

# Age-related loss of nitric oxide synthase in skeletal muscle causes reductions in calpain S-nitrosylation that increase myofibril degradation and sarcopenia

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## Summary

**Sarcopenia, the age-related loss of muscle mass, is a highly-debilitating consequence of aging. In this investigation, we show sarcopenia is greatly reduced by muscle-specific overexpression of calpastatin, the endogenous inhibitor of calcium-dependent proteases (calpains). Further, we show that calpain cleavage of specific structural and regulatory proteins in myofibrils is prevented by covalent modification of calpain by nitric oxide (NO) through S-nitrosylation. We find that calpain in adult, non-sarcopenic muscles is S-nitrosylated but that aging leads to loss of S-nitrosylation, suggesting that reduced S-nitrosylation during aging leads to increased calpain-mediated proteolysis of myofibrils. Further, our data show that muscle aging is accompanied by loss of neuronal nitric oxide synthase (nNOS), the primary source of muscle NO, and that expression of a muscle-specific nNOS transgene restores calpain S-nitrosylation in aging muscle and prevents sarcopenia. Together, the findings show that *in vivo* reduction of calpain S-nitrosylation in muscle may be an important component of sarcopenia, indicating that modulation of NO can provide a therapeutic strategy to slow muscle loss during old age.**

**Key words:** aging; calpain; nitric oxide; sarcopenia; skeletal muscle.

## Introduction

Sarcopenia, the loss of muscle mass during aging, is an inevitable process that predisposes the elderly to debilitating injuries. Sarcopenic muscle exhibits numerous defects in structure, including disarray of myofibrils and Z-disks (Volpi *et al.*, 2004). There are also changes in the levels of expression, turn-over and function of the dihydropyridine receptor and calcium pump protein that play key roles in regulating cytosolic calcium levels (Renganathan *et al.*, 1997; Ferrington *et al.*, 1998). Those changes

in channel function are associated with elevations in resting calcium levels in senescent skeletal muscle (Frayssé *et al.*, 2006) that further perturb homeostasis, in part by increasing activation levels of calcium-dependent enzymes.

Most muscle mass loss during aging reflects the loss of myofibril proteins (Evans, 1997) that comprise nearly 80% of muscle cell volume. Thus, myofibril protein cleavage is an important component of muscle wasting. The ubiquitin/proteasome proteolytic system has been implicated in virtually all models of muscle wasting, including muscle unloading, sepsis and muscular dystrophies, suggesting that the ubiquitin/proteasome system would be involved in sarcopenia. The finding that expression of the proteasome 26S subunit increases in aging muscle (Altun *et al.*, 2010) supports that expectation. However, the ubiquitin/proteasome system does not cleave myofilaments in intact myofibrils indicating that there is an initial proteolytic modification of myofilaments by some other proteolytic system (Solomon & Goldberg, 1996).

Several findings suggest that calcium-dependent proteases (calpains) can cause initial proteolytic modification of some myofibrillar proteins at the onset of myofibril breakdown (Wei *et al.*, 2004). First, calpains cleave substrate molecules at a small number of specific sites (Goll *et al.*, 2003), so that proteolysis by calpains may be sufficient for targeting fragments to the ubiquitin/proteasome system. Also, muscle fiber bundles treated with purified calpain showed disrupted Z-disks and M-lines in myofibrils, although no degradation of thin or thick filaments was apparent (Dayton *et al.*, 1976). Similarly, myofibrils treated with calpains showed loss of  $\alpha$ -actinin, a major structural protein of Z-disks, but no effect on myosin heavy chain (MyHC) (Reddy *et al.*, 1975). Calpains also degrade regulatory proteins that are components of thin filaments. In particular, troponin-T (TnT) and troponin-I (TnI) are calpain substrates (Reddy *et al.*, 1975; Ishiura *et al.*, 1979). However, the initial proteolytic modification of thick filaments, the most massive component of myofibrils, that leads to their loss in muscle wasting is unknown, unless the myofibrils are first subjected to oxidative modification. Recent findings show that the susceptibility of myofibrillar proteins to calpain-mediated cleavage is significantly influenced by their prior oxidative modification. Exposure of myofibrils to hydrogen peroxide increases susceptibility of MyHC, actin, TnI and  $\alpha$ -actinin to cleavage by calpains (Smuder *et al.*, 2010), which may be important in aging muscle in which there is increased oxidative stress.

Several mechanisms regulating calpain activity *in vivo* can influence proteolysis during muscle wasting. For example, expression of calpains-1/2 is elevated in muscle during sepsis (Williams *et al.*, 1999) and reduction of cytosolic calcium concentrations decreases proteolysis in septic muscle (Hotchkiss & Karl, 1994). Calpain activity *in vivo* may also be modulated by changes in the expression or activity of calpastatin, the endogenous inhibitor of calpains-1/2. For example, reductions in calpastatin activity, but not calpastatin concentration, occur during muscle wasting in sepsis (Wei *et al.*, 2004). The increase in calcium-dependent proteolysis in the muscles of tumor-bearing rodents also relates to decreased calpastatin expression, rather than changes in calpain (Costelli *et al.*, 2001).

Calpain-2 activity is also modulated by nitric oxide (NO) binding to cysteine in the catalytic domain of the protease, through a process called S-nitrosylation, which can influence enzyme activity. S-nitrosylation of

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cysteine(s) in calpain-2 inhibits proteolysis of protein in purified protein assays and treatment of muscle cells with NO donors prior to calpain activation prevents the formation of calpain specific cleavage products *in vitro* (Koh & Tidball, 2000). However, whether S-nitrosylation of calpains occurs *in vivo* or whether changes in calpain S-nitrosylation are a significant component in sarcopenia or other muscle diseases is unknown.

Recent work has also shown that activation of caspase-3, a cysteine protease, can promote calpain activation in muscle and that calpain and caspase-3 act in series to contribute to diaphragm atrophy during mechanical ventilation (Nelson *et al.*, 2012). However, calpain-mediated and caspase-3-mediated muscle wasting also occurs in diaphragms during sepsis, but the two proteolytic systems then operate independently and in parallel (Supinski *et al.*, 2009). Thus, although caspase-3 activation may provide an additional mechanism to regulate calpain activity *in vivo*, the role of caspase-3 in calpain-mediated muscle wasting varies with the conditions under which wasting occurs.

In this investigation, we tested whether modulation of calpain activity in muscle *in vivo* affects sarcopenia by assaying the effects of muscle-specific overexpression of calpastatin on sarcopenia. We then tested whether S-nitrosylation of calpains influences their proteolysis of specific structural and regulatory proteins in myofibrils. Finally, we tested whether changes in calpain S-nitrosylation occur during muscle aging *in vivo* and investigated whether modulation of nNOS expression *in vivo* influenced calpain S-nitrosylation in aging muscle and thereby slowed sarcopenia. Collectively, our findings provide new insights into the molecular mechanisms that underlie sarcopenia and suggest potential strategies for slowing age-related muscle wasting.

