SOLUBLE ACTIVIN RECEPTOR TYPE IIB INCREASES FORWARD PULLING TENSION IN THE *mdx* MOUSE

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ABSTRACT: *Introduction:* In this study we investigated the action of RAP-031, a soluble activin receptor type IIB (ActRIIB) comprised of a form of the ActRIIB extracellular domain linked to a murine Fc, and the NF-κB inhibitor, ursodeoxycholic acid (UDCA), on the whole body strength of *mdx* mice. *Methods:* The whole body tension (WBT) method of assessing the forward pulling tension (FPT) exerted by dystrophic (*mdx*) mice was used. *Results:* RAP-031 produced a 41% increase in body mass and a 42.5% increase in FPT without altering the FPT normalized for body mass (WBT). Coadministration of RAP-031 with UDCA produced increases in FPT that were associated with an increase in WBT. *Conclusions:* Myostatin inhibition increases muscle mass without altering the fundamental weakness characteristic of dystrophic muscle. Cotreatment with an NF-κB inhibitor potentiates the effects of myostatin inhibition in improving FPT in *mdx* mice.

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The primary objective of these studies was to assess the efficacy of the soluble activin type IIB receptor, RAP-031, in enhancing muscle function using a reliable noninvasive procedure that rapidly evaluates the deficit in limb tension development in the *mdx* and other mouse models of Duchenne muscular dystrophy.[1–10] As described on the TREAT-NMD website,[11] the whole body tension (WBT) procedure measures several variables related to the tension generated during a forward pulling maneuver. These variables include: the forward pulling tension (FPT) (in grams) averaged over the top 5 or top 10 values (FPT5, FPT10, respectively); the top 5 and top 10 FPTs normalized to the body mass (WBT5, WBT10, respectively); the ratio WBT10/WBT5, which is termed the functional reserve (FR); and the proportional decline per pull (PDP), which is the negative slope of the proportional decline in FPT over the top 10 ordered FPTs.[9,11] A recent evaluation of the reliability of this technique showed that the WBT5 and WBT10 values exhibit an intertrial reliability of approximately 0.8 (Pearson correlation coefficient) in comparison to a value of 0.6 for the 4-limb wire grid hang test.[10] Previous experiments also indicated that the WBT measure in nondystrophic and *mdx* mice correlated with measurements of gastrocnemius tension development,[1] and that two distinct and effective inhibitors of the nuclear factor–kappaB (NF-κB) pathway in *mdx* muscle produce a 12–21% increase[9] in WBT5 and WBT10, corresponding to a maximum recovery of approximately 25%. These results indicate that the WBT procedure provides a relatively rapid method for screening potential therapeutic compounds and combination therapies using the *mdx* mouse model for Duchenne muscular dystrophy.

In this study, the WBT method was used to assess the utility of a treatment that increases muscle mass by inhibiting ActRIIB activation (RAP-031). Previous studies using other means of inhibiting myostatin, one of the ligands that signals via ActRIIB to negatively regulate muscle mass, have indicated substantial increases in dystrophic muscle mass and tension development.[12,13] The primary purpose of this study was to utilize the normalized FPT measures (WBT5, WBT10) to more clearly determine whether RAP-031–induced inhibition of the ActRIIB pathway produces general improvement in the fundamental weakness characteristic of dystrophic limb muscle.[1]

Previous studies showed that a variety of inhibitors of the NF-κB pathway are useful in improving the structure and function of dystrophic muscle without altering muscle mass in the *mdx* mouse.[9,14–18] Some of these inhibitors have been shown to increase FPT normalized to body mass, suggesting that the fundamental weakness characteristic of dystrophic muscle may be diminished by treatment with NF-κB inhibitors that do not increase body mass.[9] These results suggest that cotreatment with RAP-031 and an effective inhibitor of the NF-κB pathway may be particularly useful at both increasing muscle mass and reducing limb muscle weakness. To investigate this possibility, parallel studies were conducted to determine the combined efficacy of RAP-031 with ursodeoxycholic acid (UDCA), an NF-κB inhibitor that increases limb tension without increasing dystrophic muscle mass.[9]

The results indicate that treatment of 30-day-old *mdx* mice with RAP-031 for up to 90 days

