Protein modification during biological aging: selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca^{2+} -ATPase in skeletal muscle

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The accumulation of covalently modified proteins is an important hallmark of biological aging, but relatively few studies have addressed the detailed molecular–chemical changes and processes responsible for the modification of specific protein targets. Recently, Narayanan et al. [Narayanan, Jones, Xu and Yu (1996) *Am. J. Physiol.* **271**, C1032–C1040] reported that the effects of aging on skeletal-muscle function are muscle-specific, with a significant age-dependent change in ATP-supported Ca^{2+} -uptake activity for slow-twitch but not for fast-twitch muscle. Here we have characterized in detail the age-dependent functional and chemical modifications of the rat skeletal-muscle sarcoplasmic-reticulum (SR) Ca^{2+} -ATPase isoforms SERCA1 and SERCA2a from fast-twitch and slow-twitch muscle respectively. We find a significant age-dependent loss in the Ca^{2+} -ATPase activity (26 % relative to Ca^{2+} -ATPase content) and Ca^{2+} -uptake rate specifically in SR isolated from predominantly slow-twitch, but not from fast-twitch, muscles. Western immunoblotting and amino acid analysis demonstrate that, selectively, the SERCA2a isoform progressively accumulates a significant amount of nitro-

tyrosine with age ($\approx 3.5 \pm 0.7$ mol/mol of SR Ca^{2+} -ATPase). Both Ca^{2+} -ATPase isoforms suffer an age-dependent loss of reduced cysteine which is, however, functionally insignificant. *In vitro*, the incubation of fast- and slow-twitch muscle SR with peroxynitrite (ONOO^-) (but not NO/O_2) results in the selective nitration only of the SERCA2a, suggesting that ONOO^- may be the source of the nitrating agent *in vivo*. A correlation of the SR Ca^{2+} -ATPase activity and covalent protein modifications *in vitro* and *in vivo* suggests that tyrosine nitration may affect the Ca^{2+} -ATPase activity. By means of partial and complete proteolytic digestion of purified SERCA2a with trypsin or *Staphylococcus aureus* V8 protease, followed by Western-blot, amino acid and HPLC–electrospray-MS (ESI-MS) analysis, we localized a large part of the age-dependent tyrosine nitration to the sequence Tyr²⁹⁴–Tyr²⁹⁵ in the M4–M8 transmembrane domain of the SERCA2a, close to sites essential for Ca^{2+} translocation.

Key words: calcium, nitric oxide, peroxynitrite, superoxide, thiol modification.

INTRODUCTION

Biological aging is a complex process, featuring species- and tissue-specific rates and molecular mechanisms of age-related physiological and molecular changes [1]. Age-dependent alterations of motor function, such as slowing of movement and muscle weakness, are generally associated with increased muscle contraction and half-relaxation times [2]. However, detailed knowledge of the molecular mechanisms responsible for the age-related changes of muscle performance is still lacking. The ‘free-radical theory of aging’ has forwarded the hypothesis that an increased steady-state level of reactive oxygen species available for biomolecule modification may be responsible for age-related dysfunctions [3]. In particular long-lived proteins are regarded as an important target for such reactive oxygen species, causing the accumulation of chemical modifications eventually associated with the loss of function [4].

The sarcoplasmic-reticulum (SR) Ca^{2+} -ATPase is primarily responsible for muscle relaxation by transporting cytosolic Ca^{2+} into the lumen of the SR coupled to ATP hydrolysis [5,6]. This protein is characterized by a relatively long half-life in the cell ($\approx$

