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Activating transcription factor 3 regulates chemokine expression in contracting C_2C_{12} myotubes and in mouse skeletal muscle after eccentric exercise

R. Fernández-Verdejo [a], A.M. Vanwynsberghe [a], T. Hai [b], L. Deldicque [a], M. Francaux [a, *]

[a] Institute of Neuroscience, Université Catholique de Louvain, Louvain-la-Neuve, Belgium
[b] Department of Biological Chemistry and Pharmacology, Ohio State University, Columbus, OH, USA

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ABSTRACT

Activating transcription factor (ATF) 3 regulates chemokine expression in various cell types and tissues. Herein, we studied this regulation in contracting muscle cells *in vitro*, and in skeletal muscle after muscle-damaging exercise *in vivo*. C_2C_{12} myotubes with normal or low ATF3 levels (atf3_siRNA) were electrically stimulated (EPS). Also, ATF3-knockout (ATF3-KO) and control mice ran downhill until exhaustion, and muscles were analyzed post-exercise. EPS increased ATF3 levels in myotubes ($P < 0.01$). Chemokine C-C motif ligand (*ccl*) 2 mRNA increased post-EPS, but atf3_siRNA attenuated the response ($P < 0.05$). Atf3_siRNA up-regulated *ccl6* basal mRNA, and down-regulated *ccl9* and chemokine C-X-C motif ligand (*cxcl*) 1 basal mRNAs. Post-exercise, ATF3-KO mice showed exacerbated mRNA levels of *ccl6* and *ccl9* in soleus ($P < 0.05$), and similar trends were observed for *ccl2* and interleukin (*il*) 1β ($P < 0.09$). In quadriceps, *il6* mRNA level increased only in ATF3-KO ($P < 0.05$), and *cxcl1* mRNA showed a similar trend ($P = 0.082$). Cluster of differentiation-68 (*cd68*) mRNA, a macrophage marker, increased in quadriceps and soleus independently of genotype ($P < 0.001$). Our data demonstrate that ATF3 regulates chemokine expression in muscle cells *in vitro* and skeletal muscle *in vivo*, but the regulation differs in each model. Cells other than myofibers may thus participate in the response observed in skeletal muscle. Our results also indicate that ATF3-independent mechanisms would regulate macrophage infiltration upon muscle-damaging exercise. The implications of chemokine regulation in skeletal muscle remain to be determined.

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1. Introduction

Activating transcription factor (*atf*) 3 gene is up-regulated by stimuli that perturb homeostasis. Its protein product, ATF3, modulates the inflammatory response to these stimuli [1]. ATF3 down-regulates inflammatory markers in hearts exposed to aortic banding-induced pressure overload [2], and in epithelial cells exposed to gram-negative bacteria [3]. In contrast, ATF3 up-regulates inflammatory markers in hearts exposed to phenylephrine-induced pressure overload [4], and in macrophages exposed to gram-positive bacteria [5]. Thus, ATF3 regulates inflammation in a context-specific manner.

Exercise perturbs skeletal muscle homeostasis, inducing *atf3* expression [6]. We previously reported that ATF3-KO mice show exacerbated expression of inflammation-related genes in muscle after running on a flat treadmill [7]. This response was accompanied by signals of macrophage infiltration. Interleukin (*il*) 6, *il1β*, chemokine C-C motif ligand (*ccl*) 2, *ccl9*, and chemokine C-X-C motif ligand (*cxcl*) 1 were among the affected genes. In skeletal muscle, the up-regulation of cytokines and chemokines, like *il6*, *il1β*, tumor necrosis factor α (*tnfα*), *ccl2* and *ccl6*, indicate pro-inflammatory responses and macrophage invasion [8,9]. The ATF3 role in skeletal muscle thus appears to be the attenuation of an inflammatory response induced by exercise. Notably, since skeletal muscle contains various cell types, its inflammatory response includes responses of all those cells [10]. Whether the attenuation of inflammation-related genes induced by ATF3 occurs specifically in contracting myofibers is unknown.

Eccentric exercise provokes muscle damage, inflammation, and

Abbreviations: Qua, quadriceps; Sol, soleus.

* Corresponding author. Place Pierre de Coubertin 1, bte L8.10.01, 1348 Louvain-la-Neuve, Belgium.
E-mail address: marc.francaux@uclouvain.be (M. Francaux).

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leukocyte infiltration [11]. This exercise also induces a drastic up-regulation of *atf3* mRNA level in skeletal muscle [12]. Thus, during eccentric exercise ATF3 might also regulate the inflammatory response, *e.g.* cytokine and chemokine expression. Since chemokines are crucial for leukocyte infiltration and muscle regeneration [10,13], ATF3 could also influence these processes.

