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Nandrolone reduces activation of Notch signaling in denervated muscle associated with increased Numb expression

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ABSTRACT

Nandrolone, an anabolic steroid, slows denervation-atrophy in rat muscle. The molecular mechanisms responsible for this effect are not well understood. Androgens and anabolic steroids activate Notch signaling in animal models of aging and thereby mitigate sarcopenia. To explore the molecular mechanisms by which nandrolone prevents denervation-atrophy, we investigated the effects of nandrolone on Notch signaling in denervated rat gastrocnemius muscle. Denervation significantly increased Notch activity reflected by elevated levels of nuclear Notch intracellular domain (NICD) and expression of Hey1 (a Notch target gene). Activation was greatest at 7 and 35 days after denervation but remained present at 56 days after denervation. Activation of Notch in denervated muscle was prevented by nandrolone associated with upregulated expression of Numb mRNA and protein. These data demonstrate that denervation activates Notch signaling, and that nandrolone abrogates this response associated with increased expression of Numb, suggesting a potential mechanism by which nandrolone reduces denervation-atrophy.

1. Introduction

Androgens and their synthetic analogs have well established anabolic actions on skeletal muscle [1] and have been demonstrated to reduce muscle loss caused by microgravity [2], immobilization [3] and spinal cord injury [4]. We have reported that nandrolone reduces atrophy of denervated muscle associated with significant decreases in the expression of muscle atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF1) [5]; nandrolone was administered together with testosterone at a dose resulting in high physiological concentrations of testosterone. Henceforth, we refer to this treatment as 'nandrolone'. In these studies, nandrolone was initiated either at the time of nerve transection or 29 days thereafter. When initiated at day 29, nandrolone reduced muscle atrophy and expression of MAFbx and MuRF1 at 35 and 56 days; when initiated at the time of nerve transection nandrolone had no effect on atrophy or expression of MAFbx or MuRF1 at 7 or 14 days [5]. The precise mechanisms underlying the anabolic actions of androgens on skeletal muscle are poorly understood. In mouse models of aging-related sarcopenia, testosterone reduced levels of processed myostatin and increased Notch activation [6].

Notch signaling is initiated by the binding of Notch ligands to the Notch receptor, a cell surface receptor with a single membrane spanning domain [7]. Binding of ligand releases the intracellular domain of the Notch receptor (NICD) into the cytoplasm by sequential enzymatic cleavage, followed by its nuclear translocation. The NICD induces expression of the Hey family of transcriptional repressors, including Hey1, which function as downstream effectors of Notch signaling [8–10]. Notch signaling plays an important role in tissue morphogenesis both during development and postnatal repair of injured skeletal muscle when it is critical to satellite cell activation and proliferation [11–14]. A subsequent decline in Notch signaling activity is necessary for differentiation of progenitor cells into fusion competent myoblasts [11–14]. Among the signals known to switch off Notch signaling is increased expression Numb [15]. The objective of this study was to determine the effects of nandrolone on Notch signaling in denervated skeletal muscle using nuclear levels of NICD and expression of Hey1 as measures of Notch signaling and to evaluate the potential role of Numb in modulating Notch signaling in denervated muscle.

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2. Materials and methods

2.1. Animals and tissue collection

Procedures with experimental animals were approved by the Institutional Animal Care and Use Committee at the James J. Peters VA Medical Center and were conducted in accord with accepted standards of humane animal care. Tissues used in the current study were originally collected as part of studies of the effects of nandrolone on denervation atrophy and expression of MAFbx and MuRF1 [5]. Details of the animal model are describe elsewhere [5]. Briefly, administration of nandrolone (0.75 mg/kg/wk) plus testosterone (2.8 mg/kg/day), or vehicle (propylene glycol), was begun either at day 0 (7 day group) or day 29 (35 and 56 day groups) after transection of the left sciatic nerve at the level of the humoral head. Test agents were administered as a continuous infusion by Alzet miniosmotic pumps implanted subcutaneously [5]. Nandrolone was administered together with testosterone to control for effects of nandrolone to reduce circulating testosterone levels [5]. Rat gastrocnemius muscles were collected at 7, 35, and 56 days after denervation, flash frozen in liquid nitrogen, and stored in sealed tubes at −80 °C. Frozen samples were cut from frozen muscle for subsequent biochemical analysis in both the original study and this one. Four samples per group were analyzed for the current study. Samples were chosen for the current study were those with the largest amounts of muscle tissue remaining.