14–15 days) and is turned over even more slowly in aged rats [7]. Specifically for homogenates from fast-twitch skeletal-muscle fibres, a significant age-related decrease of the rate of Ca^{2+} -uptake and loading capacity of the SR has been reported [8], rationalized by a potential inactivation of the SR Ca^{2+} -ATPase. However, in isolated SR vesicles the SR Ca^{2+} -ATPase displayed no difference, irrespective of age [9–11]. An increased rate of inactivation of the ‘old’ protein was only observed when SR vesicles were exposed to mild heating at 37 [9,11] or 40 °C [10]. It was initially suggested that alterations of the membrane environment cause the higher sensitivity of the ‘old’ SR Ca^{2+} -ATPase to heat inactivation [9]. Subsequent studies indeed demonstrated a slightly different phospholipid composition for ‘old’ as compared with ‘young’ membranes [12]. However, these differences affect neither the physical properties of bulk and protein-associated lipids nor the rotational dynamics of the protein [12], membrane properties that directly influence Ca^{2+} -ATPase activity. A more recent chemical analysis of the fast-twitch, SERCA1, isoform of the SR Ca^{2+} -ATPase isolated from old rats reveals a significantly lower content of reduced cysteine (≈ 1.5 mol of cysteine/mol of Ca^{2+} -ATPase) [10]. However,

Abbreviations used: Caps, 3-(cyclohexylamino)propane-1-sulphonic acid; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DEA/NO, diethylamine/nitric oxide, sodium salt; EDL, extensor digitorum longus; ESI-MS, electrospray-ionization MS; LDS, lithium dodecyl sulphate; NEM, *N*-ethylmaleimide; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid; SR, sarcoplasmic reticulum; NO_2 , nitrogen dioxide; ONOO^- , peroxynitrite; $\text{O}_2^{\cdot-}$, superoxide radical; V8 protease, *Staphylococcus aureus* V8 protease; C_{12}E_9 , poly(oxyethylene) 9-lauryl ether; a.m.u., atomic mass units; NOS, nitric oxide synthase; PITC, phenyl isothiocyanate.

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comparable initial activities of 'old' and 'young' Ca^{2+} -ATPase, when tested under optimum assay conditions, would suggest that the modified cysteine residues are not critically important for protein activity. Specifically the slow-twitch, SERCA2a, isoform of the SR Ca^{2+} -ATPase accumulates nitrotyrosine as a result of biological aging [13]. The presence of a nitrated protein in aged muscle tissue suggests the involvement of NO-derived species in age-related modifications, especially as these species are also known for their high reactivity towards cysteine residues [14].

NO is an important modulator of muscle contraction through the formation of cGMP and its interaction with reactive oxygen species such as superoxide radical ($\text{O}_2^{\cdot-}$) [15]. The reaction of NO with $\text{O}_2^{\cdot-}$ (reaction 1) [16,17] yields the highly cytotoxic [18] species peroxynitrite (ONOO^-), which may be responsible for the selective nitration of the SERCA2a isoform [13].

Relatively few studies have characterized the specific molecular-chemical processes diagnostic for, and promoting, the biological aging of muscle. The present paper focuses on the characterization and localization of the age-dependent post-translational modifications of the slow-twitch muscle isoform of the SR Ca^{2+} -ATPase, SERCA2a, and their potential impact on the functional integrity of this protein. Specifically, it will be demonstrated that: (i) selective nitration of the SERCA2a isoform occurs *in vivo*, in part on the sequence Tyr²⁹⁴-Tyr²⁹⁵ within the channel-like domain provided by the transmembrane helices M4-M8; (ii) selective *in vitro* nitration of the SERCA2a isoform can be achieved by the exposure of SR membranes to ONOO^- ; and (iii) nitration may affect the Ca^{2+} -ATPase activity.