Therefore, we aimed to: 1) determine the effects of ATF3 on chemokine expression in electrically stimulated muscle cells *in vitro*; 2) determine the role of ATF3 in the inflammatory response of skeletal muscle to a single bout of eccentric exercise *in vivo*; and 3) determine the influence of ATF3 in the performance to repeated bouts of eccentric exercise *in vivo*.

2. Materials and methods

2.1. In vitro experiment

C_2C_{12} myotubes were prepared as described before [14]. After 24 h in differentiation medium (DMEM, 2% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin), cells were transfected for 72 h with ON-TARGETplus Non-targeting Pool (D-001810-10, Dharmacon, Roserberg, Sweden) or Mouse atf3_siRNA (L-058604-01, Dharmacon) using Lipofectamine® RNAiMax (Thermo Fisher Scientific, Wilmington, DE, USA). After additional 72 h in differentiation medium, EPS was applied on C_2C_{12} myotubes using the C-PACE EP Cell Culture Stimulator (Ion Optix, Milton, MA, USA). We used an EPS protocol demonstrated to mimic endurance exercise without damaging cells even after 8 h of stimulation [15]. Since this protocol was specifically tested in C_2C_{12} myotubes [15], we selected these cells as our *in vitro* model. Briefly, myotubes were incubated in differentiation medium containing 5.5 mM glucose, and then 2-ms pulses of 20 V at 1 Hz were applied for 4 h. Three hours post-EPS, myotubes were washed with PBS and recovered in lysis buffer for protein extraction, or in Trizol® for RNA extraction.

2.2. In vivo experiments

The Committee for Ethical Practices in Animal Experiments of the Université Catholique de Louvain approved the procedures. The housing conditions were in accordance with the Belgian Law of May 29, 2013 on the protection of laboratory animals.

We used ATF3-KO mice in the C57BL/6JRj background as described before [7]. ATF3-KO and their control (Con) littermates were maintained in a controlled environment (14:10 h light:dark cycle, 22–24 °C) and fed *ad libitum* with standard chow and water. Two independent experiments were performed.

2.2.1. Single bout of eccentric exercise

Male ATF3-KO and Con mice (20-25-wk-old) ran on an inclined (−18°) treadmill (Exer 3/6; Columbus Instruments, Columbus, OH, USA). The speed was set at 5 m/min, and increased 1 m/min every 5 min. After 30 min, the speed increments were applied every 15 min until exhaustion. Mice were anesthetized by an intraperitoneal injection of ketamine/xylazine 28 h post-exercise. Quadriceps (Qua) and soleus (Sol) were isolated. Muscles were frozen in liquid nitrogen and then powdered with a mortar and pestle, or embedded in optimum cutting temperature compound (VWR, Leuven, Belgium) and then frozen in cold isopentane.

2.2.2. Repeated bouts of eccentric exercise

This experiment was performed with the sole purpose of determining whether ATF3 influences performance upon repeated eccentric exercise. Male ATF3-KO and Con (15-19-wk-old) ran on an inclined (−18°) treadmill once a day for 13 consecutive days. The speed was set at 8 m/min and increased by 1 m/min every 2 min. When speed reached 16 m/min, it was maintained until exhaustion. Distance run and body mass were compared between genotypes the first and last days.

2.3. Determination of putative ATF3 binding sites

The server ConTra v3 was used [16]. We analyzed gene promoters, up to 1000 bp from the transcription start site. Stringencies of 0.95 and 0.85 were used for the core and the matrix matches, respectively. The position weight matrices V$ATF3_Q6_01, M01863 and V$ATF3_Q6, M00513 were considered. We also searched for Nuclear factor kappa-B (NFκB) binding sites, since NFκB regulates various chemokines in murine cells [17]. For NFκB, the position weight matrices V$NFKB_Q6,M00194, V$NFKB_C,M00208, and V$NFKB_Q6_01, M00774 were considered.

2.4. Western blotting

Protein extraction and dosage were performed in C_2C_{12} myotubes and skeletal muscle as described before [14]. A previously reported protocol was used for western blotting [7]. Antibodies from Sigma-Aldrich (St. Louis, MO, USA) were used for α-tubulin (TG199, 1:2000 dilution) and ATF3 (HPA001562, 1:1000 dilution).

2.5. Real-time qPCR

RNA extraction was performed in C_2C_{12} myotubes and skeletal muscle using Trizol® (Invitrogen, Vilvoorde, Belgium). Reverse transcription and qPCR were done as described previously [7]. Most primer sequences have been published [7]. For beta-2 microglobulin (*b2m*) we used forward 5′-GGTCGCTTCAGTCGTCAGCA-3′, and reverse 5′-GCAGTTCAGTATGTTCGGCTTCCC-3'; for *ccl6* we used forward 5′-CTGGCCTCATACAAGAAATGGAAA-3′, and reverse 5′-GTGGCATAAGAGAAGCAGCAGT-3'. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression, using as housekeeping genes hypoxanthine guanine phosphoribosyl transferase (*hprt*) and *b2m* for myotubes and skeletal muscles, respectively. Housekeeping genes were unaffected by the experimental conditions in each model.

