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Protein thiol oxidation does not change in skeletal muscles of aging female mice

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ABSTRACT

Oxidative stress caused by reactive oxygen species is proposed to cause age related muscle wasting (sarcopenia). Reversible oxidation of protein thiols by reactive oxygen species can affect protein function, so we evaluated whether muscle wasting in normal aging was associated with a pervasive increase in reversible oxidation of protein thiols or with an increase in irreversible oxidative damage to macromolecules. In gastrocnemius muscles of C57BL/6J female mice aged 3, 15, 24, 27, and 29 months there was no age related increase in protein thiol oxidation. In contrast, there was a significant correlation ($R^2 = 0.698$) between increasing protein carbonylation, a measure of irreversible oxidative damage to proteins, and loss of mass of gastrocnemius muscles in aging female mice. In addition, there was an age-related increase in lipofuscin content, an aggregate of oxidised proteins and lipids, in quadriceps limb muscles in aging female mice. However, there was no evidence of an age-related increase in malondialdehyde or F₂-isoprostanes levels, which are measures of oxidative damage to lipids, in gastrocnemius muscles. In summary, this study does not support the hypothesis that a pervasive increase in protein thiol oxidation is a contributing factor to sarcopenia. Instead, the data are consistent with an aging theory which proposes that molecular damage to macromolecules leads to the structural and functional disorders associated with aging.

Key words: Aging; Oxidative Stress; Protein Thiol Oxidation; Carbonyl; lipofuscin; Muscle; cysteine oxidation; aging; muscle; mice; Reactive oxygen species

INTRODUCTION

Aging in muscles is characterised by a loss of muscle mass and a gradual decline in muscle function, a condition known as sarcopenia (Ryall et al. 2008; Burks and Cohn 2011; Baraibar et al. 2013). Many factors have been proposed to contribute to sarcopenia including altered metabolism of myofibres (Srikanthan et al. 2010; Baraibar et al. 2013), loss of myofibres (McKiernan et al. 2012), an impaired autophagic system (Wohlgemuth et al. 2010), increased apoptosis (Marzetti et al. 2012), denervation of myofibres (Chai et al. 2011), hormonal decline (Sakuma and Yamaguchi 2012) or environmental factors including malnutrition (Jeejeebhoy 2012) and physical inactivity (Fonseca et al. 2012). Increased oxidative stress induced by reactive oxygen species (ROS) has also been implicated in muscle wasting of normal and aged skeletal muscles (Arthur et al. 2008; Cerullo et al. 2012; Curtis et al. 2012; Forbes et al. 2012). ROS have a diverse range of actions in cells because the different forms of ROS have differing chemical properties (Barbieri and Sestili 2012; Arthur et al. 2008). The reactions of some ROS, such as hydroxyl radicals, results in irreversible oxidative damage to proteins and lipids, which can disrupt cellular functions and integrity (Imlay 2008; Reid and Moylan 2011).

There is evidence for increased oxidative damage to proteins in aged muscle tissue (Feng et al. 2008; Xu et al. 2010). For example, carbonylated proteins of mitochondria were higher in skeletal muscle of aged (26 months) rats relative to younger (12 months) rats (Feng et al. 2008) and also higher in muscles of 18 and 28 month old mice (53% and 60% respectively) compared with young 3 month old mice (Jackson et al. 2011a). Other reports have linked increased protein carbonyl levels to reduced muscle strength in older women (Howard et al. 2007). There is conflicting evidence on the extent of increased oxidative damage to lipids in muscles. Lipid peroxidation, a feature of oxidative injury resulting from the irreversible

oxidation of membrane lipids, is widely assessed by measuring malondialdehyde (MDA) or isoprostanes (Soffler et al. 2010; Padalkar et al. 2012). These have been linked, respectively, to degenerative muscular disorders such as Duchene Muscular Dystrophy (DMD) and Becker muscular dystrophy (Grosso et al. 2008) and muscle wasting in tumour bearing rats (Barreiro et al. 2005). There are conflicting studies on the extent to which MDA changes in aging muscles of mice with no age related differences (Yarian et al. 2005) and age related increases (Jackson et al. 2011b). For isoprostanes, there is no report to our knowledge, of measurements in the skeletal muscle of aging mice.

