**ACTN3** genotype and modulation of skeletal muscle response to exercise in human subjects

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**Abstract**

**ACTN3** genotype on exercise; hypertrophy signaling; glycogen; fiber types

**α-actinin-3 and α-actinin-2 are skeletal muscle specific proteins, that act as structural components of the Z-disc where they anchor thin actin filaments from adjacent sarcomeres. α-Actinin-2 is present in all muscle fiber types, whereas α-actinin-3 is confined to fast-twitch (type II) fibers exclusively (37, 50). In addition to their structural role, α-actinins also interact with skeletal muscle proteins with signaling and metabolic functions, and recent studies have indicated a specific role for the α-actinin-3 isoform in these interactions (42, 45, 51).

The α-actinin-3 protein is encoded by the **ACTN3** gene on chromosome 11 (6). A common polymorphism in this gene, R577X (rs1815739), results in a premature stop codon and lack of α-actinin-3 protein in individuals who are homozygous for the **ACTN3** null allele (XX genotype) (37). This genotype is found in ~16–19% of the general populations of European and Asian origin, and is much less frequent in populations of African origin (53). Deficiency of the α-actinin-3 protein does not result in an evident muscle dysfunction, suggesting that the other isoform, α-actinin-2, largely compensates for its absence. This conclusion is reinforced by observations of overexpression of α-actinin-2 in association with absence of α-actinin-3 in mouse and human muscle (32, 36).

However, there are circumstances in which the presence of α-actinin-3 (RR or RX genotype) is of importance, as exemplified by differences in genotype frequencies between various groups of elite athlete cohorts compared with controls. In several independent studies, the frequency of the R allele has been found to be significantly higher in sprint/power-oriented athletes compared with controls, indicating that the presence of α-actinin-3 provides an advantage for these athletes (4, 29). A large study performed by Ahmetov et al. (2) showed that the presence of α-actinin-3 might also be of advantage for different groups of endurance-oriented athletes, when a power component is involved in their sport.

Although the mechanism underlying the association between the **ACTN3** genotype and athletic performance has not been elucidated, studies on Actn3 knockout (KO) mice provide valuable information on how the absence of α-actinin-3 affects skeletal muscle properties. These studies have demonstrated that the KO mice have a lower muscle mass as a result of a significant decrease in the size of fast type IIB fibers, which are the fibers that in wild type (WT) mice exhibit the highest levels of expression of α-actinin-3 (31). It has also been reported that the KO mice have muscles with slower contractile properties and higher levels of oxidative enzymes as well as a higher endurance exercise capacity than do WT mice (31). Other characteristics of the KO mice are higher muscle glycogen content, reduced activity of glycogen phosphorylase, and higher glycogen synthase levels (42).

One explanation for the influence of the **ACTN3** genotype on performance may be the involvement of α-actinin in the regulation of muscle mass. An important function of the α-actinins in the Z-disc structure is to act as tension sensors (12, 22, 25). Mechanically induced tension plays an important role in the signaling events that mediate hypertrophy (24). Based on recent observations that a lack of the α-actinin-3 isoform alters the elastic properties of myofibrils (8, 45), it is conceivable that the sensing of mechanical stretch during muscle contraction may be affected by the **ACTN3** genotype. Such differences in mechanical sensing may lead to differences
in hypertrophy signaling and result in ACTN3 genotype-associated differences in muscle mass.

Another potential explanation for the association between the ACTN3 genotype and athletic performance is related to differences in fiber type composition between endurance-trained and sprint/power-trained athletes. The latter most often have a high percentage of fast-twitch muscle fibers, which are beneficial for optimal performance in these sports (1, 17, 47). It has been proposed that α-actinin-3 might, through an interaction with the signaling protein calcineurin, promote the formation of fast-twitch fibers (50). The possible involvement of α-actinin-3 in this process may explain the association between the RR-genotype and sprint/power athletic performance. However, in the Actn3 KO mouse model, no changes in muscle fiber type composition, as defined by the myosin heavy chain isoform have been observed, despite a pronounced shift in the contractile characteristics of fast muscle fibers toward properties of slow oxidative fibers (31). Rather, the demonstrated difference between the Actn3 KO and WT mice involves metabolic changes such as an increase in the activities of multiple enzymes in the muscles of KO mice, thus promoting oxidative metabolism (18, 31).

