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Research article

Actin nitrosylation and its effect on myosin driven motility

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Abstract: The cytoskeletal protein actin can be nitrosylated, and others have shown that nitrosylation of actin can affect actin filament polymerization. However, the effects of nitrosylation on its interactions with the motor protein myosin are unknown. We therefore measured the effect of S-nitrosylation on the interactions of several actin isoforms with myosin. We used a modified coumarin switch assay to determine the number of nitrosylated cysteines in α -skeletal muscle, α -smooth muscle, and non-muscle (β and γ) actin in response to *in vitro* treatment with nitroso-L-cysteine—an endogenous nitric oxide (NO) donor. We also measured actin filament velocity over heavy meromyosin (HMM) using an *in vitro* motility assay, the isometric force generated by HMM using a laser trap, and the actin activated ATPase rates of HMM. We found that all three isoforms of actin were nitrosylated equally at ~2 sites per monomer. Nitrosylation of skeletal muscle α -actin reduced the velocity of actin filaments over HMM in a dose dependent fashion. The sliding velocities of all actin isoforms over HMM were reduced equally by ~24% when nitrosylated with 50 μ M donor. Our data are consistent with actin nitrosylation causing an increase in the time myosin remains bound to actin during its hydrolytic cycle.

Keywords: nitric oxide; nitrosylation; actomyosin; laser trap; motility; nitrosothiol

1. Introduction

Nitric oxide (NO) has long been established to influence muscle contraction [1,2]. In demembranated striated muscle, NO donors reduce Ca^{2+} sensitivity [3-5], ATPase activity [4,5], velocity of shortening [4], and isometric force [4,5]. Similar effects have been observed in skinned cardiac myofilaments when treated with NO donors [6]. Skinned fibers lack membranes, receptors, and calcium handling systems, thus the observed effects were presumably mediated by nitrosylation. Nitrosylation is the S-nitrosation of cysteine thiols in proteins, when regulation of those proteins is

presumed to be a result. The nitrosylated proteins (nitrosoproteins) responsible for these changes are largely unknown.

Both actin and myosin are nitrosoproteins [7,8], and are the major constituents of the contractile apparatus of both muscle and non-muscle cells. The major isoforms of actin are highly conserved, with >90% sequence homology. However, among the differences between isoforms is the placement and number of cysteine residues [9,10]. These cysteines are normally reduced [11], but α -actin can be nitrosylated both *in vivo* and *in vitro* [8], and β -actin has also been shown to be nitrosylated *in vivo* [12,13]. There is disagreement as to the identify of the nitrosylated cysteines in actin; the conserved cysteines cys374 [14] and cys285 [13] have been independently identified as being nitrosylated, but never together in a single study.

The effect of NO and nitrosothiols on the mechanics and biochemistry of myosin have been established using techniques of molecular mechanics; nitrosylation of myosin decreases by half the velocity with which it moves actin and doubles the isometric force [7]. Less is known about its effects on actin. Nitrosylation of actin reduces the rate with which short actin filaments can anneal [14], and therefore affects polymerization. However, actin is also a cofactor for the activation of the myosin motor enzyme; thus nitrosylation of actin might influence myosin function. Here we explore the effects of nitrosylation of α -skeletal, α -smooth, and β/γ -non-muscle actin on the biomechanics of the actomyosin complex. Our results show that nitrosylation of actin slows myosin driven motility. However, the effect of actin nitrosylation on actomyosin biochemistry and biomechanics is modest in comparison to the effects of myosin nitrosylation.

2. Material and methods

Ethics Statement. Rat skeletal muscle tissues were obtained from animal carcasses, having been sacrificed by another investigator under a protocol (#3594) approved by the University of Virginia Institutional Animal Care and Use Committee. No animals were sacrificed specifically for this study.

Proteins. Skeletal heavy meromyosin (HMM) and α -actin were prepared as described by us previously [15]. Gizzard smooth α and non-muscle (human platelet) β and γ actin were obtained commercially (Cytoskeleton Inc.) and resuspended as specified by the manufacturer. For motility assays, filaments were stabilized and labeled with TRITC-phallotoxin. For isometric force assays, actin was biotinylated as described previously [16].