Oxidative damage to proteins and lipids can also result in the formation of a non-biologically degradable aggregate, described as lipofuscin (Terman et al. 2006) which can be quantified by morphometric analysis (Tohma et al. 2011). Lipofuscin likely reflects cumulative oxidative damage and is widely considered to be a reliable marker of aging in many post-mitotic tissues (Brunk and Terman 2002a). Examples include linear increases of lipofuscin with aging in human beta cells (Cnop et al. 2010) and human retinal pigment epithelial cells (Family et al. 2011). In muscles, lipofuscin is increased in aged mice (27 months)(Tohma et al. 2011) and aged humans (reviewed in Nakae et al. 2004).

In contrast to irreversible oxidative damage to proteins and lipids, other ROS, such as hydrogen peroxide, can cause a biologically reversible oxidation of reduced protein thiols (El-Shafey et al. 2011; Terrill et al. 2012). This is of interest because many *in vitro* studies with isolated proteins, cultured cells or isolated myofibres show that thiol oxidation can affect the function or activity of numerous proteins, including membrane channel proteins, metabolic enzymes, phosphatases and transcription factors as well as contractile proteins (Moran et al. 2001; Winterbourn and Hampton 2008; Hill and Bhatnagar 2012; Wang et al. 2012). Consequently, elevated ROS in muscles *in vivo* have the potential to cause cellular

dysfunction through the reversible oxidation of thiols on a multitude of proteins. For example, increased reversible protein thiol oxidation has been linked to muscle fatigue, a transient condition which decreases the capacity of muscles to contract (Enoka and Duchateau 2008; Dutka et al. 2012) and elevated protein thiol oxidation in muscles of mdx mice (El-Shafey et al. 2011) has been linked to muscle dysfunction (Terrill et al. 2012; Terrill et al. 2013).

The extent to which reversible protein thiol oxidation increases in aged muscle has not been directly quantified. However, indirect evidence of protein thiol oxidation comes from converse studies examining the level of reduced thiols in plasma or tissue. Total reduced thiol levels in plasma, which includes reduced protein thiols (PSH), were lower in middle aged (46-65 years old) and elderly (66-89 years old) humans relative to younger people (22-45 years old) (Cakatay et al. 2008), implying that oxidised thiols might be increased. The total reduced thiol content in mitochondria of aged (15 month) and senescent (26 month) rat hearts was also lower (about 20 % and 30 % , respectively) when compared to younger (6 month) hearts (Tatarkova et al. 2011). In contrast, no significant changes in total reduced thiol levels were found between young (5 month) and aged (24 month) rat livers (Aydin et al. 2010). A confounding factor in interpreting changes in reduced thiols in these studies is that the decrease in total reduced thiol content could be caused by a loss of proteins containing reduced thiols from the tissue or irreversible oxidation of thiols to sulfonic acid derivatives, rather than increased reversible thiol oxidation.

Oxidative stress has been proposed to lead to a catabolic state in cells with chronic exposure leading to muscle wasting (Li et al. 1998; Moylan and Reid 2007): this is of considerable interest to the age related situation of sarcopenia. We hypothesized that if there was a chronic increase in oxidative stress in aged muscle, then this would cause an increase in reversible protein thiol oxidation and, as a result of pervasive protein dysfunction, cause muscle wasting.

As a test of this hypothesis, the present study examines the extent to which protein thiol oxidation increased with age in muscles of aged female mice. As irreversible oxidative damage has been proposed as a mechanism to cause age related loss of muscle mass (Jang et al. 2010), additional markers of oxidative damage (protein carbonyls, malondialdehyde, F₂-Isoprostanes and lipofuscin) were also measured in muscle. Our data do not support the hypothesis, as there was no evidence for increased reversible protein thiol oxidation. Instead, as explained in the discussion, the data are consistent with an aging theory which proposes that molecular damage to macromolecules leads to the structural and functional disorders associated with aging.