2.6. Hematoxylin and eosin staining

Cryosections were cut on a cryostat (HM560; Thermo Fischer Scientific, Wilmington, DE, USA), fixated to SuperFrost® slides (VWR) and stained with hematoxylin and eosin. The percentage of myofibers with central nuclei was used as marker of structural damage, as previously described [18,19]. The mean ± SD of fibers analyzed per mouse were 6026.6 ± 869.2 in Qua, and 662.9 ± 112.2 in Sol.

2.7. Statistics

Data are shown as means ± SD. The analyses were performed with Prism 7.0a (GraphPad Software, La Jolla, CA, USA). Outliers were identified by the Tukey method adjusting for sample size [20]. Data were analyzed using two-way ANOVA, with Bonferroni *post-hoc* in case of significant interactions between the independent variables. P-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of electrical stimulation and ATF3 on chemokine expression in C_2C_{12} myotubes

EPS up-regulated *atf3* mRNA and protein levels in C_2C_{12} myotubes (Fig. 1*A*, *B*). This response was almost totally blunted by the

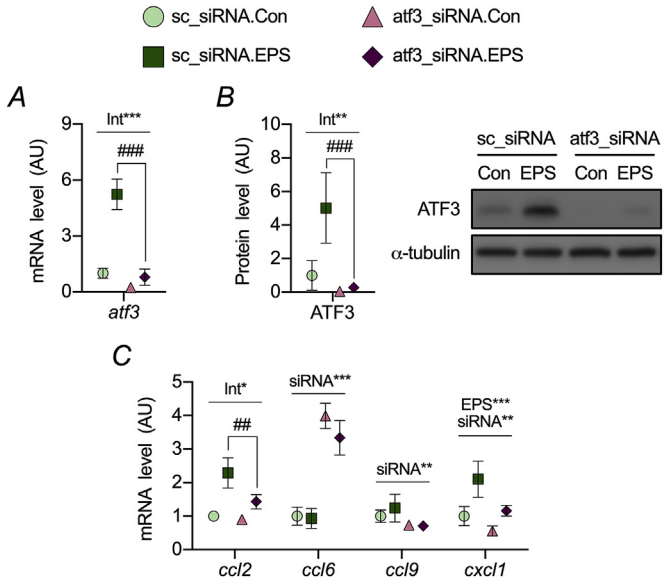

Fig. 1. Effects of electrical stimulation (EPS) and ATF3 on chemokine expression in C_2C_{12} myotubes.
C_2C_{12} myotubes were transfected with scramble (sc_siRNA) or atf3_siRNA, and then exposed to EPS or kept as control (Con). *A-B*, *atf3* mRNA (*A*) and protein (*B*) levels. *C*, chemokines mRNA levels. The error bars are not shown if they are smaller than the symbol. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for the effect of siRNA, EPS, or interaction siRNA × EPS (Int); $n = 4$ independent experiments. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ for *post-hoc* test.

atf3_siRNA, demonstrating the effectiveness of the transfection (Fig. 1*A*, *B*). The mRNA content of chemokines was differentially affected by EPS and ATF3 (Fig. 1*C*). *Ccl2* mRNA increased after EPS, but the increase was attenuated by atf3_siRNA. The mRNA level of *ccl6* and *ccl9* was not affected by EPS. However, atf3_siRNA increased *ccl6* mRNA and decreased *ccl9* mRNA. Finally, atf3_siRNA decreased *cxcl1* mRNA, but did not modify its up-regulation induced by EPS.

3.2. Analysis of putative binding sites for ATF3 and NFκB on chemokine genes

We found putative ATF3 binding sites in the promoter region of all the chemokines studied. These sites were in close proximity to NFκB putative binding sites (Table 1).

3.3. Effects of a single bout of eccentric exercise on structural damage and inflammatory markers in skeletal muscle

Maximal distance run was similar between genotypes (Con: 880.3 ± 346.6 m vs. ATF3-KO: 700.5 ± 86.5 m; $P = 0.246$, unpaired Student's *t*-test, $n = 6$). No structural damage was observed in Qua (Fig. 2*A*). In contrast, the percentage of myofibers with central nuclei increased in exercised Sol, independently of genotype (Fig. 2*B*).

In Qua, the mRNA level of *ccl2*, *ccl6*, *ccl9*, and *il1β* was up-regulated post-exercise, independently of genotype (Fig. 2*C*, *D*). A similar trend was observed for *tnfα* mRNA ($P = 0.079$; Fig. 2*D*). Notably, *il6* mRNA was only up-regulated in exercised ATF3-KO mice (Fig. 2*D*), and *cxcl1* mRNA had a similar trend ($P = 0.082$; Fig. 2*C*). The macrophage-specific marker cluster of differentiation-68 (*cd68*) mRNA increased post-exercise in both genotypes (Fig. 2*E*).

