Age-Related Decline in Actomyosin Function

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To understand the molecular basis of the functional decline in aging muscle, we examined the functional (actomyosin ATPase) and chemical (cysteine content) changes in actin and myosin purified from the muscles of young (4- to 12-month-old) and old (27- to 35-month-old) Fisher 344 rats. Using the soluble, catalytically active myosin fragment, heavy meromyosin (HMM), we determined the maximum rate ($V_{\text{max}}$) and actin concentration at half $V_{\text{max}}$ ($K_m$) of the actomyosin ATPase, using four combinations of actin and HMM from old and young rats. $V_{\text{max}}$ and $K_m$ were significantly lower when both actin and HMM were obtained from old rats than when both proteins were obtained from young rats. The number of reactive cysteines in HMM significantly decreased with age, but no change was detected in the number of reactive cysteines in actin. We conclude that aging results in chemical changes in myosin (probably oxidation of cysteines) that have inhibitory effects on the actin-activated myosin ATPase.

Skeletal muscle weakness is one of the most significant factors limiting the activities of daily living in the elderly (1). The age-related degeneration of muscle has been associated with changes in the interaction between actin and myosin, as indicated by the age-related decrease in force and speed of shortening of permeabilized muscle fibers and of myofibrils from humans as well as animals (2–6).

Among the hypotheses that have been developed to explain age-related functional changes in proteins, the one most relevant for this work involves oxidative modification of proteins by reactive oxygen species, resulting in inhibition of biological functions (7). We are particularly interested in the oxidative state of the cysteines in myosin and actin, as cysteine thiols are an important oxidation target associated with aging (8,9). Skeletal muscle myosin contains 40 cysteines (10), including two highly reactive cysteines, Cys 707 and Cys 697. Modification of these two cysteines has been shown to have dramatic effects on the functional properties of myosin, particularly in interactions with actin; spectroscopic probes attached to these sites are sensitive to ATPase activity and to actin binding (11–16). Actin contains five cysteines (17), including the highly reactive Cys 374, which is located in the region involved in the interaction with myosin. Modification of Cys 374 has been shown to affect functional interaction with myosin (18), and spectroscopic probes attached to this site are sensitive to functional interactions with myosin (19,20).

Investigations elucidating the mechanisms of the age-related degeneration of muscle have typically focused on myosin (21–24), but there is abundant evidence that modifications of actin affect actomyosin ATPase and in vitro motility (20,25–27). Therefore, to determine independently the roles of actin and myosin in age-related changes in muscle contractility, it is necessary to perform mechanical measurements on muscle from young and old animals, then purify actin and myosin from these same animals and determine separately their functional properties in the actomyosin ATPase reaction. Until the present study, this set of experiments has not been done.

In the present study, we tested the hypothesis that the age-related deterioration of muscle contractility is due to changes in the interaction between actin and myosin during the actomyosin ATPase cycle. It is difficult to study quantitatively these interactions using intact myosin, because it aggregates at low ionic strength where the interaction with actin is maximized. Therefore, quantitative measurements of the actin–myosin interaction are usually performed using soluble, catalytically active myosin fragments: two-headed heavy meromyosin (HMM) and one-headed subfragment 1 (S1). Kinetic studies have established that the mechanism of interaction between purified actin and soluble myosin heads in solution is essentially the same as that between actin and intact myosin in myofibrils (28). In the present study we purified actin and myosin from the muscles of young and old rats, prepared HMM, and determined two key parameters of the actomyosin ATPase, $V_{\text{max}}$ (the maximum rate) and $K_m$ (actin concentration at half $V_{\text{max}}$). We complemented these functional studies by measuring age-related changes in the content of reactive cysteine residues in purified actin and myosin. This approach represents a new step in exploring the mechanisms of age-related decline of actomyosin function.

**METHODS**

**Animals**

Fisher 344 male and female rats aged 4–12 months (young) and 27–35 months (old) were obtained from the aging colony maintained by the Minneapolis Veterans Administration. The 50% survival rate for the Fisher 344 rat strain is about 24 months. All animals were housed in pathogen-free conditions and received food and water ad libitum. The animal care protocol was approved by the University of Minnesota Institutional Animal Care and Use
Committee. Animals were deeply anesthetized and killed, and the semimembranosus, semitendinosus, adductor magnus, and quadriceps muscles were dissected.