METHODS AND MATERIALS

Animals

Muscles used for analysis were from 3, 15, 24, 27 and 29 month old female C57Bl/6J mice where the extent of muscle mass loss had previously been described (Shavlakadze et al. 2010; Chai et al. 2011). In particular, gastrocnemius muscles were chosen for analysis because by 27 months and 29 months of age there was a 26% and 29%, respectively, decrease in muscle mass from 24 months of age. For the lipofuscin study, quadriceps muscles were utilised where loss of muscle mass was evident by 24 months of age. In brief, all animal procedures were carried out in accordance with the guidelines of the National Health and Medical Research Council of Australia, the Animal Welfare Act of Western Australia and were approved by the Animal Ethics Committee at the University of Western Australia. Mice were fed a standard laboratory diet and killed by cervical dislocation under anaesthesia. Gastrocnemius muscles were rapidly removed, placed into polyethylene tubes and immediately frozen in liquid nitrogen. Tissues were stored at -80°C until analysis for protein thiol oxidation, protein

carbonyls, malondialdehyde and isoprostanes. Quadriceps muscles were removed from each mouse, embedded in tragacanth gum (Sigma Aldrich G1128) on a small cork block and then frozen in isopentane cooled in liquid nitrogen. Tissues were stored at -80°C until analysis for lipofuscin.

Two tag fluorescence labelling of thiol proteins

The fluorescent two-tag labelling technique involved the sequential labelling of reduced and oxidized protein thiol groups using two separate fluorescent tags on the same protein sample as described in detail elsewhere (Armstrong et al. 2011). In brief, the protein pellets were extracted with 20% TCA in acetone and re-suspended in 50 μ l of SDS buffer (0.5% SDS and 0.5 M Tris pH 7.0), in the presence of 0.5 mM BODIPY FL-N-(2-aminoethyl) maleimide (tag1) in the dark. All samples were sonicated, incubated for 30 min at room temperature in the dark with continuous vortexing, centrifuged, and the labelled protein solution precipitated with cold ethanol to remove excess dye (Flm, tag 1). The protein pellet was then redissolved in 50 μ l of SDS buffer (pH 7.0), reduced with the addition of 4 mM tris (2-carboxy-ethyl) phosphine hydrochloride (TCEP) and incubated for 1 h at room temperature in the dark. After reduction, samples were labelled with 0.25 mM Texas red - C2-maleimide (TRm, tag 2) and incubated for 30 min at room temperature in the dark. Ethanol precipitation and re-solubilisation in SDS buffer was repeated 4 times to remove excess unreacted dye (tag 2). Samples were resolubilized in 100 μ L of SDS buffer and the intensity of the fluorescence was measured for both tag 1 and tag 2 (tag 1; excitation 485 nm, emission 520 nm; and tag 2; excitation 595 nm, emission 610 nm). Ovalbumin pre-labelled with a known quantity of tag 1 and 2 was used as a standard to quantify labelled protein thiols. Protein concentration (mg/ml) was determined using the Bio-Rad DC Protein Assay. The percentage of protein thiol oxidation was expressed as the concentration of oxidized thiols (tag 2) to total protein thiols

(tag 1 plus tag 2). The inter-assay average coefficient of variance (CV) was 7.3% for percentage of oxidised protein thiols (n=8).

SDS PAGE and gel analysis of specific proteins

In order to separate proteins labelled by the 2 tag technique, SDS PAGE was performed using the Bio-Rad Mini Protean III system with gel and buffer compositions based on those described previously (Kohn and Myburgh 2006). Electrophoresis was carried out at 25 mA with voltage not exceeding 150 V for 3 h. Each gel was scanned (Typhoon Trio; GE Health, Australia) for fluorescence (FLm, ex 485 nm, em 520 nm; TRm, ex 595 nm, em 610 nm). The bands were quantified by densitometry using ImageJ (version 1.41 software (U.S. National Institutes of Health, Bethesda, MD, USA, available at <http://rsb.info.nih.gov/nih-image/>) using the integrated density function after first subtracting the background. To assess the protein thiol oxidation state of specific protein bands, a normalisation factor for differences in fluorescent intensities was used with 60 μ M FLm and 60 μ M Trm bound to albumin with a ratio of volume 4:1 (El-Shafey et al. 2011). The signals of sample proteins, actin and tropomyosin, were multiplied using the ratio obtained from the normalisation factor.

