Protective role of α-actinin-3 in the response to an acute eccentric exercise bout

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Submitted 4 September 2009; accepted in final form 25 May 2010

Vincent B, Windelinckx A, Nieless H, Ramaekers M, Van Leemputte M, Hespel P, Thomis MA. Protective role of α-actinin-3 in the response to an acute eccentric exercise bout. J Appl Physiol 109: 564–573, 2010. First published May 27, 2010; doi:10.1152/japplphysiol.01007.2009.—The ACTN3 gene encodes for the α-actinin-3 protein, which has an important structural function in the Z line of the sarcomere in fast muscle fibers. A premature stop codon (R577X) polymorphism in the ACTN3 gene causes a complete loss of the protein in XX homozygotes. This study investigates a possible role for the α-actinin-3 protein in protecting the fast fiber from eccentric damage and studies repair mechanisms after a single eccentric exercise bout. Nineteen healthy young men (10 XX, 9 RR) performed 4 series of 20 maximal eccentric knee extensions with both legs. Blood (creatine kinase; CK) and muscle biopsy samples were taken to study differential expression of several anabolic (MyoD1, myogenin, MRF4, Myf5, IGF-1), catabolic (myostatin, MAFbx, and MURF-1), and contraction-induced muscle damage marker genes [cysteine- and glycine-rich protein 3 (CSRP3), CARD, HSP70, and IL-6] as well as a calcineurin signaling pathway marker (RCAN1). Baseline mRNA content of CSRP3 and MyoD1 was 49 ± 12 and 67 ± 25% higher in the XX compared with the RR group (P = 0.01–0.045). However, satellite cell number was not different between XX and RR individuals. After eccentric exercise, XX individuals tended to have higher serum CK activity (P = 0.10) and had higher pain scores than RR individuals. However, CSRP3 (P = 0.058) and MyoD1 (P = 0.08) mRNA expression tended to be higher after training in RR individuals compared with XX α-actinin-3-deficient subjects. This study suggests a protective role of α-actinin-3 protein in muscle damage after eccentric training and an improved stress-sensor signaling, although effects are small.

α-actinin-3 deficiency; biochemical markers of muscle damage; anabolic/catabolic responses; satellite cell; calcineurin pathway

THE α-ACTININ-3 PROTEIN is a member of the actin-binding protein family consisting of four proteins related to dystrophin. The two skeletal muscle isoforms (α-actinin-2 and α-actinin-3) are major structural components of the Z line in the sarcomere. However, the precise physiological role of α-actins in the sarcomeres is not clear yet. Binding partners of α-actins are not only structural proteins of the contractile apparatus but also metabolic enzymes and signaling proteins such as calsticans (30). This indicates a role for α-actins in thin filament organization and in the interaction between the myocyte cytoskeleton and the sarcolemma. In addition, the binding pattern of α-actins is compatible with a role in regulation of myofiber differentiation and contraction (37). The ACTN3 gene (11q13–q14) encodes for the α-actinin-3 protein. The protein is expressed almost exclusively in type II muscle fibers (37). In about 18% of the Western European population, a complete deficiency of this protein occurs due to homozygosity for a premature stop codon (577X) polymorphism in the ACTN3 gene. This polymorphism (chromosome 11 position 66084671, C→T, rs1815739) is not associated with major muscular dysfunction (30). Still, a number of studies in humans (31, 38, 51) as well as in mice (8) have indicated that α-actinin-3 may be implicated in modulation of muscle force output at high contraction velocities.

Because ACTN3 is implicated in anchoring thin actin filaments to the Z line (37), it has been postulated that the protein may play a role in maintenance of the structural integrity of sarcomeres and muscle cells during lengthening muscle contractions (30). However, Clarkson et al. (12) did not find a higher plasma creatine kinase (CK) response following strenuous eccentric exercise in ACTN3 R577X homozygotes than in RR homozygotes. However, elevation of plasma CK activity may not be a sensitive enough parameter to detect small differences in the degree of exercise-induced local muscle damage between both genotype groups.