Single Fiber Contractile Measurements

Individual fiber segments (~2 mm long) from the permeabilized bundles were isolated and studied at 15°C as described in detail previously (2). Fiber segments were mounted in relaxing buffer (7.0 mM EGTA, 5.4 mM MgCl₂, 20 mM imidazole [pH 7.0], 14.5 mM creatine phosphate, 4.7 mM ATP, CaCl₂ to achieve pCa of 9, and enough KCl to achieve ionic strength of 180 mM). Sarcomere length was set to 2.5 μm, the diameter was measured at three places along the length of the fiber, and then maximal isometric force was determined in activating solution. Unloaded maximal shortening velocity, Vₒ, was determined by the slack test, in which fibers were activated by exposure to Ca²⁺ (calcium added to the relaxing buffer to bring pCa to 4.5) and then at peak isometric force rapidly shortened (slacked) by 10%–20% of fiber length such that force dropped to zero. The time between zero force and force redevelopment was measured. This procedure was performed 5 times at different slack distances. The slack distances were then regressed against the corresponding times of force redevelopment, and the slope of that line (in millimeters per second) was divided by fiber length (millimeters per fl) to obtain Vₒ (fiber length in millimeters per second).

Purification of Myosin and Actin

Each set of experiments consisted of determining actin-activated HMM ATPase under physiological conditions, myosin and HMM ATPase under “high-salt” conditions, and cysteine content in actin and HMM. For each set, two rats (one old and one young) were killed. Muscles were quickly dissected and immediately placed in ice-cold solution containing 0.2 M sucrose, 0.1 mM EDTA, and 10 mM imidazole (pH 7.0); they were subsequently homogenized on ice in rigor buffer containing 0.1 M KCl, 20 mM imidazole (pH 7.0), 0.5 mM EDTA, and the following protease inhibitors: 20 μM PMSF (phenylmethanesulfonyl fluoride), and 10 μg/ml each of aprotinin, leupeptin, antipain, pepstatin, chymostatin, TLCK (Nα-p-Tosyl-l-lysine-chloromethyl ketone), TPCK (N-p-Tosyl-l-phenylalanine chloromethyl ketone), and BAAE (Nα-Benzoyl-l-arginine ethyl). The homogenate was centrifuged for 5 minutes at 3000 g, supernatant was discarded, and the pellet was extracted for 10 minutes on ice with Guba-Straub solution (0.3 M KCl, 3 mM MgCl₂, 10 mM Tris (pH 7.5), and 2.5 mM ATP, at 25°C). The extract was first polymerized with 0.1 M KCl for 1 hour at room temperature, then the KCl concentration was increased to 1 M, and the extract was incubated for 30 minutes at 37°C and centrifuged 30 minutes at 300,000 g. The pelleted actin was suspended in G-buffer, dialyzed against G-buffer to remove KCl, and centrifuged for 10 minutes at 300,000 g to remove aggregates. After polymerization with 3 mM MgCl₂, actin was ultracentrifuged for 30 minutes at 300,000 g, and the final pellet was suspended in F-buffer (3 mM MgCl₂, 10 mM Tris, pH 7.5) containing 0.2 mM ATP. All centrifugations were performed at 4°C.

All experiments using actin, myosin, and HMM purified from one pair of rats were completed within 4 days, including the day when the animals were killed. For every given pair of young and old rats, the purification of actin and myosin as well as all experiments with purified proteins were performed in parallel to ensure that all conditions were identical, except for the rats’ ages. For results presented in this paper, 8 pairs of rats were used.

Proteins purified from the young rat are designated “young actin” (Aₒ) and “young HMM” (Mₒ), and the proteins purified from the old rat are designated “old actin” (Aₗ) and “old HMM” (Mₗ). Due to the variability of available muscle tissue and the amount of purified proteins, in two of the paired experiments using old and young HMM and in one of the paired experiment using young and old actin, the proteins from the young rat were substituted with rabbit actin and myosin. Control experiments showed that the actin-activated ATPase rates for young rat proteins were the same as those for rabbit proteins.

