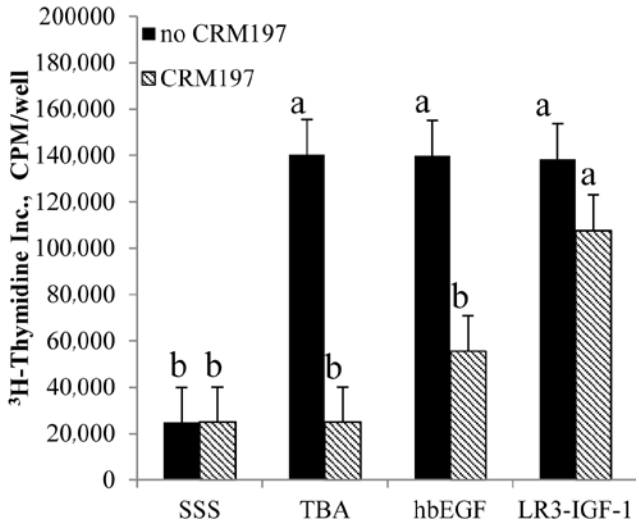

Figure 5. Effect of inhibition of heparin binding epidermal growth factor-like growth factor using 5 ng CRM197/mL on proliferation (^{3}H-thymidine incorporation [Inc.]) in bovine satellite cell (BSC) cultures treated with Dulbecco's modified Eagle medium (DMEM)/1% IGF binding protein-3–free swine serum (SSS) plus 25 ng LR3-IGF-1/mL, 5 ng heparin-binding epidermal growth factor–like growth factor (hbEGF)/mL, or 10 n*M* trenbolone acetate (TBA). Cells were plated as described in the Materials and Methods and after 72 h in culture, medium was removed, cultures were rinsed 3 times with DMEM, and cultures were fed with test media consisting of DMEM/1% SSS plus the indicated additions of hbEGF, TBA, and/or CRM197. Cultures treated with CRM197 were preincubated for 30 min with CRM197 before addition of hbEGF or TBA to the culture. Incorporation of ^{3}H-thymidine was measured at 96 h as described in the Materials and Methods. ^{a,b}Bars with different letter designations are significantly different from each other ($P < 0.001$). Data represent least squares means ± SEM from 9 separate assays using BSC isolated from at least 3 different animals. Each assay contained triplicate determinations. CPM = counts per minute.

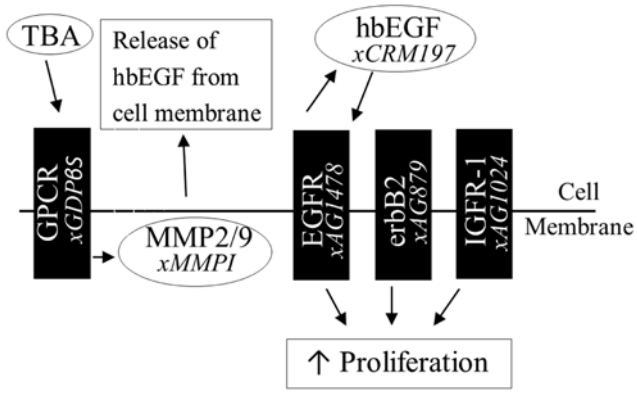

Figure 6. Hypothesized cellular mechanism of trenbolone acetate (TBA) in proliferating bovine satellite cells. Through inhibition of various receptors and intracellular molecules our research demonstrates that TBA activates G protein–coupled receptors (GPCR), which then activates matrix metalloproteinases 2 and 9 (MMP2 and MMP9) resulting in the release of heparin-binding epidermal growth factor–like growth factor (hbEGF), which then activates both the epidermal growth factor receptor (EGFR) and erbB2 resulting in increases in cell proliferation. The IGF-1 receptor (IGF-1R) is also involved in these processes, although it remains unclear how they fit into this mechanism. The different inhibitors are shown in the figure and are located beneath the molecule they inhibit next to an *x* in italics. The inhibitors that were used in the present study are guanosine 5′-O-2-thiodiphosphate (GDPβS), which generally inhibits all GPCR, a MMP2 and MMP9 inhibitor (MMPI), an inhibitor of hbEGF (CRM197), and the tyrosine kinase inhibitors AG1478, AG879, and AG1024, which inhibit the EGFR, erbB2, and IGF-1R, respectively.

proliferation (Fu et al., 2012). Additionally, this result has been observed in testosterone-induced proliferation of other nonmuscle cell types (Thomas et al., 2006; Gutkind, 1998; Shihan et al., 2014). Inhibition of GPCR using GDPβS results in nonspecific inhibition of all GPCR through its actions as a guanosine diphosphate analog that competitively inhibits G protein activation by guanosine triphosphate (**GTP**) and GTP analogs as well as its ability to inactivate G_i proteins (Eckstein et al., 1979; Piacentini et al., 1996). Consequently, our data does not allow us to identify the specific GPCR that is involved in TBA-stimulated BSC proliferation. However, our results showing that inhibition of GPCR does not suppress IGF-1–stimulated BSC proliferation indicate that inhibition of GPCR activity or activities does not have a nonspecific inhibitory effect on BSC proliferation in the presence of other mitogenic stimuli such as LR3-IGF-1.