Abbreviations: ActRIIB, activin receptor type IIb; FPT, forward pulling tension; FPT5, mean of top 5 forward pulling tensions; FPT10, mean of top 10 forward pulling tensions; FR, functional reserve; NF-κB, nuclear factor–kappaB; PDP, proportional decline per pull; PDTC, pyrrolidine dithiocarbamate; RAP-031, soluble ActRIIb–F_c fusion protein; UDCA, ursodeoxycholic acid; WBT, whole body tension; WBT5, mean of top 5 forward pulling tensions divided by body mass; WBT10, mean of top 10 forward pulling tensions divided by body mass

Key words: activin receptor type IIB, Duchenne muscular dystrophy, mdx mouse, ursodeoxycholic acid, whole body tension

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substantially increased body mass and FPT but did not significantly influence the FPT normalized to body mass (WBT5, WBT10). The 90-day treatment did, however, lead to a small but significant increase in FR (WBT10/WBT5) associated with a corresponding reduction in PDP, thus indicating that prolonged RAP-031 treatment may reduce the characteristic weakening that occurs following a forward pulling maneuver.[9] In addition to increasing FPT and body mass, cotreatment with RAP-031 and NF-κB inhibitor UDCA for 30 days produced a significant increase in WBT10. These findings indicate that treatment with the ActRIIB inhibitor RAP-031 produces roughly proportional increases in muscle mass and tension generation without altering the fundamental weakness characteristic of dystrophic muscle. Cotreatment with NF-κB inhibitor UDCA potentiated the beneficial effects of RAP-031 by producing further increases in limb tension that were associated with relatively small additional increases in body mass.[9]

METHODS

Drug Administration. *mdx* mice, aged 30 days, were used for all of the RAP-031 and UDCA studies. RAP-031 *mdx* mice were treated twice weekly with subcutaneous RAP-031 (10 mg/kg) or an appropriate volume of vehicle (10 mM Tris-buffered saline, pH 7.2). *mdx* mice treated with both RAP-031 and UDCA were exposed to 10 mg/kg twice weekly of RAP-031 along with daily intraperitoneal administration of 40 mg/kg UDCA, as described by Siegel et al.[9] The vehicle for the UDCA (Sigma #5127) studies was a 1.02% NaCl solution (pH 8.4). In the combined treatment studies, *mdx* mice received either both experimental injections (RAP-031 and UDCA) or both vehicle injections.

Determination of Whole Body Tension. WBT determinations were obtained using the techniques described by Siegel et al.[9] and on the TREAT-NMD website.[11] All FPTs were obtained by a single investigator who was blinded to the identity of the mice being examined. The determinations were made during the same time period, between 1:00 and 4:00 P.M., with the mice maintained on a 12-hour light–dark cycle with darkness at 7:00 P.M. The mice were housed individually in cages for the duration of the experiment and were not exposed to an exercise regimen. Vehicle- and drug-treated groups were age- and gender-matched (equal numbers of male and female *mdx* mice in vehicle- and drug-treated groups). The vehicle and experimental *mdx* mice were tested at 30, 60, and 90 days of treatment. The data were analyzed to determine the effect of drug treatment on the following variables: (a) FPT5, which represents the mean tension (g) of the top 5 ordered FPTs; (b) FPT10, the mean tension of the top 10 FPTs. (c) WBT5 and WBT10, which are the FPT5 and FPT10, respectively, divided by the body mass; (d) FR, which is the WBT10/WBT5; and (e) PDP, which is the proportional decline in FPT over the top 10 ordered FPTs.

Percent recovery was obtained by dividing the effect associated with drug treatment (i.e., drug-treated *mdx*–vehicle-treated *mdx*) by the initial deficit of the variable in the vehicle-treated *mdx* mice (untreated nondystrophic–vehicle-treated *mdx*), as described by Gillis.[6,19]

Statistics. SigmaPlot (versions 3.1 and 9.0) was used. Mean and standard error are shown and were analyzed using *t*-tests or Mann–Whitney rank-sum tests as appropriate. Significance was defined at the 0.05 level.