Protein Concentration

Protein concentration was measured by ultraviolet absorption, assuming molar absorption coefficients of 0.53 mg ml⁻¹ cm⁻¹ for myosin at 280 nm, 0.65 mg ml⁻¹ cm⁻¹ for HMM at 280 nm, and 0.63 mg ml⁻¹ cm⁻¹ for actin at 290 nm.

ATPase Measurements

Actin-activated HMM ATPase was measured at 25°C in 3 mM MgCl₂, 10 mM Tris (pH 7.5), and 2.5 mM ATP, at a constant concentration of HMM (0.69 μM myosin heads) and with varying concentrations of actin (from 3.5 μM to 59 μM); concentration of the liberated phosphate was determined by the Fiske–Subbarow method (33). Vₘₐₓ and Kₘ of the acto-HMM ATPase were determined by fitting the data with the Michaelis-Menten equation:

\[ v = \frac{V_{\text{max}}}{(1 + K_{\text{m}}/[A])}, \]

where v is the measured ATPase rate and [A] is actin concentration, using the software package Origin 7 (OriginLab, Northampton, MA). The high-salt ATPase activity of myosin or HMM was measured at 25°C in 0.6 M KCl,
were purchased from Sigma-Aldrich (St. Louis, MO).

The differences for all paired data. The differences were determined using the semimembranosus fibers dissected from the same animals which were the source of muscles for the other combinations. We determined: (a) the age-related changes in actin by comparing the activation of old and young actin, young HMM, Vmax values (15.69 ± 0.96 s⁻¹) were not significantly different from for young acto-HMM (A₀M₀, 9.90 ± 1.11 μM), and this difference was also statistically significant (n = 7, t = 3.164 > t_{crit} = 2.447).

Figure 1 shows only two of the four combinations of actin and HMM from a pair of rats. To assess which of the two proteins contributes most to the observed changes in actin-activated ATPase, we also performed experiments with two other combinations. We determined: (a) the age-related changes in HMM by comparing the activation of old and young HMM (designated AₙMₙ), in this case, the data from young actin and young HMM (designated AₙMₙ) are compared with the data from old actin and old HMM (designated A₀M₀). In this experiment, aging results in a significant decrease in Vmax, but no significant decrease in Km (see Figure 1 legend). After this experiment was performed on all seven pairs of rats, the mean Vmax for old acto-HMM (A₀M₀, 12.15 ± 0.85 s⁻¹), was 21% lower than that for young acto-HMM (AₙMₙ, 15.25 ± 0.75 s⁻¹), and the t test on the paired data sets showed that this decrease was statistically significant (n = 7, t = 11.00 > t_{crit} = 2.447). The mean Km for the old acto-HMM (A₀M₀, 7.51 ± 1.05 μM) was 24% lower than that for the young acto-HMM (AₙMₙ, 9.90 ± 1.11 μM), and this difference was also statistically significant (n = 7, t = 3.164 > t_{crit} = 2.447).

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50 mM Tris (pH 7.5), and 10 mM EDTA (K-ATPase) or 10 mM CaCl₂ (Ca-ATPase); protein concentrations were 0.025 mg/ml (K-ATPase of myosin and HMM), 0.3 mg/ml (Ca-ATPase of HMM), and 0.4 mg/ml (Ca-ATPase of myosin). The concentration of the liberated phosphate was determined using the malachite green method (34).

**Determination of Reactive Cysteine**

The reactive cysteine side chains were quantitated at 1 μM HMM and 5–8 μM actin in the presence of 0.1 M NaHPO₄ (pH 8), 1 mM EDTA, 1% SDS, and 10 mM Tris (pH 7.5) by the DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid)) method (35,36). DTNB (0.5 mM) was added to the prepared protein samples, and after a 15-minute incubation at 25°C the absorbance was measured at 412 nm. The cysteine content (mol/mol protein) was calculated using the absorption coefficient of the free TNB anion as 14,150 M⁻¹ cm⁻¹ (37). Control experiments using a standard solution of cysteine showed that buffers, including SDS, did not affect the results.

**Reagents**

The solutions were made in MilliQ water and degassed for ~30 minutes. All buffers, ATP, and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO).

**Data Analysis**

The values of Vmax, Km, K-ATPase, Ca-ATPase, and cysteine content were obtained in paired experiments, where the results for each pair of young and old rats were expressed by calculating the ratio (old rat value) / (young rat value) and the difference (young rat value) – (old rat value). After the experiments with all 8 pairs of rats were completed, the ratios were averaged and presented on the figures as mean ± SEM. The significance of the age-related changes was determined by performing a t test (α = 0.05) on the differences for all paired data. The differences were regarded as significantly different when t > t_{crit}.

