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Trenbolone enhances myogenic differentiation via enhancing β-catenin signaling in muscle-derived stem cells of cattle

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Abstract

Testosterone is a key hormone regulating animal growth and development, which promotes skeletal muscle growth and inhibits fat deposition; however the underlying mechanisms remain poorly defined. Because canonical Wingless and Int (Wnt)/β-catenin signaling promotes myogenesis, we hypothesized that testosterone regulates myogenesis through enhancing the β-catenin signaling pathway and the expression of its targeted genes. Muscle-derived stem cells were prepared from the skeletal muscle of fetal calf at day 180 of gestation, and treated with or without trenbolone (10 nM), a synthetic analog of testosterone, in a myogenic medium. Trenbolone treatment increased the protein levels of MyoD and myosin heavy chain, as well as the androgen receptor content. The myogenic effect of trenbolone was blocked by cyproterone acetate, a specific inhibitor of androgen receptor, showing that the myogenic effect of trenbolone was mediated by the androgen receptor. Immunoprecipitation (IP) showed that androgen receptor and β-catenin formed a complex, which was increased by trenbolone treatment. Trenbolone activated AMP-activated protein kinase, which might phosphorylate β-catenin at Ser 552, stabilizing β-catenin. Indeed, both cytoplasmic and nuclear β-catenin levels were increased following trenbolone treatment. As a result, β-catenin mediated transcriptional activity was enhanced by trenbolone treatment. In conclusion, these data provide evidences that testosterone increases cellular β-catenin content which promotes the expression of β-catenin targeted genes and myogenesis in the muscle-derived stem cells of cattle.

Keywords

AMPK; β-catenin; cattle; myogenesis; testosterone; trenbolone

1. Introduction

Skeletal muscle and fat are most important tissues regarding meat animal production, and appropriate development of both tissues directly impact the efficiency and quality of animal production. Both skeletal muscle cells and adipocytes are derived from the same pool of mesenchymal stem cells during the early developmental stages (1). Promoting myogenesis from stem cells increases lean mass and lean/fat ratio; enhancing intramuscular adipogenesis increases intramuscular fat and improves the eating quality of meat but fat deposition

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elsewhere leads to waste. Hence, it is essential to regulate stem cell differentiation in order to enhance lean growth and improve meat quality.

Testosterones are important sex hormones regulating animal growth and development, which is known to enhance myogenesis while inhibits adipogenesis (2-5). Testes develop during the early to mid-gestation in ruminant animals, which secrete testosterones to regulate fetal development (6,7). In addition, testosterones are also known to enhance muscle growth postnatally. Trenbolone acetate is a synthetic analog of testosterone which is widely used in the US and Canada (8). Implanted trenbolone in steers enhances animal growth performance and increases muscle mass (9-12). To date, however, the underlying mechanisms for the biological effect of testosterone on muscle growth are not very clear.

Wingless and Int (Wnt)s are a family of secreted glycoproteins that regulate cell proliferation and differentiation (13). Wnts exert their effect mainly through the β-catenin pathway; once Wnt signaling is activated, β-catenin translocates into nuclei and binds to the T cell factor/lymphoid-enhancing factor (TCF/LEF) to regulate the expression of target genes (14). Wnt/β-catenin signaling pathway is required for myogenesis (15-17). Activation of the Wnt signaling pathway enhances myogenesis and inhibits adipogenesis in cultured mesenchymal stem cells (18).

AMPK is a heterotrimeric enzyme with α, β, and γ subunits (19,20). The α subunit is the catalytic unit, the γ subunit has a regulatory function, and the β unit provides anchorage sites for the α and γ subunits (21). AMPK is known as a key regulator of energy metabolism (19,20), and is increasingly recognized as a key mediator of cell differentiation (22,23). Activation of AMPK promotes myogenesis (24). Our previous study shows that AMPK cross-talks with β-catenin through phosphorylation of β-catenin at Ser 552 (25). In this study, we hypothesized that testosterone activates AMPK and enhances β-catenin signaling which promotes myogenesis.

