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Oxidative hotspots on actin promote skeletal muscle weakness in rheumatoid arthritis

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Abstract

Skeletal muscle weakness in patients suffering from rheumatoid arthritis (RA) adds to their impaired working abilities and reduced quality of life. However, little molecular insight is available on muscle weakness associated with RA. Oxidative stress has been implicated in the disease pathogenesis of RA. Here we show that oxidative post-translational modifications of the contractile machinery targeted to actin result in impaired actin polymerization and reduced force production. Using mass spectrometry, we identified the actin residues targeted by oxidative 3-nitrotyrosine (3-NT) or malondialdehyde adduct (MDA) modifications in weakened skeletal muscle from mice with arthritis and patients afflicted by RA. The residues were primarily located to three distinct regions positioned at matching surface areas of the skeletal muscle actin molecule from arthritis mice and RA patients. Moreover, molecular dynamic simulations revealed that these areas, here coined “hotspots”, are important for the stability of the actin molecule and its capacity to generate filaments and interact with myosin. Together, these data demonstrate how oxidative modifications on actin promote muscle weakness in RA patients and provide novel leads for targeted therapeutic treatment to improve muscle function.

Introduction

Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory diseases. Skeletal muscle weakness is a frequent complication in patients with RA; it has debilitating consequences on the ability to work and leads to reduced quality of life for the afflicted individuals (1-3). Altered intrinsic muscle function, including a reduction in muscle strength without corresponding loss of muscle mass has been reported in RA patients (2, 4-6). However, little molecular insight is available on RA-induced muscle weakness.

Oxidative stress arises from a disproportionate amount of reactive oxygen/nitrogen species (ROS/RNS), e.g. peroxynitrite (ONOO⁻) that has been implicated in the pathogenesis of RA (7-12). ONOO⁻ can induce oxidative post-translational protein modifications by nitrating tyrosine residues (13) and facilitate the formation of malondialdehydes adducts (MDA) on basic amino acids, such as histidine (13, 14). Increased level of 3-nitrated tyrosines (3-NT) and MDA adducts have been observed in rodent models with arthritis and in patients with RA (7, 12, 15-17). The ROS/RNS sources behind arthritis-induced oxidative stress are not fully established...
in skeletal muscle (18), but nitric oxide synthase (NOS), NADPH oxidases (NOX) and mitochondria are suggested downstream targets of pro-inflammatory cytokines in inflammatory processes (18-21). Skeletal muscle is composed of bundles of muscle fibers and each fiber consists of thousands of myofibrils that feature the sarcomeres with the actomyosin contractile machinery (18). Previous work in rodents indicate that arthritis-induced muscle weakness is associated with higher levels of oxidative modifications on actin (15, 16, 18, 22). However, details of how oxidative modifications of actin interfere with the force producing machinery have remained elusive.

Here we show that oxidative post-translational modifications of skeletal muscle actin (α-actin) and myofibrils lead to impaired actin polymerization and decreased force production. Furthermore, using mass spectrometry, we identified the actin 3-NT-modified residues as well as those carrying MDA in weakened skeletal muscle from mice with arthritis and from RA patients. These residues were predominantly located in three regions of the actin molecule, and remarkably enough the same regions were recognized in the samples from both the arthritis mice and RA-patients. Polymerization studies and molecular dynamic simulations were carried out to analyze functional consequences and provide atomistic details of actin after oxidative stress modifications in these regions. This demonstrated the importance of these regions for actin intra- and inter-domain bonding and myosin interaction, and provides a molecular explanation to the observations made in arthritis mice and RA patients. Consequently, these regions should be useful starting points for the design of novel specifically targeted therapeutics to treat RA-associated muscle weakness.

**Results**

**Muscle weakness in mice with arthritis**

To evoke arthritis, unilateral injections of complete Freund's adjuvant (CFA) into the knee or ankle of mice were used (Fig. 1a). In line with other arthritis models, increased levels of oxidative nitration markers (3-NT) were observed on muscle proteins in adjacent muscle (Fig. 1b-c interossseous, Fig. 1d-e gastrocnemius) at peak inflammation, i.e. two weeks post CFA injections with an ankle or knee diameter twice as large as the control leg (Supplementary Fig 1a-b). As compared with the control leg (Ctrl), the arthritic leg developed muscle weakness with both extensor digitorum longus (EDL, Supplementary Fig 1c-d.) and single muscle fibers from flexor digitorum brevis (FDB) producing lower ex vivo muscle-specific force (i.e. force per cross-sectional area) (Fig. 1f). There was no difference in the cross-sectional area between
the non-arthritic and arthritic EDL or FDB muscle and the inflammation did not affect total
body weight (Fig. 1g, Supplementary Fig. 1c-f). Furthermore, the non-arthritic leg showed
similar force production as muscle from untreated wild-type mice, hence the induced arthritis
does not interfere with contralateral force production (Supplementary Fig. 1c).

Ca^{2+} release from the sarcoplasmic reticulum (SR) enables contraction by uncovering active
sites on actin for myosin binding (23). Thus, the higher the intracellular free Ca^{2+} concentration
([Ca^{2+}]_i), the greater the force generated until saturation of [Ca^{2+}]_i and all actomyosin
interactions are activated. However, at the stimulation frequencies used, no difference in tetanic
[Ca^{2+}], or Ca50 ([Ca^{2+}], at 50\% Pmax, 0.8±0.2 vs 0.7±0.2 μM, n.s.) was found between arthritis-
induced and Ctrl muscle fibers (Fig. 1h-i). This indicates that SR Ca^{2+} release and myofibrillar
Ca^{2+} sensitivity are functioning normally in fibers from arthritis muscles.