**RESULTS**

**Contractility of Single Muscle Fibers**

Age-related changes in muscle contractility were determined using the semimembranosus fibers dissected from the same animals which were the source of muscles for the purification of actin and myosin. Specific tension of the fibers from the old animals (82.7 ± 2.0 kN/m²) was 77% of that of the fibers from the young animals (106.8 ± 2.8 kN/m²), and the unloaded shortening velocity Vₒ of the fibers from the old animals (7.5 ± 0.5 fl/s) was 78% of that of the fibers from the young animals (9.6 ± 0.4 fl/s). These results are consistent with the age-related decline in muscle function previously observed in our laboratories (4,5). To investigate the biochemical basis of this decline, we measured the ATPase activities of the purified actin and myosin.

**Effects of Aging on Actin-Activated HMM ATPase**

Figure 1 is representative of a paired experimental set, in which we determined Vmax and Km of the actin-activated HMM ATPase. In this case, the data from young actin and young HMM (designated AₙMₙ) are compared with the data from old actin and old HMM (designated A₀M₀). In this experiment, aging results in a significant decrease in Vmax, but no significant decrease in Km (see Figure 1 legend). After this experiment was performed on all seven pairs of rats, the mean Vmax for old acto-HMM (A₀M₀, 12.15 ± 0.85 s⁻¹), was 21% lower than that for young acto-HMM (AₙMₙ, 15.25 ± 0.75 s⁻¹), and the t test on the paired data sets showed that this decrease was statistically significant (n = 7, t = 11.00 > t_{crit} = 2.447). The mean Km for the old acto-HMM (A₀M₀, 7.51 ± 1.05 μM) was 24% lower than that for the young acto-HMM (AₙMₙ, 9.90 ± 1.11 μM), and this difference was also statistically significant (n = 7, t = 3.164 > t_{crit} = 2.447).

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significantly different \((n = 6, t = 0.029 < t_{\text{crit}} = 2.571)\). Similarly, the corresponding \(K_m\) values \((10.32 \pm 1.11 \mu M\) for \(A_{o}M_{o}\) and \(9.54 \pm 1.29 \mu M\) for \(A_{y}M_{o}\)) \((n = 6, t = 0.910 < t_{\text{crit}} = 2.571)\) were not significantly different \((n = 6, t = 0.910 < t_{\text{crit}} = 2.571)\). We conclude that the age-related changes in actomyosin function are due primarily to the changes in myosin.

**Age-Related Changes in the ATPase of Myosin in the Absence of Actin**

Because myosin is implicated in the functional effects of aging, we obtained insight into the age-related changes in myosin by measuring the high-salt \(K^-\) and \(Ca^-\) ATPase activity of purified myosin and HMM. It has been shown that ATPase activities measured under these conditions are quite sensitive to the modification (e.g., oxidation) of myosin’s two reactive cysteines, Cys 707 and Cys 697. Increased modification of Cys 707 results in a decrease in the K-ATPase and increase in the Ca-ATPase, whereas oxidation of both Cys 707 and Cys 697 inhibits both ATPases \((11,15,38)\) (Figure 3).

In the present study, we detected an age-related decrease of K-ATPase by about 10\%, from \(10.23 \pm 0.76 \text{ s}^{-1}\) to \(9.10 \pm 0.53 \text{ s}^{-1}\), which was statistically significant \((n = 8, t = 3.189 > t_{\text{crit}} = 2.363)\). The Ca-ATPase activities of old HMM \((0.83 \pm 0.05 \text{ s}^{-1})\) and young HMM \((0.82 \pm 0.07 \text{ s}^{-1})\) were not significantly different \((n = 8, t = 0.201 < t_{\text{crit}} = 2.365)\). Essentially the same results were observed for intact myosin, where K-ATPase of young myosin, \(12.29 \pm 1.26 \text{ s}^{-1}\) was significantly \((n = 8, t = 3.054 > t_{\text{crit}} = 2.365)\) higher than that of old myosin, \(11.60 \pm 1.11 \text{ s}^{-1}\), whereas the Ca-ATPase activities were unaffected \((0.77 \pm 0.03 \text{ s}^{-1}\) for young, \(0.76 \pm 0.04 \text{ s}^{-1}\) for old; \(n = 8, t = 0.920 < t_{\text{crit}} = 2.365)\). Thus, digestion of myosin to HMM does not affect age-related differences in the enzymatic properties of myosin. These results suggest age-related oxidation of cysteine residues in myosin, but the lack of activation of Ca-ATPase argues against specific modification of Cys 707.