It is well documented that unaccustomed eccentric muscle contractions cause sarcomere disruption (17, 18) and muscle cell breakdown, which initiates a wide spectrum of intracellular responses, including inflammation, and an exaggerated myocellular repair/remodeling response (48). Against this background, different biochemical “markers” of structural muscle damage may be used to detect differential myocellular response to unaccustomed lengthening muscle contractions between ACTN3 genotype groups. Thus cysteine- and glycine-rich protein 3 (CSRP3) and cardiac ankyrin repeat protein (CARP), two titin-interacting proteins that are believed to play a role in the sensing of mechanical strain during muscle contraction (19, 27), are well chosen as damage response markers. Like ACTN3, CSRP3 (also known as muscle LIM protein, or MLP) is also localized in the Z disk of sarcomeres, where it is bound to telethonin and interacts with titin via titan cap (T-cap). Upregulation of the CSRP3 and CARP genes has been found to be exaggerated during early recovery from lengthening muscle contractions (25, 27, 33). There are also data to indicate that heat shock proteins (HSPs) are involved in remodeling and reinforcement of the myofibrillar structures after disruptions in these ultrastructures. Translocation of small HSPs (HSP27 and αB-crystallin) from the cytosol to the Z disk and intermediate structures after eccentric exercise merits their function as stabilizers and protectors of myofibrillar structures.
to an eccentric exercise bout in humans. In this study we give the effect of the ACTN3 R577X genotype on the acute responses profile (resistance exercise) or increased expression profile (28). Shows an early upregulation and later downregulation expression specific RING finger-1 (MURF-1) (35, 46). MAFbx/atrogin-1 ligases muscle atrophy F-box (MAFbx/atrogin-1), and muscle-considered to be regulated by the muscle-specific ubiquitin degradation involves the ubiquitin proteasome pathway and is have been found (dependent on training load); however, the inflammatory response triggered by contraction-induced muscle damage also involves upregulation of the expression of several myokinases, including IL-6 (6, 21, 28). IL-6 expression has been found to be increased after concentric exercise (7, 28) and eccentric treadmill running (6), and a recent study also found specific exercise-induced upregulation of IL-6 in type II muscle fibers (21), which is also the site of ACTN3 expression.

Muscle damage logically also elicits a muscle remodeling response, which involves activation of various intracellular repair mechanisms. Activation, proliferation, and differentiation of satellite cells plays a pivotal role in this process, which is modulated by locally produced insulin-like growth factor 1 (IGF-1) (44). Differentiation of satellite cells into myoblasts involves the regulation of skeletal muscle-specific proteins belonging to the family of myogenic regulatory factors (MRFs). Satellite cells express MRFs, whose members include myogenin, myogenic differentiation 1 (MyoD1), myogenic factor 5 (Myf5), and MRF4, which probably play an important role in exercise-induced muscle regeneration. The expression program of MRFs during satellite cell activation, proliferation, and differentiation involves rapid upregulation of MyoD1 already within 12 h following muscle overloading or injury. This process occurs before satellite cell proliferation would be expected, typically 24–48 h following the stimulus. MyoD1 and Myf5 are key factors for the acquisition of myogenic identity and are rapidly upregulated in activated satellite cells and myoblasts. Myogenin and MRF4 are expressed following satellite cell proliferation and during their differentiation program. There is evidence of increased expression levels of myogenin (440 and 300%) and MRF4 (120%) within 2–24 h after resistance training in humans (2, 45, 56). However, following a 3-day repeated eccentric exercise protocol, significant increases in myogenin expression were accompanied with a decrease in MyoD expression (14). It was previously hypothesized that ACTN3 R577X could function through indirect interactions with calcineurin via binding to calcarcins (30, 37). RCAN1 (also known as MCIP1) mRNA expression is used as an indicator of calcineurin signaling in vivo (16, 54) and has been shown to be upregulated during recovery from eccentric exercise (33).

Increased rates of protein synthesis up to 48 h after exercise have been found (dependent on training load); however, the rate of protein breakdown is also increased, albeit to a lesser degree, leading to an improved net protein balance. Protein degradation involves the ubiquitin proteasome pathway and is considered to be regulated by the muscle-specific ubiquitin ligases muscle atrophy F-box (MAFbx/atrogen-1), and muscle-specific RING finger-1 (MURF-1) (35, 46). MAFbx/atrogen-1 mRNA is specifically downregulated after lengthening muscle contractions (25). Depending on the exercise mode, MURF-1 shows an early upregulation and later downregulation expression profile (resistance exercise) or increased expression profile (28).

Against this background, this study aimed to investigate the effect of the ACTN3 R577X genotype on the acute responses to an eccentric exercise bout in humans. In this study we give insights in the genotype-specific response of mRNA expression of genes involved in anabolic/catabolic pathways and a set of biochemical markers of muscle damage (CARP, CSRP3, HSP70, IL-6). The interaction of α-actinin-3 with a marker of the calcineurin pathway (RCAN1) is further investigated. In addition, we explore fiber type-specific satellite cell number differences between genotype groups. We proposed the following hypotheses: 1) individuals lacking the stabilizing influence of α-actinin-3 experience more muscle damage (CK, strength loss, pain) in response to eccentric exercise; and 2) more muscle damage in XX individuals causes a higher degree of activation of the postexercise repair response.