2.2. Reagents and antibodies

Primers and probes used for real-time PCR were obtained from the Assays on Demand catalog of Applied Biosystems (Forster City, CA). Antibodies against β-tubulin were obtained from AbCam (Cambridge, MA); antibodies against Notch intracellular domain (NICD) and Numb were obtained from Cell Signaling (Danvers, MA). Anti-Hey1 and histone H1 antibodies were purchased from Santa Cruz Inc. (Santa Cruz, CA).

2.3. Quantitative real-time PCR

Extraction from tissues of total RNA, and quantitative real time PCR, were performed as described previously [5] using a thermocycler (Model 7500; Applied Biosystems). For each sample, real-time PCR determinations were performed in triplicate, and the mean for the crossing points of triplicates was used in subsequent calculations. mRNA levels were expressed as fold-change using the $2^{-\Delta\Delta Ct}$ method [16]. Data were normalized relative to 18s RNA.

2.4. Protein extraction and Western blot analysis

Gastrocnemius muscle (25 mg) was homogenized in 500 μL of buffer [150 mM sodium chloride, 3.2 mM Na_2PO_4, 0.8 m K_2PO_4(pH 7.4),1% NP-40, 0.5% sodium deoxycholate, 0.5% sodium dodecylsulfate] using a Polytron. Homogenates were cleared by centrifugation in a microcentrifuge at 14,000 rpm for 5 min. Proteins from the cytosolic and nuclear fractions were isolated using a commercial kit purchased from PIERCE (Rockford, IL), according to the manufacturer's instructions. Protein content was assayed using a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin as a standard. Western blotting was performed as previously described [17] using the following antibodies: NICD (1:1000); Hey1 (1:250); and Numb (1:1000). β-Tubulin and histone H1 were used as the internal controls for Western blot analyses of cytoplasmic or total proteins, and nuclear fractions, respectively. Immunostaining was visualized by enhanced chemiluminescence and recorded with photographic film or a Kodak IS4000 imaging system. Scanning densitometry of digitized images was performed using Imagequant TL (GE Life Sciences, Piscataway, NJ). Intensities of bands were normalized relative to β-tubulin or histone H1 as indicated then expressed as fold-change relative to the Sham-denervated vehicle control.

2.5. Statistics

Data are expressed as mean values ± SEM. The significance of differences among sham, vehicle- and nandrolone-treated groups at specific time-points was determined using two-way analysis of variance (ANOVA) with a Bonferroni test post hoc to determine the significance of differences between specific pairs of means. Statistical calculations were performed using Prism 4.0 (GraphPad Software, San Diego, California). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Nandrolone inhibited denervation-induced Notch signaling activity

Western blot analysis showed that NICD levels significantly increased by more than 2.5-fold in nuclear fractions from denervated gastrocnemius muscles from the vehicle group at 7 and 35 days compared with the levels in sham-denervated muscle at these time points (Fig. 1A and B) and remained increased by approximately 1.8-fold at 56 days, suggesting an increased activity of Notch signaling after denervation. In denervated muscle from nandrolone-treated animals, however, nuclear NICD levels were significantly reduced, by more than 2-fold, at 7 and 35 days compared to denervated muscle from the vehicle group (Fig. 1A and B).

Levels of Hey1 mRNA were significantly increased by nearly 2-fold at 35 and 56 days in denervated muscle from the vehicle group compared to that from the sham-denervated group (Fig. 2A). Hey1 protein levels were increased by approximately 1.5-fold at all time points in denervated muscle from the vehicle group compared to the sham-denervated group (Fig. 2B and C). As compared to the denervation-vehicle group, nandrolone significantly reduced Hey1 mRNA levels at 35 and 56 days, and Hey1 protein levels at 7, 35 and 56 days (Fig. 2A–C).