RESULTS

RAP-031 Produces Proportional Increases in Body Mass and FPT without Altering WBT. Body mass and WBT determinations were obtained at 30-day intervals in a group of *mdx* mice that were treated for 90 days with RAP-031, beginning at 30 days of age. After 30 days of treatment, the body mass of the RAP-031–treated mice was approximately 24% greater than the mass of the vehicle-treated mice (Fig. 1A1; $P < 0.001$), and the FPT5 (Fig. 1B1) and FPT10 (Fig. 1C1) had significantly ($P < 0.001$) increased by 33% and 36%, respectively. Because nondystrophic mice at 2–4 months of age exhibit FPT10 values that are approximately 15.5 times their body weight of 30 g,[9] the FPT10 values in the RAP-031–treated *mdx* mice after 30 days of treatment (Fig. 1C1) represent a recovery[6,19] in forward pulling strength of approximately 25%. The WBT5 (Fig. 2A1) and WBT10 (Fig. 2B1) values of the RAP-031–treated mice at this age, however, were not significantly increased above the values seen in the corresponding vehicle-treated *mdx* mice. After 60 days of treatment, body mass had significantly ($P < 0.001$) increased by 32% (Fig. 1A2), FPT5 by 25% (Fig 1B2), and FPT10 by 31.5% (Fig. 1C2). No significant increases in either WBT5 or WBT10 were observed (Fig. 2A2 and B2). After 90 days of treatment, body mass was significantly ($P < 0.001$) increased by 41% (Fig. 1A3). At this point, FPT5 (Fig. 1B3) and FPT10 (Fig. 1C3) were significantly ($P < 0.001$) increased by 31.5% and 42.5%, respectively. Neither the WBT5 (Fig. 2A3) nor the WBT10 (Fig. 2B3) were significantly elevated after 90 days of RAP-031 treatment, even though the recovery in FPT10 after 90 days reached approximately 37% (Fig. 1C3).

The proportional increases in FPT10 produced by RAP-031 after 30–90 days of treatment (Fig. 1C)