MATERIALS AND METHODS

Subjects

Twenty healthy young men (age: 22.0 ± 0.4 yr; body weight: 71.0 ± 2.0 kg) volunteered to participate and gave written consent after being fully informed about the study protocol. Anthropometric characteristics (means ± SE) of the subjects are shown in Table 1. No associations between the ACTN3 R577X genotype and any of the anthropometric measurements were found, indicating similar physical characteristics between both groups. The protocol was approved by the Ethics Committee of the Faculty of Medicine at Katholieke Universiteit Leuven. Subjects were recruited from among the local student population. Inclusion criteria were 1) male, 2) age between 18 and 30 yr, and 3) in good health. Exclusion criteria were 1) acute or chronic disease, 2) regular use of any medication during a period of 6 mo before the study, 3) any medical condition that might contraindicate high-intensity exercise, and 4) a history of regular resistance training in a period of 12 mo before the study. Participants were selected from a larger group of subjects (n = 106) that were screened for the ACTN3 R577X genotype. Subjects were instructed not to participate in any high-intensity exercise during the 2 days preceding the experimental sessions.

Study Protocol

The chronology of events is presented in Fig. 1. Subjects reported to the research center about 2 wk before the experimental session. During the pretest, knee extensor strength was measured, and subjects also participated in a habituation session to get familiar with the experimental exercise protocol to be performed. The pretest consisted of measurement of various strength characteristics of the right quadriceps muscle on a self-constructed computerized isokinetic dynamometer (described in Muscle Strength Measurements).

First, maximal isometric knee extension torque was measured at a knee angle of 45°. This was followed by dynamic torque assessments by a series of maximal concentric contractions at 100, 200, and 300°/s, respectively. Finally, eccentric torque was measured during a maximal contraction with muscle lengthening induced by the dynamometer at a velocity of −60°/s.

Table 1. Anthropometric characteristics by ACTN3 R577X genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>577RR</th>
<th>577XX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>21.3 ± 0.5</td>
<td>21.8 ± 0.7</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>71.7 ± 3.4</td>
<td>71.0 ± 2.4</td>
</tr>
<tr>
<td>Height, cm</td>
<td>180.0 ± 1.5</td>
<td>178.0 ± 2.8</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.1 ± 1.0</td>
<td>22.4 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE for 577RR (n = 9) and 577XX (n = 10) genotypes.
Subjects were instructed to report to the research center (randomized by ACTN3 genotype) on the experimental day in the fasted state. One RR subject dropped out at this stage due to illness; data are therefore presented for 9 RR and 10 XX subjects. On arrival, subjects rested for 30 min before the first muscle biopsy was taken from the vastus lateralis of the right quadriceps muscle. A venous blood sample (10 ml; BD Vacutainer serum tube) was taken from an antecubital vein. The warming up consisted of 15 min of cycling on an ergometer at a moderate intensity. Immediately after the warming up, subjects conducted the eccentric exercise bout on a self-constructed programmable dynamometer (described in Muscle Strength Measurements). The exercise consisted of 8 sets of unilateral knee extensions, 4 sets for each leg (alternating), with each set consisting of 20 maximal repetitions (45 s of rest between sets). Before the start of each eccentric contraction, the subject’s leg was extended passively by the dynamometer. An auditory signal announced the start of each movement of the dynamometer arm, which induced knee flexion (from 5 to 85° knee angle, with 0° as the extended position) at an angular velocity of −60°/s. The subjects were instructed to maximally counteract the knee flexion imposed by the dynamometer. Again, an auditory signal marked the end of the contraction, and the dynamometer returned the subject’s leg to the starting position. Subjects were verbally encouraged to produce maximal force during each contraction. After the exercise bout, subjects rested in the laboratory for 6 h. One hour after the end of the exercise bout, a second muscle biopsy through the same incision and a blood sample were taken. Subjects then received a standardized meal (750 kcal). At the end of the 6-h recovery period, a third muscle biopsy (new incision in the vastus lateralis of the left quadriceps muscle) and blood sample were taken. Before leaving the research center, subjects were asked to score their perceived muscle soreness and pain on a visual analog scale (VAS). On departure from the research center, subjects were asked not to take analgesic medication during the coming days (49). Twenty-four and 48 h after the exercise session, the subjects returned to the research center for another venous blood sample and to fill out the VAS. On the third day, 48 h after the exercise session, subjects again reported to the laboratory for the posttest evaluation of knee extension torques, which was identical to the pretest.

**Muscle Strength Measurements**

Knee extension torque measurements and the eccentric exercise protocol were performed on a self-constructed computerized isokinetic dynamometer (servomotor SEW Eurodrive CM90; Bruchol, Germany). Subjects were positioned in a 30° backward inclined chair with the upper body and upper leg fixed. Quadriceps strength was measured while subjects pushed against a lever arm, which was fixed to the lower leg above the ankle joint. Torque (Nm) was continuously measured at a sampling rate of 1,000 Hz using a calibrated torque transducer (type 1605; Lebow Products, Troy, MI; 0.05% accuracy level), which was mounted in the axis of the machine that was aligned with the axis of the knee joint.