Donor preparation and treatment. SNO-L-cysteine (SNO-cys) (10 mM) was prepared as described in [17] and stored in the dark at -80 °C. SNO-cys was diluted immediately before use with PBS acidified to pH 6.5, and diluted to final concentrations with actin buffer (25 mM Imidazole with 0.1 mM EGTA, 25 mM KCl, 4 mM MgCl₂, pH 7.4) less than 30 seconds before it was combined with actin. Actin filaments were treated with 0.05, 0.5, 5, or 50 μ M donor in reducing agent-free solutions and incubated on ice under vacuum for 20 minutes to allow complete decomposition of the donor and to remove liberated NO.

Quantifying nitrosylation. The coumarin switch assay for measuring S-nitrosylation is described elsewhere [7,18], and was performed with modifications. F-actin (20 μ g) was dialyzed into a DTT free buffer, diluted with PBS to 0.4 μ g/mL, and exposed to either SNO-cys (50 μ M) or an equivalent volume of acidified PBS as a control. Protein was precipitated with 90% cold acetone (-20 °C) for 20 minutes and then centrifuged for 10 minutes to remove excess donor. The precipitate was resuspended in HENS buffer (250 mM HEPES, 1 mM DTPA, 0.1 mM neocuproine, and 2% SDS) with 20 mM

methyl methanethiosulfonate (MMTS) and incubated at 50 °C for 20 minutes to block reduced cysteines. The protein was then precipitated twice from HENS buffer to remove excess MMTS before resuspending in labeling buffer (250 mM HEPES with 2% SDS, 4 mM ascorbate, 1 mM N-[6-(7-Amino-4-methylcoumarin-3-acetamido)hexyl]-3'-(2'pyridyldithio)propionamide (AMCA-HPDP), and 10 µM cupric sulfate). Samples were incubated at room temperature in the dark for one hour before precipitating to remove excess label. Pellets were dissolved in 4X electrophoresis sample buffer (Invitrogen), and resolved on a 12% gel (Invitrogen). Gels were imaged under ultraviolet light (Biorad ChemiDoc, 302 nm excitation, 530 nm emission). Gels were subsequently stained with colloidal coomassie (Invitrogen) to measure total protein. Bands were quantified using Image LabTM Software (Bio-Rad).

We introduced two important variations for quantifying the number of nitrosylated cysteines in a given protein. First, for each actin protein of interest, we included a control sample that was not treated with SNO-cys, a SNO-cys treated sample, and an untreated sample that was not blocked with MMTS during the switch procedure. The unblocked sample yields fluorescence proportional to the total reduced cysteine complement of the protein (Figure 1A). Second, to accurately determine the background signal, several different volumes of protein (2, 4, and 8 μ L) were run on a gel for each sample. Fluorescence and total protein band measurements increase linearly with the amount of the protein in the band; thus linear regression was used to fit each of these as a function of protein load (volume loaded in each lane, Figure 1B). All samples were normalized for variations in loading by taking the ratio of the fluorescence slope and the total protein slope (*f*/*t*) from those regression fits. The fraction of cysteines modified in an actin monomer was then calculated as

$$\frac{(f/t)_{SNO-cys} - (f/t)_{control}}{(f/t)_{-MMTS} - (f/t)_{control}}$$
 Equation 1

Actin-Activated ATPase Assay. The actin-activated ATPase assay was performed as described by Guo and Guilford with modifications [15]. Actin (0–20 μ M) was combined with 45 μ g/mL HMM in actin buffer and incubated at 30 °C. The reaction was initiated by the addition of 1 mM ATP and terminated at various times by adding 25 μ L of the reaction mixture to 25 μ L of stop solution (60 mM EDTA, pH 6.5, 6.6% SDS). Inorganic phosphate concentrations were determined by adding 100 μ L of freshly prepared color developing solution (2% ammonium molybdate in 4N H₂SO₄, with 0.5% FeSO₄). Absorbance was read at 650 nm. Standards were prepared from K₂HPO₄.