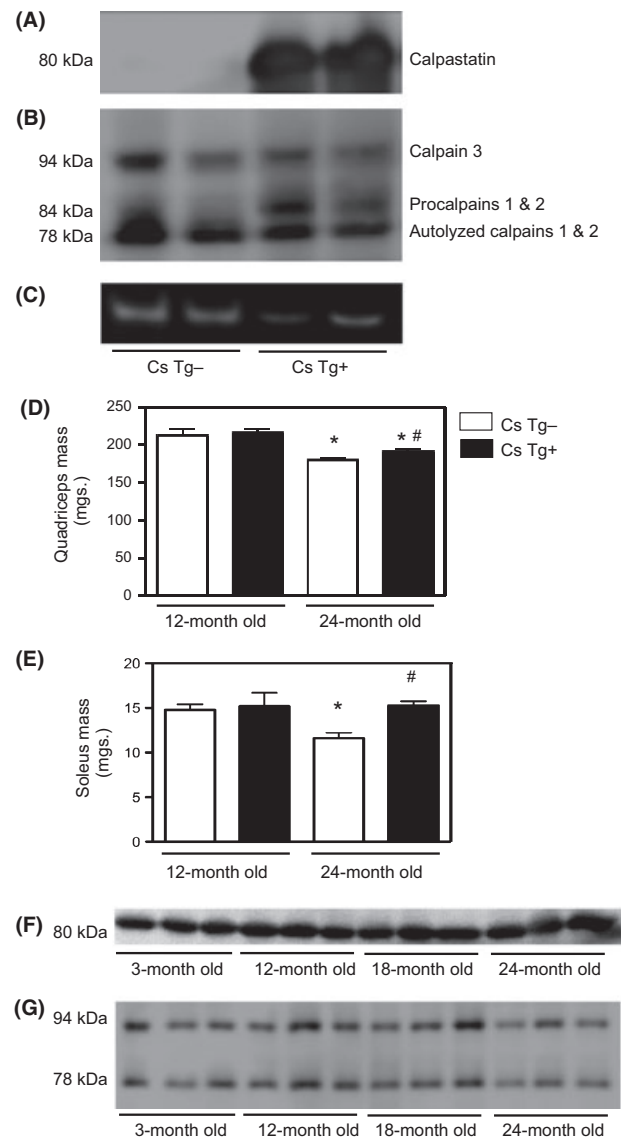
## Results

### Calpastatin over-expression inhibits calpain activity in aging skeletal muscle

Expression of a muscle-specific, calpastatin transgene caused more than a 50-fold increase in calpastatin concentration in skeletal muscles of 2-year-old mice (Fig. 1A). Transgene expression did not significantly affect the expression of calpain-3, apparent at 94 kDa in Western blots, or affect the concentration of autolyzed calpains-1 and 2 in skeletal muscle, apparent at 78 kDa (Fig. 1B). However, the quantity of inactive, unautolyzed, procalpains-1 and -2 at 84 kDa was greater in calpastatin transgenic muscle samples (Fig. 1B), indicating that elevation in calpastatin inhibited the autolytic protease activities of calpain in aging muscle. Furthermore, zymograms showed that cleavage of substrate by calpains was reduced in muscle extracts from calpastatin transgenic mice compared to wild-type (WT) mice, further substantiating that the increase in calpastatin reduced protease activity in old muscle (Fig. 1C).

### Calpastatin overexpression slows sarcopenia

Muscle mass decreases that occurred in quadriceps, which exclusively expresses MyHC-2, and soleus, which expresses both MyHC-1 and MyHC-2, were reduced in calpastatin transgenic mice, indicating that calpain-mediated proteolysis contributes to sarcopenia (Fig. 1D,E). However, muscle aging did not increase the concentration of calpains or calpastatin in muscle, indicating that age-related increases in calpain-mediated proteolysis were not attributable to changes in calpain-1/2 or calpastatin expression (Fig. 1F,G).



**Fig. 1** Increases in calpain activity but not expression contribute to sarcopenia. (A) Western blot of extracts of quadriceps from 2-year-old mice [WT (CsTg-); calpastatin transgenic (CsTg+)] that was probed with anti-calpastatin; (B) Western blot of extracts of quadriceps from 2-year-old, CsTg- or CsTg+ mice that was probed with anti-calpain. The increased proportion of unautolyzed, pro-calpain at 84 kDa in CsTg+ mice reflects calpain inhibition attributable to elevations of calpastatin in CsTg+ muscles. (C) Zymogram of extracts from quadriceps from 2-year-old, CsTg- or CsTg+ mice showing the reduction of substrate cleavage in the gel at 78 kDa, reflecting the decrease in calpain activity in CsTg+ muscles. D and E. Decreases in muscle mass in wild-type quadriceps (D) and soleus (E) between 12-months and 24-months of age were reduced by calpastatin transgene expression. \*Indicates significantly different from 12-month-old muscle of same genotype.  $P < 0.05$ .  $n = 6$ . #Indicates significantly different from WT muscle at same age.  $P < 0.05$ .  $n = 6$ . (F) Calpastatin Western blot shows there is no significant change in calpastatin expression in WT quadriceps during aging. (G) Calpain Western blot shows that there is no significant change in calpain expression in WT muscle during aging.

### nNOS expression in skeletal muscle declines with aging

Because increases in calpain-mediated proteolysis in aging muscle were not attributable to changes in the expression of calpains or calpastatin,

we tested whether there were age-related changes in the expression of other enzymes that could regulate the calpain activity. Previous studies showed that NO can inhibit calpain activity, leading us to test whether nNOS expression changed in old muscle. Quantitative PCR (QPCR) data for transcript levels of nNOS $\alpha$ , which is expressed in nerve and muscle cells, and nNOS $\mu$ , which is a muscle-specific isoform, show large decreases in expression in skeletal muscles between 1- and 2 years of age (Fig. 2A,B). In quadriceps, expression levels of total nNOS declined significantly ( $P < 0.05$ ) by 37% during this period, while nNOS $\mu$  declined by 41%. In soleus, the decline in nNOS expression was also significant ( $P < 0.05$ ) and even greater. Expression of nNOS $\alpha$  decreased by 78%, and nNOS $\mu$  decreased by 97%. Western blots of whole muscle homogenates also show similarly large decreases in nNOS protein levels during muscle aging (Fig. 2C).