In Sol, exercise-induced up-regulation of *ccl6* and *ccl9* mRNA was higher in ATF3-KO than Con mice (Fig. 2*F*). *Il1β* mRNA presented a similar trend ($P = 0.083$; Fig. 2*G*). Although exercise increased *ccl2* mRNA in both genotypes ($P < 0.01$), the response tended to be higher in ATF3-KO ($P = 0.053$; Fig. 2*F*). The mRNA of *cd68* increased post-exercise in both genotypes (Fig. 2*H*).

3.4. Effects of repeated bouts of exhaustive eccentric exercise on exercise performance and body mass

Distance run was similar between genotypes the first day, and decreased ≈50% after 13 days, independently of genotype (Fig. 3*A*). A similar response was observed for the body mass, with a decrease of ≈2.5% in both genotypes (Fig. 3*B*).

4. Discussion

We showed that: 1) ATF3 is induced by EPS and regulates chemokine mRNA expression in C_2C_{12} myotubes; 2) ATF3-KO mice show a more marked up-regulation of some inflammation-related genes, but appear to have normal macrophage infiltration in skeletal muscle after eccentric exercise; and 3) the repetition of exhaustive eccentric exercise bouts decreases performance to the same extent in control and ATF3-KO mice. Our results suggest ATF3 attenuates inflammation of skeletal muscle upon muscle-damaging eccentric exercise. We cannot currently discern how much the use of different models explains the different results *in vivo* vs. *in vitro*. However, the ATF3-dependent regulation of chemokines in skeletal muscle seems not attributable only to myofibers, but other cells would be implicated as well. Finally, ATF3 seems not essential for macrophage infiltration or muscle recovery upon eccentric exercise.

Contraction induces *atf3* gene expression in skeletal muscle, as shown by models of nerve stimulation [21], eccentric contractions [22], and exercise [6,12]. However, the cell type responsible for this effect is unknown. We have previously shown in mice that endurance exercise increases ATF3 protein levels 3 h post-exercise [7]. Here, we electrically stimulated C_2C_{12} myotubes with a protocol that mimics endurance exercise *in vitro* [15], and analyzed the responses 3 h after. EPS increased *atf3* mRNA and protein levels in C_2C_{12} myotubes, suggesting part of the ATF3 up-regulation in contracting skeletal muscle occurs in myofibers.

During exercise, contracting myofibers produce and release chemokines to exert different actions, including leukocyte chemoattraction [10]. ATF3 seems to regulate this process. Our previous [7] and current data in whole muscle suggest that ATF3 attenuates chemokine expression. However, our present *in vitro* findings demonstrate that ATF3 have different effects on C_2C_{12} myotubes. For instance, our previous [7] and current data show *ccl2* mRNA level is exacerbated in soleus of ATF3-deficient mice post-exercise. Contrarily, in *atf3*-deficient myotubes, *ccl2* mRNA level was attenuated after electrical stimulation. This suggests the response in the whole muscle differs from the response in isolated myofibers. Since we used whole-body knockout mice, *atf3* expression and subsequent chemokine regulation could be affected in other cell types. Therefore, a cross-talk among the cells composing the whole muscle should exist, leading to the response measured at the tissue level. Cross-talk between muscle cells and immune cells has already been proposed in skeletal [10] and cardiac [4] muscles. Caution is needed however, because myotubes might not be totally representative of adult myofibers [23]. In any case, our data suggest ATF3 might regulate chemokine expression in a cell-specific manner.

We identified putative ATF3 binding sites in the promoter region of the chemokine genes. These sites were in close proximity to putative binding sites for the chemokine-regulating transcription factor NFκB. A previous study in C_2C_{12} myotubes demonstrated that

Table 1
Putative transcription factor (TF) binding sites on chemokine genes.