**Analyses of Blood Samples**

**ACTN3 R577X SNP genotyping.** DNA was extracted using the Chemagen DNA blood kit on an automated Chemagen Magnetic Separation Module I (Chemagen, Baesweiler, Germany) and a Multiprobe I robotic station (PerkinElmer, Waltham, MA). Genotyping was performed using a TaqMan single-nucleotide polymorphism (SNP) genotyping assay (Applied Biosystems) containing a 20× mix of unlabeled PCR forward and reverse primers as well as VIC- and FAM-labeled allele discrimination probes (assay ID: C_590993_1_). Real-time quantitative PCR (qPCR) was carried out in a 20-µl reaction mixture with 5 µl of DNA, 4 µl of RNase-free water, 1 µl of 20× TaqMan SNP genotyping assay mix, and 10 µl of the 2× TaqMan universal PCR master mix (Applied Biosystems). Amplification and detection were performed using the ABI PRISM 7300 sequence detection system (Applied Biosystems). Thermal cycling conditions were 10 min at 95°C, followed by 40 two-step cycles, including 15 s of denaturation at 92°C and 60 s of annealing/extension at 60°C. All reactions were set up manually, and allele calling was done using SDS 1.3 software.

**Creatine kinase.** CK assay in blood samples was done according to standard laboratory procedures (Enzyline CK NAC Optimise 20 kit; bioMerieux) (AU 5400; Olympus). The measurement and reporting were done at 37°C. The internal reference values are 25–170 U/l for women and 25–195 U/l for men. Three subjects (2 RR and 1 XX) had basal CK activity above these reference values and were excluded from further statistical analyses.

**Analyses of Muscle Biopsy Samples**

**Tissue collection.** Muscle biopsies were taken from the vastus lateralis muscle through a 5-mm incision in the skin under local anesthesia (2–3 ml of lidocaine) using a Bergström-type needle. After being freed from any visible nonmuscle material, the part of the muscle sample was immediately frozen in liquid nitrogen, and the remaining part was mounted in embedding medium cooled in isopentane. Samples were stored at −80°C until further analyses.

**RNA extraction and reverse transcription.** Total RNA was extracted using TRIzol (Invitrogen) reagent from 15–20 mg of frozen muscle tissue. Spectrophotometric analyses (Gensesys 10 UV) of RNA concentration and purity (UV 260/280 and UV 260/230 ratios) were performed, and extracts were further stored at −80°C until used for real-time PCR analysis at a later date. Total RNA was reverse transcribed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. cDNA conversion was carried out in a 20-µl reaction volume including 2 µl of RT-PCR buffer, 0.8 µl of dNTP mix, 2 µl of random primers, 1 µl of MultiScribe reverse transcriptase, 1 µl of RNase inhibitor, and 13.2 µl of total RNA and nuclease-free water. Because total RNA concentrations were variable between individual samples, a variable volume of total RNA and nuclease-free water was used for reverse transcription to produce a final cDNA concentration of 50 ng/µl. Thermal cycling conditions included 10 min at 25°C, 120 min at 37°C, and 5 s at 85°C. cDNA samples were stored at −20°C until further analyses.

**Real-time qPCR.** Primers and probes for real-time qPCR were designed by Applied Biosystems and supplied as a Gene Expression Assay mix containing a 20× mix of unlabeled PCR forward and reverse primers as well as TaqMan MGB probe. FAM dye-labeled assay IDs were Hs99999905_m1 (GAPDH), Hs0187842_m1 (β2-microglobulin), Hs00159528_m1 (MyoD1), Hs00231165_m1 (MRF4), Hs00217574_m1 (Myf5), Hs00193363_m1 (myostatin), Hs00321167_m1 (myogenin), Hs00153126_m1 (IGF-1), Hs01120957_m1 (RCAN1), Hs00369714_m1 (MAFbx/atrogin-1), Hs00283297_m1 (MURF1), Hs01733171_m1 (CARP), Hs00185787_m1 (CSRP3), Hs00174131_m1 (IL-6), and Hs00359147_s1 (HSP70). Real-time qPCR was carried out in a 20-µl reaction mixture with 2 µl of diluted cDNA (20 ng), 7 µl of RNase-free water, 1 µl of 20× TaqMan gene expression assay mix, and
10 μl of the 2′X TaqMan gene expression Master Mix, containing ROX as a passive reference dye (Applied Biosystems). Amplification and detection were performed using the ABI PRISM 7300 sequence detection system (Applied Biosystems). Thermal cycling conditions included an initial 2 min at 50°C and 10 min at 95°C, followed by 40 two-step cycles including denaturation of 15 s at 95°C and annealing/extension of 60 s at 60°C. Each gene was analyzed with the incorporation of a negative control. All reactions were performed in triplicate. To compensate for variations in input RNA amount and efficiency of reverse transcription, normalization factors were determined based on the geometric mean of the expression levels of multiple reference genes (GAPDH and β2-microglobulin) as described by Vandesompele et al. (50) using qBase software (20). Gene expression data after training are represented with resting samples before training assigned the arbitrary value of 1.0 and all other samples expressed relative to this value. Based on earlier studies on exercise-induced time courses of changes in our genes of interest, mRNA expression levels were studied at 6 h postexercise (28, 56). To study earlier exercise-induced responses in MAFbx, MURF1, and RCAN1 (28, 40), expression levels were studied at both 1 and 6 h postexercise.