Motility assay. The DTT-free motility assay is described elsewhere in detail [7]. Briefly, 60 µg/mL HMM in 10 mM DTT was injected into a nitrocellulose-coated flow cell and incubated at room temperature for one minute. The flow cell was washed with 1 mg/mL polyvinylpyrrolidone (PVP 40) in actin buffer (25 mM ionic strength) to remove the remaining reducing agent and to block the surface [2]. After incubation at room temperature for one minute, actin filaments were incubated in the flow cell for one minute, followed by actin buffer and motility buffer (actin buffer with 0.5% methyl cellulose, glucose/glucose oxidase/catalase oxygen scavenger, and 1 mM ATP). All solutions were degassed under vacuum for 20 minutes before use. Degassing was repeated every three flow cells or 20 minutes, whichever came first.

Motility was observed on an Olympus IX70 microscope with a 100X, 1.3 n.a. objective heated to 30 °C (Bioptechs), and imaged with an intensified CCD camera (PTI). Images were taken at 4 frames per second and analyzed using ImageJ software [19] and a semi-automated tracking algorithm [20].

Laser Trap Isometric Force Assay. An optical trap assay described by Rao et al. was used to

measure the stall force of multiple myosin heads interacting with an actin filament [16]. Briefly, 0.97 μ m streptavidin-coated microspheres (Bangs Laboratories) were fluorescently labeled with TRITC-labeled BSA. Nitrocellulose flow cells were prepared similar to the DTT free motility assay. HMM (55 ug/mL) with 10 mM DTT was injected into the flow cell and subsequently blocked with 1 mg/mL PVP40. Biotinylated actin filaments were added followed by streptavidin microspheres suspended in actin buffer with 1 mM ATP and 1% oxygen scavenger. Individual beads were trapped and brought into contact with the trailing end of a motile actin filament. Stall force measurements were taken when forward motion of the actin filament stalled for greater than 50 ms. This was repeated for a range of actin filament lengths (2–7 μ m). A micrograph was saved of each filament for subsequent measurements of length. The number of myosin heads available to interact with the actin filament was estimated from HMM surface densities [15] and the actin filament length.

Statistics. Comparisons of velocities, ATPase rates, and nitrosylated cysteine counts were made by t-test. The slopes of linear fits to force versus actin filament length data from the laser trap were compared as described in [16]. Errors are provided throughout the text as the standard error of the mean (s.e.m.).

3. Results

3.1. Two cysteines per monomer can be nitrosylated in vitro by SNO-cys

We found that skeletal actin is nitrosylated by SNO-L-cys to a fraction of 0.48 ± 0.08 (N = 3, ± s.e.m.) of total cysteines (Figure 1). This translates to approximately 2 cysteines per actin monomer (2.4 ± 0.4) based on counts of cysteines in published primary structures (UniProt).

Comparisons were made of nitrosylation in response to SNO-cys treatment of skeletal, smooth, and non-muscle actins. We found that all the actin isoforms were nitrosylated to a similar degree (p = 0.71, N = 3) *in vitro* by SNO-cys (Figure). The extent of nitrosylation is not surprising given prior research that separately identified Cys285 and Cys374 as being nitrosylation sites in actin [13,14], and these are conserved between isoforms (Table 1). These two sites have yet to be identified as nitrosylated in the same isoform, however.

Table 1. Map of reported posttranslational modifications cysteine residues in four actin isoforms. (A) Alkylation; (C) Carbonylation; (G) S-glutathionylation; (N) S-nitrosylation; (O) Other oxidation. (X) Amino acid not present in isoform.