EXPERIMENTAL

Materials

The following chemicals were obtained from their respective sources: monoclonal antibodies to SERCA1 and SERCA2a (Affinity Bioreagents, Golden, CO, U.S.A.) and to nitrotyrosine [Upstate Biotechnology, Lake Placid, NY, U.S.A., and a gift from Professor J. S. Beckman (Department of Anesthesiology, University of Alabama at Birmingham, Birmingham, AL, U.S.A.)], goat anti-mouse IgG (H + L)-horseradish peroxidase and -alkaline phosphatase conjugates (Pierce); trypsin (sequencing grade, Promega), *Staphylococcus aureus* V8 protease (V8 protease; Boehringer Mannheim); Calcium Green-5N (Molecular Probes); *N*-ethyl[¹⁴C]maleimide ([¹⁴C]NEM) (NEN Life Science Products). All reagents for gel electrophoresis and electroblotting, and prestained protein and peptides markers, were from Bio-Rad (Richmond, CA, U.S.A.); reagents and standards for amino acid analysis were from Pierce (Rockford, IL, U.S.A.). All other chemicals were analytical grade or better. Nitric oxide (NO) was generated *in situ* by the release of NO from diethylamine/NO, sodium salt (DEA/NO; Research Biochemicals International, Natick, MA, U.S.A.), essentially as described by Maragos et al. [19]. ONOO^- was synthesized as described by Pryor et al. [20]. The potassium phosphate buffer employed in the oxidation experiments was treated with 5% (w/v) Chelex-100 (Bio-Rad) for 1 h in order to minimize transition metal contaminations.

Animals and SR preparations

The animals used in this study were young adult (4–5 months), middle-aged (10 and 16 months) and old (26–28 months) Fisher 344 male rats obtained from the National Institute of Aging colonies maintained at Harlan Sprague-Dawley, Inc. (Indian-

apolis, IN, U.S.A.). Before being killed, the rats were generally allowed to adapt for 2 weeks after arrival. SR vesicles enriched in slow-twitch membranes ('slow') were prepared from slow-twitch muscles (soleus, adductor longus, vastus intermedius) and SR enriched in fast membranes ('fast') from the remaining hindlimb skeletal muscles as described previously [13]. For some experiments, as specified in the Results section, only soleus (containing 80% of slow-twitch fibres [21]) and extensor digitorum longus (EDL) (containing nearly all, > 95%, fast-twitch fibres) were used for SR preparations, and in this case the last centrifugation step was eliminated. SR vesicles were suspended in a medium consisting of 0.3 M sucrose and 20 mM Mops, pH 7.0, and stored at -70°C . Protein concentration was determined by using the Pierce (Rockford, IL, U.S.A.) bicinchoninic acid ('BCA') protein assay and BSA as a standard.

Functional assays

Total, Ca^{2+} -dependent and basal ATPase activities were measured at 25°C by colorimetric determination of P_i in the presence or absence of the ionophore A23187 as described by Lanzetta et al. [22]. Ca^{2+} transport was determined fluorimetrically on a FluoroMax-2 fluorimeter (JOBIN YVON-SPEX Instruments, Edison, NJ, U.S.A.) using the low-affinity chelator Calcium Green as described in [23]. Excitation was set at 488 nm and fluorescence emission was monitored at 530 nm. Using a calibration curve (free Ca^{2+} range from 2 nM to 2 mM) we found an apparent dissociation constant, K_d , of $6.8 \pm 0.5 \mu\text{M}$ for Ca^{2+} binding at Calcium Green concentrations of 100 nM. Ca^{2+} uptake was measured at 25°C in a magnetically stirred solution in a cuvette containing 0.05–0.1 mg/ml of SR protein, 0.1 M KCl, 5 mM MgCl_2 , 100 mM Mops, pH 7.0, 2 mM EGTA, 5 mM oxalate, 0.5 μM proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone and 6.9 μM of free Ca^{2+} , calculated with the program developed by Fabiato and Fabiato [24]. The reaction was started by the addition of 1 mM ATP. The calibration curve was used to convert fluorescence intensity to free (unbound) Ca^{2+} concentration at the identical instrument settings.

Determination of total free thiol groups in the SR vesicles and purified Ca^{2+} -ATPase

The total content of free thiol groups of the SR vesicles and purified SR Ca^{2+} -ATPase was quantified using [¹⁴C]NEM, as described previously [10].

Chemical oxidation

The oxidation reactions were carried out using SR vesicles containing 2 mg/ml SR protein, 10 mM phosphate buffer and 100 mM NaCl at 25°C in a total volume of 250 μl . The initial pH was adjusted to 7.4 and shifted less than 0.2 units after the addition of the ONOO^- stock solution and did not change with the addition of DEA/NO. Oxidation reactions were initiated by addition of various concentrations of ONOO^- or DEA/NO and run for 30 min. In a control experiment, 200 μM ONOO^- was added to a reaction medium without protein. The ONOO^- was allowed to completely decompose in this reaction mixture before SR vesicles were added (reverse-order-of-addition experiment).