Furthermore, a study performed by Quinlan et al. (42) provided evidence that glycogen metabolism might be altered in association with ACTN3 deficiency. That study showed that several enzymes involved in glycolytic metabolism are upregulated, glycogen phosphorylase (GPh) activity is markedly reduced, and muscle glycogen content is increased in the KO mice (42). On the basis of these findings, the authors propose that alterations in GPh activity, in the absence of α-actinin-3, may be an important factor in the association between ACTN3 genotype and human performance.

Studies of the association between ACTN3 genotype and properties of skeletal muscle in humans, such as muscle mass, fiber type composition, and metabolic adaptations related to glycogen metabolism, which have been pinpointed as being associated with ACTN3 deficiency. That study showed that several enzymes involved in glycolytic metabolism are upregulated, glycogen phosphorylase (GPh) activity is markedly reduced, and muscle glycogen content is increased in the KO mice (42). On the basis of these findings, the authors propose that alterations in GPh activity, in the absence of α-actinin-3, may be an important factor in the association between ACTN3 genotype and human performance.

<table>
<thead>
<tr>
<th>ACTN3 genotype distribution</th>
<th>Study I</th>
<th>Study II</th>
<th>Study III</th>
</tr>
</thead>
<tbody>
<tr>
<td>XX = 10</td>
<td>XX = 7</td>
<td>XX = 0</td>
<td>XX = 5</td>
</tr>
<tr>
<td>RX = 28</td>
<td>RX = 9</td>
<td>RX = 8</td>
<td>RX = 4</td>
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<tr>
<td>RR = 17</td>
<td>RR = 12</td>
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<tr>
<td>n</td>
<td>55</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>Age, yr</td>
<td>27 ± 2</td>
<td>23 ± 2</td>
<td>28 ± 3</td>
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<tr>
<td>Weight, kg</td>
<td>74 ± 9</td>
<td>75 ± 8</td>
<td>84 ± 7</td>
</tr>
<tr>
<td>Height, cm</td>
<td>179 ± 6</td>
<td>178 ± 7</td>
<td>184 ± 5</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.2 ± 2</td>
<td>23.3 ± 2</td>
<td>24.7 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SD.
Table 2. Training volume, fiber-type composition, and cross-sectional fiber area (CSA) across ACTN3 R577X genotypes

<table>
<thead>
<tr>
<th></th>
<th>Genotype</th>
<th>Sample</th>
<th>Training Volume</th>
<th>Type I Fibers</th>
<th>Type II Fibers</th>
<th>Mean Fiber Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>all</td>
<td>RR</td>
<td>wk</td>
<td>%</td>
<td>%</td>
<td>μm²</td>
</tr>
<tr>
<td>n</td>
<td>83</td>
<td>29</td>
<td>39</td>
<td>51.4 ± 13</td>
<td>32.0 ± 10</td>
<td>4,564 ± 1,695</td>
</tr>
<tr>
<td>Training Volume</td>
<td>29</td>
<td>39</td>
<td>15</td>
<td>50.6 ± 13</td>
<td>31.8 ± 9</td>
<td>4,349 ± 1,539</td>
</tr>
<tr>
<td>Type I Fibers</td>
<td>39</td>
<td>15</td>
<td>15</td>
<td>15.5 ± 11</td>
<td>10.7 ± 7</td>
<td>4,660 ± 1,789</td>
</tr>
<tr>
<td>CSA of type I fibers, μm²</td>
<td>15</td>
<td>15</td>
<td>2.8 ± 4</td>
<td>3.3 ± 4</td>
<td>2.2 ± 4</td>
<td>4,663 ± 1,028</td>
</tr>
<tr>
<td>n</td>
<td>67</td>
<td>26</td>
<td>9</td>
<td>2.6 ± 3</td>
<td>1.2 ± 3</td>
<td>4,813 ± 1,436</td>
</tr>
</tbody>
</table>