	Cys10	Cys17	Cys217	Cys257	Cys272	Cys285	Cys374
Skeletal α-actin	A [23–25]	Х			Х		A [25,26]
							C [27]
							G [28,29]
							N [14]
Smooth α -actin					Х		
Non-muscle β-	Х	A [26]			G [28]	N [13]	O [26,30]
actin					O [26,28]		
Non-muscle γ-	X	A [26]			O [26,28]		O [26]
actin							



Figure 1. Nitrosylation of actin by 50 μ M SNO-L-cys. A: Skeletal muscle actin was nitrosylated by exposure to SNO-L-cys (L) compared the control (C). The control and treated samples were compared to a sample that was not blocked (NB), leaving all available cysteines exposed to the AMCA (coumarin) label; this allowed us to determine the fraction of cysteines modified by SNO-L-cysteine. The nitrosylation signals were normalized to the total protein. B: Three different protein loads were used for each sample, and normalized nitrosylation signals from treated (squares) and control (triangles) were subjected to linear regression. The slopes were used to determine the quantitative level of nitrosylation relative to NB (circles), as shown in Equation 1. C: Skeletal muscle (SK), smooth muscle (SM), and non-muscle (NM) actin isoforms were nitrosylated to a similar extent by exposure to SNO-cys over the control (p = 0.71). Quantification was performed as described in the methods and in Equation 1. Error bars are standard error of the mean.

3.2. Nitrosylation of actin slows motility by HMM in a dose-dependent manner

The *in vitro* motility assay reproduces much of the *in vivo* function of actomyosin, yet does so in a highly defined environment. We used this assay to determine the velocity of fluorescent actin filaments over HMM with and without nitrosylation of actin. Skeletal α -actin in a reducing agent-free buffer was treated with various concentrations of SNO-cys. We observed a dose-dependent response of the skeletal actin filament velocities to SNO-L-cys (Figure 2). There was a significant decrease in velocity of samples treated with 0.5 and greater μ M donor (p < 0.05, N = 5) compared to control. For the samples treated with 50 μ M donor, the mean velocity was decreased by 24±7% from control.

Skeletal HMM is known to be responsive to SNO-L-cys [7]. We therefore conducted a control experiment to assure that the observed effects of SNO-L-cys were from nitrosylation of the actin filaments and not an effect of residual donor on HMM. After treatment of filaments with donor, 200 μ L aliquots were passed through a centrifugal concentrator (Millipore, 10 kDa MWCO) to remove excess donor. Filaments were resuspended in 200 μ L of actin buffer and degassed for 20 minutes. Flow

cells were loaded as before. A comparable decrease was seen as in the previous experiments $(16\pm9\%)$. Thus the reduction in velocity is due to a nitrosylation of actin, and not to carry over of donor.



Figure 2. Dose response velocities of SNO-L-cys-treated skeletal actin over HMM. Velocities were measured from the *vitro* motility assay and are displayed as mean filament velocities. There was a significant decrease in the overall velocities in the samples treated with $\geq 0.5 \mu$ M donor compared to control. **p* < 0.05. Error bars represent standard error of the mean.

3.3. The motility of the major isoforms of actin respond similarly to nitrosylation

Skeletal, smooth, and non-muscle actin isoforms differ in their complement of cysteines. Thus nitrosylation sites and responses to nitrosothiols may differ between them. To test this possibility, α -skeletal, α -smooth, and β/γ -non-muscle actins were treated with 50 μ M donor and observed in the *in vitro* motility assay. There was no significant difference between the control velocities of each isoform (Figure 3), which is consistent with previous reports [21]. Each isoform exhibited a significant decrease in mean velocity compared to control when treated with SNO-cys (p < 0.05 for each, N = 5). Interestingly, there was no difference in the response to SNO-cys between isoforms (p = 0.13) when data were normalized to daily controls.

3.4. Actin-activated enzyme kinetics of myosin and myosin stall force are only modestly altered by nitrosylation of actin

Actin-activated ATPase assays were performed with skeletal muscle HMM in the absence of reducing agents (Figure 4). There were modest (non-significant) reductions in V_{max} and K_m associated with nitrosylation of actin by 50 μ M SNO-L-cys. The estimated V_{max} for the control and treated actin were 66 ± 9 and 57 ± 10 μ M/min, respectively (p = 0.3, N = 3)—a 14% reduction. The estimated K_m for actin activation of HMM were 7 ± 2 and 4 ± 2 μ M for the control and treated samples, respectively (p = 0.15). It is important to note that these differences are based on the standard errors of the fitted parameters rather than point to point comparisons.