Heat inactivation

Heat-stability of native SR vesicles was determined by monitoring Ca^{2+} -ATPase activity during an incubation period of up to 4 h at 40°C . The incubation medium contained 20 mM Mops, pH 7.0,

5 mM MgCl₂, 0.4 mM CaCl₂ and 2 mg/ml SR protein. At various times, aliquots of 100 µg of SR protein were withdrawn and assayed for Ca²⁺-ATPase activity.

Proteolysis

Limited tryptic digestion was performed as previously described [25]. V8 proteolysis of SR proteins (2 mg/ml) was carried out at 37 °C for 24 h at a protein/protease ratio of 50:1 in 10 mM phosphate buffer, pH 7.6, containing 10 mg/ml poly(oxyethylene) 9-lauryl ether (C₁₂E₉), 150 mM NaCl and 10 mM EDTA. Proteolytic activity was stopped by heating the samples for 2 min at 100 °C.

Gel electrophoresis

SDS/PAGE was performed using a 5 % Laemmli gel with a 3 % stacking gel [26], which resolved both the fast-twitch and the slow-twitch isoforms of the Ca²⁺-ATPase. For subsequent amino acid analysis, large peptide fragments of the Ca²⁺-ATPase or SR proteins were separated by Tricine/SDS/PAGE using either 0.75 mm 10 % mini gels or 1.5 mm 16.5 % preparative gels (10 % acrylamide/3 % methylenebisacrylamide spacing gel, 4 % stacking gel [27]). The samples were dissolved in 2 % SDS/50 mM Tris/HCl (pH 6.8)/15 % (w/v) sucrose/0.05 % Bromophenol Blue, containing either 2.5 % β-mercaptoethanol (reducing conditions) or 5 mM NEM (alkylating conditions), and incubated for 60 min at 30 °C. After electrophoresis, gels were either stained for protein with Coomassie Blue R-250 or electroblotted. The relative amount of Ca²⁺-ATPase in the SR preparations was determined from densitometric measurements (Sigma Scan software) of the protein band migrating with an apparent molecular mass of 110 000 (SERCA1) or 95 000 (SERCA2a).

Electroblotting

The protein or peptide bands were electrotransferred (2 h at 100 V, Mini Trans Blot electrophoretic-transfer cell, Bio-Rad) from gels either to nitrocellulose sheets (Bio-Rad, 0.20 or 0.45 µm pore size) using 25 mM Tris/HCl (pH 8.0)/195 mM glycine/20 % methanol, or to PVDF membranes (Bio-Rad, 0.2 µm pore size) in 10 mM Caps buffer, pH 11, containing 10 % methanol. After the transfer, the PVDF membrane was rinsed with water, stained with 0.1 % (w/v) Coomassie Blue R-250 in 20 % (v/v) methanol for 1 min, followed by destaining in 40 % (v/v) methanol containing 10 % (v/v) acetic acid for 10 min [28]. Finally, the PVDF membranes were rinsed with water and areas of interest were excised and used for amino acid analysis or N-terminal sequencing. Nitrocellulose membranes were used for immunodetection.

Amino acid analysis

Amino acid analysis was done after hydrolysis of the pure Ca²⁺-ATPase (obtained by electroblotting, see above) in 6 M HCl (24 h at 110 °C), followed by derivatization with phenyl isothiocyanate (PITC), as previously described for SERCA1 [10]. Detection and quantification of 3-nitrotyrosine and 3,5-dinitrotyrosine was achieved relative to defined concentrations of authentic standards.