It has been shown numerous times that MMP2 and MMP9 are involved in proliferation and migration of many different cell types including muscle SC (Newby, 2006; Montarras et al., 2013). Several studies have demonstrated in both human and bovine cells that MMP2 and MMP9 are active in SC (Guérin and Holland, 1995; Balcerzak et al., 2001). Matrix metalloproteinases 2 and 9 increase cell proliferation by cleaving hbEGF from the cell membrane, resulting in activation of the EGFR (Luttrell et al., 1999; Prenzel et al., 1999; Liu and Armant, 2004; Chantrain et al., 2006; Almendro et al., 2010). In the current study, we have demonstrated that inhibition of MMP2 and MMP9 is able to significantly inhibit TBA-induced BSC proliferation, indicating that MMP2 and MMP9 are involved in the molecular pathway involved in TBA-mediated BSC proliferation. Inhibition of MMP2 and MMP9 not only inhibits TBA-stimulated BSC proliferation but also inhibits hbEGF- and LR3-IGF-1–stimulated proliferation of BSC. These data raise the question of whether or not MMP2 and MMP9 are specifically involved in the proteolytic release of hbEGF that results in increased proliferation. Several studies have shown that MMP are involved in extracellular matrix alteration involved in both proliferation and migration of SC (Montarras et al., 2013). Although our data support a role for MMP2 and MMP9 in TBA-mediated BSC proliferation, it does not conclusively establish the specific responsibility of these molecules in this process. Further research needs to be conducted to determine the exact role of MMP2 and MMP9 in TBA-mediated increases in muscle growth.

Results from the present study demonstrate that exogenous hbEGF stimulates proliferation of cul-

tured BSC and that treatment with CRM197 inhibits this stimulation. Numerous studies have shown that CRM197 specifically binds hbEGF, inhibiting its ability to bind the EGFR (Mitamura et al., 1995). As such, our results showing that CRM197 inhibits TBA-stimulated BSC proliferation indicate that hbEGF is required for TBA to stimulate proliferation of BSC cultures. Our results showing that CRM197 treatment does not inhibit proliferation in BSC cultures treated with LR3-IGF-1 establish that CRM197 does not have a general inhibitory effect on BSC proliferation. Consequently, it appears very likely that CRM197 specifically inhibits TBA-stimulated BSC proliferation by binding and inactivating hbEGF.

Testosterone has been shown to elicit effects in several different tissue types through EGFR signaling (Gupta, 1996; Walker, 2009; Latour et al., 2013). More specifically, the EGFR has been shown to play a role in testosterone-induced effects within skeletal muscle (Hamdi and Mutungi, 2010; Sen et al., 2011). Similarly, in the present study, we have demonstrated that when the EGFR is inhibited by AG1478, TBA-mediated increases in BSC proliferation are significantly reduced down to the control level. Although, to date, no work has previously looked at whether erbB2 is affected by testosterone, many studies have been done analyzing the role of erbB2 and its interactions with other erbB family members (Tzahar et al., 1996; Liebmann, 2011). Previous research has shown that erbB2 is the preferred dimerization partner of EGFR and that erbB2 is a lateral signal transducer to the other erbB family members (Tzahar et al., 1996; Graus-Porta et al., 1997; Liebmann, 2011). Based on finding from these studies as well as the findings of our own study, it is likely that both the EGFR and erbB2 are necessary for TBA to increase proliferation rates of BSC cultures.

Local production of IGF-1 in skeletal muscle is thought to have a significant role in supporting normal muscle growth (Sjogren et al., 1999). Previous studies have shown that testosterone treatment increases IGF-1 mRNA levels in human skeletal muscle (Gayan-Ramirez et al., 2000; Lewis et al., 2002). Additionally, treatment with a TBA and E2 implant for as few as 7 d has been shown to increase IGF-1 mRNA levels in the LM of yearling steers when compared with nonimplanted steers (Johnson et al., 1998; Dunn et al., 2003; Pampusch et al., 2003). Previous work from our lab shows that BSC cultures grown in 10% FBS have increased IGF-1 mRNA levels when treated with TBA; however, when cultures are plated in 1% SSS, treatment with TBA has no effect on IGF-1 mRNA levels (Kamanga-Sollo et al., 2004, 2008). Despite this result, in both the present study and previous studies that have been conducted in our lab, we have demonstrated that TBA-stimulated proliferation requires the IGF-1R in cultures grown in 1% SSS (Kamanga-Sollo et al., 2008). These results have been obtained using 2 different specific inhibitors of the IGF-1R, AG1024 and JB1 (Kamanga-Sollo et al., 2008). This indicates that TBA increases proliferation of BSC cultures through a mechanism involving the IGF-1R; however, it is not due to increased IGF-1 mRNA expression levels within the BSC. Recent findings suggest that the EGFR may mediate expression of the IGF-1R, although a direct mechanism regarding the involvement of the IGF-1R in our system remains unclear (Riedemann et al., 2007; Reiter et al., 2014).

Our current results show that GPCR, MMP2 and MMP9, hbEGF, EGFR, erbB2, and IGF-1R are required for TBA-stimulated BSC proliferation, indicating that TBA increases proliferation of BSC through a nongenomic mechanism. These data in conjunction with reports that TBA activates GPCR as well as reports stating that in nonmuscle cells, GPCR result in activation of MMP2 and MMP9, which cleaves hbEGF from the cell membrane through proteolytic activity, resulting in EGFR and erbB2 activation, suggest that a mechanism involving GPCR and EGFR/erbB2 transactivation may be involved in TBA-mediated increases in BSC proliferation. This hypothesis is further supported by our findings that the EGFR and erbB2 are required for TBA-stimulated BSC proliferation. The exact role that MMP2 and MMP9 and the IGF-1R have in these processes remains unclear and requires further study. In conjunction with the proposed nongenomic mechanism of TBA-stimulated proliferation, it is important to remember that TBA-mediated proliferation of BSC also requires the AR and these nongenomic and genomic mechanisms are likely not independent of one another, although their exact relationship remains to be further elucidated.

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