Caffeine treatment [5 mM] and high stimulation frequency (120 Hz) effectively release all
Ca^{2+} stored in the SR and tetanic [Ca^{2+}], will reach a level high enough to maximally activate
the contractile machinery (24). Indeed, the presence of caffeine at 120 Hz stimulation
potentiated SR Ca^{2+} release (Fig. 1j), but had no effect on the force production of either control
fibers or the CFA-treated fibers (Fig. 1k). Caffeine enhanced SR Ca^{2+} release in control muscle
fibers did not have any notable effect on force production because the tetanic force prior to
caffeine application is close to maximal. That the lower force in CFA-treated muscle was not
improved by caffeine exposure shows that the weakness is the result of impaired myofibrillar
function, rather than impaired SR Ca^{2+} release or reduced SR Ca^{2+} storage. Thus, CFA-induced
muscle weakness appears to be the result of defects in the contractile machinery.

Declined myofibrillar force and compromised filament formation

Because muscle weakness is associated with oxidative stress (15, 16, 18, 22, 25-27), we
investigated whether inducing oxidative modifications of individual myofibrils (Fig. 2a)
interfered with force production. Treatment with 5-amino-3-(4-morpholinyl)-1,2,3-
oxadiazolium chloride (SIN-1), an NO and O_2•• donor which forms ONOO^- (28), resulted in
markedly weaker myofibrils than untreated myofibrils (Fig. 2b-c), as measured with atomic
force cantilever (16). To avoid formation of nitrosylated cysteine residues, dithiothreitol (DTT)
was present during these measurements, and consequently the SIN-1 treated myofibrils showed
increased levels of oxidative nitration (3-NT) due to the presence of ONOO^- (Fig. 2d).
A major component of myofibrils is filamentous actin (F-actin) (29), which is formed by polymerization of monomeric actin (G-actin). The polymerization process results in formation of asymmetric helical actin filaments, each characterized by a fast (barbed) and slow (pointed) polymerizing end (30) and extensive structural cooperativities coupled to ATP hydrolysis and interaction with filament binding proteins (30-33). Net assembly of ATP-bound G-actin occurs at barbed ends and is followed by nucleotide hydrolysis and subsequent structural transitions of the newly added subunits (33). At steady-state condition, net dissociation of the ADP-carrying monomers occurs from the pointed end. Consequently, the filament is polymorphic, structurally representing an ensemble of different states (34) and with each actin subunit in contact with four other subunits, the structure is highly combinatorial and susceptible to protein modification and various molecular interactions.

To investigate whether oxidative modification of monomeric actin by SIN-1 affects the polymerization process, monomeric actin was pre-incubated with SIN-1 and polymerization was then initiated by addition of salt (MgCl$_2$ + KCl) under standardized conditions in the presence of DTT (Supplementary Fig. 2a-b). We found the rate of polymerization of the oxidatively modified actin to be significantly slower than that of unmodified actin (Fig. 2e-f), which is in agreement with previous reports (35, 36). Moreover, the effect of SIN-1 was dose-dependent as demonstrated by gradual reduction of both filament formation rate and final steady-state polymerization level (Fig. 2g-h). Hence, the polymerization capacity of the modified actin was drastically compromised by the SIN-1 treatment. Prolonged incubation (48 hours) at room temperature after initiation of polymerization led to extensive decline in the steady-state level of filamentous actin of samples exposed to SIN-1 compared to control samples (31% and 7% respectively). This suggests that ADP-actin dissociating from the filament pointed end under these conditions exhibit impaired capacity to undergo nucleotide exchange and be recharged with ATP for another round of polymerization, and/or filaments assembled from modified actin were inherently more unstable than filaments consisting of unmodified subunits.

The oxidative modifications target three distinct regions at the actin molecule

To explore how oxidative modifications of myofibrillar actin and of the isolated protein cause decreased force production and impaired polymerization, mass spectrometry was used to identify the location and modification of the altered amino acid residues. This approach
identified two variants of oxidative alterations, 3-NT (-NO₂; +46 Da) and MDA (-C₃H₅O; +54 Da) consistently occurring in myofibril actin, the purified protein and in skeletal muscle actin from CFA-treated mice (Supplementary Table 1). Important in this context is that the amino acid sequence of α-actin from mice, rabbits and humans is identical (Supplementary Table 2). The positions of the 3-NT and MDA modified amino acids in the crystal structure of ATP-bound actin (Fig. 3a) are visualized in Fig. 3b-d and all modifications of the three different samples are listed in (Supplementary Table 1).

Remarkably, a majority of the possible MDA and 3-NT modifications in α-actin from CFA mice and the SIN-1 treated purified protein are either localized to identical positions or in close proximity of each other. The localization similarity suggests that the conserved actin structure exposes distinct regions sensitive to oxidative stress (Fig. 4a-c). The first such hotspot is located in subdomain (SD)-1, where residues H101, Q360 and Y362 underwent -MDA, -MDA, and -3NT modifications, respectively (Fig. 4a). The second region found to be targeted in all actin samples resides in SD2 (Fig. 4b) where H40, Q41 and Y53 went through -MDA, -MDA, and -3NT alterations, respectively, which all are likely to impact on the flexing of the polypeptide chain proposed to occur upon ATP-hydrolysis (37) and actin polymerization (38). Moreover, H40 and Q41 are located in the so called DNase-binding loop (D-loop) which comprises residues 38-53 and plays a critical role in longitudinal inter-subunit contacts in the filament (30, 38) and partake in the interaction with myosin (39). In congruence with these observations, residue Y53 has previously been shown to be accessible for nitration in vitro by ONOO⁻ (40), and post-translational modification by phosphorylation of this residue is known to cause slower polymerization and impaired filament stability (41-43).