Nitrotyrosine immunoprecipitation

Large peptide fragments obtained after mild V8 proteolysis of SR proteins (2 mg/ml) were precleared (45 min, 4 °C) with 15 µl

of Gamma Bind Plus Sepharose (Pharmacia) and the supernatant was incubated overnight at 4 °C with 10 µg of monoclonal anti-nitrotyrosine antibody. Immune complexes were precipitated (1.5 h, 4 °C) with 50 µl of Gamma Bind Plus Sepharose, washed three times with the appropriate buffer for proteolysis, centrifuged for 15 s at 15 000 g and resuspended in SDS/PAGE sample buffer [16 mM Tris/HCl (pH 6.8)/2.5 % glycerol/0.5 % SDS/200 mM β-mercaptoethanol/0.001 % Bromophenol Blue] [29], heated (10 min, 40 °C), and immediately applied to either 10 or 16.5 % Tricine gels. After separation by SDS/PAGE, the peptides were electrotransferred to nitrocellulose for Western-blot analysis (detection of nitrated peptides, see below) or to PVDF membrane (for N-terminal sequencing). N-terminal sequencing of the nitrated peptides was done by the Instrumentation Facility of the Beckman Research Institute (Division of Immunology), City of Hope, CA, U.S.A..

Western immunoblotting of Ca²⁺-ATPase isoforms and nitrotyrosine-containing large peptide fragments

After electrotransfer of proteins or peptides to nitrocellulose membranes, Western blotting was done as previously described [13]. The primary antibodies for SERCA1, SERCA 2a and nitrotyrosine were diluted 1:10 000, 1:500, and 1:1000 respectively. The secondary goat anti-mouse IgG-horseradish peroxidase- or -alkaline phosphatase-linked antibody was diluted 1:2000 or 1:1000 respectively. Colour development was accomplished using the peroxidase substrate 4-chloro-1-naphthol (3 mg/ml in methanol) or 1-Step[™] (Nitroblue Tetrazolium chloride/S-bromo-4-chloroindol-3-yl phosphate) substrate (Pierce). Quantification of the signals was done by computerized densitometry (area × intensity). Amounts of 25–100 µg of SR protein provided a linear response for the quantification of the SERCA1 and SERCA2a proteins. The specificity of the anti-nitrotyrosine antibody was confirmed via two experimental controls [30]. First, the primary anti-nitrotyrosine antibody was placed for 1 h into a solution containing 10 mM 3-nitrotyrosine/0.1 M Tris-buffered saline and 0.05 M Tris/HCl, pH 7.4, before using it in the Western-blot experiment (antigen-competed antibody). Secondly, after electroblotting, the nitrocellulose membrane was treated for 10 min with 1.0 M sodium dithionite in 0.1 M PBS, pH 9.0.

Sample preparation for HPLC-ESI-MS analysis of tryptic peptides

Highly purified, delipidated (by size-exclusion chromatography) Ca²⁺-ATPase has a great tendency for aggregation [31]. In order to prevent aggregation during the first step of purification of the Ca²⁺-ATPase from SR vesicles, we generated large tryptic fragments of the SR Ca²⁺-ATPase by mild proteolysis with trypsin (see above) and fractionated them by size-exclusion chromatography (SEC). Aliquots of peptide fragments corresponding to 2 mg of SR protein in 0.5 ml were diluted 1:2 with solubilizing buffer containing 1 % (w/v) LDS, 0.1 M Li₂SO₄, 0.05 M lithium acetate, pH 4.5, and 5 mM NEM (for alkylation of free thiol groups), and incubated for 60 min at room temperature. After filtration through a 0.45 µm-pore-size Millipore filter, aliquots of 200 µg of SR protein were separated by SEC on a Shimadzu HPLC system equipped with a TSK G 2000 SW column (TOSO HAAS, Montgomeryville, PA, U.S.A.), eluted with solubilizing buffer minus NEM at 0.4 ml/min, essentially as described in [32]. The column was calibrated with molecular-mass standards in the range 12.4–200 kDa. The peaks were monitored at 280 nm and collected manually every 0.5 ml. Collected fractions were analysed by SDS/PAGE/Western im-