Values are means ± SD. No significant differences across the ACTN3 R577X genotypes were detected in either males or females in the ANOVA. CSA was not determined in study III and is based on a subgroup of a total sample. Results from studies I, II, and III are combined and presented subdivided by sex and genotype. Mean fiber area was calculated from the formula: \[\text{mean fiber area type I} = \frac{\text{type I} \times \text{mean fiber area type I} + \text{type IIA} \times \text{mean fiber area IIA} + \text{type IIB} \times \text{mean fiber area of type IIB}}{100}\].

Exercise protocols

In study II, subjects performed one bout of 30-s sprint exercise (Wingate test) on a mechanically braked cycle ergometer (Cardionics, Bromma, Sweden), and in study III the subjects performed three bouts of the 30-s cycle sprints with a 20-min rest between the bouts. The cycle sprints were performed at maximal propelling speed against a resistance of 7.5% of the subject's body mass (5).

Muscle biopsies

Sampling procedure. Muscle samples were obtained from the middle portion of the vastus lateralis muscle using percutaneous needle biopsy technique (7). In study I the muscle samples were obtained only at rest. In study II and study III the samples were obtained at rest and following sprint exercise. The first biopsy sample was obtained randomly in either right or left leg prior to the first sprint. The second muscle sample was obtained from the opposite leg. In study II the postexercise muscle samples were freeze-dried and used for dissection of single fibers. Approximately 100 single-fiber fragments were dissected from each biopsy sample. These were classified histochemically as fiber type I or II (15) and thereafter divided into separate pools of type I and type II. The mean weight of the pools was 40 μg (range 15–75 μg).

Quantitative glycogen analysis. Muscle samples were freeze-dried and dissected free from visible blood and connective tissue and homogenized by hand in a glass homogenizer in 0.1 M phosphate buffer, pH 7.7 with 0.5% BSA. For the determination of glycogen content in study I aliquots of the crude muscle homogenate were boiled in 1.5 M HCl for 1 h to break down glycogen to glycosyl units. In study II the fiber pools used for glycogen analysis were digested by adding 20 μl of 1M KOH and vigorously mixing and warming the samples for 15 min at 50°C, after which amyloglycosidase was added to break down glycogen to glycosyl units (23). The extracts were neutralized by addition of 0.25 M HCl. Glycogen content was thereafter measured in both types of extracts by analyzing glucose using a fluorometric enzymatic method (28).

Western blot. In study III, the muscle samples obtained at rest and 140 min following the last of three 30-s Wingate cycling bouts were used for Western blot analysis to study the phosphorylation of AktSer473, mTORSer2448, p70S6kThr389, rpS6Ser235/236, and AMPKThr172. The freeze-dried muscle samples were homogenized in an ice cold lysis buffer using a glass homogenizer. The protein content was determined by a Bio-Rad Bradford protein assay and diluted to a final concentration of 2 μg/μl containing 25% Laemmli buffer. An SDS-PAGE (Criterion precast, 4–20% gradient gels; BioRad) was loaded with 30 μg of protein in each well and was run for 72 min at 200 V. Subsequently, the proteins were transferred to a PVDF membrane during 3 h in ice cold buffer, blocked in TBS containing 5% milk for 1 h at room temperature, and incubated overnight at 4°C in specific primary antibodies purchased from Cell Signaling Technology (Boston, MA): p-AktSer473 (1:1,000), p-mTORSer2448 (1:500), p70S6kThr389 (1:1,000), p-rpS6Ser235/236 (1:1,000), p-AMPKThr172 (1:1,000). The following day the membranes were washed in TBST, incubated in secondary antibody (1:5,000) for 1 h at room temperature, further washed, and finally visualized using enhanced chemiluminescence reagents (Pierce Supersignaling west femto sensitivity substrate, Thermo Fisher Sci, Rockford, IL) and a Molecular Imager ChemiDocTM XRS system. Quantity One version 4.6.3 software (Bio-Rad Laboratories) was used to quantify the resulting blots. For detailed description of the Western blot procedures see Ref. 14.
Genotyping