2. Material and methods

2.1. Antibodies and chemicals

Antibodies against β-catenin (#9587), β-tubulin (#2146), androgen receptor (#3202), phospho-AMPK α subunit (#2535), AMPK α subunit (#2532) and histone 3 (#9715) were purchased from Cell Signaling (Danvers, MA); anti-MyoD (#5117) was purchased from GenScript (Piscataway, NJ). Desmin and MHC antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). IRDye 800CW goat anti-rabbit secondary antibody and IRDye 680 goat anti-mouse secondary antibody were purchased from LI-COR Biosciences (Lincoln, NE). Trenbolone acetate and cyproterone acetate were purchased from Sigma (St. Louis, MO), and dissolved on DMSO.

2.2. Cell culture

Bovine muscle-derived stem cells (MDSC) were prepared from the skeletal muscle of a male fetus at around day 180 of gestation obtained from a cow (Angus breed) slaughtered in the Meat Laboratory at University of Wyoming for teaching purposes and three replicate experiments were conducted on these cells. Muscle-derived stem cells were separated from

the *Longissimus dorsi* (Ld) muscle as previously described (26). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37 °C. When cells were 100% confluent, FBS was replaced by 2% horse serum for myogenic induction (27). When appropriate, cells were also treated with trenbolone (10 nM) and/or trenbolone (10 nM) + cyproterone acetate (100 nM).

2.3. Transfection

Transfection was performed using Lipofectamine (Invitrogen, Carlsbad, CA) according to the instruction from the manufacturer. Briefly, when MDSC reached 95% confluence, cells were changed to no antibiotic medium and transfected with TOP-Flash or FOP-flash at the ratio of DNA (μg): Lipofectamine (μl) =1:3.

2.4. Isolation of Nuclei and cytosol

Nuclei and cytosol proteins were isolated using a nuclear extract kit from Active Motif North America according to the manual instruction (Carlsbad, CA).

2.5. Real-time quantitative PCR (RT-PCR)

Total RNA was extracted using Trizol (Sigma, Saint Louis, MO) followed by DNase I (#M0303s) digestion (New England Biolabs, Ipswich, MA) and l μg total RNA was used for cDNA synthesis using a reverse transcription kit (Bio-Rad, Hercules, CA). Real-time PCR (RT-PCR) was carried out using CFX RT-PCR detection system (Bio-Rad, Hercules, CA) with a SYBR Green RT-PCR kit from Bio-Rad (Hercules, CA) and following cycle parameters were used: 20 sec at 95 °C, 20 sec at 55 °C, and 20 sec at 72 °C for 36 cycles. Primer sequences and their respective PCR fragment length were as following: androgen receptor (PCR product size: 186 bp), forward: 5′-AGGCGCTTCTACCAGCTCACCA-3′, and reverse: 5′-ACGATAGCGCTTGACTTTACCCG-3′; β-catenin (151 bp) forward: 5′-TGGTGTGCCAAGTGGGTGGC-3′, and reverse: 5′-AGGCGAACAGCGTTCTGGGC-3′; and 18S rRNA (118 bp) forward: 5′-CCTGCGGCTTAATTTGACTC-3′, and reverse: 5′-AACTAAGAACGGCCATGCAC-3′. After amplification, a melting curve (0.01 C/sec) was used to confirm product purity and agarose gel electrophoresis was performed to confirm that only a single product of the right size was amplified. The final primer concentration was 200 nM for each gene. The amplification efficiency was 0.90 – 0.99, and the difference between internal reference and experimental gene was less than 5%. Results are expressed relative to 18S rRNA using delta CT method.

2.6. Immunoprecipitation

After washing with cold PBS, MDSC were lysed with ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2.5 mM sodium pyrophosphate, 1 μg/mL leupeptin, 1 mM Na_3VO_4 and 100 mM NaF. Lysates were collected and microcentrifuged at 14,000 × g for 10 min at 4 °C; 200 μL supernatant was mixed with 20 μL Protein A Sepharose bead slurry (50%) (Rockland Inc, Gilbertsville, PA) for 2 hours and then centrifuged to remove non-specific binding of proteins with Protein A Sepharose beads. The resulting supernatant (150 μL) was mixed with anti-β-catenin antibody (1:50 dilution) and incubated with rocking at 4 °C overnight.