### nNOS overexpression slows sarcopenia

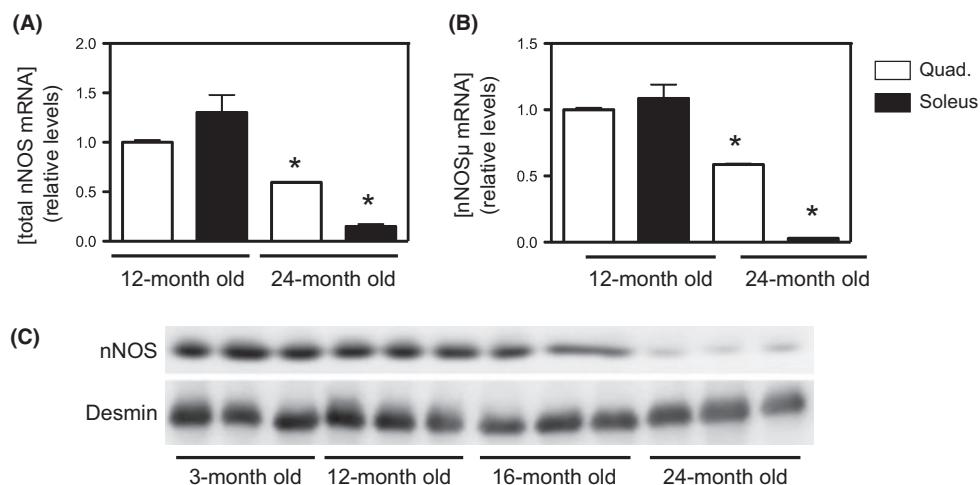
Because our findings showed that nNOS expression in skeletal muscle declined dramatically as muscle aged and that calpain-mediated proteolysis contributed significantly to sarcopenia and because NO is a negative regulator of calpain activity, we tested whether overexpression of nNOS in muscle slowed sarcopenia. We confirmed by Western blots that a muscle-specific, nNOS transgene continued to be expressed at high levels in old muscle (Fig. 3A). Expression of the nNOS transgene provided significant ( $P < 0.05$ ) protection against muscle mass loss between the ages of 12- and 24 months. Although WT quadriceps mass declined by 17% during this period, there was no significant mass lost in quadriceps of nNOS Tg+ mice (Fig. 3B). An even more prominent effect of transgene expression in soleus occurred, in which wild-type muscles lost 22% of mass, while no significant mass loss occurred in nNOS Tg+ soleus (Fig. 3C). This protective effect of the transgene was also observed in assays of fiber cross-sectional area (Fig. 3D) where cross-sectional area of muscle fibers in wild-type soleus decreased significantly ( $P < 0.05$ ) by 25%, while no change occurred in cross-section of nNOS Tg+ fibers during this period (Fig. 3E). Our findings indicate that the differences in muscle mass between 24-month-old WT and nNOS Tg+ mice was primarily attributable to differences in fiber size, not fiber number. The number of fibers per mid-belly muscle cross-section did not differ between WT and nNOS Tg+ soleus (Fig. 3F). Furthermore, nNOS transgene expression had no effect on the relative proportion of type 1 and type 2 muscle fibers in soleus (Fig. 3G).

### Caspase activation increases in aging muscles

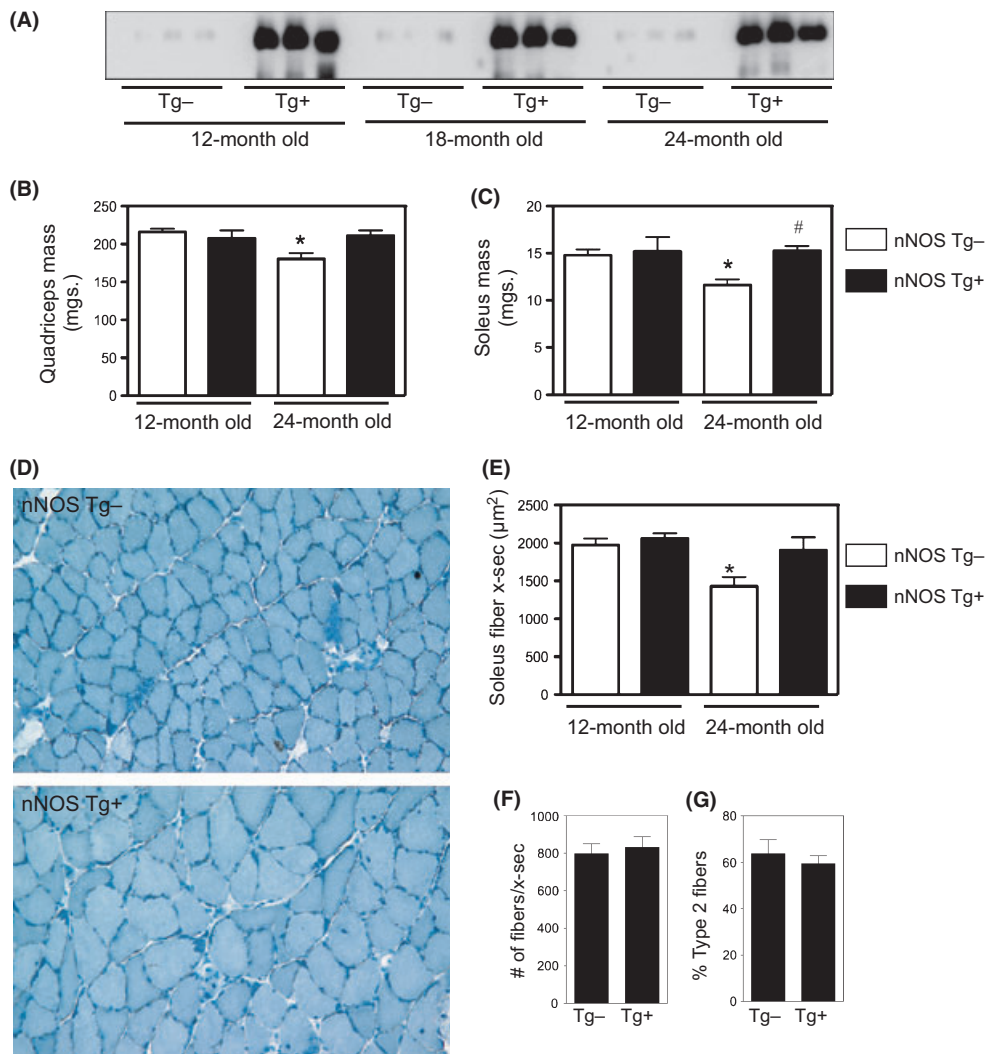
Recent findings have shown that in at least some models of muscle wasting, caspase-3 activation can increase calpain activation during muscle atrophy (Nelson *et al.*, 2012). We tested whether caspase-3 activation increased during sarcopenia by assaying for shifts between the proportion of muscle caspase-3 that was present as inactive procaspase-3 (32 kDa) versus the activated form, reflected by the quantity of the 17 kDa subunit present in the activated form. Western blotting showed a dramatic reduction of the proenzyme accompanied by a large increase in the active form in senescent muscle (Fig. S1), showing that caspase-3 activation increases during sarcopenia.

### Calpain cleaves MyHC-2b and other structural and regulatory proteins in intact myofibrils

Myofibrillar proteins comprise nearly 80% of muscle cell volume and the decline in muscle mass during aging largely reflects their loss. Thus, their initial proteolytic modification may provide an important control point in determining whether there is a loss of functional muscle mass. We assayed whether calpain cleaves proteins in intact myofibrils. Peptide maps of the calpain digests were performed by SDS-PAGE and revealed prominent calpain substrates at 235, 200, 190, 160, 55, 31 and 23 kDa. Prominent proteolytic fragments were identified at 135 and 80 kDa, with less prominent fragments at 145, 141, 110, 103, 96 and 65 kDa (Fig. 4A). Western blotting was used to test the identity of many of the substrates and proteolytic fragments and revealed that MyHC (200 kDa), desmin (55 kDa), TnT (31 kDa) and TnI (23 kDa) were major substrates for calpain in intact myofibrils (Fig. 4B). Major proteolytic fragments were identified at both 135 kDa and 80 kDa as MyHC-2b. The putative fragment observed at 75 kDa was identified as the exogenous calpain that was added to the myofibril preparation. No proteolysis of titin, actin or tropomyosin (Tm) was observed (data not shown). Interestingly, our findings show that calpain digests of myofibrils produced a small (circa 2 kDa) reduction in  $\alpha$ -actinin mass (Fig. 4B). Collectively, these findings indicate that the major structures in myofibrils that are disrupted by calpain-mediated proteolysis are thick filaments (MyHC), the intermediate filament scaffold (desmin) and the troponin complex (TnT and TnI) and that proteolytic modification of  $\alpha$ -actinin could disrupt interactions between thin filaments and the Z-disk. Thus, calpain-mediated proteolysis of myofibrillar components could contribute importantly to the loss of skeletal muscle mass and