The third hotspot is localized to Y294, N296, N297 (modified: -3NT, -MDA, -MDA) in SD3 (Fig.4c). This is the exact location and kind of oxidative modifications (3-NT and MDA) we found in α-actin from mice with CFA-generated arthritis and SIN-1 treated myofibrillar actin. In addition, the SIN-1-treated monomeric actin showed modifications in this site (Y294/-3NT and N297/-MDA), again pointing to the conserved nature of the actin molecule and its susceptibility to oxidative modification to certain specific regions irrespective of the species origin or preparation of the protein. We conclude that the α-actin molecule has three oxidative hotspots formed by a few clustered amino acid residues accessible for MDA and -3NT modification.
The non-muscle β/γ-actin isoforms are also expressed in skeletal muscle, but generally not involved in the contractile machinery (44-46). Prior to MS-analysis, actin was trypsin digested which created eleven unique peptides when comparing skeletal muscle α-actin (ACTS) and β/γ-actin (ACTB/G). Thus, trypsin digestion enables identification of modifications specific for β/γ-actin in skeletal muscle from CFA mice. Eight modified residues were identified and six of them were located in peptides unique for β/γ-actin (Supplementary Table 3). MS-analysis of non-muscle β/γ-actin from the knee joint of CFA mice was also performed. Six modifications (SD1: H87/MDA, Q360/MDA*, Y362/3NT* and in SD3: Y218/3NT, Y294/3NT* and N296/MDA*) were identified. Two of them were exclusive for β/γ-actin and four of them (*) matched modified residues found in α-actin (Supplementary Table 3). This shows that 3NT and MDA modifications are not stringent to α-actin in skeletal muscle from mice with arthritis, but also are present in β/γ-actin in both muscle and non-muscle tissue.

**Human RA patients exhibit muscle weakness and matching oxidative hotspots on actin**

We next investigated if the findings from mice with arthritis were translational to human patients with RA. Female patients with seropositive RA on stable disease-modifying antirheumatic drug (DMARD) treatment without or in combination with stable low-dose glucocorticoids and displaying a moderate disease activity (average disease activity score (DAS) of 3.3±0.4; n=11, 53.3±1.5 years old, 8.9±0.4 years disease duration, Fig. 5a, Supplementary Table 4) had significantly weaker leg muscles than age- and weight-matched healthy women (Fig. 5b).

The quadriceps cross-sectional area (CSA) of patients with RA was not significantly different to the quadriceps CSA of healthy individuals (Fig. 5c). Further, there were no significant differences in total amount of daily physical activity or the degree of physical intensity (47) performed between patients with RA and healthy control individuals (Fig. 5d-e). Thus, the force decline cannot be explained by a decrease in muscle mass or general muscle inactivity. Instead, the data suggests that the muscle weakness exhibited in RA patients is the result of arthritis-induced intrinsic muscle dysfunction, as observed in mice with arthritis (Fig. 1f).

Muscle biopsies from RA patients were analyzed with mass spectrometry to identify oxidative modifications and revealed eleven consistent oxidative modifications on actin in total (in SD1: H87/MDA; H101/MDA; Q360/MDA, in SD2: H40/MDA; Q41/MDA; Q59/MDA, in SD3: H275/MDA; Y294/3NT; N297/MDA, and in SD4: Y218/3NT; Q246/MDA. (Fig. 5f)).
Intriguingly, these modifications were located at matching hotspots, and in some cases even at identical residues in α-actin from mice with arthritis (Fig. 5g-i). Specifically, in hotspot 1: in SD1, H101/MDA and Q360/MDA, in hotspot 2: SD2, H40/MDA and in hotspot 3: in SD3, Y294/3NT and N297/MDA were found at precisely the same location in α-actin from mice with arthritis and patients with RA (Fig. 5g-i, red ribbons). The “conserved” location of these α-actin hotspots for oxidative modification reflects the highly conserved nature of all vertebrate actin and illustrates the strength of the mouse model as used above for understanding molecular mechanisms behind human diseases where conserved proteins play a crucial role for the pathogenesis.

Residues important for actin filament formation, stability and myosin interaction are 3-NT and MDA modified in RA patients

Molecular dynamic simulations (48) of F-actin in ADP-form were carried out to reveal structure details of the polymer at atomic level, particularly with respect to the modified residues identified from patients with RA. Root mean square fluctuations (RMSF) assesses flexibility of the different domains (Fig. 6a); SD1 and SD3 had limited flexibility (except for the residues at the N-terminus); the flexible D-loop region in SD2 showed a large RMSF and holds the second hotspot including the MDA modified H40 and Q41; SD4 had two regions of higher RMSF, including Q246/MDA. The other modified residues were located in less dynamic regions. The simulations of solvent-accessible surface area (SASA) suggest solvent exposure of MDA-modified H40, Q43, Q246 and Q360 (Fig. 6b), in accordance with those found to be MDA-modified and identified by mass spectrometry of biopsies from RA patients. Cryo-electron microscopy analysis have highlighted these residues as important for intramolecular bonding (interactions within the actin monomer), filament inter-subunit interactions and for actomyosin interactions during the power stroke and coupled force production (30, 33, 39, 49). Specifically, H40 and Q41 in the D-loop of SD2 are involved in longitudinal bonding with residues in SD1 and SD3 in the adjacent subunit towards the pointed end of the filament, and lateral interactions with SD3 in the neighboring subunit across the filament axes (30, 39). Additionally, residue Q246 in SD4 interacts with residues in the adjacent subunit (SD3) at its pointed end, further contributing to filament stability (Fig. 6c).

The MDA modified H275 and N297, and the Y294/3NT are all proximal to a tight electrostatic network with the D-loop in the adjacent subunit by H173, D286 and E270 (33). Thus,
modification of one or more of these residues could interfere with longitudinal intramolecular bonding. In contrast to the residues discussed above, H275, N297, and Y294 that are all in the less dynamic SD3, were found to be buried throughout the whole simulations (Fig. 6b). The biochemistry that makes these residues accessible to chemical modifications despite not being annotated as solvent-accessible remains elusive. However, the local physico-chemical microenvironment in the molecule, including the capacity to propagate tyrosyl radical intermediates and lipid peroxyl radicals is known to influence the susceptibility of residues to modifications, and hence buried tyrosines can be accessible independently of SASA (13).