Genomic DNA was extracted from peripheral blood using the QIAamp DNA extraction kit (Qiagen), from muscle samples by TaqMan Sample-to-snp kit (Applied Biosystems), or from saliva using a self-collection kit from Oragene (DNA Genotek, Ottawa, Ontario, Canada). Genotyping for the identification of the R577X mutation in the ACTN3 gene was performed by an allelic discrimination assay with fluorogenic probes (Perkin-Elmer ABI Prism 7700 Sequence Detection system, Applied Biosystems). The primer sequences were ACAGATCGTTCAAGCACAAC (forward) and ACCTCGGATGCCCATGATG (reverse), and the TaqMan probe sequences were TCCTCCTCGTCAAGC and CGCTTCATGC (polymorphism in bold). The probes were labeled with fluorescent dyes VIC and FAM, respectively.

Statistics

Results are presented as mean ± SD. Factorial ANOVA with ACTN3 genotype as independent variable and fiber type composition, CSA and basal glycogen levels as dependent variables was employed to evaluate the potential differences between the ACTN3 genotypes. ANOVA analysis of fiber type composition and CSA were performed separately for males and females. Multiple regression analysis was applied to evaluate the contribution of ACTN3 genotype, sex, and training volume as predictors of fiber type composition and CSA. Coefficient of determination (R^2) indicating the percentage of the variance explained by each predictor and the P value is stated in the result section. Due to limited number of subjects with XX genotype in study II, changes in glycogen content in pools of type I or type II fibers across ACTN3 genotypes were statistically analyzed by Kruskal-Wallis nonparametric test.

RESULTS

Fiber type composition and muscle fiber area

The fiber type composition and the CSA across the ACTN3 genotypes according to sex are presented in Table 2. No significant differences in the percentage of type I, type IIA, or type IIB fibers were observed across the ACTN3 genotypes in either males or females (ANOVA, P > 0.05). Multiple regression analysis showed that training volume (P < 0.001, R^2 = 0.15) and sex (P < 0.001, R^2 = 0.10), but not ACTN3 genotype (P = 0.57, R^2 = 0.02) significantly explained the percentage of type I fibers. The percentage of type IIA fibers was dependent on training volume (P = 0.04, R^2 = 0.03) but was independent of sex (P = 0.09, R^2 = 0.02) or genotype (P = 0.68, R^2 = 0.01). Moreover, the percentage of type IIB fibers was significantly explained by training volume (P < 0.001, R^2 = 0.10) and sex (P = 0.02, R^2 = 0.04); however, there was no significant contribution of genotype (P = 0.63, R^2 = 0.01). Finally, the percentage of type IIC fiber was explained by training volume (P = 0.06, R^2 = 0.03) and sex (P = 0.01, R^2 = 0.05), with no significant contribution of genotype (P = 0.52, R^2 = 0.02). Similarly, the CSA of type I, type IIA, type IIB nor type IIC fibers did not differ significantly between the ACTN3 genotypes in either males or females (ANOVA, P > 0.05). A multiple regression analysis showed that the CSA of type I, type IIA, type IIB, and type IIC was significantly explained by sex, whereas the contributions of training volume and genotype were not significant.

Hypertrophy signaling

The activation of the Akt/mTOR signaling pathway, 140 min after three bouts of Wingate cycling, was analyzed in study III. The phosphorylation of mTOR, p70S6k, rpS6, and Akt was significantly higher after exercise than it was at rest. The relative exercise-induced increase in the phosphorylation of mTOR and p70S6k was significantly smaller in the XX than it was in the RR + RX genotype (ANOVA, P = 0.03 and P = 0.01, respectively), whereas no significant difference was observed regarding the increase in rpS6 and Akt across genotypes (Fig. 1). AMPK phosphorylation levels 140 min after exercise were not different from the phosphorylation levels observed at rest (data not shown).