Protein carbonylation

Protein carbonylation content was quantified using 2, 4-dinitrophenylhydrazine (DNPH) as described elsewhere (Hawkins et al. 2009). In brief, gastrocnemius muscles were homogenised in 20% cold TCA in acetone and proteins pellets were precipitated by centrifugation at 8000 g for 10 minutes at 4 °C. Pellets were resuspended in 2 M HCl with and without DNPH. Both samples were incubated for 30 minutes at room temperature in the dark. Proteins were washed with ethyl acetate/ethanol (1:1) and dissolved in 6 M guanidine

hydrochloride and absorbance was measured at 370 nm. Carbonyl groups were estimated by measuring the absorbance at 370 nm of the DNPH derivatives in the suspension and subtracting the absorbance of the unlabelled proteins. Protein concentration (mg/ml) was determined using the Bio-Rad protein assay. The final data were expressed as nmol of carbonyl per mg soluble protein.

F₂-Isoprostanes

Isoprostanes were quantified as previously described (Mori et al. 1999). In brief, approximately 10 mg of frozen gastrocnemius muscle and 2 ml of chloroform, methanol and BHT solutions were added in that order. Samples were homogenised and after incubation at room temperature for 30 minutes and centrifuging, the supernatants were dried and mixed with 50 µL of internal standards (15-F₂₊-IsoP-d4, 8-F₂₊-IsoP-d4, 2 ng) and 200 µL of DDI water. After adding 250 µL of 1 M potassium hydroxide (KOH), samples were heated for 30 minutes at 40°C, 2 mL sodium acetate (pH 4.6) was added, centrifuged and applied to Certify II columns under vacuum, and washed with 2 mL methanol: water (1:1), (5-6 mmHg) and 2 mL ethyl acetate: hexane (1:3) (5-6 mmHg). Samples were collected with 2 mL ethyl acetate: methanol (9:1) (5-6 mmHg), dried and derivatised. The derivatised F₂-iso-prostane fraction was treated with a mixture of pentafluorobenzylbromide (40 ml, 10% (v/v) in acetonitrile) and N,N -diisopro-pylethylamine (20 ml, 10% (v/v) in acetonitrile) at room temperature for 30 minutes, dried under N₂ and treated with N,O-bis- (trimethylsilyl)trifluoroacetamide-1% trimethylchlorosilane (99:1, 20 ml) and anhydrous pyridine (10 ml) at 45°C for 20 min to yield the trimethylsilylethers and then dried with N₂. Samples were reconstituted in 30 µL of isooctane and processed for gas chromatography as previously described Mori (1999).

Malondialdehyde (MDA)

Malondialdehyde levels were determined as previously described (El-Shafey et al. 2011). Gastrocnemius muscles were homogenized in 0.1 M HClO₄ as an aqueous acid extraction medium. Sample supernatant (150 µl) or MDA standard was derivatized by thiobarbituric acid (TBA) at 50°C for 1 h. After cooling, 200 µl of butanol was added and mixed vigorously, the butanol layer was separated by centrifugation at 8000 g for 5 min, transferred to auto sampler vials and analysed by HPLC (UltiMate 3000 LC system, Dionex) on a reverse phase column (Acclaim 120; C18 column; 5 µm; 4.6 x 150 mm; Dionex). HPLC was used to detect the (TBA)₂-MDA adduct because of its high analytical sensitivity and specificity. A MDA standard solution was prepared (Wang et al. 2002). The limit of detection was 0.02 µM (based on three standard deviations of the blank measurements), with MDA concentrations in tissue samples ranging between 0.3 – 0.9 µM. All samples were run in duplicate and the level of MDA was expressed as nmol/mg soluble protein.

Lipofuscin

Lipofuscin was quantified using a non-subjective method as previously described (Tohma et al. 2011). Briefly sections were analysed with a fluorescent LEICA DM RB/E microscope equipped with Q imaging micropublisher 3.3 RTV camera, a 100 W mercury lamp, 450-490 nm excitation filter (blue) and a 515 nm emission barrier filter. All sections were scanned using an automatic stage control setting which generated a grid structure of images covering the entire section. Images of each section were captured using Imagepro (version: 6.2.0424 for windows 2000/XP). Quantification of lipofuscin granules was performed with ImageJ. Threshold levels were determined and applied to images to discriminate lipofuscin from

background signal and the area occupied by lipofuscin was expressed as a percent of total image area ((area of granules / total image area) x100).

Statistical methods

All data are expressed as mean±SE. Muscles were not pooled. For each age, muscles from 6 mice were analysed, except for 15 months where n=5. Statistical analyses used SPSS software (version 16.1). Means were compared using t -tests or one-way ANOVA with LSD post-hoc testing where appropriate and differences were considered to be statistically significant at p less than 0.05. Excel and SPSS software were used to calculate correlations.