Finally, examination of close contacts (hydrogen and non-hydrogen bonds) was performed to further expose what functional effects MDA and 3-NT modifications might impose on the rest of the protein. It was found that H101, H275 and Y218 would lose hydrogen bonds with drastic consequences for their interaction with nearby residues (Fig. 6d). Furthermore, each of the eleven residues identified with oxidative modifications in skeletal muscle actin from patients with RA exhibits ~35-95 close contacts between non-hydrogen atoms separated by less than 4Å (Fig. 6e), and altering the local chemical environment by the bulky covalent MDA (C\textsubscript{3}H\textsubscript{3}O) or 3-NT (NO\textsubscript{2}) is likely to dramatically impede these contacts by creating local steric restrictions and triggering of conformational changes (Fig 6f-h). Together the above results demonstrate the presence of oxidative modifications of residues in skeletal muscle actin from patients with RA, which are critical to actin filament stability and myosin interaction, and therefore represent strong contributing causes to the muscle weakness from which these patients suffer.

Discussion

Impaired muscle capacity has a negative influence on the quality of life to RA patients and is a contributing factor to long-term sick-leaves associated with RA and therefore important also from a socioeconomic perspective (1-6, 50, 51). Here we show that oxidative stress-induced intrinsic muscle dysfunction is a major contributor to muscle weakness associated with RA and is specifically due to modifications of actin with impeding consequences for actin filament formation, stability and actomyosin force generation. The modified residues are found in discrete regions of the actin molecule, referred to as oxidative hotspots that provide novel molecular insights into how oxidative stress promotes skeletal muscle weakness in RA without loss of muscle mass or reduced overall physical activity in the patients.
With 3-NT and MDA oxidative modifications being non-enzymatic mechanisms, the selectivity for the targeted residues on actin *in vivo* is not evident (13, 52, 53). There is currently no consensus regarding the selectivity of oxidative modifications. However, the local microenvironment and the structural features of the protein are believed to affect the selectivity, i.e. residues located near charged amino acids or on loop structures appear more prone to become modified (13, 52, 54, 55), which harmonizes with our results. **Supplementary Fig. 3** shows that our hotspots are primarily located in loop regions and that the modifications were all closely located to charged amino acid residues, i.e. to Arginine (R), Lysine (K), Aspartic acid (D), Glutamic acid (E) and Histidine (H). Due to the short biological half-life of ROS/RNS (e.g. ~5-20 ms for ONOO⁻, ~1μs for O₂⁻*), the protein also needs to be in close vicinity of the subcellular sites of ROS/RNS generation for the modification to occur (52, 56). Indeed, NOX2 and the Mical flavoprotein monooxygenase has been shown to interact with actin and directly affect actin dynamics and cause instability of F-actin, respectively (57-59). These enzymes diffusely release ROS/RNS and the actin isoforms represent only one set of proteins that are modified by these ROS/RNS generators. Thus, the specificity and selectivity of these ROS/RNS sources for particular actin residues remain poorly understood.

In addition to actin, other proteins directly involved in skeletal muscle sarcomere function, such as myosin, tropomyosin and troponin might be targeted by oxidative stress that may interfere with muscle force production. Nevertheless, in line with previous reports (15, 16, 22, 60), our results emphasize actin as a prime target for intracellular oxidative stress modifications. This appears valid also for non-muscle actin with 3NT and MDA modifications found in joints from arthritis mice (**Supplementary Table 3**). Moreover, specific actin oxidation has been reported in brain regions from patients with Alzheimer’s disease, in skin biopsies from Friedrich’s ataxia patients (61, 62) and in spinal cords from an inflammatory mouse-model of pain (63). Moreover, the oxidative modifications identified here were consistently present on α-actin from RA patients on DMARD treatment in combination with or without low doses of glucocorticoids (see **Supplementary Table 4**). The patients in our study used conventional synthetic DMARDs (mostly methotrexate) with a broad immunosuppressive activity, and biological DMARDs that targets a specific protein structure with pro-inflammatory activity in the attempt to lower the rheumatic disease activity, e.g. rituximab (Mabthera), etanercept (Embrel), abatacept (Orencia), tocilizumab (RoActerma). This implies that muscle weakness is a comorbidity that cannot be counteracted by DMARDs and glucocorticoids alone and suggests that supplementary pharmacological treatment targeted to improve intrinsic muscle function is needed.
Currently there is a serious deficit in available clinical therapeutic approaches to counteract muscle weakness. However, the data presented here provide new leads for the development of such targeted treatments. It is well known that ROS/RNS have both beneficial (23, 64-66) and detrimental (25, 67-69) effects on skeletal muscle function. Antioxidant treatment of illnesses related to oxidative stress ought therefore to be directed towards the negatively affected protein functions, instead of having a broad scoped effect on ROS/RNS signaling as is common with antioxidant treatment today. Hence, the use of general antioxidants e.g. vitamin E, vitamin C and N-acetylcysteine (NAC) which will allocate to the intracellular space and interact with surrounding molecules in a nonspecific and uncontrolled manner is a likely reason to the failure of numerous clinical trials (70-72), including a recent attempt to reduce RA disease activity with NAC (73). Therefore, the aim should be to specifically target proteins identified as being negatively affected by the oxidative stress. In this regard, an SOD2/catalase mimetic (EUK-134) that supposedly targets the mitochondria has shown promising effects in the attempt to prevent muscle weakness in rats with arthritis (15).