Then, protein A Sepharose bead slurry (50%, 20 μL) was added and continued to incubate with rocking for 2 additional hours. Immunoprecipitates were collected and washed with 100 μL lysis buffer three times. Then, an equal volume of 2 × SDS loading buffer was added and boiled for 5 min. Immunoblotting was conducted using an antibody against androgen receptor.

2.7. Luciferase reporter activity assay

To measure the transcriptional activity of TCF/LEF, MDSC seeded in 24 well-plates were transfected in triplicates with a TCF/LEF reporter (Top-flash, Addgene plasmid 12456) or control vector (Fop-flash, Addgene plasmid 12457) (28). β-Galactosidase vector (Promega, Madison, WI) was transfected with an internal control. 24 hr following transfection, cells were lysed and the luciferase activity was measured with Bright-Glo Luciferase Assay System (Promega, Madison, WI). Luciferase activity was normalized to the β-galactosidase activity.

2.8. Immunoblotting Analyses

Cells were homogenized in 200 μl extraction buffer containing 20 mM Tris-HCl (pH 7.4 at 4 °C), 2% SDS, 1% Triton X-100, 5.0 mM EDTA, 5.0 mM EGTA, 1 mM DTT, 100 mM NaF, 2 mM sodium vanadate, 0.5 mM phenylmethylsufonyl fluoride, 10 μL/mL leupeptin, and 10 μl/ml pepstatin. Supernatant was mixed with an equal amount of sample loading buffer containing 150 mM Tris-HCl (pH 6.8), 20% glycerol, 2 mM 2-mercaptoethanol, 0.004% (wt/vol) bromophenol blue and boiled for 3 min. Samples were subjected to SDS-PAGE and immunoblotting as previously described using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Band density was normalized according to the β-tubulin content (29).

2.9. Statistics

For all cell culture studies, at least 3 replicate experiments were conducted. All data were expressed as mean ± standard errors (SE). Data were analyzed independently using the GLM of SAS (SAS Inst., Inc., Cary, NC) and Tukey's Studentized Range test was used to determine significant differences among means. $P < 0.05$ was considered to be significant.

3. Results

Trenbolone stimulates myogenic differentiation in cattle derived stem cells

In order to determine the composition of cattle MDSC, we conducted immunocytochemical staining with an anti-desmin antibody. As shown in Fig. 1A, the majority of cells were desmin positive (88.0±3.0 %), indicating they were myogenic cells. Those unstained cells are likely to be uncommitted multipotent cells, fibroblasts and pre-adipocytes.

MDSC were treated with or without trenbolone (10 nM) and trenbolone plus cyproterone acetate (100 nM) (30). Key myogenic regulatory factors such as MyoD (an early myogenic marker) and Myosin Heavy Chain (MHC, a late myogenic marker) were analyzed by immunoblotting. Compared with the control group, both MyoD and MHC protein levels were up-regulated by trenbolone treatment ($P < 0.05$); blocking androgen receptor by

cyproterone acetate offset the myogenic effect of trenbolone (Fig. 1BC). We also conducted immunocytochemical staining using an anti-MHC antibody. More myotubes were formed in the trenbolone group (Control versus treatment: 5.7±0.6% versus 13.6±0.5%; $P < 0.01$; Fig. 1D).

Trenbolone exerts myogenic effect mainly through androgen receptor

We analyzed androgen receptor expression using both immunoblotting and real-time PCR. Trenbolone enhanced both mRNA expression and protein content of the androgen receptor. Blocking androgen receptor by cyproterone acetate did not attenuate androgen receptor expression due to trenbolone treatment ($P < 0.05$, Fig. 2AB), indicating that other mechanisms existed which enhanced androgen receptor expression following androgen treatment.

Androgen receptor interacts with β-catenin and promotes β-catenin translocation

Given the importance of β-catenin signaling in regulating myogenesis, we further analyzed whether androgen receptor interacts with β-catenin using immunoprecipitation. Androgen receptor formed a complex with β-catenin which was enhanced by trenbolone treatment ($P < 0.05$, Fig. 3A). Because both β-catenin and androgen need to go into nuclei to regulate gene expression, we then analyzed the sub-cellular location of β-catenin by separating nuclei and cytosol proteins. Trenbolone treatment increased both nuclei and cytosol β-catenin levels ($P < 0.05$, Fig. 3BC).