3.2. Nandrolone induced Numb expression

Levels of Numb mRNA and protein were not significantly different when comparing sham-denervated and denervated-vehicle

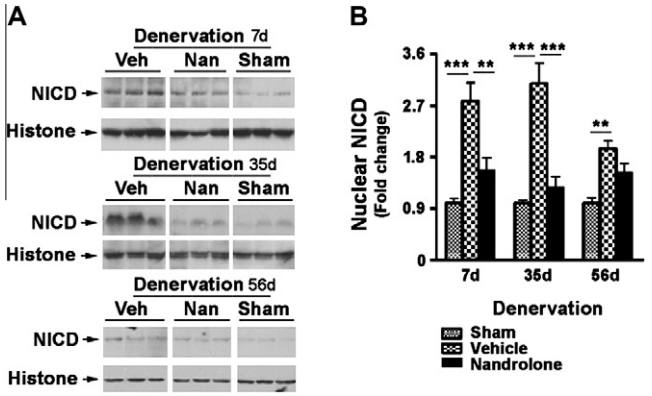

Fig. 1. Nandrolone prevented denervation-induced activation of Notch Receptor. (A) Nuclear protein was isolated from gastrocnemius muscles and subjected to Western blot analysis using an antibody against NICD, then stripped and reprobed with antibody against histone H1. (B) Bands for Notch in A were quantified by scanning densitometry and normalized to histone H1. $^{**}p < 0.01$, and $^{***}p < 0.001$.

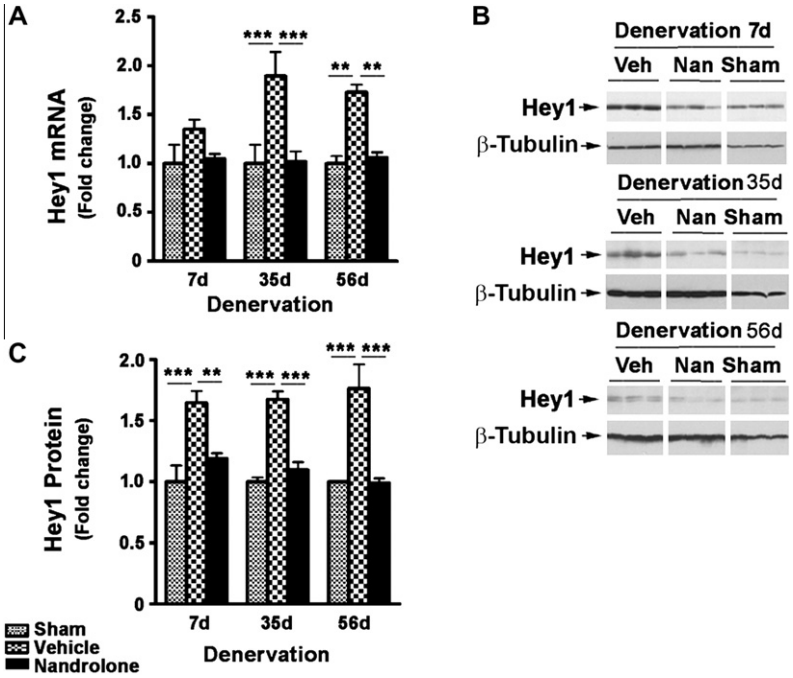

Fig. 2. Nandrolone prevented denervation-induced increases in expression of Hey1. (A) Hey1 mRNA levels in rat gastrocnemius muscles were determined by quantitative real-time PCR. (B) Western blot analysis of total protein isolated from rat muscles using an antibody against Hey1. The blots were stripped and reprobed with β-tubulin antibody. (C) Bands for Hey1 in D were quantified by scanning densitometry and normalized to β-tubulin. Data are mean values ± SEM for four animals per group; $^{**}p < 0.01$, and $^{***}p < 0.001$.