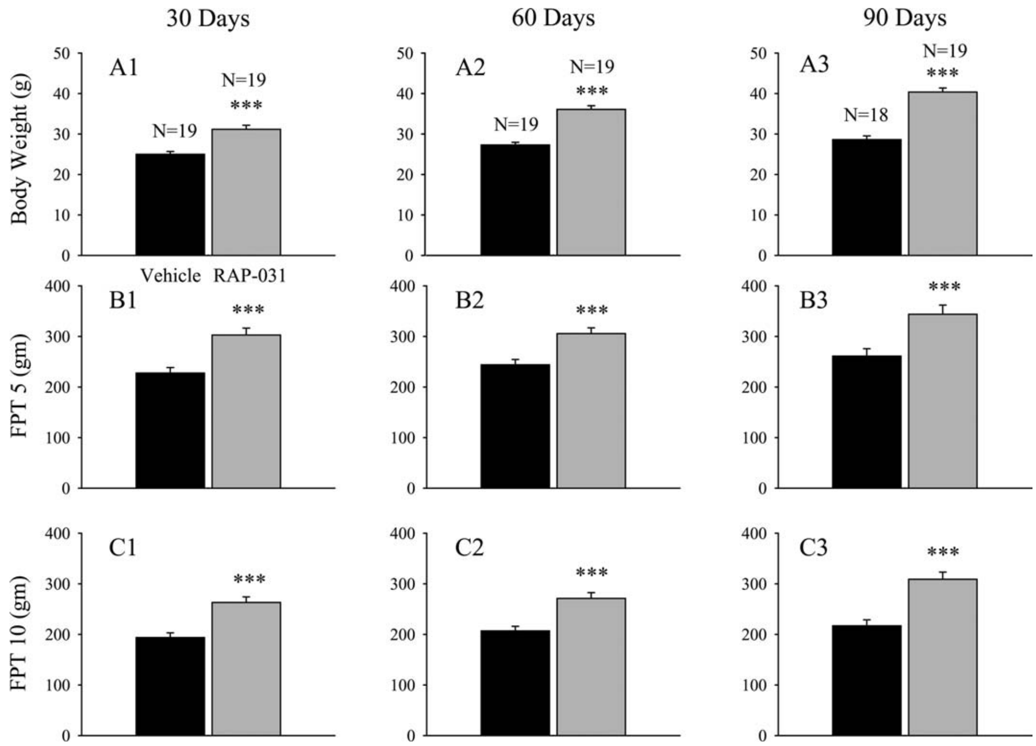

FIGURE 1. RAP-031 treatment produces roughly proportional increases in body mass **(A)**, FPT5 **(B)**, and FPT10 **(C)** in young adult (30-day-old) *mdx* mice treated for 30 **(A1, B1, C1)**, 60 **(A2, B2, C2)**, or 90 days **(A3, B3, C3)**. Black histobars: vehicle treated; gray histobars: RAP-031 treated. N represents the number of *mdx* mice in each group. ***$P < 0.001$ vs. vehicle-treated *mdx* mice.

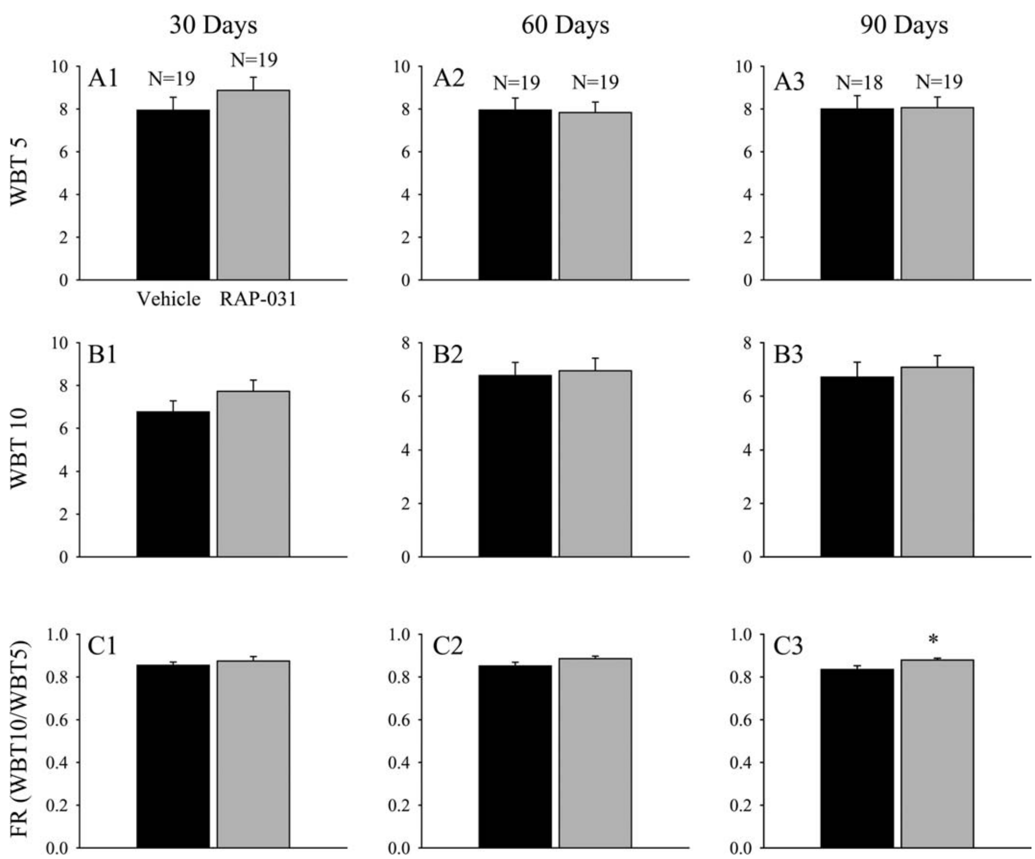

FIGURE 2. RAP-031 treatment does not influence FPT normalized to body mass. **(A1–A3)** WBT5 values at 30, 60, and 90 days, respectively. **(B1–B3)** WBT10 values at 30, 60, and 90 days. **(C1–C3)** FR values at 30, 60, and 90 days. Black histobars: vehicle treated; gray histobars: RAP-031 treated. N represents the number of *mdx* mice in each group. *$P < 0.05$ vs. vehicle-treated *mdx* mice. Note that the FR is significantly increased in the RAP-031–treated mice after 90 days of treatment.