Gene	TF	Position relative to the TSS	Strand	Core score	Matrix score	Sequence[a]	Conservation[b]
ccl2	ATF3	−55 to −45	(−)	0.955	0.929	ccTGACTccac	Rat, ghinea pig, chimp, orangutan, rhesus, marmoset
	NFkB	−157 to −142	(+)	1.000	0.938	ttcctGGAAAcacccg	Rhesus, marmoset
ccl6	ATF3	−286 to −296	(+)	0.955	0.893	tgagAGTCAag	–
		−738 to −748	(−)	0.955	0.923	tgTGACTctct	Rat
	NFkB	−745 to −760	(+)	0.958	0.910	ctcttGGGAAgtccat	Rat
ccl9	ATF3	−978 to −988	(−)	0.955	0.944	caTGACTtagt	–
	NFkB	−25 to −40	(−)	1.000	0.950	tggggcTTTCCaaagt	Rat, rabbit, chimp, rhesus, horse, marmoset
		−840 to −853	(−)	0.962	0.893	agggaggATCCCct	–
cxcl1	ATF3	−219 to −209	(+)	1.000	0.893	cagcCGTCAct	Rat
		−682 to −672	(+)	0.955	0.854	ccttAGTCAaa	Rat
	NFkB	−72 to −57	(−)	1.000	1.000	cgggaaTTTCCctggc	Tenrec, rat, cow, horse, hedgehog, shrew, chimp, orangutan, rhesus, marmoset
		−73 to −60	(+)	1.000	0.964	ccGGGAAtttccct	Tenrec, rat, cow, horse, hedgehog, shrew, chimp, orangutan, rhesus, marmoset
		−73 to −60	(−)	1.000	0.940	cgggaatTTCCCtg	Tenrec, rat, cow, horse, hedgehog, shrew, chimp, orangutan, rhesus, marmoset
		−72 to −61	(+)	0.973	0.972	cGGGAAtttccc	Tenrec, rat, cow, horse, hedgehog, shrew, chimp, orangutan, rhesus, marmoset
		−72 to −61	(−)	0.973	0.876	gggaatTTCCCt	Tenrec, rat, cow, horse, hedgehog, shrew, chimp, orangutan, rhesus, marmoset
		−691 to −676	(−)	1.000	0.927	gtggacTTTCCttagt	Rat
		−604 to −591	(+)	1.000	0.938	tcGGGAAgttccca	Rat, dog
		−604 to −591	(−)	1.000	0.947	cgggaagTTCCCaa	Rat, dog
		−604 to −593	(+)	0.973	0.868	cGGGAAgttccc	Rat, dog
		−604 to −593	(−)	0.973	0.868	gggaagTTCCCa	Rat, dog

[a] The core sequence is in capital letters.
[b] The binding site is conserved if it localizes in the same relative position as for the mouse, and fulfills the stringency levels. TSS, transcription start site.