**Fig. 2** Neuronal nitric oxide synthase expression and concentration declines in aging muscle. (A and B) QPCR results of total nNOS (A) and nNOS $\mu$  (B) in WT quadriceps and soleus show large reductions in nNOS during aging. Expression level in 12-month quadriceps was set at 1.0 unit. \*Indicates significantly different from 12-month-old muscle.  $P < 0.05$ ,  $n = 5$ . (C) nNOS Western blot shows that nNOS concentration in WT quadriceps decreases during aging. The same blot was reprobbed with anti-desmin to confirm equal loading of lanes.



**Fig. 3** Increased nNOS expression in muscle reduces sarcopenia. (A) Western blot of extracts of quadriceps from WT (Tg-) or nNOS transgenic (Tg+) mice was probed with anti-nNOS and confirms that high levels of nNOS expression persist in the muscle of transgenic mice at 2 years. (B and C) Decreases in muscle mass in wild-type quadriceps (B) and soleus (C) between 12-months and 24-months of age were prevented by nNOS transgene expression. \*Indicates significantly different from 12-month-old muscle of same genotype.  $P < 0.05$ .  $n = 6$ . #Indicates significantly different from WT muscle at same age.  $P < 0.05$ .  $n = 6$ . (D) Cross-sections of soleus from WT (nNOS Tg-) or nNOS transgenic (nNOS Tg+) muscles from 2-year-old mice. The fiber size in nNOS Tg+ samples is greater than in nNOS Tg- samples. Bar = 50 μm. (E) Measurements of fiber cross-sectional areas of every fiber in entire cross-sections of nNOS Tg- and nNOS Tg+ soleus show that the age-related decline in fiber size that occurred in WT mice was prevented by transgene expression. \*Indicates significantly different from 12-month-old muscle of same genotype.  $P < 0.05$ .  $n = 6$ . (F and G) The number of fibers (F) and the proportion of fibers that expressed MyHC-2 (G) were counted for entire, cross-sections of 2-year-old mice. nNOS transgene expression did not affect number of fibers or their contractile phenotype in aged mice.

function during aging, if muscle calpain activity were elevated during senescence.

### Calpain preferentially cleaves MyHC-2b in the head and mid-rod regions in intact myofibrils

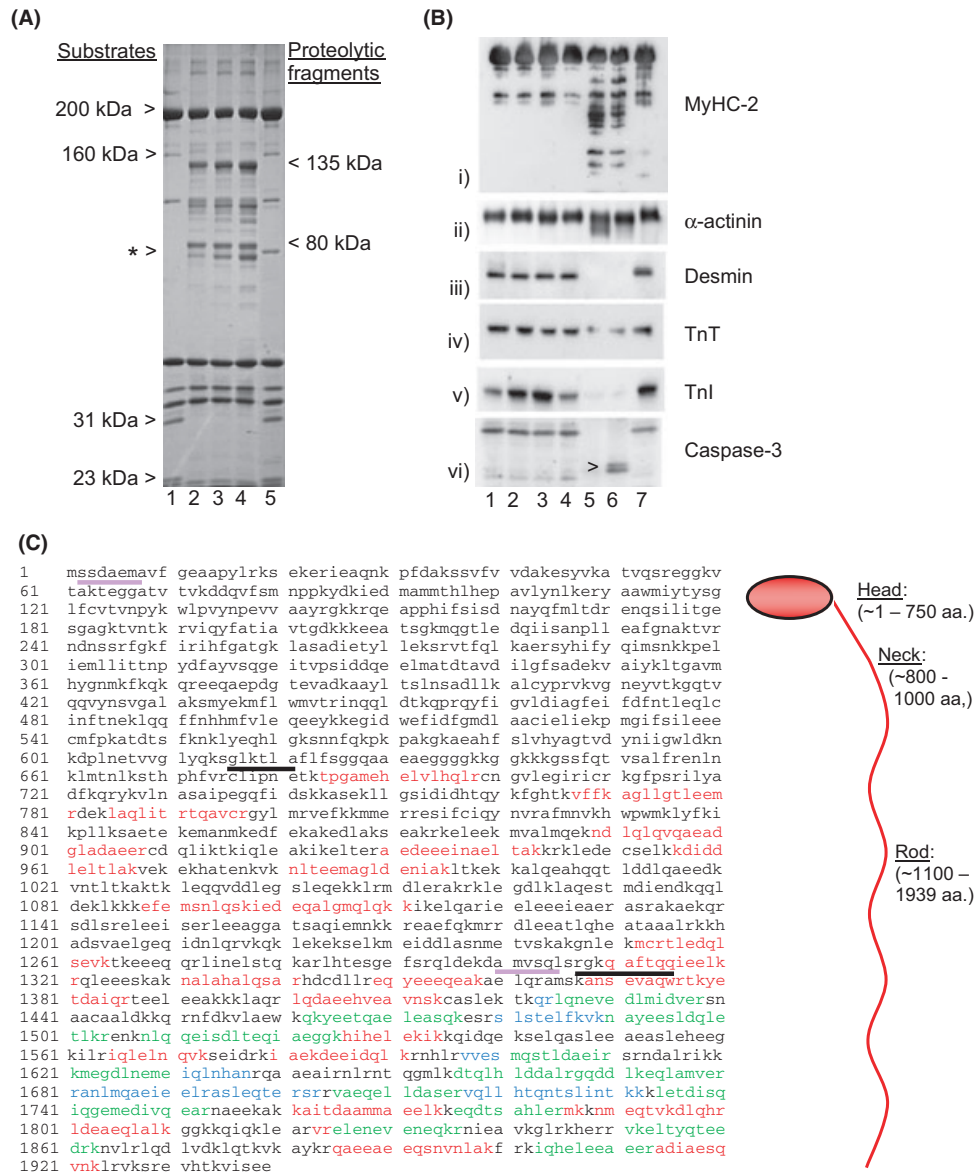
The finding that MyHC-2 is a substrate in myofibrils for calpain proteolysis led us to assay the specific sites of MyHC-2 cleavage. Mass spectrographic data of tryptic fragments of the 135 kDa and 80 kDa MyHC polypeptides provided high confidence matches with MyHC-2b, indicating preferential cleavage of this MyHC isoform by calpain. By mapping the tryptic fragments onto the full-length MyHC-2b sequence, we found that these fragments were formed by MyHC cleavage in the head region and mid-rod region of the MyHC-2b molecule (Fig. 4C). N-terminal sequencing of both the 135 kDa and 80 kDa fragments by Edman degradation

identified the specific cleavage site for the 80 kDa fragment at R(1308) and the 135 kDa fragment at G(616) (Fig. 4C). These data show that calpain cleavage at G(616) will proteolyse MyHC specifically in the myosin motor domain, proximal to the ATP-binding sites.