Here, we have identified a limited set of oxidative hotspots in actin which seriously interfere with its function when undergone 3-NT and MDA modifications, two common markers of oxidative stress. Thus, to develop actin-targeted compounds with the capacity to reduce the local chemical environment, analogous to targeted synthetic DMARDs, which are designed to attack a particular molecular structure may be a way to counteract RA-induced muscle weakness. Notably, oxidative stress-induced loss of force production without a reduction in muscle mass has been reported in rodent models of heart failure and cancer, entailing that intrinsic muscle weakness might be a present comorbidity across several chronic diseases. Moreover, since actin is a highly conserved protein essential to all cells and targeted by oxidative stress in both muscle and non-muscle tissue, the development of an actin-directed drug has the potential to be of a wider use than to improve muscle function in patients suffering from muscle weakness.
Methods

Study Approval
All animal experiments complied with the Swedish Animal Welfare Act, the Swedish Welfare ordinance and applicable regulations and recommendations from the Swedish authorities. The study was approved by the Stockholm North Ethical Committee on Animal Experiments (N108/13 and N273/15). All human experiments were approved by the regional ethical review board in Stockholm (2014/516-31/2) and complied with the Declaration of Helsinki. Oral and written informed consent were obtained from all patients and control subjects prior to participation in the study.

Animals
In all studies, mice were handled and euthanized in accordance with approved institutional, national and international guidelines. All efforts were made to follow the Replacement, Refinement and Reduction guidelines. Arthritis was induced in female C57BL/6JRj (Janvier) mice by intra-articular injection of complete Freund’s adjuvant (CFA 10mg/ml, Chondrex) in the ankle (5μl) or in the knee joint (10μl). Injections were performed on animals anesthetized with isoflurane. Mice were housed at the local animal facility with a 12h light-dark cycle, and were provided with standard rodent chow and water ad libitum. Mice were monitored daily to ensure that a sufficient health and nutritional status was maintained. Knee and ankle joint diameter were measured with a caliper every other day. Mice were sacrificed 14 days post injection by cervical dislocation.

Patients with RA and healthy controls
Eleven women with seropositive RA diagnosis according to the criteria of the American College of Rheumatology (ACR, 1987) participated in the study. All patients were on stable anti-rheumatic treatment. RA-disease activity was determined by calculating DAS. Detailed information about all patients included in this study can be found in Supplementary Table 4. The control group were eleven age-matched (age range 45-62 years) women without inflammatory disease. All participants were subjected to Biodex force measurements and a CT-scan of the legs, and an accelerometer analysis of their daily activity. Biopsies of the quadriceps vastus lateralis muscle were obtained from a subset of the RA patients by the Bergström percutaneous needle biopsy technique (74).
**Ex vivo force measurements**

EDL muscles from both legs (arthritic and non-arthritic) were rapidly excised under the microscope after the sacrifice, as described previously (75). Muscles were kept in a Tyrode solution (temperature was set at 31°C) containing (in mM): 121 NaCl, 5 KCl, 1.8 CaCl₂, 0.4 NaH₂PO₄, 0.5 MgCl₂, 24 NaHCO₃, 0.1 EDTA, and 5.5 glucose, gassed with 95% O₂/5% CO₂ to achieve a bath pH of 7.4. The muscles were mounted between a force transducer and an adjustable holder (World Precision Instruments). Muscles were stimulated with supramaximal current pulses (0.5 ms duration; 150% of current required for maximum force response) via plate electrodes lying parallel to the muscles. Muscles were set to the length at which tetanic force was maximum (optimal length L₀) and the force-frequency relationship was determined using the following stimulus frequencies: 1, 20, 30, 40, 50, 70, 100, 120, and 150 Hz (300 msec tetanic duration). At least 1 min of recovery separated electrical stimulations. Electrically stimulated force production was expressed as absolute force (mN) and as specific force (kN/m²). Muscle cross-sectional area (CSA) was assessed by dividing muscle mass by the product of muscle length and muscle density (1.06 g/cm³).

Single intact flexor digitorium brevis (FDB) fibers were obtained by dissection and thereafter mounted in a chamber between a force transducer and an adjustable holder, as described previously (16). Fibers were superfused with Tyrode bubbled with 95% O₂/5% CO₂ to achieve a bath pH of 7.4. The fiber length was adjusted to obtain maximum tetanic force. The diameter of the fiber at this length was used to calculate the cross-sectional area. The fiber was stimulated with supramaximal electrical pulses (0.5 ms in duration) delivered via platinum electrodes placed along the long axis of the fiber. [Ca²⁺], was measured with the fluorescent Ca²⁺ indicator indo-1. The mean fluorescence of indo-1 at rest and during tetanic contractions was measured and converted to [Ca²⁺] using an intracellularly established calibration curve (76).

**Immunoblots**

Muscles (gastrocnemius, tibialis anterior (TA) and interosseous (IO)) were homogenized in buffer containing (in mM): 10 Tris-maleate, 100 KCl, 2 MgCl₂, 2 EGTA, 2 Na₂P₂O₇, 1 NaVO₄, 25 KF and protease inhibitor (Roche, 1 tablet/50ml). The lysates were cleared by 10 min centrifugation at 3300 rpm (4 °C). Equal amounts of proteins were separated by electrophoresis and transferred onto membranes (Immobilon-FL, Millipore). Membranes were incubated with primary antibody (anti-3NT ab52309, Abcam; anti-MDA ab2764-2, Abcam) and then washed and incubated with infrared-labelled secondary antibody (IRDye 680 and IRDye 800 LI-COR
biosciences). Immunoreactive bands were analyzed with LI-COR Odyssey Infrared Imaging System and normalized against total protein or actin.