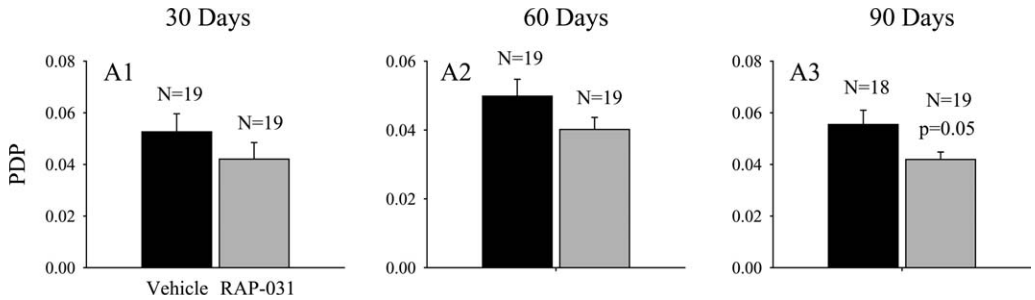

FIGURE 3. The effect of RAP-031 treatment on the PDP at 30 **(A1)**, 60 **(A2)**, and 90 **(A3)** days. Black histobars: vehicle treated; gray histobars: RAP-031 treated. N represents the number of *mdx* mice in each group.

were consistently slightly larger than the corresponding increases in FPT5 (Fig. 1B). This apparent trend was further evaluated by examining the FR, which is equal to WBT10/WBT5 (Fig. 2C1–C3). The results indicate that the FR was significantly ($P < 0.05$) increased after 90 days of RAP-031 treatment (Fig. 2C3). The PDP, which represents the normalized decline in FPT over the top 10 ordered values, was consistently lower in the RAP-031–treated *mdx* mice and, at 90 days, the decrease just failed to reach statistical significance (Fig. 3A3; $P = 0.05$).

In summary, these results indicate that RAP-031 treatment produced proportional increases in body mass and FPT and did not significantly influence either the WBT5 or WBT10. However, the treatment produced slightly larger proportional increases in FPT10 that resulted in a significant increase in FR (Fig. 2C3) associated with a reduction in PDP after 90 days of treatment (Fig. 3A3).

Treatment with RAP-031 and UDCA Increases WBT10. Previous experiments in this laboratory indicated that a 30-day treatment of 30-day-old *mdx* mice with UDCA produced a 21% increase in both WBT5 and WBT10 without corresponding increases in body mass and did not influence the FR or PDP.[9] In the present study, a group of 30-day-old *mdx* mice were cotreated with RAP-031 and UDCA, and the results were compared with a control group treated with the appropriate vehicle solutions. Cotreatment with these agents for 30 days produced substantial increases in body mass (by 30%) and FPT, and a significant ($P < 0.05$) 32% increase in WBT10 (Fig. 4), which corresponded to a recovery[6,19] of approximately 27%. Neither the FR (Fig. 4F) nor the PDP were influenced by the 30-day treatment with both RAP-031 and UDCA.

DISCUSSION

The WBT method for assessing the FPT of mice measures the combined tension generated by all of the limbs along with that generated by the abdominal and thoracic musculature and the distal limbs as the mice produce a forward pulling maneuver while continually gripping a tubular wire mesh.[1–10] An advantage of the WBT procedure over the