### Caspase-3 is a component of myofibrils that influences calpain-mediated cleavage of myofibrillar proteins

Because we unexpectedly observed that caspase-3 is present in purified myofibril preparations (Fig. 4B), we assayed whether any of the proteolytic modifications that we observed in peptide maps of calpain-treated myofibrils were influenced by the activation of myofibrillar caspase-3. As shown in Fig. 4B, caspase-3 inhibition produced no detectable difference in calpain-mediated proteolysis of desmin, TnT or Tnl. However, caspase-3 inhibition decreased calpain-2 cleavage of

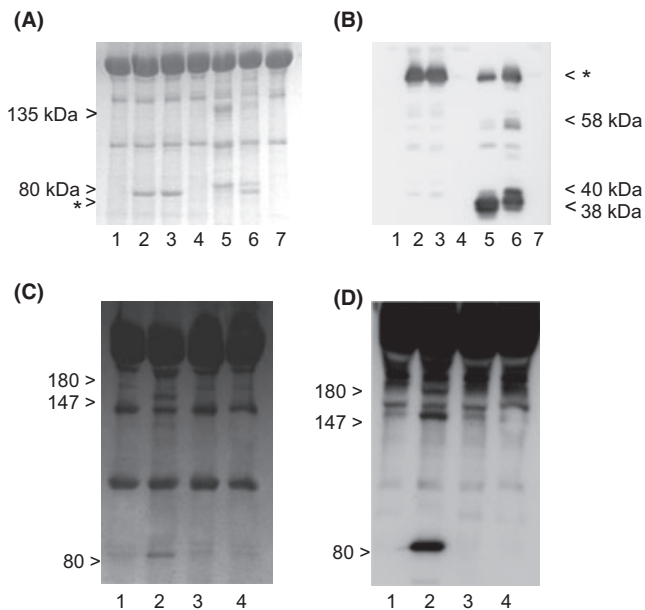




**Fig. 4** Structural and regulatory proteins in myofibrils are cleaved by calpain-2. (A) Myofibrils purified from WT, mouse muscles were subjected to calpain-2 proteolytic or control conditions and the samples separated by SDS-PAGE and stained with Coomassie blue. Treatment conditions were as follows: Myofibrils + calcium (lane 1). Myofibrils + calcium + 0.3 U calpain-2 (lane 2). Myofibrils + calcium + 0.5 U calpain-2 (lane 3). Myofibrils + calcium + 1.0 U calpain-2 (lane 4). Myofibrils + EDTA (lane 5). \*Indicates exogenous calpain-2 added to the preparation. Masses of proteolytic substrates identified in the treatment are listed on the left. Masses of proteolytic fragments are listed on the right. (B) Myofibrils purified from WT, mouse muscles were subjected to calpain-2 proteolytic or control conditions and then analyzed by Western blots using the antibodies to: (i) MHC-2b, (ii)  $\alpha$ -actinin, (iii) desmin, (iv) TnT, (v) TnI, (vi) caspase-3. Treatment conditions were as follows: Myofibrils + EDTA (lane 1). Myofibrils + EDTA + 0.5 U calpain-2 (lane 2). Myofibrils + EDTA + 0.5 U calpain-2 + Ac-DEVD-CHO (a caspase-3 specific inhibitor) (lane 3). Myofibrils + EDTA + Ac-DEVD-CHO (lane 4). Myofibrils + calcium + 0.5 U calpain-2 (lane 5). Myofibrils + calcium + 0.5 U calpain-2 + Ac-DEVD-CHO (lane 6). Myofibrils + calcium + Ac-DEVD-CHO (lane 7). The symbol ">" than indicates 28 kDa fragment of caspase-3. (C) Full-length amino acid sequence for MHC-2b showing map of tryptic fragments of calpain-2 cleavage products from myofibril digests. Red peptides: tryptic fragments that were identified by mass spectroscopy in the 135 kDa fragment of myofibrils treated with calpain-2. Blue peptides: tryptic fragments identified by mass spectroscopy in the 80 kDa fragment obtained in calpain-2 digests. Green oligopeptides: tryptic fragments identified in both the 135 kDa and 80 kDa fragments. Black underlined sequences were identified by Edman degradation and sequencing as the N-termini of the 135 kDa fragment (GLKTLA) and the 80 kDa fragment (RGKQAFATQ) produced by calpain-2 treatments. Lavender underlined sequences were identified by Edman degradation and sequencing as the N-termini of the 147 kDa fragment (SSDAEMA) and 80 kDa fragment (AMVSQL) produced by caspase-3 treatments.

$\alpha$ -actinin and MyHC-2 in myofibrils (Fig. 4B). This indicates that myofibrillar caspase-3 activation can increase calpain activity and calpain-mediated proteolysis of myofibrillar MyHC-2 and  $\alpha$ -actinin. Further evidence that myofibrillar caspase-3 can increase calpain activity was provided by the observation that caspase-3 inhibition reduces calpain autolysis (Fig. 5A) and affected the mass of proteolytic

fragments of calpain-2 formed during its autolysis; calpain-2 immunoblots of calpain-2 digests of myofibrils in the presence of caspase-3 inhibitor yielded a 40 kDa fragment of calpain-2 that was not present in the absence of caspase-3 inhibitor (Fig. 5B). These observations further substantiate that these proteolytic systems interact in processing myofibrillar proteins. Our findings also show that presence of caspase



**Fig. 5** Calpain-2 and caspase-3 interact in cleaving myofibrillar proteins. (A and B) Myofibrils from WT, mouse muscles were subjected to calpain-2 proteolytic or control conditions. The treatment conditions were as follows: Myofibrils + EDTA (lane 1). Myofibrils + EDTA + 0.5 U calpain-2 (lane 2). Myofibrils + EDTA + 0.5 U calpain-2 + Ac-DEVD-CHO (lane 3). Myofibrils + EDTA + Ac-DEVD-CHO (lane 4). Myofibrils + Ca + 0.5 U calpain-2 (lane 5). Myofibrils + Ca + 0.5 U calpain-2 + Ac-DEVD-CHO (lane 6). Myofibrils + Ca + Ac-DEVD-CHO (lane 7). Note that the density of the 135 kDa band in Lane 5 is greater than in Lane 6, indicating that addition of caspase inhibitor decreases MHC cleavage to a 135 kDa fragment by calpain-2. Also note that the 75 kDa exogenous calpain-2 (indicated by \*) that is present in Lanes 2 and 3 is absent in Lane 5, indicating autolysis in the presence of calcium. However, addition of caspase inhibitor to the preparation in lane 6, protects the 75 kDa exogenous calpain-2. The 80 kDa band coincides with the MyHC-2b fragment produced by calpain cleavage. (B) Myofibrils were subjected to the same treatments as in (A), but the samples were used for Western blotting with antibodies to calpain. Note the presence of exogenous, calpain-2 in Lanes 2, 3, 5 and 6 (indicated by \*). Note also that the density of the exogenous calpain-2 band is less in Lane 5, reflecting autolysis. However, the quantity of 75 kDa calpain-2 is greater in Lane 6 than Lane 5, indicating that caspase inhibitor reduces calpain-2 autolysis. Note also that Lane 6 shows a 40 kDa calpain fragment that is not present in Lane 5, suggesting that caspase-3 inhibition influences the site of calpain autolysis. (C) Coomassie blue stained gel of myofibrils subjected to caspase-3 treatment or control conditions. Treatment conditions were: Myofibrils (lane 1). Myofibrils + 52 000 U mL<sup>-1</sup> of caspase-3 (lane 2). Myofibrils + 50  $\mu$ M caspase-3 inhibitor (lane 3). Myofibrils + caspase-3 + caspase-3 inhibitor (lane 4). (D) Western blot using anti-MyHC-2b on myofibril preparations treated identically as (C). Note that the prominent, proteolytic fragments appearing in lane 2 of (C) coincide with anti-MyHC-2b reactive bands in (D) at 180, 147 and 80 kDa. The identity of the caspase-3-generated proteolytic fragments as cleavage products of MyHC-2b was confirmed by mass spectroscopy.