munoblotting (see above) for the presence of tyrosine-nitrated peptides. The highest amount of nitrated fragments A₁ and B of the Ca²⁺-ATPase were detected in fraction 6.0–6.5 ml. This fraction was combined from ten runs and used for further analysis. Collected peptides were concentrated using Microcon microconcentrators (10 kDa cut-off), treated with acidified acetone to remove LDS and salts [33], and dried. The pellet was resuspended and subjected to exhaustive tryptic digestion [trypsin/peptide ratio 1:10 (w/w)]. For this purpose, 0.5–1.0 mg of purified large tryptic fragments of the Ca²⁺-ATPase were first incubated with 50 mM NH₄HCO₃ (pH 8.2), 1 M urea, and 1 mM dithiothreitol for 15 min at 50 °C, and finally in the presence of 0.05–0.1 mg of trypsin for 16–24 h at 37 °C. The peptides were chromatographed immediately or stored at 4 °C for not more than 24 h.

HPLC–ESI-MS analysis of tryptic peptides

The identification of tryptic peptides was carried out by microbore HPLC on-line-coupled to an electrospray mass spectrometer [Autospec-Q tandem hybrid mass spectrometer; obtained from VG Analytical (now Micromass UK Ltd.), Wythenshaw, Manchester, U.K.] equipped with an OPUS data system. The mass range was scanned in the positive mode from 500–2500 atomic mass units (a.m.u.) with a scan rate of 8 s/decade (= 8 s/10 mass units) (resolution of 1500). The microbore HPLC instrumentation consisted of two pumps (Micro-Tech Scientific, Sunnyvale, CA, U.S.A.), a dynamic mixing chamber with a volume of 20 µl (Micro-Tech Scientific), and a model 8125 injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 15 µl sample loop. Separations were performed on a 150 mm × 1.00 mm C₁₈ column [Zorbax, 5 µm particle size, 30 nm (300 Å); Hewlett–Packard, Palo Alto, CA, U.S.A.] at a flow rate of 50 µl/min, followed by a post-column split diverting 8 µl/min into the mass spectrometer and 42 µl/min into a collection vial. The peptides were monitored at 214 nm with a UV detector equipped with a micro flow cell (model 200; Linear Instruments, Fremont, CA, U.S.A.). Separation of the peptides was achieved with the following steps: 2 min isocratic elution with 98% mobile phase A, containing a 98:2:0.1 (by vol.) mixture of water, acetonitrile and trifluoroacetic acid (TFA), and 2% mobile phase B, containing a 10:90:0.09 (by vol.) mixture of the same solvents, followed by a linear gradient increasing mobile phase B from 2 to 20% within 10 min, followed by a linear gradient increasing mobile phase B at 1%/min over 70 min, and concluded with a linear gradient increasing mobile phase B at 0.5%/min within 15 min. Collected fractions of interest were concentrated and stored at –70 °C for further analysis of nitrotyrosine by UV–visible spectroscopy.

Statistics

For each age, six groups of animals containing three to six rats were killed for the SR preparations. The values in the Figures and Tables are expressed as means ± S.E. Groups were compared by Student's *t*-test analysis to determine statistically significant differences.

RESULTS

Characterization of SR preparations

We used SR vesicles isolated from skeletal muscle of young adult (5 months), middle-aged (10 and 16 months) and old (26–28 months) Fisher 344 rats. Two distinct preparations were (i) 'slow', enriched in slow-twitch fibres (isolated from soleus, adductor longus and vastus intermedius muscles), and (ii) 'fast',

enriched in fast-twitch fibres (isolated from the remaining rat hindlimb muscles). Results from assays of abundance, isoform typing and functional properties of the Ca²⁺-ATPase protein are presented in Table 1. Data were collected for most assays from a total of 72 individual animals, consisting of six paired preparations of each age group. In agreement with our previous study characterizing age-related alterations in SR membranes isolated from mixed-fibre-type muscles of Fisher 344 rats [10], we found no significant age-related changes in the relative content of total Ca²⁺-ATPase protein based on densitometry of Coomassie Blue-stained gels (Figure 1A and Table 1) or in the content of the fast-twitch isoform SERCA1 and slow-twitch isoform SERCA2a, as determined by Western-blot analysis (Figures 1B and 1C, and Table 1, entries 1–4) for each fibre-type-specific preparation. Both types of our SR preparations were functionally active; however, we observed different extents of ionophore stimulation, ≈ 3.6-fold for 'fast' and 2.2-fold for 'slow' SR. When assayed under identical conditions (i.e. in the presence of oxalate), the coupling ratio, expressed as ratio of Ca²⁺ transported per ATP hydrolysed (determined from the ratio of Ca²⁺ uptake and Ca²⁺-dependent ATPase activity) was nearly 2-fold lower for 'slow' as compared with 'fast' SR. This probably reflects fibre-type-specific differences in lipid composition and lipid/protein ratio resulting in less tightly sealed vesicles of 'slow' SR [34].