groups (Fig. 3A–C). Expression of Numb mRNA was significantly increased in the denervated-nandrolone group by nearly 2-fold at 7 days and more than 2-fold at 35 days when compared to either the sham-denervation or denervation-vehicle groups. The induction of Numb mRNA by nandrolone was reduced at 56 days (Fig. 3A). Numb protein was also significantly increased in denervated muscle from the nandrolone group by approximately 2-fold at 7 and 35 days compared to the sham-denervation or denervation-vehicle groups but levels of this protein were not altered in the denervation-nandrolone group at 56 days (Fig. 3B and C).

4. Discussion

4.1. Alterations in Notch signaling after denervation

The findings of elevated levels of NICD and Hey1 expression in denervated muscle indicate activation of Notch signaling. Thus, the findings demonstrate that increased Notch signaling was present after nerve transection at 7 and 35 days after denervation and persisted, to a lesser extent, at 56 days. The mechanism by which activation of Notch occurred in denervated muscle is unclear. We are not aware of other reports describing levels of Notch signaling in muscle paralyzed by nerve transection or other neurological deficits. Because Notch has been found to promote proliferation of muscle progenitor cells [11–14], it is noteworthy that previous reports described proliferation of satellite cells after nerve transection that began within 48 h after denervation and continued for at least 30 days, resulting in significant increases in satellite cell numbers during the initial period after nerve transection [18–20]. Of interest, it has been reported that after longer periods of denervation, satellite cell numbers are reduced [21]. One contributing factor may be apoptosis of satellite cells in denervated muscle [22]. Another possible explanation for this decrease is suggested by our findings that Notch signaling declines at 56 days.

4.2. Effects of nandrolone on Notch signaling and Numb

Findings that nandrolone reduced nuclear levels of NICD and reduced expression of Hey1 at 7 and 35 days indicate that nandrolone reduced Notch signaling in denervated muscle at these times. Notably, NICD levels and Hey1 expression were reduced by nandrolone in denervated muscle to levels observed in sham-denervated animals. One explanation for the nandrolone-induced reductions in Notch signaling in denervated muscle may be the marked nandrolone-induced increase in Numb mRNA and protein levels. Numb is an adapter protein and Notch inhibitor that targets Notch and NICD for degradation [15]. Thus, it seems likely that upregulation of Numb by nandrolone contributed to the nandrolone-induced reductions in Notch signaling observed in denervated muscle. The above findings establish Numb as an androgen-responsive gene, at least in denervated muscle. Little is known about the regulation of Numb either at the transcriptional or post-transcriptional level. Possible mechanisms are suggested by an initial analysis of the Numb promotor (C. Cardozo, unpublished) which suggests the presence of several potential androgen response elements and multiple TCF/LEF sites within the first 2 kb upstream of the transcriptional start site.

One potential consequence of increased Numb expression is myogenic differentiation of satellite cells. Numb has been shown to promote differentiation of satellite cells of the myogenic lineage, whereas the absence of Numb maintains cells in the intermediate progenitor stage (12). How upregulation of Numb alters the biology of denervated muscle will be an interesting area for future studies. Our findings do not permit conclusions about the role of these changes in Notch signaling and Numb expression in effects of nandrolone on muscle mass after nerve transection. This is because the effects of nandrolone on Notch signaling and Numb expression occurred earlier after nerve transection than did protection against continue loss of muscle mass. Specifically, upregulation of Numb and downregulation of Notch signaling were