Pain Scales

A VAS was used to assess the subjective perception of muscle soreness following the exercise bout (41). Subjects were asked to rate their perception of pain on a horizontal 10-cm-long axis ranging from 0 (no pain) to 10 (unbearable pain) for each leg separately. This scale included the response. A probability level (P < 0.05) was considered statistically significant, although trends (0.05 < P ≤0.10) are also reported. All data are means ± SE.

RESULTS

Effect of ACTN3 Polymorphism on Muscle Strength

Maximal knee extension torques at different contraction velocities (−60°/s eccentric, to +300°/s concentric contractions) were measured before (pretest) and 48 h after (posttest) the exercise bout involving intermittent maximal eccentric muscle contractions. As shown in Fig. 2, knee extension torque substantially decreased as contraction velocity increased from −60° to +300°/s. Torques were lower during the posttest than the pretest in both XX and RR individuals, most prominently during the dynamic contractions at 100° and 200°/s (P < 0.05). The decrease in torque production at 100°/s was independent of the experimental group. During dynamic contractions at 200°/s, only XX individuals showed a decrease from the pretest to the posttest (P < 0.05). Still, knee extension torque at the highest velocity (300°/s) tended to be higher in RR than in XX individuals (P = 0.10). Absolute muscle torques were similar between XX and RR individuals in either the pretest or the posttest.

Effect of ACTN3 Polymorphism on CK

Blood CK activity was measured at rest and at 6, 24, and 48 h after the exercise bout. As shown in Fig. 3, CK activity increased after exercise in both XX and RR individuals (P = 0.002), reaching peak values at 24 h postexercise. Compared with rest, peak CK activity was approximately twofold higher in RR individuals, whereas it was approximately fourfold higher in the XX group (genotype × time interaction effect, F = 2.3, P = 0.09). Independent sample t-test revealed no significant differences between the two genotype groups at any of the measured time points. At 24 h postexercise, a trend could be observed indicating a higher CK activity in XX compared with RR individuals (P = 0.10).

Effect of ACTN3 Polymorphism on Anabolic/Catabolic mRNA Expression

The mRNA expression of several anabolic (MyoD1, MRF4, IGF-1, Myf5, and myogenin) and catabolic pathway genes was decreased by 10.22 ± 0.32.247 on July 11, 2017 http://jap.physiology.org/ Downloaded from
(myostatin and two ubiquitin ligases, MAFbx and MURF-1) was measured using real-time qPCR before and after the eccentric exercise bout (Figs. 4 and 5). At baseline and compared with RR individuals, XX individuals had 49% higher mRNA expression of MyoD1 (P = 0.01). All other mRNA levels were similar between groups.

Six hours postexercise and compared with the XX group, MyoD1 expression levels tended to be higher (78% 36%) in the RR group (P = 0.08). mRNA levels of the other target genes were similar between the groups. As shown in Fig. 5A, the eccentric exercise bout markedly increased the expression level of myogenin (~4-fold), whereas mRNA content of MyoD1 and MRF4 increased to a smaller degree (~2-fold; P < 0.05). IGF-1, Myf5, and myostatin expression levels were unaffected by the exercise bout.

MRNA content of two ubiquitin ligases (MAFbx and MURF-1) were measured at 1 and 6 h postexercise (Fig. 6A). Changes in expression of MAFbx and MURF-1 were similar between the genotype groups. For MAFbx, an overall decrease in expression after exercise was found: expression levels decreased 31% at 1 h and 79% at 6 h postexercise compared with rest (P < 0.01). The expression of MURF-1 showed a different response to the exercise bout. One hour after exercise, MURF1 mRNA levels increased approximately threefold compared with baseline (P < 0.01). Six hours after exercise, MURF-1 mRNA levels decreased 23% below baseline values (P < 0.01).

**Effect of ACTN3 Polymorphism on mRNA Expression of Biochemical Markers of Muscle Damage**

The mRNA expression of several contraction-induced muscle damage markers (CSRP3, HSP70, IL-6, and CARP) was evaluated before and after the eccentric exercise bout (Figs. 4 and 5). At rest, XX individuals had 67% 25% higher expression of CSRP3 compared with RR individuals (P = 0.045). All other mRNA levels did not differ between genotype groups.