inhibitor affects calpain-mediated cleavage of caspase-3. Myofibrils subjected to calpain digestion in the presence of Ac-DEVD-CHO yielded a 28 kDa caspase-3 fragment that was not present in calpain digests performed in the absence of caspase-3 inhibitor (Fig. 4B). This suggests that the interaction of caspase-3 with caspase-3 inhibitor protects a proteolytic site from calpain or that caspase-3 undergoes autolysis after activation by calpain-2.

#### Caspase-3 cleaves myofibrillar MyHC-2b

The finding that the extent of myofibrillar MyHC-2b cleavage to 80 kDa and 135 kDa fragments by calpain-2 was decreased by caspase-3

inhibition suggested that caspase-3 lysis of calpain-2 increased its activation and thereby increased MyHC-2b cleavage. However, an alternative explanation would be that caspase-3 acts directly on MyHC-2b, to produce proteolytic fragments of the same mass as those produced by calpain cleavage. We tested this by incubating myofibrils with caspase-3 in the presence or absence of caspase-3 inhibitor. Peptide maps showed that caspase-3 proteolysis of myofibrillar proteins produced proteolytic fragments at 80 kDa, 147 kDa and 180 kDa (Fig. 5C). Western blots confirmed that caspase-3 cleaved MyHC-2 to 80 kDa, 147 kDa and 180 kDa fragments (Fig. 5D). In addition, mass spectroscopy data showed that these fragments were high probability matches to MyHC-2b, suggesting that the 80 kDa fragment that was generated by calpain-2 treatments of myofibrils could be a fragment of MyHC-2b that was produced by myofibrillar caspase-3 that was activated by exogenous calpain-2 in experimental treatments. However, N-terminal sequencing data obtained by Edman degradation of the 80 kDa fragment produced by caspase-3 treatment of myofibrils showed that the cleavage site was distinct from the cleavage site at which calpain-2 acted to produce the 80 kDa fragments. However, the cleavage sites for the two proteases were immediately adjacent to one another (Fig. 4C).

#### Nitric oxide S-nitrosylates calpain-2 and caspase-3 and inhibits proteolysis of myofibrillar MyHC-2b

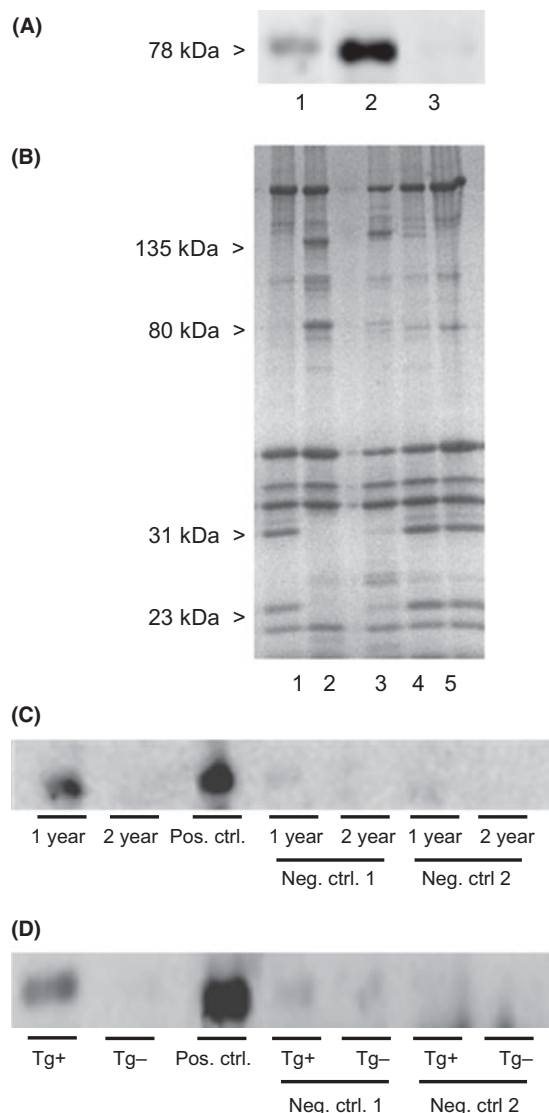
Because NO can S-nitrosylate calpain-2 and caspase-3 (Rossig *et al.*, 1999; Maejima *et al.*, 2005) and our findings show that these proteases cleave myofibrillar MyHC-2b, we assayed whether NO decreased myofibril proteolysis by either calpain-2 or caspase-3. First, the biotin-switch assay was used to show that NO generated by NO donors produced S-nitrosylated calpain-2 and caspase-3 (Fig. 6A; Fig. S1B). Assays were then performed to test whether NO donors decreased proteolysis of myofibrillar proteins. Peptide maps showed that the presence of NO donors during calpain-2 treatments of myofibrils prevented the proteolysis of TnT or TnI and prevented the cleavage of MyHC-2b to its 80 kDa or 135 kDa fragments (Fig. 6B). Similarly, the presence of NO donors during caspase-3 treatments of myofibrils prevented proteolysis of MyHC-2b to its 80 kDa, 147 kDa and 180 kDa fragments (Fig. S1C).

#### S-nitrosylation of calpain in muscle declines during aging

The findings that nNOS levels decline in aging muscle and that calpain S-nitrosylation inhibits its activity and that calpain-mediated cleavage of muscle proteins contributes to mass loss in aging muscle supported the hypothesis that the loss of muscle nNOS in aging could lead to a decrease in calpain S-nitrosylation *in vivo*. We tested this possibility in biotin-switch assays of quadriceps from 1-year-old and 2-year-old mice. Although detectable levels of S-nitrosylated calpain were observed in extracts of 1-year-old muscle, no S-nitrosylated calpain was identified in 2-year-old muscle (Fig. 6C), suggesting that S-nitrosylation may be a functionally significant regulator of calpain activity in muscle *in vivo*.