Trenbolone stabilizes β-catenin possibly via activation of AMPK

Because both nuclei and cytosol β-catenin levels were increased with the treatment of trenbolone, we asked why trenbolone increased the β-catenin level. Real-time PCR was used to evaluate β-catenin mRNA expression following trenbolone treatment. As shown in Fig. 4A, β-catenin mRNA expression did not change between groups with and without trenbolone treatment.

β-Catenin is phosphorylated at Ser 552 by protein kinase A (PKA), protein kinase B (Akt) and AMPK, which enhances β-catenin contents and mobilization from the cytoskeleton pool (31-35). Therefore, we further analyzed whether trenbolone activates AMPK. Addition of trenbolone activated AMPK ($P < 0.05$, Fig. 4B), and the phosphorylation of β-catenin at Ser 552 was also increased ($P < 0.05$, Fig. 4C), which was correlated with increased β-catenin content (Fig. 3BC).

Trenbolone treatment enhanced β-catenin mediated transcriptional activity

β-Catenin interacts with transcriptional factors of the TCF/LEF-1 family to regulate downstream target gene expression. To evaluate whether trenbolone treatment increased β-catenin mediated gene expression, TOP flash reporter and control FOP flash plasmids were transfected into cells. Trenbolone treatment increased the TOP flash transcriptional activity ($P < 0.05$, Fig. 5), showing that trenbolone enhanced β-catenin mediated gene expression.

Discussion

In animals, prenatal muscle growth is characterized by the generation of new myocytes which fuses to form new muscle fibers, while postnatal muscle growth is the outcome of hypertrophy of existing muscle fibers. A pool of multipotent cells exist inside muscle, which are capable to differentiate into myocytes, adipocytes and fibrogenic cells (36). These cells are commonly referred to as MDSC which are slightly different from satellite cells which exist in mature muscle and are mostly committed myogenic cells (37). At the early developmental stage, the concentration of MDSC in muscle is very high, which reduces as muscle development continues (36). To obtain a high ratio of multipotent cells, we prepared MDSC from the skeletal muscle of fetal cattle. The composition of prepared cells was analyzed by using immunofluorescence staining; the large portion of these isolated cells was myogenic cells as indicated by the presence of a myogenic marker, desmin. Those unstained cells are likely composed of MDSC, pre-adipocytes and fibroblasts.

To determine the myogenic effect of trenbolone, we first analyzed the expression of MyoD and MHC. MyoD is a key mediator of initial myogenesis, while MHC is the marker of mature myotubes (38,39). We observed the expression of MyoD was enhanced in cattle MDSC after trenbolone treatment. Immunocytochemistry staining with anti-MHC also indicated that trenbolone promoted the formation of myotubes. When the androgen receptor was blocked by cyproterone acetate, the myogenic effect of trenbolone was blocked. These data indicate that trenbolone promotes myogenesis in cultured cattle cells, which is at least partially mediated by the androgen receptor. Then we analyzed androgen receptor expression. Similar with previous reports (40,41), trenbolone increased the androgen receptor expression at both protein and mRNA levels. However, blocking androgen receptor did not abolish the enhancement of androgen receptor expression by trenbolone, suggesting non-androgen receptor-mediated effects exist (42,43).

Wnt/β-catenin signaling pathway works through both frizzled and low-density lipoprotein receptor-related proteins (LRP), which leads to the inactivation of glycogen synthase kinase 3β (GSK-3β) and prevention of β-catenin degradation. In nuclei, β-catenin binds to T-cell factor (TCF) lymphoid-enhancer binding factor (LEF) transcription factors to induce Wnt targeted genes, including genes involved in myogenesis (44), Pax 3 and Gli (45,46), which then induce expression of MyoD and Myf-5 (47-50). β-Catenin was a primary mediator of the canonical Wnt/β-catenin signaling pathway, and increase in the cellular β-catenin content alone increases myogenesis (51,52).

In a recent study using C3H10T1/2 cells, androgen was shown to form a complex with β-catenin to promote myogenesis (53), which was confirmed in this cattle MDSC study. Beyond this observation, we observed that androgen treatment increased cellular β-catenin protein levels in both cytosol and nuclei, which had not been observed previously. This observation indicates that the increase in the formation of androgen receptor and β-catenin complex may be due to the increase in their cellular contents following androgen treatment, not necessarily due to their increased affinity with androgen receptor as previously reported (53). When we are writing this paper, a study appeared that using a genome-wide microarray screen for pro-myogenic genes in C2C12 cells, Axin and Axin2, two negative regulators of

β-catenin, were down-regulated by androgen (54), consistent with our observation of increased β-catenin protein level following trenbolone treatment in cattle MDSC.