As shown in Fig. 5, B and C, all four genes are markedly upregulated after an eccentric exercise bout. CARP expression levels increased about 150-fold, whereas mRNA levels of HSP70 increased ~15-fold and levels of CSRP3 and IL-6, about 4-fold (all P < 0.05). At 6 h after exercise, RR individuals had 87% 39% higher CSRP3 levels than XX individuals (P = 0.058). No other differences between the genotype groups were observed.

**Effect of ACTN3 Polymorphism on RCAN1 mRNA Expression**

Response of RCAN1 mRNA expression to exercise was measured by real-time qPCR at 1 and 6 h postexercise (Fig. 6B). At 1 h postexercise, the mRNA content of RCAN1 had increased ~33-fold (P < 0.01). Six hours after exercise,
RCAN1 expression levels were still 16-fold higher than baseline values ($P < 0.01$). The increase in RCAN1 mRNA levels after exercise was similar in both genotype groups.

**Effect of ACTN3 Polymorphism on Satellite Cell Content**

Satellite cell content was measured by Pax7 staining in baseline muscle biopsy samples (Fig. 7). As shown in Table 2, the number of satellite cells was similar between type I and type II fibers, and there was no difference between the genotype groups in either fiber type ($P > 0.05$).

Subjective perception of muscle soreness and pain was evaluated using a VAS. General pain sensation peaked at 6 h postexercise and then gradually decreased at 24 and 48 h (data not shown). XX individuals reported a 1.7-fold (±0.4) higher pain sensation in the right leg ($P = 0.048$) 6 h postexercise. Twenty-four and 48 h postexercise, the XX group still tended to report higher pain scores than the RR group ($P = 0.06$). Other VAS scores were similar between RR and XX individuals.

**DISCUSSION**

The primary aim of this study was to determine whether the ACTN3 R577X polymorphism affects indicators of muscle damage (CK activity, drop in muscular strength due to damaged muscle fibers and muscle soreness) and mRNA expression levels of anabolic/catabolic pathway genes, biochemical markers of mechanical-induced muscle damage and RCAN1 after a single bout of eccentric exercise. We showed that indicators of muscle damage (CK activity and VAS scores) are generally higher in 577XX individuals postexercise. However, contrary to our expectations, homozygotes for the 577R allele
tended to show higher repair responses, as indicated by increased expression of MyoD1 \( (P = 0.08) \) and CSRP3 \( (P = 0.058) \) compared with XX carriers. Furthermore, in this study we also have demonstrated for the first time that 577XX individuals have higher expression levels of MyoD1 \( (P = 0.01) \) and CSRP3 \( (P = 0.045) \) compared with RR individuals in baseline conditions. Genotype-specific differences in these baseline and response mRNAs could not be attributed to differences in baseline satellite cell composition.

Given the invasive nature of the biopsy protocol, genotype groups were small (RR: \( n = 9 \); XX: \( n = 10 \)). In addition, the expected differences in muscle characteristics due to genotypic differences also are expected to be small. Earlier reports estimated the ACTN3 R577X genotype to explain only 2.2% of the total variance in baseline muscle strength in adult women, 2.6% of the total variance in 40-m sprint speed in adolescent boys, and 3.5% of total variation within baseline CK activity (12, 31). Results with statistical evidence for trends in genotype-dependent differences \( (P < 0.10) \) are therefore also discussed to avoid false negative findings and highlight possibly relevant associations.

CK Activity, Pain, and Strength Response

After the eccentric exercise bout, 577XX individuals tended to have higher peak CK values compared with RR individuals. This finding is in contrast with Clarkson et al. (12), who found no association of the R577X polymorphism with the change in blood proteins (CK and myoglobin) postexercise. We hypothesized that structural differences in ACTN3-deficient fibers could increase muscle damage and hence demonstrate greater increases in CK response to eccentric exercise. Although our data are not conclusive, they seem to support this hypothesis. We might have observed larger differences between genotype groups if the eccentric protocol used higher contraction speeds that challenged \( \alpha \)-actin-3-deficient fast fibers to a larger extent. However, we prioritized our protocol for standardization and (velocity) control of eccentric contractions. Compared with studies using other acute eccentric training bouts, our protocol did result in a similar time course and CK blood response levels (13), although the protocol of Clarkson et al. (12), which focused on controlled rhabdomyolysis, did report much higher and later peaks in CK activity. Although the measurement of CK activity in serum is widely used as an indicator of muscle damage (1, 53), more specific and sensitive markers [e.g., \( \alpha \)-actin (34), skeletal troponin I (47)] might need to be studied to detect more subtle differences in muscle damage between ACTN3 R577X genotype groups.

Another indicator of exercise-induced muscle damage is delayed onset of muscle soreness (4). We evaluated the degree of postexercise muscle soreness by the use of VAS scores (13, 41). Interestingly, subjective assessment of muscle soreness revealed a similar trend as observed in the CK response to exercise. 577XX individuals experienced more muscle soreness following the bout of eccentric muscle contractions than RR individuals.