**Myofibrillar force measured with atomic force cantilevers**

Myofibrils were isolated from rabbit psoas muscle as described previously (16, 77) and suspended in rigor buffer (10 mM Imidazole, 100 mM KCl, 2 mM MgCl$_2$, 1 mM EGTA, pH 7.2) with DTT (1 mM). Myofibrils were incubated with SIN-1 (10mM, 10 min) and transferred to the experimental chamber placed on the top of an inverted microscope equipped with a system of atomic force cantilevers (AFC). The chamber was washed with rigor buffer followed by relaxing buffer (pH 7, pCa$^{2+}$ 9 : 20 mM Imidazole, 7.2 mM EGTA, 13.74 µM CaCl$_2$, 5.4 mM MgCl$_2$, 68.7 mM KCl, 5.6 mM ATP, 14.4 mM creatine phosphate). An atomic force cantilever with a known stiffness was attached to one end of a myofibril and a glass needle to the other end of the myofibril acts as a force transducer. The length of the myofibril sarcomere was adjusted to 2.8 µm and contraction was induced by addition of activating buffer with pCa$^{2+}$ 4.5 (20 mM Imidazole, 7.2 mM EGTA, 7 mM CaCl$_2$, 5.4 mM MgCl$_2$, 52.3 mM KCl, 5.7 mM ATP, 14.4 mM creatine phosphate, pH 7). The deflection of the cantilever due to myofibrillar contraction was measured by shining a laser light upon the cantilever, which then was translated to force in kN/µm$^2$, as described previously (16, 77).

**Actin polymerization assay**

Actin polymerization was measured using the pyrenyl-assay (78). Monomeric actin (Cytoskeleton AKL99) in 5mM Tris- HCl pH 7.6 at RT, 0.1mM CaCl$_2$, 0.5mM ATP, 0.5mM DTT (G-buffer) was incubated with SIN-1 at indicated concentrations for 15 min at room temperature. Actin concentration was determined by absorbance measurement at 290 nm using $E= 0.63$ ml/mg x cm as described by Houk and Ue (79). 10 min prior to initiation of polymerization, EGTA and MgCl$_2$ were added to final concentrations of 0.2 mM and 50 µM, respectively, to replace actin-bound Ca$^{2+}$ for Mg$^{2+}$ (80). Polymerization was induced by addition of MgCl$_2$ and KCl to 1 and 100 mM, respectively (final concentrations). The assay was performed in 96-well plates with 8 µM actin in the presence of 4% pyrene-actin and in a final volume of 130 µl (accomplished by pre-addition of G-buffer). The process was followed by excitation at 365 nm and emission detection at 410 nm (Fluoroskan II, Labsystems). Steady-state fluorescence was measured 24h and 48h after polymerization initiation.
Mass spectrometry analysis

Mass spectrometry (MS) was performed on purified rabbit skeletal muscle actin (Cytoskeleton AKL99), isolated myofibrillar actin from rabbit psoas muscle (myofibrillar actin), actin from CFA mice and actin from patients with RA (human RA actin). Samples were digested by trypsin (overnight), cleaned on a C-18 HyperSep plate and dried in a speedvac centrifugal evaporator. The peptides were then separated by chromatography in a C18 column (Silica Tip 360µm OD, 75µm ID, New Objective) connected to the UltiMate™ 3000 RSLCnano chromatography system (Thermo Scientific). Ionized peptides by electrospray were injected into an Orbitrap QExactive Plus mass spectrometer (Thermo Fisher Scientific). The survey MS spectrum was acquired at the resolution of 60.000 in the range of m/z 200-2000.

MS/MS data was extracted with RawtoMGF software and analyzed using Mascot (Matrix Science, version2.5.1) and X!Tandem (thegpm.org; version CYCLONE 2010.12.01.1) software. Fragment ion mass tolerance and parent ion tolerance were set to 0.020 Da and 9.0 PPM, respectively. Variable modifications of amino acids were specified as tyr-nitration, cys-nitrosylation and MDA modifications, with fixed modifications formed by the sample preparation specified as cys-carbamidomethylations. Carbonylation (DNP) could not be detected as it requires a targeted approach with labeled reaction sites. Validation of the MS/MS based peptide and proteins identifications was performed in Scaffold (version Scaffold_4.4.5, Proteome Software Inc.) and protein probabilities were assigned by the protein prophet algorithm (81). All post-translational modifications (PTMs) were validate and localized by the Scaffold PTM software. The UCSF-Chimera program (73), version 1.1rc was used to visualize the PTMs identified with MS. The monomeric G-actin model in its ATP bound state was obtained from the 2ZWH (RSCS Protein Databank) protein crystal structure source 61 (37). All modifications are presented with their one-letter code and specifier. The homology between of the skeletal muscle actin sequences was confirmed with ClustalW (82) sequence alignment (EMBL-EBI).

Human accelerometer and force measurements

The daily activity of patients with RA and control subjects was recorded with accelerometers (GT, ActiGraph). Accelerometer data was collected from the subjects over seven consecutive days and analyzed with Actilife software. Total daily activity was calculated by dividing the total minutes of activity by the validated wearing period (e.g. >90 minutes of consecutive inactivity were classified as non-worn periods and were filtered out from data analysis by using
a previously defined algorithm) (81). The degree of activity was categorized as sedentary, light,
moderate and vigorous activity (47). A Biodex System 4 Pro™ (Biodex Medical Systems Inc.)
was used to measure isometric force from *quadriceps femoris* of RA patients and healthy
control persons. The subjects were carefully positioned in the Biodex chair and performed three
repetitions of maximal isometric contractions (at knee angle 120°). The specific force (kN/m²)
was calculated by converting the peak torque (Nm) into kN by using the define length of the
cantilever in which the leg was fixed, which was then divided by the cross sectional area (CSA)
of the quadriceps muscle as determined by a CT-scan of the leg. Image J was used to measure
CSA of the quadriceps at the intermediate of the femur bone.