RESULTS

The effect of aging on reversible protein thiol oxidation

The extent of protein thiol oxidation was measured in gastrocnemius muscles of mice at ages 3, 15, 24, 27 and 29 months (Fig. 1). Between 3 and 27 months there were no significant changes in the levels of oxidised protein thiols (PSox), but there was a significant increase at 29 months (Fig. 1A). For reduced protein thiols (PSH), the changes were more complex, with a marked decrease (33%) in reduced protein thiols evident between 15 and 24 months. Reduced protein thiols remained low at 27 months but by 29 months were comparable to the levels seen in younger adult (3 and 15 months old) mice (Fig. 1B). Two calculated parameters were also examined: total protein thiol content ($PS_{tot} = PSH + PS_{ox}$) and percentage protein thiol oxidation ($PS_{ox}\% = PS_{ox}/[PSH + PS_{ox}] \times 100$). The changes for total protein thiol content reflected the changes for reduced protein thiol content (Fig. 1C). For percentage protein thiol oxidation, there was a significant increase at 24 months (Fig. 1D). There was no

significant correlation between muscle mass and percentage protein thiol oxidation ($R^2 = 0.0038$).

The changes in total protein thiol content (Fig. 1C) could reflect changes in protein composition, protein amount in aged muscle, or irreversible oxidation of protein thiols.

Therefore, we examined the extent to which aging affected the level of protein thiol oxidation of two abundant cytoskeletal proteins, previously shown to be susceptible to thiol oxidation; actin and tropomyosin (Passarelli et al. 2008), with representative images for proteins labelled with fluorescent dyes shown (Fig. 2A,B). No consistent pattern was observed for age related changes in thiol oxidation of these proteins. Actin showed significantly increased thiol oxidation only in the 29 month old muscles (Fig. 2C). Thiol oxidation of tropomyosin did not change during aging (Fig. 2D).

The effect of aging on irreversible oxidative damage

Oxidative damage to proteins in ageing gastrocnemius muscles, as measured by the protein carbonylation assay, was unchanged between 3 and 15 month. There was a trend for an increase at 24 month, although this was not significant ($p=0.28$), with a significant increase at 27 and 29 month, relative to mice aged 24 month or younger (Fig. 3A). For mice aged between 15 and 29 month, there was a significant correlation between protein carbonyl content and loss of muscle mass ($R^2 = 0.698$, $P<0.05$) (Fig. 3B). Two different assays were used to examine the effect of aging on oxidative damage to lipids within gastrocnemius muscle of mice aged 3 or 29 months. Measurement of malondialdehyde and F_2 -Isoprostanes provided no evidence for increased oxidative damage to lipids (Fig. 3 C and D) in aged mice (29 months) relative to younger mice (3 months).

To examine the effect of aging on lipofuscin accumulation, quadriceps muscles were analysed, instead of gastrocnemius muscles, because the collection procedure for lipofuscin analysis differed from the other analytical techniques. Lipofuscin quantified on entire cross-sections of frozen muscles was barely detectable in 3 month old quadriceps muscles (Fig. 4A), but was conspicuous in 29 month old muscles as autofluorescent granules (Fig. 4B). An increase in lipofuscin content was evident by 15 month of age with a further increase by 24 and 27 months (Fig. 4C). However, lipofuscin content at 29 months was lower than at 27 months. Loss of muscle mass was evident in aged quadriceps muscles as early as 24 months, rather than only by 27 months as seen for gastrocnemius muscles (Shavlakadze et al. 2010).

DISCUSSION

The major finding of this study is that reversible protein thiol oxidation was not consistently increased with aging and was not correlated with age-related loss of muscle mass. Therefore, this study does not support the hypothesis that a pervasive increase in protein thiol oxidation is a contributing factor to sarcopenia. However, irreversible oxidative damage, as measured by protein carbonylation and lipofuscin content, showed a steady age-related increase and was associated with loss of muscle mass. These observations are consistent with previous work proposing increased irreversible oxidative damage as a contributing factor towards sarcopenia (Meng and Yu 2010; Semba et al. 2007; Barreiro et al. 2006).