In the posttest, both genotype groups exhibited similar relative strength declines in relative isometric, eccentric, and dynamic contractions at 100°/s. However, for dynamic contractions at 200°/s, only XX individuals had significant decreases in knee extension torque. Clarkson et al. (12) previously reported the absence of an association between ACTN3 R577X and the drop in muscle strength after eccentric elbow exercise. These authors, however, evaluated changes in maximal isometric voluntary contractions (12), whereas genotype-dependent responses in our protocol were only present in dynamic contractions at 200°/s. The specific localization of \( \alpha \)-actin-3 in fast muscle fibers might explain the velocity-specific effects seen in our study, whereas no genotype-dependent effects on strength losses were seen in isometric strength (this study and Ref. 12).

Differences in mRNA Expression Response Levels

Six hours after eccentric exercise, we observed an ~80% greater upregulation of MyoD1 and CSRP3 in the RR group compared with the XX group. This was contrary to our expectations, because the lower plasma CK activity in RR individuals indicates a lower degree of muscle damage. We speculate that higher CSRP3 and MyoD1 expression levels could indicate an increased signaling response toward mechanical strain in the Z line in RR individuals. CSRP3, a Z-line protein present in multiple cellular compartments, translocates to the nucleus on mechanical stress, where it interacts with the MyoD-E-protein heterodimer, enhancing the DNA-binding activity of this transcription complex (3, 19, 24, 25). However, it must be noted that the higher relative postexercise MyoD1 and CSRP3 mRNA levels are at least partly related to the lower baseline levels. The effect of MyoD1 expression...
ion on satellite cell characteristics could not be examined, since enhancement of the satellite cell pool can only occur as early as 4 days postexercise (23).

None of the other markers, inflammation, and target genes involved in anabolic/catabolic pathways differed significantly in postexercise response between the two ACTN3 genotype groups. Overall, the eccentric exercise produced a significant increase in mRNA expression of HSP70, IL-6, CSRP3, CARP, MyoD1, MRF4, and myogenin mRNA, whereas expression levels of Myf5, IGF-1, and myostatin remained unchanged compared with rest. Although exact comparisons of mRNA expression changes are difficult due to differences in contraction type or protocol of the training bout and sampling time points, these findings are similar to previous reports in literature (22, 25, 28, 45, 56). In the study by Yang et al. (56), Myf5 mRNA did not significantly change 4–8 h after running or resistance training exercise, in the presence of significant changes in other MRFs. The expected downregulation of myostatin was only significantly observed 8 h after running exercise (28) or was not observed 24 h after eccentric exercise (22). Also, IGF-1 mRNA responses were unchanged at least for the IGF-1Ec isoform (including isoform b and c) up to 48 h after high-resistance knee extension exercise (45). Despite the lack of significant changes in expression of myostatin, mRNA levels of two ubiquitin ligases did show altered expression after exercise. Varying results concerning the effects of exercise on MAFbx and MURF-1 expression have been reported (10, 25, 57). Mascher et al. (35) reported, similar to our own findings, MURF1 mRNA to be increased 2 h after leg press exercise, whereas MAFbx was unchanged early after exercise but downregulated 48 h later. In our study, we observed decreased expression levels already by 1 h after exercise.

It was previously hypothesized that the ACTN3 (R577X) polymorphism functions through altered interaction with calcineurins and therefore influences calcineurin signaling (55). Calcineurin and its downstream transcription factor, nuclear factor of activated T cells (NFAT), play an important role in the hypertrophy response through a decrease in myostatin expression and altered expression of unknown targets (36). Based on the premise that expression of RCAN1 provides an indicator of the state of activation of the calcineurin signaling pathway, our data show no evidence of a significant role of α-actinin-3 in altering calcineurin signaling after an eccentric training bout. Overall, the eccentric exercise bout induced a substantial (33-fold) increase in RCAN1 expression already 1 h postexercise. Five hours later, expression levels were still 16-fold higher compared with baseline. Norrbom et al. (40) also reported increased RCAN1 (MCIP1) mRNA levels 2 h after concentric knee extension exercise; however, they noted only a fourfold increase compared with preexercise values. Hence, the marked increase in RCAN1 after the eccentric exercise bout adds further proof to the increased anabolic signaling found after exercise.

Baseline Differences

At baseline, absolute muscle torques were similar between the two genotype groups. For relative knee extension torque (relative to isometric torque), however, we found that RR individuals at high contraction velocities (300°/s), but not at low contraction velocities, tended to have higher muscle strength than XX individuals. This finding confirms our previous report showing increased muscle performance at high contraction velocities in RR individuals (51). Literature data with regard to the association between muscle strength and ACTN3 genotype are equivocal (11, 12, 15). However, differences in strength protocols (contraction mode and velocities) and studied populations (ethnicity, sample size, and sex) can underline the discrepancy in findings.