'Fast' SR preparations from all four age groups were enzymically active with nearly identical Ca²⁺-dependent ATPase activities and Ca²⁺ transport rates (Table 1, entries 8 and 9). The slight increase (≈ 8%) in the rate of Ca²⁺ uptake in SR from 26–28-month-old animals can be explained by the increased Ca²⁺-binding capacity of SR membranes that we (results not shown) and others have observed [35].

Although our 'slow' preparations consisted of only ≈ 50% of the slow-twitch isoform, SERCA2a, we were able to detect functional differences between the two distinct fibre-type preparations as well as age-related differences among 'slow' SR preparations. 'Slow' SR exhibited a much lower Ca²⁺-dependent ATPase activity (entry 6) and Ca²⁺-uptake rate (entry 9) (based on total SR protein) as compared with 'fast', due, in part, to the decreased abundance of the Ca²⁺-ATPase in slow-twitch muscle, isoform composition and regulatory inhibition of the SERCA2a by phospholamban [36,37]. In addition, between the ages of 10 and 16 months we find an approx. 18 or 26% age-related decrease of ionophore-stimulated Ca²⁺-dependent ATPase activity of 'slow' SR if activity is based on total SR protein (entry 6) or calculated relative to the content of the SR Ca²⁺-ATPase (entry 8) respectively. 'Slow' SR from 16- or 26–28-month-old rats also shows lower rates (≈ 30%; *P* < 0.05) of Ca²⁺ uptake compared with SR from 5- and 10-month-old animals (entry 9), in agreement with data previously reported by Narayanan et al. [35]. To further delineate the differential effect that aging has on the Ca²⁺-ATPase from slow- and fast-twitch fibres, we isolated SR from single muscles abundant in fast-twitch fibres (EDL) and slow-twitch fibres (soleus) from 5- and 28-month-old rats. In this case, the measured Ca²⁺-uptake rate of soleus SR from 28-month-old animals was 50% lower than that of SR from 5-month-old animals. The significantly larger decrease in the Ca²⁺-uptake rate of soleus SR suggests a prominent role of the SERCA2a and its modification in this age-related phenomenon. This is further supported by the fact that no age-related difference was evident in the Ca²⁺-transport function of SR from EDL muscle.

Previously we reported no initial difference in the Ca²⁺-dependent ATPase activity of mostly 'fast' SR isolated from 5- and 26–28-month-old rats [10,11]. However, exposure to elevated temperatures (37–40 °C) causes a progressive loss of activity

Table 1 Characterization of SR vesicles isolated from fast and slow-twitch skeletal muscles of Fisher 344 strain rats