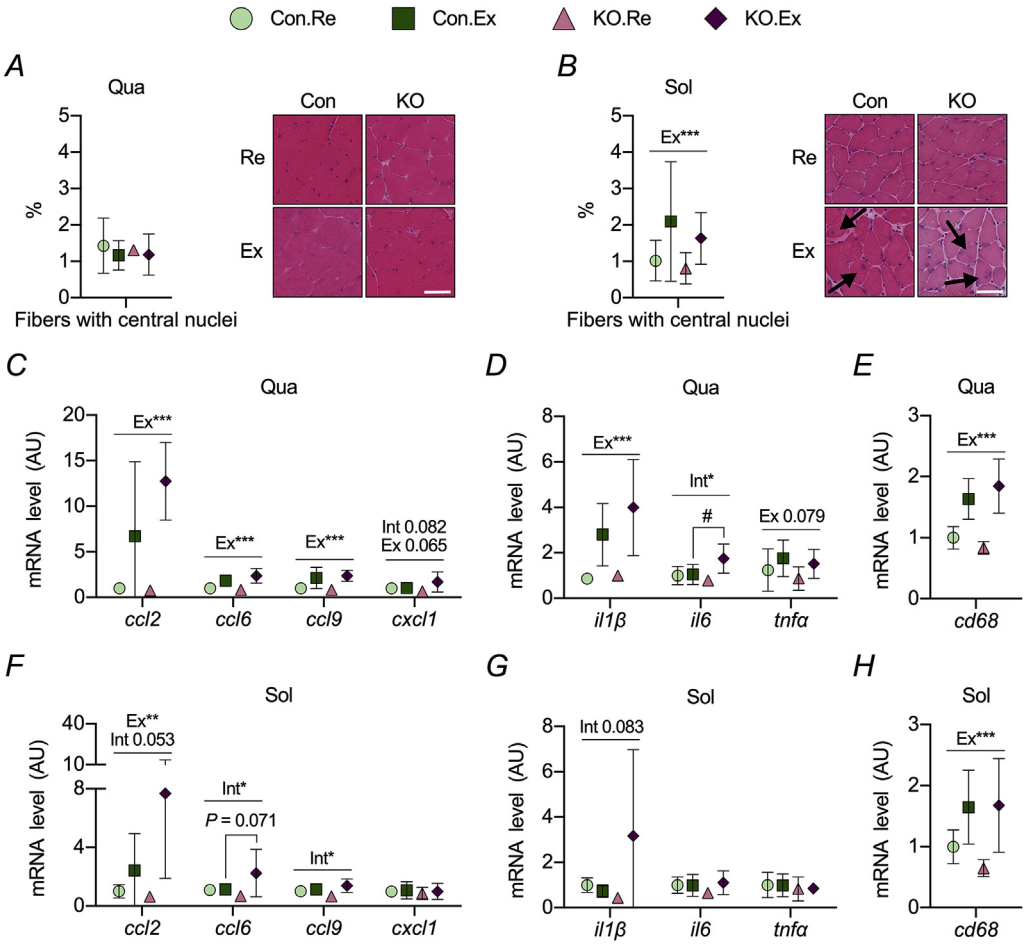

Fig. 2. Effects of a single bout of eccentric exercise on structural damage and inflammatory markers in skeletal muscle.
ATF3-KO (KO) and control (Con) mice exercised (Ex) or remained at rest (Re). *A-B*, percentage of myofibers with central nuclei in Qua (*A*) and Sol (*B*). Representative images are shown (arrows indicate affected fibers; bar, 50 μm). *C-E*, mRNA of chemokines (*C*), cytokines (*D*) and *cd68* (*E*) in Qua. *F-H*, mRNA of chemokines (*F*), cytokines (*G*) and *cd68* (*H*) in Sol. The error bars are not shown if they are smaller than the symbol. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for the effect of exercise (Ex), genotype (Ge), or interaction exercise × genotype (Int); $n = 4–6$. $^{\#}P < 0.05$ for *post-hoc* test.

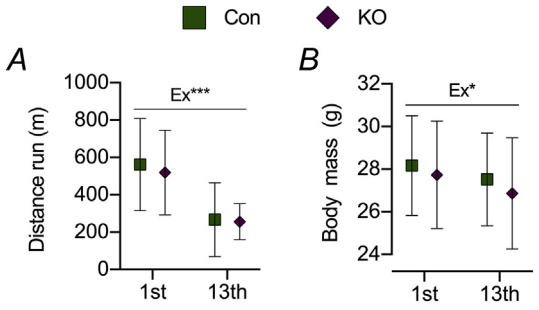

Fig. 3. Effects of repeated bouts of exhaustive eccentric exercise on exercise performance and body mass.
ATF3-KO (KO) and control (Con) mice exercised once a day for 13 consecutive days. Maximal distance run (*A*) and body mass (*B*) were compared the first (1st) and last (13th) days. $^{*}P < 0.05$, and $^{***}P < 0.001$ for the effect of exercise (Ex); $n = 6$.

NFκB activation was necessary for the up-regulation of *ccl2* expression upon EPS [15]. Here, we demonstrate that ATF3 is also necessary. This suggests ATF3 and NFκB might interact to induce *ccl2* expression upon EPS, and this could also be the case for other chemokines. Future studies should analyze how ATF3 and NFκB interact to regulate chemokine expression upon muscle contraction.

Reports in rodents have shown muscle damage, elevated inflammatory markers, and leukocyte infiltration, 24–48 h after downhill running [18,24,25]. We therefore performed our analyses 28 h post-exercise. In soleus, eccentric exercise increased the percentage of fibers with central nuclei, indicating regeneration post-damage. No such effect was detected in quadriceps. However, markers of inflammation and macrophage infiltration increased in exercised quadriceps and soleus. These findings suggest our protocol was an adequate stimulus for both muscles, but soleus was more affected. Previous reports have also shown that slow-twitch muscles, like soleus, are more affected by eccentric exercise [19,25]. Importantly, there was no difference in the muscle damage between control and ATF3-KO mice.

We have previously demonstrated that ATF3 attenuates chemokine/cytokine expression in skeletal muscle after non-damaging exercise [7]. Here, we used exhaustive eccentric exercise, since this exercise modality damages muscles and up-regulates inflammatory chemokines/cytokines [11]. In quadriceps, *il6* mRNA content increased post-exercise only in ATF3-KO, and a trend was observed for *cxcl1*. In soleus, we observed a more marked up-regulation of *ccl6* and *ccl9* mRNA in ATF3-KO post-exercise. Trends for the same response were found for *ccl2* and *il1β*. Therefore, our data demonstrate that ATF3 attenuates the expression of inflammation-related genes in skeletal muscle also upon muscle-damaging exercise. This effect is more pronounced in soleus than quadriceps, agreeing with our previous report [7]. The different fiber type composition [26], recruitment during exercise [27], and/or level of damage post-eccentric exercise (Fig. 2*A*, *B*), might explain these findings.

In our previous report, we showed that non-damaging exercise associated with signals of macrophage infiltration in soleus of ATF3-KO mice [7]. This result was surprising, since only minor leukocyte infiltration is expected upon non-damaging exercise. Here, we used downhill running to test whether in an exercise known to provoke leukocyte infiltration, ATF3 would also play a role. Downhill running associated with signals of macrophage infiltration, agreeing with previous reports [18,24,25]. Notably, control and ATF3-KO mice responded similarly. This suggests that in the context of exercise-induced muscle damage, ATF3-independent mechanisms regulate macrophage infiltration. Indeed, *atf3* mRNA level had returned to resting levels 28 h after eccentric exercise in our control mice (not shown). The response to muscle damage probably involves many cells and signals that finely tune the infiltration of macrophages and other leukocytes [10]. Future studies using flow cytometry will be useful to precisely determine the role of ATF3 on leukocyte infiltration.