Glycogen

Glycogen content in biopsy samples obtained at rest did not differ across the ACTN3 genotypes (ANOVA, P > 0.05). The resting glycogen levels were (mean ± SD, mmol glycosyl units per kg dry muscle) 641 ± 203 in the RR (n = 44), 599 ± 174 in the RX (n = 50), and 597 ± 224 in the XX (n = 18) genotypes.

In study II, the breakdown of glycogen during exercise was studied by analyzing glycogen content before and immediately after 30 s of Wingate cycling. In this unique study the glycogen level was analyzed in dissected pools of type I and type II fibers. The decrease in glycogen levels with 30-s Wingate cycling in type I and type II fibers, subdivided according to the ACTN3 genotype, is presented in Fig. 2. The decrease in glycogen content during exercise was significantly different across the ACTN3 genotypes in type II fibers (Kruskal-Wallis test P = 0.03) but not in type I fibers (Kruskal-Wallis test P = 0.38).

DISCUSSION

The results of the present investigation supported our hypothesis that the ACTN3 genotype modulates response to exercise, although no differences across the genotypes were detected at baseline. We showed here that the lack of α-actinin-3 in individuals with the XX genotype was associated with less pronounced activation of the signaling events that mediate muscle hypertrophy, which is in agreement with the finding of a decreased size of fast type IIB fibers in the KO mice (31). In addition, our results indicate that the lack of α-actinin-3 in individuals with the XX genotype may constrain glycogen utilization during sprint exercise, thereby promoting the maintenance of high glycogen levels, which is also in agreement with previous findings indicating a link between type IIB fibers and glycogen content (32). This link is particularly relevant since the glycogen content of the type IIB fibers in the XX group was significantly lower than in the RR and RX groups.

Fig. 1. Percentage change in the phosphorylation of AktSer473, mTORSer2448, p70S6kThr389, and rpS6Ser335/236 from baseline to 140 min after sprint exercise, by ACTN3 genotype. Values are the mean ± SE. °P values indicate the level of statistical significance in ANOVA when pooled values of RR and RX are compared with XX.
line with the higher glycogen content observed in the KO mice (42).

Reduced muscle mass has been observed in the KO mice in muscles that normally express α-actinin-3 (31, 44). In humans, lower muscle mass has been observed in older women with α-actinin-3 deficiency (48, 57, 58). A possible mechanism underlying the effect of α-actinin-3 on elite athlete performance may be its potential influence on muscle mass. Mechanically induced tension plays an important role in the signaling events that mediate hypertrophy (24). The findings that α-actinins are involved in sensing tension during muscle contraction and link it with signaling mechanisms (6, 12, 41) may be an explanation of how the lack of α-actinin-3 contributes to variations in muscle mass. The α-actinins form several different complexes at the Z-disc, one of which is a putative mechanosensing complex, in which the α-actinins interact with titin/Tcap/muscle LIM protein (22, 26). Factors that affect the integrity of the complex in response to stretch have been suggested to influence the signaling response (19, 20). The two α-actinin isoforms, α-actinin-2 and α-actinin-3, differ in their interaction with titin (45) and how they affect muscle fiber elasticity (8). Thus it is conceivable that the ACTN3 genotype impact the stability of the mechanosensing complex, thereby affecting the signaling pathways that lead to hypertrophy. The mTOR pathway is an important signaling pathway in the regulation of muscle mass. Recent studies have provided evidence that mTOR signaling is activated through the enzyme phospholipase D and suggested a model in which mechanical stimuli induce an increase in phosphatidic acid which binds to and activates mTOR (for references see Ref. 24). Interestingly, the interaction between titin and α-actinin is regulated by phospholipids (56). The results of the present study, which showed lower activation of the mTOR signaling pathway in the XX compared with the RR/RX genotype, support our hypothesis that hypertrophy signaling is influenced by the ACTN3 genotype. This conclusion is also reinforced by the fact that no ACTN3 genotype-associated differences in Akt phosphorylation were observed in the present study, as it has been shown that activation of the mTOR signaling pathway mediated by mechanical sensing may act independently of Akt/PI3K activation (55). Based on the reasoning presented above, the results of this study support the hypothesis that, in response to exercise, presence of α-actinin-3 promotes the hypertrophy of skeletal muscle.