The next question is why androgen enhances β-catenin cellular content. The cellular β-catenin content is regulated mainly by degradation (55,56). β-Catenin is phosphorylated at Ser 552 and Ser 675 by protein kinase A (PKA), protein kinase B (Akt) and AMPK (31-35), which enhances β-catenin contents and mobilization from the cytoskeleton pool (31,32). Therefore, we analyzed β-catenin phosphorylation at Ser 552, which was increased by androgen treatment. We further analyzed kinases responsible for this phosphorylation. We observed that AMPK activity was enhanced by androgen, which may explain the increase in the cellular β-catenin levels.

In conclusion, our findings demonstrate that testosterone enhances myogenesis in cattle MDSC at least partially through enhancing β-catenin signaling pathway, which is known to be important for myogenesis. Such enhancement is likely due to the phosphorylation of β-catenin at Ser 552 which enhances its stability and promotes its mobilization from the cytoskeleton pool. Our study provides an additional mechanism for the promotion of muscle growth by testosterone.

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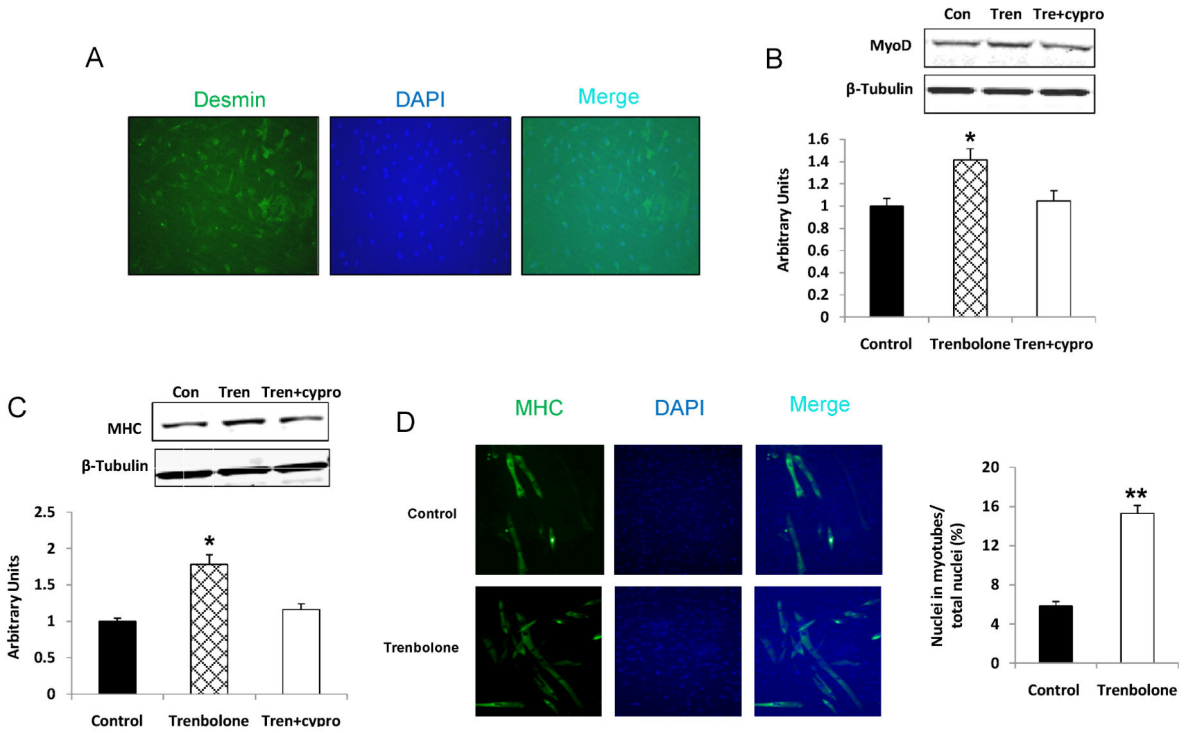

Fig. 1.