#### nNOS transgene expression in muscle increases calpain S-nitrosylation in aged skeletal muscle

Because our findings support the hypothesis that loss of nNOS from senescent muscle reduces inhibition of calpain activity and thereby increases muscle wasting, we tested whether the prevention of muscle wasting in old, nNOS Tg+ muscles was accompanied by increased calpain S-nitrosylation. Using the biotin-switch assay, we found that nNOS transgene expression in 2-year-old mouse quadriceps increased



**Fig. 6** S-nitrosylation of calpain inhibits cleavage of myofibrillar proteins and nNOS transgene expression restores calpain S-nitrosylation in aging muscle *in vivo*. (A) Anti-calpain Western blot of calpain-2 subjected to treatment with NO donor followed by biotin switch to isolate S-nitrosylated calpain-2. Lane 1: calpain-2 from a preparation that was not exposed to NO donor. Lane 2: myofibrils + calcium + calpain. Lane 3: myofibrils + calcium + calpain + 2 mM SNAP. Lane 4: myofibrils + calcium + calpain + 5 mM SNAP. Lane 5: myofibrils + calcium + calpain + 5 mM SNAP + calpain-inhibitor-1. (C) Anti-calpain blot of S-nitrosylated proteins isolated from muscle extracts by the biotin switch. Muscle extract from 1-year-old mice (1 year.) contained S-nitrosylated calpain, but no S-nitrosylated calpain was detectable in muscle extracts from 2-years-old mice (2 year). Positive control samples (Pos. ctrl.) were extracts that were treated with SNP before the biotin switch. The first set of negative controls (Neg. ctrl. 1) included identical samples from 1-year-old mice (1 year.) or 2-years-old mice (2 year.) used in the biotin switch, but sodium ascorbate was eliminated from assay. The second set of negative controls (Neg. ctrl. 2) used the same samples, but biotin-HPDP was eliminated from the assay. (D) Anti-calpain blot of S-nitrosylated proteins isolated from muscle extracts from 2-years-old, nNOS transgenic mice (Tg+) or 2-years-old, WT mice (Tg-) showing that expression of the nNOS transgene caused S-nitrosylation of calpain in muscle *in vivo*. Control preparations were performed identically to those shown in Fig. 6C.

S-nitrosylation of calpain (Fig. 6D). In contrast, caspase-3 S-nitrosylation was not detectable in muscle extracts from either WT or nNOS transgenic mice at either 1 year or 2 years of age (data not shown).

## Discussion

Our findings show that calpain inhibition slows sarcopenia and decreases the proteolysis of myofibril-associated structural and regulatory proteins. Further, we show that increased expression of calpastatin or nNOS greatly reduces sarcopenia and provide the first evidence of an *in vivo*, age-related shift in S-nitrosylation of a regulatory protein, calpain, that appears to contribute significantly to the senescence-related loss of tissue. These findings indicate that previous reports of an increase in calcium-dependent proteolytic activity in aging muscle (Dargelos *et al.*, 2007) could reflect reductions of calpain S-nitrosylation that were secondary to reductions in nNOS expression. Collectively, these findings reveal a new mechanism that underlies muscle wasting during old age and suggest that therapeutic interventions that target S-nitrosylation of skeletal muscle calpain could slow sarcopenia.

Other findings suggest that NO mediated signaling that does not involve calpain inhibition is important in affecting wasting of non-senescent muscle during disuse atrophy, cancer or denervation. Paradoxically, NO production by muscle increases during muscle disuse or following denervation (Suzuki *et al.*, 2007), despite reductions in nNOS concentration (Nguyen & Tidball, 2003; Suzuki *et al.*, 2007). Perhaps, the elevation in NO production when nNOS concentration is reduced reflects the higher cytosolic calcium concentrations in disuse-atrophied muscles (Ingalls *et al.*, 1999), which would increase nNOS activity. Inhibition of nNOS in animals experiencing disuse atrophy slows atrophy and prevents increases in the expression of transcripts involved in muscle atrophy, such as Foxo3a, MuRF1 and atrogin-1 (Suzuki *et al.*, 2007). Those findings indicate that in the disuse model, NO promotes atrophy by driving the expression of proteins associated with atrophy rather than inhibiting atrophy through calpain S-nitrosylation. Conversely, NO production can also influence muscle mass by positively affecting expression of myofibrillar proteins in muscle experiencing increased loading. For example, rapid increases in muscle mass and elevated expression of MyHC that normally occur during increased muscle loading are significantly reduced in animals that were treated with NOS inhibitors during the period of increased loading (Sellman *et al.*, 2006). Thus, elevations in NO production in muscles experiencing modified loading can promote either wasting or growth, at least in non-senescent muscles.

Loss of nNOS from aging skeletal muscle may contribute to muscle dysfunction through numerous mechanisms that are independent of NO modulation of muscle atrophy through calpain-dependent or independent mechanisms. For example, aging muscle shows defects in synaptic structure at neuromuscular junctions (NMJs) (Gutmann & Hanzlikova, 1965) and loss of nNOS in skeletal muscle is associated with structural defects at NMJs (Shiao *et al.*, 2004). In addition, aging skeletal muscle in rodents experiences increases in the numbers of inflammatory cells following injury (Ghaly & Marsh, 2010) and loss of nNOS from dystrophic skeletal muscle causes increases in muscle inflammation (Wehling *et al.*, 2001). Thus, the loss of nNOS from aging muscle may contribute to muscle dysfunction through multiple mechanisms, in addition to the effects on muscle wasting that are explored in the present study.

We were struck by the substrate preference of calpain-2 for MyHC-2b over other MyHC isoforms and expected that the preferential cleavage of the MyHC-2b could reflect differences in the primary structure of MyHC isoforms at the cleavage sites. However, homology searches of the

peptide sequences neighboring the cleavage sites showed little difference in primary structure between MyHC-2b and other muscle MyHC isoforms at the cleavage sites. Furthermore, predictions of calpain cleavage sites using a computational program (Liu *et al.*, 2011) scored the calpain cleavage sites of MyHC-2b that we identified empirically as similar to homologous domains of other striated muscle MyHC isoforms. Thus, we anticipate that the preferential cleavage of MyHC-2b in intact myofibrils reflects an isoform specific availability of the cleavage site conferred by secondary structure or by the association with binding partners in the myofibrils. We also speculated that the preferential cleavage of MyHC-2b by calpain-2 could underlie a more avid breakdown of proteins specific to the type 2 fiber phenotype, in view of previous findings reporting that type 2 muscle fibers that express MyHC-2 are preferentially lost during sarcopenia (Holloszy *et al.*, 1991). However, our data do not provide strong support for that interpretation. First, the protective effect of the calpastatin transgene on muscle wasting in the quadriceps, which is entirely type 2 fibers, was similar to the protective effect in the soleus, which is approximately 50% type 1 and 50% type 2. Likewise, there was greater protection by nNOS transgene expression in soleus versus quadriceps rather than a preferential protection of quadriceps mass. Thus, the mechanism that underlies the preferential loss of size of type 2 fibers in sarcopenia remains unknown.