We found no effect of the ACTN3 genotype on baseline CK activity. Similar findings were reported by Lucia et al. (29). However, Clarkson et al. (12) showed homozygote carriers of the X allele to express lower resting CK activity than heterozygote subjects. These baseline differences in CK activity were probably caused by a difference in muscle mass and/or physical activity level over the different genotype groups (5). It is therefore reasonable to conclude that, at baseline, there is no evidence of an association of the ACTN3 genotype with CK activity.

Interestingly, 577XX individuals showed increased expression of MyoD1 and CSRP3 at baseline compared with RR individuals. This genotype-dependent difference in baseline expression was contradictory to our expectations, since subjects were instructed not to perform any intense physical activity 48 h before the start of the study. MyoD1 plays an important role in growth and repair of muscle fibers and is expressed in activated satellite cells. However, independent of fiber type, satellite cell numbers were similar between XX and RR genotypes. Therefore, the higher level of MyoD1 expression in XX conceivably was due to increased MyoD1 expression in myonuclei, rather than to a higher degree of satellite cell activation or proliferation (9, 26). The parallel higher mRNA expression of CSRP3 in the α-actinin-3-deficient group at least confirms what could be expected given the cofactor function of CSRP3 for MyoD (3, 19).

In addition to its role in determination of myoblasts, MyoD1 is also involved in muscle-specific MHC profile and in fiber-type transitions. In rodents, MyoD1 is mainly expressed in fast-twitch glycolytic muscles such as tibialis anterior and extensor digitorum longus (26). α-Actinin-3 is found almost exclusively in fast muscle fibers (37), and we previously demonstrated that homozygosity for the 577R allele is associated with higher proportion of fast glycolytic muscle fibers (51). However, finding this increased MyoD1 and CSRP3 expression levels in XX individuals at rest was contrary to expectations.

Since everyday physical activities and activities of moderate intensity were still allowed in the 48-h period before rest biopsies were taken, it is possible that the physical activity intensity threshold for increased mRNA expression of MyoD1 and CSRP3 might therefore be lower in XX compared with RR individuals. Alternatively, although speculative, it might suggest that in α-actinin-3-deficient muscle, MyoD1 and CSRP3 need to be upregulated in the baseline state, representing a higher regeneration/remodeling potential at baseline, to compensate for a stunted response in the upregulation of MyoD1 and CSRP3 after exercise.

Why only limited effects of α-actinin-3 on the measured indicators of muscle damage were found may be explained by compensatory increases in α-actinin-2, since both are present in type II fibers. However, contradictory findings regarding this
possibility have been reported. Based on our previously reported data (51), we find average red staining for ACTN2 protein to be similar between genotype groups for each of the fiber types (type I, \( P = 0.56 \); type IIa, \( P = 0.77 \); type IIx, \( P = 0.73 \)). However, a recent study by Norman et al. (39) demonstrated that ACTN2 mRNA expression was affected by the content of \( \alpha \)-actinin-3, since ACTN2 expression was significantly higher in XX compared with RR subjects when the percentage of type IIb fibers was taken into account as a covariate. These data support the hypothesis that \( \alpha \)-actinin-2 compensates for the lack of \( \alpha \)-actinin-3, as was also reported for Actn3\(^{-/-}\) mice (32). Although data on compensation of increased ACTN2 are still equivocal, the overall higher mRNA expression of ACTN2 over ACTN3 mRNA in RR subjects is also indicative of an important role for ACTN2 in fast muscle fibers. More detailed analyses of our own previously reported data confirm that, also at the protein level, \( \alpha \)-actinin-2 content in type II fibers (61 ± 5 arbitrary units) is higher than \( \alpha \)-actinin-3 content (50 ± 5 arbitrary units) within the RR group. The limited findings of ACTN3 protection against muscle damage in this study may therefore be related in part to the presence of ACTN2 protein in both XX and RR groups.

This study focused on the effects of one isolated gene polymorphism for which subjects were first genotyped and selected. Possibly, the combined effect of ACTN3 R577X with other genetic variants (e.g., in myostatin, calcineurin B, IL-6, and others) might show larger effects on differential mRNA expression of ACTN3 over ACTN2 in RR individuals. The ACTN3 R577X polymorphism may be associated with increased muscle strength in response to mechanical stress at the Z line. Still, the overall effect of ACTN3 (R577X) polymorphism on the response to eccentric exercise seems limited.

ACKNOWLEDGMENTS

We thank the subjects who participated in this study for their effort and motivation. We also thank Karen Van Proeyen and Karolina Szulczuk for assisting with the laboratory experiments.

DISCLOSURES

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

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J Appl Physiol • VOL 109 • AUGUST 2010 • www.jap.org