**Reactive Cysteine Content in Myosin and Actin From Young and Old Muscle**

To evaluate total cysteine content in myosin’s HMM and in actin, the DTNB assay was performed on proteins unfolded by treatment with 1% SDS. In control experiments using rabbit skeletal muscle HMM, this method yielded \(31.4 \pm 1.2 \text{ cysteines/mol of HMM}\), in good agreement with the 32 cysteines predicted from the amino acid sequence \((10)\). DTNB titration of HMM from old and young rats showed an age-related decrease in free cysteine content, from \(31.11 \pm 0.95 \text{ moles per mol in young HMM}\) to \(28.03 \pm 0.89 \text{ moles per mol in old HMM}\). This difference was statistically significant \((n = 6, t = 5.793 > t_{\text{crit}} = 2.365)\) (Figure 4).

We did not detect any age-related difference in the cysteine content in actin. The cysteine content in the young actin, \(4.31 \pm 0.10 \text{ moles/mol}\), was essentially the same as that in the old actin, \(4.30 \pm 0.06 \text{ moles/mol}\) (Figure 4). This finding is in agreement with the measurements using rabbit skeletal muscle actin in our previous work and work by others \((36)\), and is consistent with 5 cysteines/mol of actin, which is predicted from the amino acid sequence \((17)\).

Control experiments demonstrated that the purification procedure did not introduce Cys modifications in actin and myosin. We performed the initial steps of the purification process (homogenization of muscle and extraction of myosin in Guba-Straub solution) in the presence of 5 mM dithiothreitol (DTT), an antioxidant specific for Cys). Neither the actin-activated ATPase activity nor the cysteine content
of purified proteins was affected by this DTT treatment. Furthermore, the cysteine content of the young as well as the old HMM purified by our standard procedure (in the absence of DTT) was not affected by a subsequent reduction at 10 mM DTT, indicating that the detected decrease in free cysteine thiols is due to irreversible modifications occurring in vivo.

**DISCUSSION**

**Age-Related Changes in Myosin Function and Chemical State**

To perform systematic studies of age-related changes in the functional and chemical properties of actin and myosin, we have purified actin and myosin from the hamstring and quadriceps muscles of young and old animals. These muscle were chosen because the previous research in our laboratories showed that their contractility decreases with aging and that this decrease is not associated with changes in the expression of myosin heavy-chain isoforms but may be associated with chemical changes in myosin, such as cysteine oxidation (4,5,39,40). The present study has two principal findings for purified actin and myosin: (1) a significant age-related decrease in the $V_{\text{max}}$ and $K_m$ of actin-activated ATPase and (2) a significant age-related decrease of free cysteine content in myosin.

The age-related decrease in $V_{\text{max}}$ (21%), detected at the level of the purified proteins, is comparable to the 22% decrease in shortening speed of muscle fibers from the same rats (see Results section) and to the 20% decrease in unloaded shortening speed of myofibrils from the semimembranosus muscle (41). The established correlation between the unloaded shortening velocity $V_o$ of the different muscle types (e.g., fast and slow myosin isoforms) and the actomyosin ATPase (42,43) suggests that the detected decrease in $V_{\text{max}}$ of the ATPase is due to changes in the properties of the myosin molecule. The analysis of the age-induced changes in acto-HMM, HMM alone, and actin alone supports the following conclusions: young actin

**Mechanism of Age-Related Changes in the Actomyosin ATPase**

The biochemical steps of the actomyosin ATPase cycle have been associated with distinct structural states of actin and myosin. The current models postulate that muscle contraction is generated upon the transition of the actin–myosin complex from states of weak interaction (AM•ATP, AM•ADP•P) to states of strong interaction (AM•ADP•P, AM, AM•ATP) (43). In terms of this model, the age-related decrease in $V_{\text{max}}$ suggests alterations in the structural states of myosin that affect the transition from weak to strong interactions. Possible transitions include disorder-to-order transitions in the catalytic and light-chain domains of myosin or changes in the internal structure of the catalytic domain (44,46). This possibility is supported by the observed correlation between changes in $V_{\text{max}}$ and mutations of specific sequences within the actin-binding region of myosin (47).