Our finding that caspase-3 inhibition reduces calpain-2-mediated cleavage of myofibrillar proteins agrees with recent work showing that caspase-3 and calpains act in series to promote muscle atrophy, in at least some instances of wasting (Nelson *et al.*, 2012). In particular, we learned that caspase-3 inhibition decreased calpain-2 cleavage of  $\alpha$ -actinin and MyHC-2 in myofibrils. Previous investigators have shown other interactions between the calpain system and the caspase-3 system. For example, pro-caspase-3 is a calpain substrate, and cleavage of pro-caspase-3 by calpain yields truncation of the N-terminal region to render a modified pro-caspase that is less readily activated (McGinnis *et al.*, 1999). Furthermore, both calpains-1/2 and caspase-3 can cleave calpastatin, so that elevations of calpain or caspase-3 activity can promote calpain activity by decreasing calpastatin-mediated inhibition of calpain (Wang *et al.*, 1998; Goll *et al.*, 2003). However, caspase-3 also functions independently in cleaving myofibrillar proteins. Recombinant caspase-3 cleaves  $\alpha$ -actin to 15, 20 and 30 kDa fragments and cleaves  $\alpha$ -actinin to a 45 kDa fragment (Communal *et al.*, 2002). Caspase-3 can also cleave actin present in actomyosin complexes and caspase-3 cleavage of actin in lysates of muscle cells or whole muscle (Du *et al.*, 2004). In addition, caspase-3 inhibitors can prevent the accumulation of 14 kDa actin fragments and reduce proteolytic rates in muscles excised from diabetic or uremic rats, indicating that caspase-3 activation may be an initial step in myofibrillar protein degradation in some pathological conditions (Du *et al.*, 2004).

Although caspase-3 is able to cleave myofibrillar proteins *in vitro* (Communal *et al.*, 2002; Du *et al.*, 2004) and promote muscle wasting in sepsis *in vivo* (Supinski *et al.*, 2009) without interacting with calpain, our findings indicate that caspase-3-mediated proteolysis is not a major contributor to sarcopenia through processes that are calpain-independent. Despite the large shift in caspase-3 from the proenzyme form to the activated form during sarcopenia, our finding that calpastatin overexpression prevented significant muscle mass loss or fiber size reduction during aging indicates that proteolysis by caspase-3 in the absence of calpain-mediated proteolysis is, at most, as a minor feature in sarcopenia. Similarly, the protective effect of the nNOS transgene on sarcopenia was accompanied by an increase in calpain S-nitrosylation, but there was no detectable caspase-3 S-nitrosylation in nNOS transgenic muscles. Although the effects of

calpastatin or nNOS transgene expression argue against a major role of caspase-3 in sarcopenia that is independent of calpains, we are unable to exclude the possibility that caspase-3-mediated proteolysis acts in series with calpain-mediated proteolysis in driving muscle wasting in sarcopenia.

In conclusion, we believe that the finding that elevated NO production in muscle causes calpain S-nitrosylation and greatly slows sarcopenia may introduce new approaches to reducing sarcopenia based on NO modulation of calpain activity. Fortunately, there are already NO-releasing pharmaceutical agents that have yielded acceptable outcomes in a Phase 1 clinical trial with muscular dystrophy patients (D'Angelo *et al.*, 2012), and perhaps those drugs may also be useful for slowing sarcopenia. However, increased muscle use also provides an immediately available, safe and proven mechanism to increase muscle nNOS expression (Tidball *et al.*, 1998), at least in young animals, that does not require drug interventions and increased muscle use through exercise is currently the most well-recognized, successful and safe intervention to reduce muscle mass loss in aging human populations.

## Experimental procedures

A more detailed account of experimental procedures can be found in the online supplemental information accompanying this article.

### Animal treatments

Experiments involving animals were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

### Myofibril isolation

Myofibrils from mouse hindlimb muscles were purified using a modification of a previous technique (Meng *et al.*, 1996; Data S1).

### Western blots

Muscle homogenates or purified myofibrils were prepared for polyacrylamide gel electrophoresis and Western blotting, using modifications of a previously described technique (Wehling *et al.*, 2001; Data S1).

### Zymograms

Zymograms of calpain activity used a previously described technique (Tidball & Spencer, 2002; Data S1).

### RNA isolation and quantitative PCR

Frozen muscles were homogenized and RNA was isolated, cDNA generated and real-time, quantitative PCR (QPCR) performed as described previously (Villalta *et al.*, 2011). Primers used for QPCR to assay expression levels of reference genes are reported in Table 1.

### Muscle fiber type and size

Soleus muscles frozen in isopentane were cross-sectioned mid-belly, and the sections were stained with hematoxylin or with an antibody to MyHC-2 that binds MyHC 2a and MyHC 2b (clone WB-MHCf). All muscle fibers in each hematoxylin-stained section were counted, and the



**Table 1** Primers used to assay nNOS isoforms and reference genes

Gene	Accession number	5'→3'		Amplicon size (bp)
nNOS (total)	NM_008712	Fwd	CGGGGAGAATGATCGAGTC	139
		Rev	CCTGGAAGGACTGCCATTC	
nNOS $\mu$	S81982	Fwd	CCATGTTGACTCTGAAGCCC	161
		Rev	GCCAGCGGTCCAGTACTTTC	
SRP14	NM_009273.4	Fwd	GAGAGCGAGCAGTTCCTGAC	196
		Rev	CGGTGCTGATCTTCCTTTTC	
RNSP1	NM_001080127.1	Fwd	AGGCTCACCAGGAATGTGAC	196
		Rev	CTTGCCCATCAATTGTCTCT	
		Rev	TACACCAGAAACCTTCCACT	

cross-sectional areas of all fibers were measured using a digital imaging system. The total numbers of MyHC-2-expressing fibers in the cross-section of each muscle were counted microscopically.

### Peptide map of myofibrils

Purified myofibrils were subjected to proteolytic digest with either calpain-2 or caspase-3 (Supporting Methods) and analyzed by SDS-PAGE to identify proteolytic substrates and fragments. In other preparations, myofibrils were incubated with calpain-2 or caspase-3, but an NO donor [sodium nitroprusside (SNP) or S-Nitroso-N-acetylpenicillamine (SNAP); Sigma-Aldrich, St. Louis, MO, USA] was added to assess whether NO affected proteolysis of myofibrils.

### Mass spectroscopy

Calpain-2 or caspase-3 digested myofibrils and their controls were separated by SDS-PAGE, and the gels were stained with Coomassie Blue. Bands that appeared in control lanes but not protease-treated lanes (substrates) or appeared in protease-treated lanes but not in control lanes (fragments) were excised, subjected to trypsinization and analyzed by mass spectroscopy (Data S1). The tryptic fragments of each sample that were identified by mass spectroscopy were mapped onto the amino acid sequence data (obtained through SwissProt) to identify the domains of substrate proteins in myofibrils that were cleaved by calpain-2 or caspase-3.

### N-terminal sequencing

Calpain-2 or caspase-3 digested myofibrils and their controls were electrophoresed by SDS-PAGE and then transferred electrophoretically to polyvinylidene difluoride membranes. Membrane transfers were stained in Coomassie Blue. Protein bands corresponding to major proteolytic fragments of MyHC were cut from the membrane and subjected to Edman degradation for N-terminal sequencing (ABI Procise 492 sequencer).

### S-nitrosylation and biotin-switch assay

Protein S-nitrosylation was assayed using a modification of a previously described technique (Forrester et al., 2009; Data S1).

### Statistics

Data are presented as mean  $\pm$  SEM. One-way analysis of variance was used to test whether differences between groups were significant at  $P < 0.05$ .

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1** Caspase-3 activation increases in aging skeletal muscle *in vivo* and S-nitrosylation inhibits caspase-3 proteolysis of MyHC-2b in purified myofibrils.

**Data S1** Experimental procedures.