Downhill running is an effective model of overtraining in rodents, decreasing exercise performance and altering body mass [28]. Since ATF3-KO mice had a higher inflammation upon eccentric exercise, we tested whether they responded worse to repeated eccentric sessions. Repeated eccentric exercise decreased performance and body mass similarly in control and ATF3-KO mice. Considering this result, we decided not to analyze molecular markers in the skeletal muscle of these mice. Our data suggest ATF3 does not influence the response to eccentric overtraining, at least regarding changes in performance and body mass.

In conclusion, ATF3 attenuates the expression of inflammation-related genes in skeletal muscle upon a single bout of muscle-damaging exercise. This molecular effect seems not to influence macrophage infiltration or exercise performance. Future work should study the physiological implications of the gene regulation induced by ATF3. Notably, our data suggest ATF3 actions at the whole muscle level depend on a cross-talk between different cell types. These results open new perspectives in the study of exercise, inflammation, and ATF3.

Conflict of interest

The authors declare no conflicts of interest.

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References

[1] T. Hai, C.C. Wolford, Y.S. Chang, ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component? Gene Expr. 15 (2010) 1–11, http://dx.doi.org/10.3727/105221610X12819686555015.
[2] H. Zhou, H. Guo, J. Zong, J. Dai, Y. Yuan, Z.Y. Bian, et al., ATF3 regulates multiple targets and may play a dual role in cardiac hypertrophy and injury, Int. J. Cardiol. 174 (2014) 838–839, http://dx.doi.org/10.1016/j.ijcard.2014.04.160.
[3] C.M. Calton, L.K. Wade, M. So, Upregulation of ATF3 inhibits expression of the pro-inflammatory cytokine IL-6 during Neisseria gonorrhoeae infection, Cell Microbiol. 15 (2013) 1837–1850, http://dx.doi.org/10.1111/cmi.12153.
[4] L. Koren, D. Alishekevitz, O. Elhanani, A. Nevelsky, T. Hai, I. Kehat, et al., ATF3-dependent cross-talk between cardiomyocytes and macrophages promotes cardiac maladaptive remodeling, Int. J. Cardiol. 198 (2015) 232–240, http://dx.doi.org/10.1016/j.ijcard.2015.06.099.
[5] C.T. Nguyen, E.H. Kim, T.T. Luong, S. Pyo, D.K. Rhee, ATF3 confers resistance to pneumococcal infection through positive regulation of cytokine production, J. Infect. Dis. 210 (2014) 1745–1754, http://dx.doi.org/10.1093/infdis/jiu352.
[6] M. McKenzie, A. Goldfarb, Aerobic exercise bout effects on gene transcription in the rat soleus, Med. Sci. Sports Exerc 39 (2007) 1515–1521, http://dx.doi.org/10.1249/mss.0b013e318074c256.
[7] R. Fernández-Verdejo, A.M. Vanwynsberghe, A. Essaghir, J.B. Demoulin, T. Hai, L. Deldicque, et al., Activating transcription factor 3 attenuates chemokine and cytokine expression in mouse skeletal muscle after exercise and facilitates molecular adaptation to endurance training, FASEB J. 31 (2017) 840–851.