Molecular dynamics simulations

The 3.3 Å resolution (PDB: 2ZWH) structure of rabbit skeletal muscle alpha F-actin with ADP
and Ca²⁺ was used as starting structure for the molecular dynamics (MD) simulations. This
structure includes all amino acid residues and has previously been used in MD-simulation
studies (83-85). Simulations and analysis were performed by the Gromacs simulation package
(86) version 5.1.2. The actin structure was solvated in a truncated dodecahedron box so that the
minimum distance between any protein atom and the edge of the box was 1.0 nm and the TIP3P
(87) water model was applied. 52 K⁺ and 39 Cl⁻ ions were added to neutralize the protein and
mimic an ionic strength of 0.15 M. The CHARMM36 force field (88) was applied for all atoms
except Ca²⁺, K⁺, and Cl⁻ for which force field parameters were taken from ref. (89-91),
respectively. Prior to the simulations, internal constraints were relaxed by energy minimization,
until the maximal force on individual atoms was smaller than 100 kJ mol⁻¹ nm⁻¹. The system
was equilibrated for 5 ns before data collection for analysis. During the MD runs, the LINCS
(92) algorithm was used to constrain the lengths of bonds. The temperature was kept constant
at 300 K by use of the velocity-rescaling thermostat (93) (τ_T = 0.1 ps). The pressure was coupled
to an external bath with the Parrinello-Rahman algorithm (94) (P_ref = 1 bar, τ_P = 1 ps). Van der
Waals forces were truncated at 1.0 nm with a plain cut-off and long-range dispersion
corrections were applied for energy and pressure. Long-range electrostatic forces were treated
using the particle mesh Ewald method (95, 96). Four replicates were run for 100 ns each.
Analysis of the simulations was carried out by automatic tools available in Gromacs. Error
estimate is a block averaging method and the error estimate is the variance between averages
of all blocks. Root mean square fluctuations (RMSF) of the Cα atoms were calculated as
averages of 2 ns blocks for the last 60 ns of the simulation time for the four 100 ns simulations.
**Statistical analysis**

The data are presented as mean ± SEM. For all statistical analysis the normal distribution was tested with a Shapiro-Wilk test and equal variance was tested with Brown-Forsythe test. In case of no equal variance a Mann-Whitney Rank sum test was applied. Differences among experimental groups were analyzed by two-tailed Student’s *t*-tests or analysis of variance (ANOVA) with appropriate post hoc and multiple comparison tests. *P* values less than 0.05 were considered significant (*P* < 0.05; **P** < 0.01; ***P*** < 0.001). All statistical analysis was performed with help of Sigma plot 13.0 (Systat Software) or Prism 7.0 (GraphPad Prism).

**Author contributions**

CIS, NB, RK, TY, BCGK, RF, PT, TG and JTL designed and conceptualized the research. MMS, MP, BA, KO, AJC, EA, ML, TRL, ER, KÄM, KS, SA, BCGK, and EK performed research. MMS, MP, AJC, EA, TRL, ER, PT, DR, RK, RF, TG and JTL analyzed data. MMS, KÄM, KS and CIS provided the animals. MMS, MP, AJC, EA, TRL, ER, PT, DR, RK, RF, TG and JTL performed statistical analyses and power calculations. MMS, RK and JTL wrote the original draft. All authors reviewed and edited the manuscript.