Furthermore, the findings from Actn3 KO mice demonstrate that the lack of α-actinin-3 is associated with reduced activity of glycogen phosphorylase and increased activity of glycogen synthase, as well as higher muscle glycogen content (42). The authors propose that these changes lead to a decreased capacity of muscle to use glycogen as a fuel. However, in agreement with the results of a study by Vincent et al. (52), the present investigation of human subjects did not confirm differences in basal glycogen levels across ACTN3 genotypes. Nonetheless, our finding that the ACTN3 genotype had an influence on the sprint exercise-induced decrease in glycogen levels in type II fibers which express α-actinin-3, but not in type I fibers (study II), speaks in favor of the suggested association between the ACTN3 genotype and glycogen utilization.

Finally, the current data suggest that the ACTN3 genotype does not play a significant role in determining muscle fiber type composition or fiber dimensions at baseline in moderately to well-trained subjects. Using our relatively large cohort consisting of males and females with variable training backgrounds, we showed that training status and sex, but not ACTN3 genotype, had a significant effect on fiber type composition. The present results were consistent with the results of an earlier study from our laboratory, which demonstrated that fiber type composition is independent of ACTN3-genotype (36). Our results were, however, in contrast with those of a study by Ahmetov et al. (3) who reported a significantly higher proportion of type I fibers in subjects with the XX genotype. The cohort investigated by Ahmetov et al. consisted of a group of physically active males merged with a group of male and female athletes. Our results were also in contrast with those of a study by Vincent et al. (50) who reported that the percentage surface area occupied by type IIX fibers and the number of type IIX fibers (which are equivalent to type IIB fibers) were greater in individuals with the RR- compared with those with the XX genotype. However, a subsequent study by Vincent et al. (52) did not confirm their previous findings of an association between ACTN3 and fiber type proportions. The potential effect of ACTN3 genotype on fiber type composition is probably small and the discrepancies observed between studies may depend on the extent to which other factors known to influence fiber type composition, such as training background and sex, varied within each study. Nonetheless, studies that used the KO mouse model suggest that adaptation of the α-actinin-3 deficient muscle occurs via signaling pathways that are involved in the regulation of the size and metabolic properties of muscle fibers, rather than their myosin heavy chain content (18, 31).

In summary, reports from several independent studies indicate that the presence of the α-actinin-3 protein provides an advantage for power- and strength-oriented athletes (4, 16, 30, 39, 54). The mechanism via which the ACTN3 genotype may influence performance is unclear and only a few studies aimed...
at elucidating this question at the cellular level in humans have been published (8, 46, 51, 52). To our knowledge, the present study is the first that demonstrated ACTN3 genotype-associated differences in the activation of the mTOR signaling pathway in human skeletal muscle. The present data showing the effects of ACTN3 genotype on glycogen utilization during sprint exercise only in type II fibers, which normally express α-actinin-3, and not in type I fibers, are also novel, but should be considered as preliminary because of the limited number of subjects with XX genotype that were identified and could be included in the analyses. In conclusion, the present findings, reinforce the proposal based on findings from the KO mouse model that ACTN3 genotype-associated differences in hypertrophy signaling and glycogen handling, provide a mechanistic explanation of how ACTN3 genotype may influence human physical performance (31, 42).

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: B.N. and E.J. designed research; B.N. and M.E. performed experiments; B.N. and M.E. analyzed data; B.N. and E.J. interpreted results of experiments; B.N. and M.E. prepared figures; B.N., M.E., H.C.R., T.x., and E.J. drafted manuscript; B.N., M.E., H.C.R., and E.J. edited and revised manuscript; B.N., M.E., H.C.R., T.x., B.G., and E.J. approved final version of manuscript.

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