The simplest interpretation of the age-related decrease in $K_m$ is increased binding affinity of actin for myosin in the weak binding states (28,48). This interpretation of $K_m$ is supported by studies on the effects of mutations in the actin-binding regions of myosin and myosin-binding regions of actin. These studies showed that the relative order of changes in $K_m$ followed the relative order of changes in the weak binding affinities (25,47). We conclude that the age-related changes result in an accumulation of the weak binding complexes A-M•ATP and A-M•ADP•P, due to structural changes in myosin, which slow down the whole actomyosin ATPase cycle. This conclusion is consistent with that derived previously from our electron paramagnetic
studies on permeabilized muscle fibers, indicating an age-related decrease in the fraction of the myosin heads in the strong-binding structural states (4).

Our proposed interpretation in terms of age-related changes in the molecular interactions between actin and myosin may be oversimplified, as both $K_m$ and $V_{max}$ are determined by multiple transitions between intermediates of the actomyosin ATPase cycle (28,48). Thus, the understanding of the age-related changes in the molecular interaction of actin and myosin requires further biochemical as well as structural studies. The present study has taken the first step toward this goal, by demonstrating that purified actin and myosin from aged rats reveal consistent biochemical age-related changes.

Age-Related Oxidative Modifications of Myosin

We detected oxidation of 3 of 32 cysteines (10) in HMM. This finding is comparable with the previously reported oxidation of about four cysteines per myosin in the gastrocnemius muscle of rat (23). These results support the hypothesis that cysteines are important oxidative targets in the aging process (8,9). The age-induced modifications of the myosin molecule are probably facilitated by its long half-life and age-related decrease in the turnover rate (49–51).

The observed ~10% decrease in K-ATPase of HMM suggests some modification of Cys 707 and/or Cys 697. However, the lack of changes in Ca-ATPase strongly suggests that the modification is not specific for these two residues (11,12). Because oxidation of cysteines is one of the possible chemical changes in myosin that results in inhibition of the actin-activated ATPase, some of the oxidized residues could be located within or in the vicinity of the functionally relevant sequences in myosin. Possible candidates include Cys 540 and Cys 402. Cys 540 is in a region important for the strong interaction with actin. Cys 402 is of particular interest because mutation of the neighboring Arg 403 to Gln causes human heart muscle dysfunction associated with familial hypertrophic cardiomyopathy (52).

The decrease in K- and actin-activated myosin ATPases could also result from oxidative modification of sites other than cysteine. Studies on rabbit skeletal myosin (53,54) have correlated inhibition of K-ATPase with modification of Lys 84 at the interface of the catalytic and lever arm domains, and with glycation of yet unspecified lysines (55). Glycation of myosin, detected in aging rats (24), has been suggested as the mechanism for the inhibition of actin sliding on aged myosin in vitro (56,57), but localization of the glycated residues remains unknown.

Age-Related Changes in Actin

We did not detect significant age-related changes in actin’s functional properties or cysteine content. However, we cannot exclude the possibility that other changes in actin occur and subsequently alter muscle function via effects on actin’s interaction with proteins other than myosin. It has been shown that the oxidative modification of actin inhibits the interaction with $\alpha$-actinin (58). The mutation-induced changes in $\alpha$-actinin binding sites of actin were implicated in the mechanism of myocyte dysfunction and heart failure in dilated cardiomyopathy (59). Future experiments are needed to elucidate age-related structural and functional changes in actin.

Summary

The present work explores the biochemical basis of previously observed age-related degeneration of muscle contractility. Using purified actin and myosin, we have found a significant age-related inhibition of the actin-activated myosin ATPase, and a significant age-induced decrease in the free cysteine content of myosin. Future experiments will focus on a more thoroughly analyzing the age-related chemical changes in both actin and myosin, localizing sites of oxidative modifications, and determining their specific roles in the impairment of functional actin–myosin interactions.

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