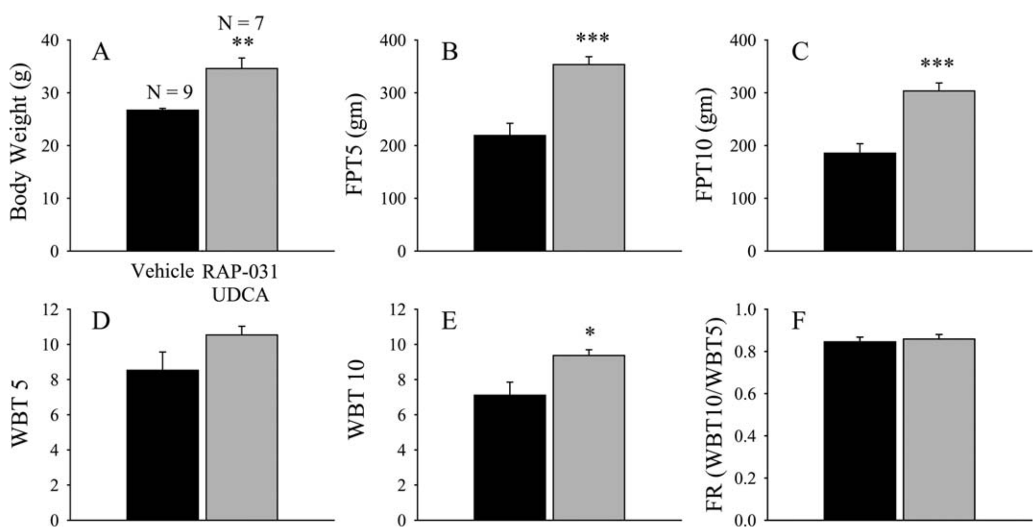

FIGURE 4. Combined RAP-031 and UDCA treatment for 30 days produced substantial increases in FPT and significant increases in WBT10. **(A)** Body weight, **(B)** FPT5, **(C)** FPT10, **(D)** WBT5, **(E)** WBT10, and **(F)** FR. Black histobars: vehicle treated, gray histobars: RAP-031 and UDCA treated. N is the number of *mdx* mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively, vs. vehicle-treated mice.

similar method of assessing fore- or hindlimb grip strength is that the WBT force produced by all of the muscles involved in generating a forward pulling maneuver is more clearly related to body mass than is the grip force, which is primarily generated by the distal limb musculature. Previous investigations of grip strength in a mouse model of amyotrophic lateral sclerosis, for example, showed that RAP-031 produced increases in grip strength that were roughly proportional to corresponding increases in body mass.[20] The results of our study are consistent with those observations. Our data show that treatment of *mdx* mice with RAP-031 produced substantial muscle hypertrophy and increases in absolute forward pulling tension but did not alter the forward pulling tension normalized to body mass (i.e., WBT5, WBT10; Fig. 2A and B).

The results are also consistent with the effect of long-term treatment of *mdx* mice with myostatin antibodies that enhanced absolute tension development but did not influence specific tension (tension normalized to cross-sectional diameter) in isolated muscle experiments.[12] Similar results were obtained by Qiao et al.,[13] who used an adenovirus delivery system to treat *mdx* mice with the myostatin inhibitor, myostatin propeptide, and produced an increase in the absolute grip strength and muscle tension, with no effect on specific twitch or tetanic tension in the tibialis anterior muscle. The WBT results showing no effect of RAP-031 on WBT (Fig. 2) suggest that this measure is the whole body equivalent of the total specific tension generated by the limb, abdominal, and thoracic musculature during a forward pulling maneuver. Consistent with results obtained using other modes of inhibiting myostatin,[12,13] our findings indicate that myostatin inhibition increases whole body strength through increases in muscle mass but it does not alter the fundamental weakness that is characteristic of dystrophic fibers.

Previous results in this laboratory indicate that two distinct inhibitors of the NF-κB pathway, pyrrolidine dithiocarbamate (PDTC) and UDCA, produced moderate increases in WBT that were not associated with increases in body mass.[9] These effects are clearly distinct from those produced by ActRIIB inhibition, which increases muscle mass and tension without altering WBT. This supports the idea that the magnitude of the tension effect is directly proportional to the amount of lean mass amassed. The mechanism of action for the functional benefits of NF-κB inhibitors is not known, but it may involve increases in the resting membrane potential,[14,21] which would be expected to increase the quantity of sarcoplasmic Ca^{2+} released during an action potential. In our study, treatment with the NF-κB inhibitor UDCA in conjunction with RAP-031 produced increases in body mass that were associated with increases in WBT10 (Fig. 4), similar to those produced by UDCA alone.[9] These results suggest that combined treatment with an effective NF-κB inhibitor and RAP-031 may increase muscle mass and FPT and, in addition, increase limb strength normalized to body mass (WBT10). Similar studies using the WBT procedure to assess various combination therapies will hopefully prove to be useful in the development and design of clinical trials for the treatment of Duchenne and related muscular dystrophies.

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