Figure 3. Response of mean velocity of actin isoforms to SNO-L-cys. Skeletal muscle (SK), smooth muscle (SM), and non-muscle (NM) actin filaments were treated with 50 μ M SNO-L-cys (open bars) and the velocities were compared to an untreated control (filled bars). **p* < 0.05 compared to control. Error bars show the standard error of the mean.



Figure 4. Actin-activated ATPase rate. Rates were measured for control (•) and nitrosylated (\circ) skeletal actin. There was not a significant difference between control and treated actin-activated ATPase rates, though the trend was to lower V_{max} in SNO-L-cys treated actin. K_m and V_{max} values were determined by fitting Michaelis-Menton for the control (solid line) and nitrosylated (dotted line) data. Error bars are within the symbols.

We used an optical trap assay as a physiologically relevant measure of myosin force production [16] to determine whether nitrosylated actin is driven by myosin with altered stall force. Trapped beads were brought into contact with the trailing end of a motile actin filament and remained in contact until the filament reached stall (Figure 5). When repeated for a range of filament lengths, the slope of the relationship between stall force and filament length is a valuable measure of force generation. Control

filaments were propelled with a force of 5.9 ± 0.4 pN/µm of actin filament length, while filaments nitrosylated with 50 µM SNO-L-Cys were propelled with a force of 6.5 ± 0.5 pN/µm of actin filament length (Figure 5). The 14% increase in the force per unit length generated by skeletal HMM on nitrosylated actin filaments was not significant (p = 0.2, N = 20).



Figure 5. Measurement of isometric force after SNO-L-cys treatment of actin. Top: A streptavidin-coated microsphere was held in the laser trap (dashed arrows) and touched to the trailing end of a moving biotinylated, TRITC-phalloidin labeled actin filament. Force generated by the ensemble of HMM (F) caused the microsphere to displace from trap center (d) until the filament stalled. F and therefore d (which we measured) increase linearly with actin filament length. Bottom: Myosin isometric force was measured when in contact with control (\bullet , solid line) and nitrosylated (\circ , dashed line) actin. Dotted lines show 95% confidence intervals of the regression fits.

4. Discussion

Our finding that that each isoform is nitrosylated at about 2 cysteines per monomer by the endogenous nitrosothiol SNO-L-cys appears to resolve an outstanding disagreement in the literature; one study identified the highly reactive cys374 in skeletal α -actin as a nitrosylated cysteine [14], while another identified cys285 in non-muscle β -actin [13], even though these two residues are present in all actin isoforms. The latter study used mass spectroscopy, which would ideally have full coverage of the actin molecule and would identify all the nitrosylated cysteines, while the other used blockade of Cys374 by alkylation. However, proteolytic peptides display a wide range of physiochemical

properties that cause them to be unevenly represented in mass spectroscopy [22]. There is little surprise that one technique or another might identify only one of two nitrosylated cysteines. Our approach is useful in that it allows accurate counting of nitrosylated cysteines when the primary structure of the protein is available, and suggests that both these residues may be nitrosylated. Our normalization procedure and use of multiple sample loadings gives the precision necessary for reproducibility.

Nitrosylation of actin by SNO-L-cys caused a small but significant decrease in the velocity of nitrosylated actin over myosin in an *in vitro* assay of motility, the mechanisms of which we discuss later. That nitrosylation caused a similar decrease in velocity of each actin isoform over myosin suggests that the cysteine(s) responsible are among those conserved between the isoforms. The mammalian actin isoforms vary slightly in their primary structures but relatively more so in the number and placement of cysteine residues [9,10]. The cysteine complements of actin isoforms and their known oxidative posttranslational modifications were summarized in Table 1.

The relative reactivity of the cysteines in actin is primarily a function of accessibility and the position of the cysteine in respect to surrounding amino acids [31]. Cys374 sits at the bottom of a hydrophobic pocket [32], but some of the immediately surrounding amino acids are basic. This arrangement has been proposed to constitute a nitrosylation motif that may promote reactions of NO or nitrosothiols [33,34]. Cys285 likewise sits in a hydrophobic pocket [10] adjacent to a lysine. This is consistent with our data, and with others' [13,14], though it is obviously not a definitive identification of both these two cysteines being nitrosylated, or as being the source of the functional changes observed here.