Trenbolone promotes myogenesis in cattle muscle-derived stem cells. Cells were staining with anti-desmin and DAPI, and myogenic cells were counted (A). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37 °C. When cells were 100% confluence, FBS was replaced by 2% horse serum for myogenic induction with trenbolone (10 nM) and trenbolone (10 nM) + cyproterone acetate (100 nM). Both MyoD (6 d) and Myosin heavy chain (MHC, 12 d) were analyzed by western blotting. Data showed that trenbolone increased both MyoD and MHC expression; blocking androgen receptor, myogenic effect of trenbolone was blocked (B, C). Cells were staining with anti-MHC and DAPI at day 12 after trenbolone treatment. Data showed that trenbolone increased the number of differentiated myotubes (D). (*$P < 0.05$; **$P < 0.01$; Mean ± SE; n = 3).

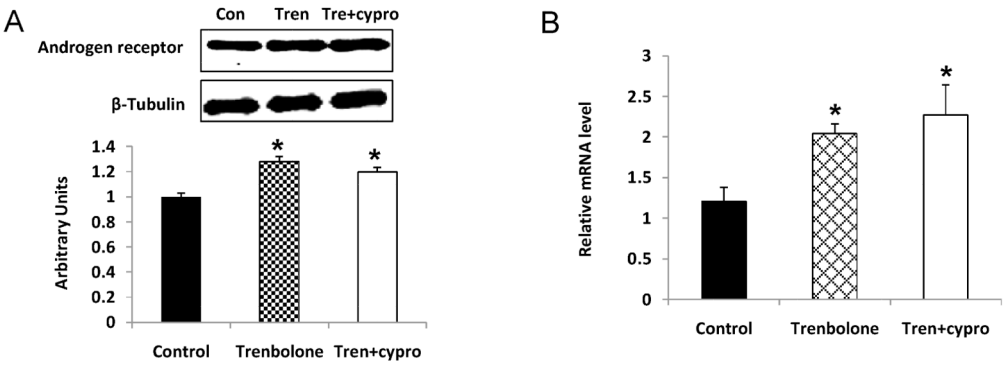

Fig. 2.

Trenbolone exerts myogenic effect mainly through androgen receptor. Cells were treated with trenbolone (10 nM) and trenbolone (10 nM) + cyproterone acetate (100 nM). Cells were collected for real-time PCR (6 h) and western blotting (24 h) analyses. Data indicate that androgen receptor was up-regulated by trenbolone at both protein and mRNA levels. (*$P < 0.05$; Mean ± SE; n = 3).

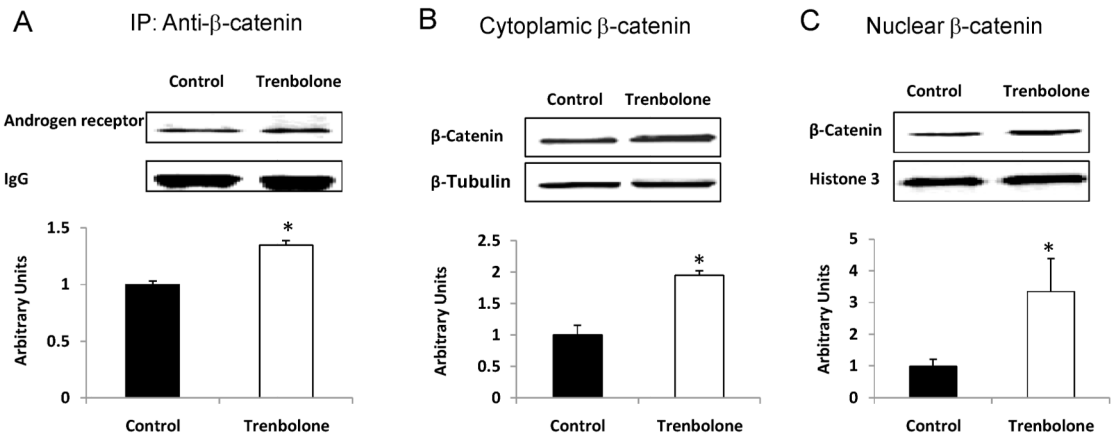

Fig. 3.