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Figure 1. Muscle weakness and accompanied oxidative modifications in skeletal muscle from mice with arthritis. (A) An illustration of the induction site of arthritis by complete freund’s adjuvant (CFA) (ankle: used for flexor digitorum brevis (FDB) muscle fiber force measurements, knee: used for extensor digitorum longus (EDL) whole muscle force measurements). Immunoblots and quantification of 3-nitrotyrosine (3-NT) in interosseous (B-C) and gastrocnemius (D-E) muscle from mice with arthritis (CFA) and healthy controls (Ctrl) (n=6). 3-NT levels were normalized to total protein coomassie stains. (F) Ex vivo specific force (contractions induced at 15-150 Hz, n=4-5) of intact individual muscle fibers from arthritis leg (pink) and healthy controls (blue). (G) Calculated cross-sectional area of FDB fibers from CFA and control legs. (H) Typical examples (120 Hz stimulation frequency, 350 ms train duration) of intracellular (tetanic) Ca$^{2+}$ (upper) and specific force (lower) in control (blue) and CFA (pink) fibers (n=4-5). (I) Mean (± SEM) force vs. intracellular Ca$^{2+}$ data obtained in 15 to 150 Hz contractions produced in control fibers and CFA fibers. Mean values of intracellular Ca$^{2+}$ (J) and specific force (K) in the presence or absence of caffeine [5mM, 2min exposure] in CFA and control FDB fibers. Data are mean ± SEM. Statistical analysis in C, D, J, K was performed using two-tailed Student’s t-test and in F by two-way ANOVA. A P value less than 0.05 was considered significant. *P< 0.05, **P<0.01, ***P<0.001.
Fig. 2. Oxidative modifications introduced by SIN-1 decrease myofibrillar force and impair actin polymerization. (A) Phase-contrast image of an isolated myofibril set up for force measurement using the atomic force cantilever (AFC). (B) Typical records of active force from AFC measurements in myofibrils at pCa$^{2+}$ of 4.5 with and without SIN-1 [10mM, 10 minutes] with DTT present [1mM DTT] (n=9-10). (C) Mean (± SEM, n=9-10) of the active isometric force produced by the myofibrils with or without SIN-1 at a sarcomere length of 2.8 μm and pCa$^{2+}$ 4.5. (D) Immunoblots and quantification of the 3-NT level on myofibrillar actin (n=9-10). (E) Typical polymerization records of the actin polymerization assay using pyrene labeled actin polymerized with control G-actin (Ctrl) or G-actin pre-incubated in SIN-1 [5, 10 or 20 mM; 15min pre-incubation time] (n=3-4). (F) Mean fluorescence intensity (± SEM, n=3-4) of the polymerization reaction at half maximum level of polymerization of Ctrl and SIN-1 G-actin [5, 10 or 20 mM, 15min]. (G) Mean (± SEM, n=3-4) polymerization rate during linear elongation stage of polymerization of Ctrl and SIN-1 G-actin [5, 10 or 20 mM, 15min]. (H) Mean fluorescence (mean ± SEM, n=3-4) of Ctrl and SIN-1 [5 mM] G-actin at steady state of G-actin polymerization, 24h and 48h after induction of the polymerization. Statistical analysis for C, D and H was performed by applying two-tailed Student’s t-test. For G, One-way ANOVA with Holm–Sidak post hoc test was used. A P value less than 0.05 was considered significant. *P<0.05, **P<0.01, ***P<0.001.
Figure 3. Oxidative 3-NT and MDA modifications on the actin monomer. (A) Overview of a skeletal muscle α-actin molecule with coloring of subdomain (SD) 1 in grey, SD2 in light blue, SD3 in dark blue and SD4 in turquoise, which are kept consistent throughout the figures. (B-D) 3-nitrotyrosine (3-NT) and malondialdehyde (MDA) oxidative modifications were identified on α-actin with mass spectrometry. SD1–SD4 are shown with the recurrent oxidative modifications highlighted in each subdomain. The actin model is adapted from the 2ZWH crystal structure of the Protein Data Bank Europe (PDBe). Tyrosine residues (Y) were nitrated (3-NT), whereas and histidine (H), glutamine (Q) and asparagine (N) were MDA modified. (B) SD1–SD4 of oxidative modified α-actin residues from mice with arthritis (CFA) (pink, n=5). (C) Overview of the oxidative modified amino acids identified in SIN-1 [5mM] treated G-actin (orange, n=3) and (D) SIN-1 [10mM] treated myofibrillar actin (brown, n=3). The models were generated with UCSF-Chimera (97).
Figure 4. Three oxidative hotspots on the actin monomer. The actin monomer with the three oxidative hotspots identified by mass spectrometry, visualized with zooms; (A) hotspot 1 in subdomain (SD)1 with histidine (H)101/MDA, glutamine (Q)360/MDA, tyrosine (Y)362/3NT; (B) hotspot 2 in SD2 with H40/MDA, Q41/MDA, Y53/3NT and (C) hotspot 3 in SD3 with Y294/3NT, asparagine (N)296/MDA, N297/3NT. Amino acids in red represents those residues that had the same modification in SIN-1 treated actin and actin from CFA mice (6 out of 9). The models were generated with UCSF-Chimera (97).
Figure 5. Patients with RA exhibit muscle weakness and matching oxidative hotspots on the actin monomer. (A) An illustration of the composite disease activity score (DAS), with a 44-joint count (red circles) to assess swelling and 53-joint count to assess pain (DAS can range from 0.23 to 9.87). Mean ±SEM DAS was 3.3 ±0.4 (n=11, see supplementary Table 3 for details). (B) Mean ±SEM of isometric specific force of quadriceps femoris from patients with RA and healthy controls (n=11 per group). (C) Cross-sectional area (CSA) of quadriceps femoris measured by CT-scans (mean ±SEM, n=11). (D) Total daily activity in minutes measured with Actilife accelerometers (mean ±SEM, n=11) and (E) percent time spent in each defined activity category. (F) Overview of the oxidative 3-nitrotyrosine (3-NT) and malondialdehyde (MDA) modified amino acids on skeletal muscle actin (SD1-SD4) identified by mass spectrometry in patients with RA (n=5). The actin model is adapted from the 2ZWH crystal structure of the Protein Data Bank Europe (PDBe). Tyrosine residues (Y) were nitrated (3-NT), whereas histidine (H), glutamine (Q) and asparagine (N) were MDA modified. (G-I) The actin monomer visualizing that the oxidative hotspots in patients with RA (green) coincides with hotspots in mice with arthritis (pink). Amino acids depicted in red ribbons represents the residues that had the identical modification on actin in RA patients and CFA mice (5 out of 11). Generated with UCSF-Chimera (97).

Statistical analysis was performed using two-tailed Student’s t-test. A P value less than 0.05 was considered significant. *P< 0.05.
Figure 6. Altered filament stability, intersubdomain interactions and myosin interaction with oxidative modifications on actin. Analysis of molecular dynamics (MD) simulations calculated from trajectories of four 100 ns MD-simulations of ATP-bound F-actin. (A) Root mean square fluctuations (RMSF) of the Cα atoms (black line with gray shadow showing the standard deviation). RMSF values are averages of 2 ns blocks, calculated for the last 60 ns of the simulation time for the four 100 ns simulations. Residues exposed to modifications are pointed out in the sequence by their respective amino acid abbreviation and color coded according to its domain. (B) Solvent-accessible surface area (SASA), SD shown as error bars. (C) F-actin (PDB: 2MVA) with four G-actin monomers (A1-A4) envisioned in light to dark shade of blue. Histidine (H)40, glutamine (Q)41, Q246 and Q360 in red spheres visualizing their intricate location for inter- and intra-molecular bonding. (D) Number of H-bonds and (E) contacts between oxidized hotspot residues and the rest of the actin protein. A contact was defined as when the distance was less than 4Å between two non-hydrogen atoms. Values are averages with error estimates from block averaging in parentheses. (F) A model of a fragment of F-actin (A1-A3) (PDB: 5H53) with malondialdehyde (MDA) added to the residues. (G-H) Hotspot 2 with and without the presence of modifications on tyrosine (Y)294 and asparagine (N)297.