The mechanism by which nitrosylation of actin reduces its velocity of motion over myosin remains an open question. However, two theories present themselves. First, nitrosylation of one or both of these residues might directly affect the binding of myosin to actin. For example, Cys374 is positioned at the C-terminus of the actin monomer in actin subdomain 1 [25]. Fluorescence quenching [35] and FRET measurements [36] show that Cys374 is adjacent to myosin's binding site. A polar nitrosothiol group on this cysteine might thus directly affect myosin binding or a rate-limiting step in its hydrolytic cycle. Oxidation of Cys374 by copper is already known to alter actin filament velocities over myosin in an *in vitro* assay [30].

An alternative possibility is that nitrosylation induces global changes in the structure of the actin filament that in turn affect myosin binding. Actin filaments are structurally polymorphic even when they are compositionally homogeneous [37-39]. Any binding partner or covalent modification could conceivably bias the structure of the actin filament. Indeed, using divalent cations to stabilize different forms of F-actin has been shown to alter cooperative binding of myosin to actin [40]. Likewise, phalloidin binding alters the flexibility of actin filaments and their velocity over myosin *in vitro* [41,42]. Situated in subdomain 3, Cys285 is involved in hydrophobic interactions that contribute to the main internal stability of the filament [10]. Nitrosylation of this residue may have far-reaching structural effects in the actin filament.

Regardless of the exact mechanism, the velocity with which myosin propels actin (V_{actin}) is often understood through a simple model, wherein V_{actin} is directly related to the distance myosin moves actin in a single step (d_{uni}) and inversely related to the time myosin is attached to actin (t_{on}) during a single cycle of ATP hydrolysis—that is, steps of a certain size occurring at a particular maximum frequency.

$$V_{actin} = d/t_{on}$$

Similarly, the time averaged force generated by myosin (F_{avg}) on actin is related to the force

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generated by myosin in a single step (F_{uni} , the step or unitary force), and the duty ratio (f) of myosin—that is, the fraction of its total cycle time myosin spends attached to actin and generating force.

$$F_{avg} = f \cdot F_{uni}$$
$$f = t_{on} / (t_{on} + t_{off})$$

where t_{off} is the detached (off) time of myosin. Thus $t_{on} + t_{off}$ is the total cycle time of myosin. The ATPase rate is an indirect reciprocal measure of this cycle time.

$$V_{max} \propto (t_{on} + t_{off})^{-1}$$

We assume that nitrosylation of actin can alter only t_{on} or t_{off} to elicit the changes we observed in our experiments, since altering either d_{uni} or F_{uni} would probably require major conformational changes in myosin. Thus the decrease in velocity that we measured is almost certainly due to a 31% increase in t_{on} .

 t_{off} is much larger than t_{on} in striated muscles, as evidenced by their low duty ratios [43]. Thus a 31% increase in t_{on} would increase the total cycle time only slightly, assuming that t_{off} remains unchanged. We would thus expect only a small decrease in the ATPase rate (an indirect measure of cycle time). Indeed, we measured only a statistically insignificant 14% decrease in the ATPase rate, implying a 17% increase in $(t_{on} + t_{off})$. We should see a concomitant increase of only 12% in both f and in F_{avg} (1.31/1.17 = 1.12). Indeed, we measured a statistically insignificant 14% increase in average force production on nitrosylated actin. While neither of these latter two measures was statistically significant, our data are wholly consistent with actin nitrosylation causing an increase in the time myosin remains attached to actin, with no other changes to the hydrolytic cycle or the mechanics of the system.

These changes are small relative to the effects of NO on myosin [7]. It is thus possible that actin serves as a SNO reservoir in cells to regulate other processes, as suggested by others [14], rather than itself being a major mechanism through which motility or contraction are regulated by nitrosylation. We cannot, for example, exlude the possibility that nitrosylated actin served as a donor in our experiments to transnitrosate myosin (direct NO transfer from one protein to another), leading to the observed effects.

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Conflict of interest

The authors have no financial conflicts of interest to declare.

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