Trenbolone increases cellular β-catenin levels. Cells were treated with trenbolone (10 nM) for 12 h. Immunoprecipitation was carried using anti-β-catenin and then androgen receptor was analyzed by western blotting. Data showed that androgen receptor formed a complex with β-catenin; trenbolone treatment increased androgen receptor protein content (A). Cells were treated with trenbolone (10 nM) for 12 h and then cytoplasmic and nuclear portions were separated. Data indicated that Trenbolone treatment increased both cytoplasmic and nuclear β-catenin levels. (*$P < 0.05$; Mean ± SE; n = 3).

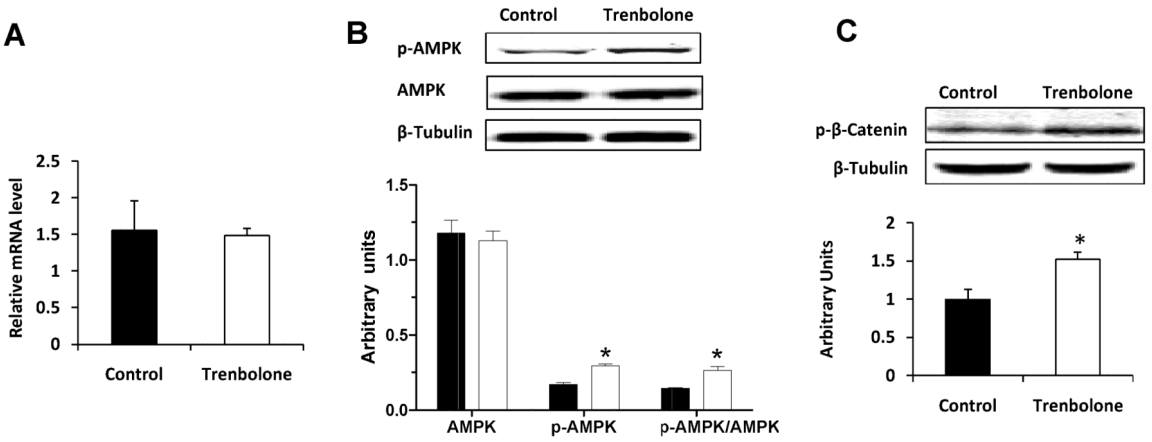

Fig. 4.

Trenbolone stabilizes β-catenin through the activation of AMPK. Cells were treated with trenbolone (10 nM) for 6 h. The mRNA expression was analyzed with real-time PCR. Data showed that β-catenin mRNA expression was not changed between control and trenbolone treated groups (A). Trenbolone promoted the activation of AMPK (B) and increased the phosphorylation of β-catenin at ser 552 (C). (*$P < 0.05$; Mean ± SE; n = 3).

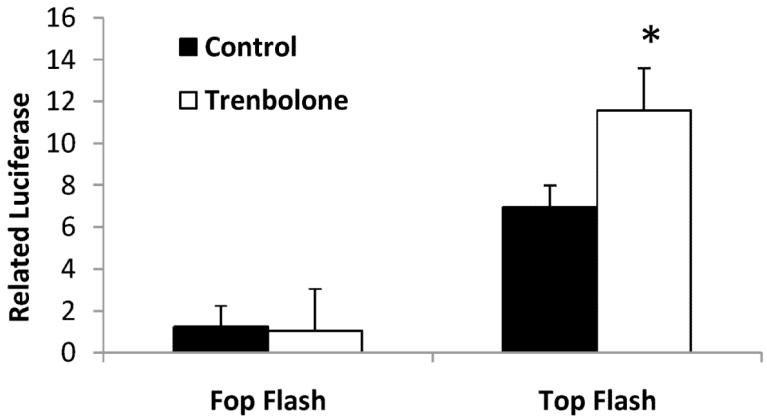

Fig. 5.

Androgen treatment enhances β-catenin mediated gene expression. Cells were transfected with FOP-flash or TOP-flash with or without trenbolone. Luciferase activity was measured after 24 h treat. No difference was detected between trenbolone and control treatments when cells were transfected with FOP flash. Trenbolone increased the luciferase activity when transfected with TOP flash. (*$P < 0.05$; Mean ± SE; n = 3).