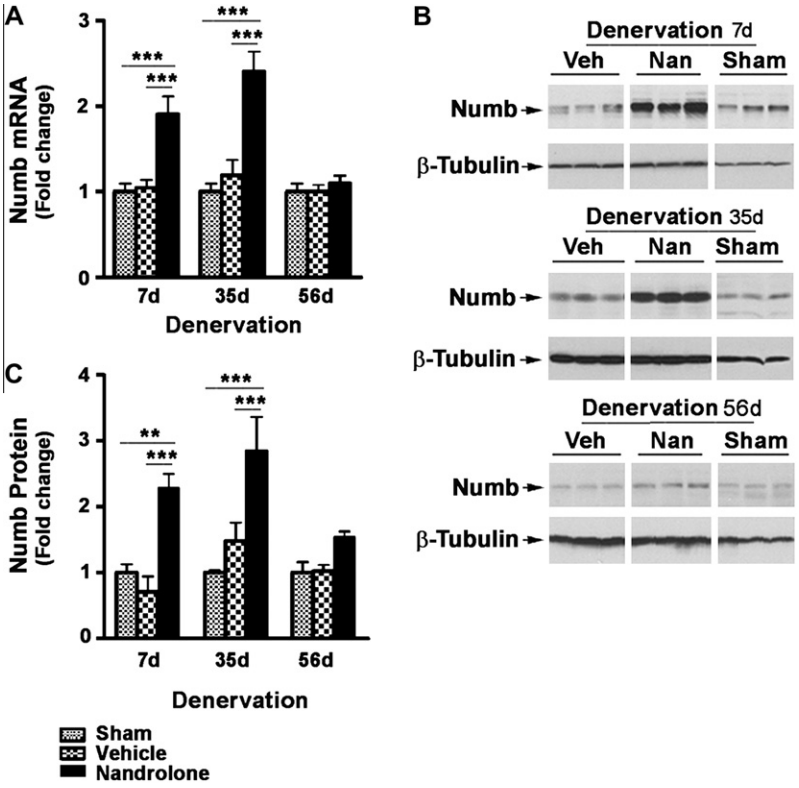

Fig. 3. Nandrolone upregulated Numb expression. (A) Numb mRNA levels in rat gastrocnemius muscles were determined by quantitative real-time PCR. (B) Total protein was isolated from gastrocnemius tissues and subjected to Western blot analysis using an anti-Numb antibody then stripped and reprobed with antibody against β-tubulin. (C) Band intensities in B were quantified by scanning densitometry and normalized relative to those for β-tubulin. Data are means ± SEM for 4 animals per group; $^{*}p < 0.01$, and $^{***}p < 0.001$.

evident by 7 days, whereas nandrolone did not protect against atrophy until 35 days [5]. The findings suggest that altered Notch signaling and/or Numb expression is not sufficient to slow denervation atrophy, but do not exclude the possibility that such changes are necessary for effects of nandrolone to preserve the mass of denervated muscle.

Our findings differ from those from studies of the effect of testosterone on neurologically intact muscle. In humans, testosterone was shown to promote the proliferation of satellite cells [23,24] and increase nuclear staining for Notch in muscle from older men without altering Numb expression [24]. In hypogonadal mice, testosterone stimulated hypertrophy of gastrocnemius muscle associated with p38 MAPK-dependent activation of Notch, and upregulation of the Notch ligand Delta 1 [25]. Why the effects of testosterone and nandrolone on Notch signaling and Numb expression should be so different in denervated versus neurologically intact muscle is not clear. One possible explanation is that the effects of these agents depends on the cause of muscle atrophy, and that they have both common and distinct mechanisms in muscle loss due to hypogonadism, age, glucocorticoids, or paralysis. The time-dependent responses of denervated muscle to nandrolone highlight the potential diversity of muscle responses to androgens, and the critical role played by the biological state of the muscle in determining responses of muscle to nandrolone or other androgens. In the current study, the change in biological state of denervated muscle over time is shown by the initial high Notch signaling early after denervation and subsequent decline in Notch signaling. The importance of biological context in determining the effects of these agents is also clearly illustrated by findings that in the denervated muscle samples used in the current study, nandrolone reduced expression of MAFbx and MuRF1 at 35 and 56 days, but not 3, 7 or 14 days [5]; additionally, a genome-wide analysis of genes regulated by nandrolone revealed that while over 100 genes were regulated by nandrolone at 7 or 35 days, only 20 were regulated by this agent at both time points [26].

Disclosures

The authors have no financial or other conflicts of interest to disclose.

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