Our data did not provide any evidence of a chronic age dependent increase in protein thiol oxidation at 27 months in female C57 mice when a loss of muscle mass was evident. One possibility was that the method was not sensitive enough to detect changes in global protein thiol oxidation caused by a putative chronic and pervasive oxidative stress in aged muscles. However, both reduced and oxidised protein thiols were measured with the level of protein

thiol oxidation expressed as a percentage to enhance the sensitivity of detection (El-Shafey et al. 2011). Using this approach, we have been able to demonstrate increased protein thiol oxidation in the muscles of dystrophic mdx mice and also dysferlin-deficient mice (El-Shafey et al. 2011; Terrill et al. 2013). Furthermore, the global protein thiol measurements of the aged normal muscles in the present study are supported by the analyses of two abundant cytoskeletal proteins (actin and tropomyosin). We have previously found that the thiol oxidation level of these proteins is increased following stimulation of isolated Extensor digitorum longus muscles from male Wistar rats muscle (unpublished data) in accordance with many observations of increased oxidative stress in response to muscle contraction (Lamb and Westerblad 2011). However, there was no evidence of increased protein thiol oxidation for these muscle proteins in gastrocnemius muscles of sedentary aged mice.

This study provides evidence linking protein carbonylation to age related muscle wasting. This finding supports previous studies that have shown increased oxidative damage to proteins with aging as measured by protein carbonylation in gastrocnemius muscles of 18 and 28 month old mice (Jackson et al. 2011b). Age related increases in protein carbonylation have also been shown in the plasma of 14 and 24 month old mice (Jana et al. 2002), in the liver of 24 month old mice (Rabek et al. 2003), and in the brain of 18 month old mice (Soreghan et al. 2003). In humans, there was increased protein carbonylation in skeletal muscle biopsies of human subjects aged over 60 years (Mecocci et al. 1999b) and in the plasma of women who were aged 65 or older (Howard et al. 2007).

In contrast to the increase in oxidative damage to proteins, there was no evidence of increased lipid peroxidation, as measured by malondialdehyde and F₂-isoprostanes, in very old muscles of female mice aged 29 months (intermediate ages were not analysed due to lack of sample). Previous reports on the extent to which lipid peroxidation was increased in aging are

ambiguous. For example, the levels of F₂-isoprostanes were similar in the brains of 4, 10, 50, and 100 week old male rats (Youssef et al. 2003) and in hind limb muscle of aged (30 months) rats. The MDA/4-hydroxyalkenals index in older rats (30 month), a measure of lipid peroxidation, was comparable to young rats (6 month) (Siu et al. 2008). In contrast, in a study utilising biopsies, malondialdehyde levels in the vastus medialis or lateralis of human subjects increased during aging, but not until after the age of 70 years (Mecocci et al. 1999a).

Malondialdehyde in the vastus lateralis muscle was also higher in both sexes from the muscle of elderly (≥ 70 years old) healthy subjects relative to younger subjects (≤ 35 years old), who underwent orthopaedic surgery under general anesthesia (Fano et al. 2001) and in the plasma of people older than 80 years (Fabian et al. 2012). The importance of the increase in lipid peroxidation in relation to loss of muscle is not clear since sarcopenia was not assessed.

However, as sarcopenia is usually evident before the age of 70 (Scharf and Heineke 2012), the increase in lipid peroxidation may well be a secondary effect of sarcopenia rather than causative of sarcopenia.

The differential response we observed in aged mouse muscles between protein carbonylation which increased, and lipid peroxidation which did not, has been observed previously for biomarkers of irreversible oxidative damage. For example, carbon tetrachloride poisoning caused an increase in plasma malondialdehyde and F₂-isoprostanes but not protein carbonyls (Kadiiska et al. 2005). In dystrophic mdx mouse muscle we have previously shown that protein carbonyls, but not malondialdehyde, were elevated relative to non-dystrophic muscle (El-Shafey et al. 2011). The differential responses in these biomarkers may reflect differences in the macro-molecular targets of ROS (Davies 2005). Such disparity emphasises the need to consider the potential for different outcomes for oxidative stress.