[8] J. Rodriguez, R. Fernández-Verdejo, N. Pierre, F. Priem, M. Francaux, Endurance training attenuates catabolic signals induced by TNF-α in muscle of mice, Med. Sci. Sport Exerc 48 (2015) 227–234, http://dx.doi.org/10.1249/MSS.0000000000000756.
[9] J. Porter, W. Guo, A. Merriam, S. Khanna, G. Cheng, X. Zhou, et al., Persistent over-expression of specific CC class chemokines correlates with macrophage and T-cell recruitment in mdx skeletal muscle, Neuromuscul. Disord. 13 (2003) 223–235.
[10] N.J. Pillon, P.J. Bilan, L.N. Fink, A. Klip, Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications, Am. J. Physiol. Endocrinol. Metab. 304 (2013) E453–E465, http://dx.doi.org/10.1152/ajpendo.00553.2012.
[11] G. Paulsen, U.R. Mikkelsen, T. Raastad, J.M. Peake, Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? Exerc Immunol. Rev. 18 (2012) 42–97.
[12] L.G. MacNeil, S. Melov, A.E. Hubbard, S.K. Baker, M.A. Tarnopolsky, Eccentric exercise activates novel transcriptional regulation of hypertrophic signaling pathways not affected by hormone changes, PLoS One 5 (2010) e10695, http://dx.doi.org/10.1371/journal.pone.0010695.
[13] G.L. Warren, T. Hulderman, D. Mishra, X. Gao, L. Millecchia, L. O'Farrell, et al., Chemokine receptor CCR2 involvement in skeletal muscle regeneration, FASEB J. 19 (2005) 413–415, http://dx.doi.org/10.1096/fj.04-2421fje.
[14] N. Pierre, R. Fernández-Verdejo, P. Regnier, S. Vanmechelen, B. Demeulder, M. Francaux, IRE1α and TRB3 do not contribute to the disruption of proximal insulin signaling caused by palmitate in C2C12 myotubes, Cell Biol. Int. 40 (2016) 91–99, http://dx.doi.org/10.1002/cbin.10542.
[15] S. Miyatake, P.J. Bilan, N.J. Pillon, A. Klip, Contracting C2C12 myotubes release CCL2 in an NF-κB-dependent manner to induce monocyte chemoattraction, Am. J. Physiol. Endocrinol. Metab. 310p (2015) E160–E170, http://dx.doi.org/10.1152/ajpendo.00325.2015.
[16] L. Kreft, A. Soete, P. Hulpiau, A. Botzki, Y. Saeys, P. De Bleser, ConTra v3: a tool to identify transcription factor binding sites across species, update 2017, Nucleic Acids Res. (2017), http://dx.doi.org/10.1093/nar/gkx376.
[17] Y. Xia, M. Pauza, L. Feng, D. Lo, RelB regulation of chemokine expression modulates local inflammation, Am. J. Pathol. 151 (1997) 375–387. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1858005/pdf/amjpathol00020-0062.pdf.
[18] S.K. Tsivitse, T.J. McLoughlin, J.M. Peterson, E. Mylona, S.J. McGregor, F.X. Pizza, Downhill running in rats: influence on neutrophils, macrophages, and MyoD+ cells in skeletal muscle, Eur. J. Appl. Physiol. 90 (2003) 633–638, http://dx.doi.org/10.1007/s00421-003-0909-0.
[19] H.K. Smith, M.J. Plyley, C.D. Rodgers, N.H. McKee, Skeletal muscle damage in the rat hindlimb following single or repeated daily bouts of downhill exercise, Int. J. Sports Med. 18 (1997) 94–100, http://dx.doi.org/10.1055/s-2007-972602.
[20] D.C. Hoaglin, B. Iglewicz, Fine tuning some resistant rules for outlier labeling, J. Am. Stat. Assoc. 82 (1987) 1147–1149, http://dx.doi.org/10.2307/2289392.
[21] W.A. LaFramboise, R.C. Jayaraman, K.L. Bombach, D.P. Ankrapp, J.M. Krill-Burger, C.M. Sciulli, et al., Acute molecular response of mouse hindlimb muscles to chronic stimulation, Am. J. Physiol. Cell Physiol. 297 (2009) C556–C570, http://dx.doi.org/10.1152/ajpcell.00046.2009.
[22] I.A. Barash, L. Mathew, A.F. Ryan, J. Chen, R.L. Lieber, Rapid muscle-specific gene expression changes after a single bout of eccentric contractions in the mouse, Am. J. Physiol. Cell Physiol. 286 (2004) C355–C364, http://dx.doi.org/10.1152/ajpcell.00211.2003.
[23] G. Ravenscroft, K.J. Nowak, C. Jackaman, S. Clément, M.A. Lyons, S. Gallagher, et al., Dissociated flexor digitorum brevis myofiber culture system - a more mature muscle culture system, Cell Motil. Cytoskelet. 64 (2007) 727–738, http://dx.doi.org/10.1002/cm.20223.
[24] N. Kawanishi, K. Kato, M. Takahashi, T. Mizokami, Y. Otsuka, A. Imaizumi, et al., Curcumin attenuates oxidative stress following downhill running-induced muscle damage, Biochem. Biophys. Res. Commun. 441 (2013) 573–578, http://dx.doi.org/10.1016/j.bbrc.2013.10.119.
[25] R. Armstrong, R. Ogilvie, J. Schwane, Eccentric exercise-induced injury to rat skeletal muscle, J. Appl. Physiol. 54 (1983) 80–93.
[26] D. Bloemberg, J. Quadrilatero, Rapid determination of Myosin Heavy Chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis, PLoS One 7 (2012) e35273, http://dx.doi.org/10.1371/journal.pone.0035273.
[27] R.R. Roy, D.L. Hutchison, D.J. Pierotti, J.A. Hodgson, V.R. Edgerton, EMG patterns of rat ankle extensors and flexors during treadmill locomotion and swimming, J. Appl. Physiol. 70 (1991) 2522–2529.
[28] B.C. Pereira, A.L. Da Rocha, A.P. Pinto, J.R. Pauli, C.T. De Souza, D.E. Cintra, et al., Excessive eccentric exercise-induced overtraining model leads to endoplasmic reticulum stress in mice skeletal muscles, Life Sci. 145 (2016) 144–151, http://dx.doi.org/10.1016/j.lfs.2015.12.037.