Irreversible oxidative damage has the potential to contribute to loss of muscle mass (Meng and Yu 2010; Arthur et al. 2008). One possible mechanism involves lipofuscin, which is considered to be a non-degradable aggregate of oxidised proteins, lipids and a small fraction of carbohydrates (Brunk and Terman 2002a). Accumulated lipofuscin is proposed to cause apoptosis via destabilisation of lysosomal membranes (Kurz et al. 2008). Lipofuscin may also impair autophagy, leading to progressively less mitochondrial recycling and thus cause cellular dysfunction in the post-mitotic cells (Brunk and Terman 2002b). Impaired autophagy has also been linked to an increase in lipofuscin-like autofluorescence (Stroikin et al. 2004), which may mean that impaired autophagy can aggravate lipofuscin accumulation and, as a consequence, cause cell death. In this context, the observed lipofuscin accumulation in aged quadriceps muscle could be a trigger for the decrease in quadriceps muscle mass observed at 27 months. Surprisingly, there was 27% decline in lipofuscin content from 27 to 29 months in quadriceps muscle. A decrease in lipofuscin content has also been observed by others where, for example, lipofuscin increased from 20 to 70 years, and then declined in the 8th decade of life in retinal pigment epithelium of humans (Delori et al. 2001). There are two possible explanations. First we speculate that if lipofuscin was triggering cell death, then the loss of myofibres would explain the decrease in lipofuscin at the oldest ages. Secondly the lower lipofuscin level might also be contributing longer lifespan as mice that reach 29 month old have lesser amount of lipofuscin than the ones that died earlier.

Although our focus was on mechanisms by which ROS could be causative of sarcopenia, our data also contributes to the wider debate regarding aging mechanisms. Since we did not observe elevated protein thiol oxidation in aged muscle, it is unlikely that widespread protein dysfunction caused by pervasive reversible protein thiol oxidation is causative of aging, at least in muscle. This observation does not exclude the possibility that specific signal transduction proteins, transcription factors or compartmentalised proteins (e.g. mitochondrial)

are oxidised as a result of ROS acting as second messengers in signalling pathways. Further work, utilizing proteomic techniques (Lui et al. 2010), for example, could be used to establish whether this is possible. Oxidative stress caused by ROS is complex because the different ROS have differing chemical properties. In this context, the increase in protein carbonyls and lipofuscin is consistent with some ROS, such as hydroxyl radicals, causing sarcopenia through irreversible oxidative damage to macromolecules. This concept is compatible with one of a mechanistic theories of biological aging which proposes that molecular damage to macromolecules would lead to the structural and functional disorders associated with aging (Moyle and Reid 2007; Rattan 2008). However, as this link is only associative, further work is required to establish whether oxidative damage to macromolecules is causative of sarcopenia.

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FIGURE LEGENDS

Figure 1. Protein thiol oxidation in aging gastrocnemius muscles of female mice.

(A) Oxidised protein thiol content (PSox), (B) reduced thiol content (PSH), (C) total protein thiol content and (D) percentage protein thiol oxidation (PSox%). Bars not sharing a common letter are significantly different ($P < 0.05$), $n=6$ except for 15 months where $n=5$. Values are expressed as means \pm SE.

Figure 2. The effect of aging on thiol oxidation of specific proteins in gastrocnemius muscles in mice. SDS–PAGE of fluorescent proteins scanned for (A) reduced protein thiols (FLm), and (B) reversibly oxidised protein thiols (TRm). Percentage thiol oxidation of (C) actin, and (D) tropomyosin was calculated from scanned images. Bar shows $p < 0.05$; $n=6$, except 15 months where $n=5$. Values are expressed as means \pm SE.

Figure 3. Measurement of three parameters of irreversible oxidative damage in gastrocnemius muscles of aging mice. (A) Protein carbonyl levels. (B) Relationship between protein carbonyl levels and muscle mass, with line of best fit shown. (C) F₂-Isoprostanes levels, and (D) MDA levels. Bars not sharing a common letter are significantly different ($p < 0.05$); $n=6$, except 15 months where $n=5$. Values are expressed as means \pm SE.

Figure 4. Quantification of lipofuscin in aging quadriceps muscles. Example fluorescent images for lipofuscin are for (A) 3 month old and (B) 29 month old age C57BL/6 mice. Scale bar is 25 μ m. Values are expressed as means \pm SE. (C) Percentage area of lipofuscin and (D) muscle wet mass as reported in Shavlakadze (2010) from aging mice. Bars not sharing a common letter are significantly different ($P < 0.05$); $n=6$, except 15 months where $n=5$.

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