		Age (months) ...	5		10		16		26–28	
Entry	Parameter	Muscle ...	Slow	Fast	Slow	Fast	Slow	Fast	Slow	Fast
1	Ca ²⁺ -ATPase (% of the total SR protein*)		23.8 ± 2.2	36.9 ± 2.0	25.4 ± 2.5	33.6 ± 2.0	27.8 ± 1.5	33.6 ± 2.8	26.1 ± 1.7	35.8 ± 1.6
2	SERCA 1a (% of the total Ca ²⁺ -ATPase†)		52.5 ± 5.0	91.7 ± 4.3	48.0 ± 2.8	89.8 ± 3.7	48.8 ± 0.7	89.4 ± 4.2	49.7 ± 5.5	90.8 ± 3.4
3	SERCA 1a (nmol/mg of SR protein)		1.14	3.06	1.11	2.74	1.23	2.73	1.17	3.02
4	SERCA 2a (% of the total Ca ²⁺ -ATPase†)		48.0 ± 3.9	8.3 ± 1.0	53.8 ± 2.8	10.5 ± 1.9	53.6 ± 1.0	10.7 ± 1.1	50.2 ± 2.8	9.2 ± 1.9
5	SERCA 2a (nmol/mg of SR protein)		1.05	0.28	1.19	0.35	1.36	0.36	1.20	0.33
6	Ca ²⁺ -dependent ATPase activity‡		2.2 ± 0.1	3.2 ± 0.4	2.3 ± 0.2	3.6 ± 0.2	1.9 ± 0.1¶	3.4 ± 0.2	1.8 ± 0.1**	3.4 ± 0.2
7	Basal ATPase activity§		0.5 ± 0.10	0.4 ± 0.02	0.5 ± 0.03	0.4 ± 0.03	0.6 ± 0.01	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.03
8	Ca ²⁺ -dependent ATPase activity		9.3 ± 0.4	8.6 ± 0.4	9.0 ± 0.7	10.8 ± 0.5	6.7 ± 0.3¶	10.0 ± 0.9	6.8 ± 0.5**	9.4 ± 0.5
9	Ca ²⁺ -uptake rate (μmol of Ca ²⁺ /min per mg of SR)		1.6 ± 0.1	4.9 ± 0.1	1.7 ± 0.2	5.0 ± 0.4	1.1 ± 0.1††	4.9 ± 0.1	1.3 ± 0.2††	5.3 ± 0.4

* Data were obtained by densitometric analysis of Laemmli SDS/5%-PAGE gels stained with Coomassie Blue; values are means (± S.E.M.) % for Ca²⁺-ATPase (SERCA 1a + SERCA 2a) relative to the total SR proteins.

† Data are obtained by densitometric analysis of Western immunoblots; values are means (± S.E.M.) % for SERCA 1a or SERCA 2a relative to the total Ca²⁺-ATPase content.

‡ Ca²⁺-dependent ATPase activity was calculated as the difference between total and basal ATPase activity, as described in the Experimental section; values are means (± S.E.M.) (μmol of P_i/min per mg of SR protein).

§ Values are means (± S.E.M.) (μmol of P_i/min per mg of SR protein).

|| Ca²⁺-dependent ATPase activity was assayed as in ‡, but values are means (± S.E.M.) (μmol of P_i/min per mg of Ca²⁺-ATPase).

¶ 16-month samples were significantly different from 5–10-months samples at the *P* < 0.016 level (*n* = 6).

** 26–28-month samples were significantly different from 5–10-month samples at the *P* < 0.002 level (*n* = 6).

†† 16- and 26–28-month samples were significantly different from 5-month samples at the *P* < 0.1 level (*n* = 6).

Figure 1 Characterization of SR preparations from fast (f) and slow-twitch (s) skeletal muscle of 5-month-old (Y) and 28-month-old (O) Fisher 344 strain rats

SR vesicles (50 μg of protein/line) from 5- and 28-month-old rats were analysed by SDS/5%-PAGE (A) and immunostaining with monoclonal antibodies to SERCA 1 (B), to SERCA 2a (C) and to nitrotyrosine (D).

which is more rapid in SR vesicles isolated from old as compared with that from young rats [10,11]. As shown in Figure 2, both 'slow' and 'fast' SR isolated from young rats exhibit an initial resistance to heat inactivation (for ≈ 1 h) in contrast with the immediate loss of activity of SR from old rats. Noteworthy is the slight increase of activity within the first hour only observed for 'slow' SR from 5-month-old rats. This is likely due to a temperature-induced dissociation of a small fraction of non-covalent Ca²⁺-ATPase aggregates. Subsequently, over the next 3 h, a time-dependent inactivation proceeded with a higher rate for 'slow' as compared with 'fast' SR for all age groups. The results presented here clearly demonstrate differences in heat-

stability between our 'slow' and 'fast' SR preparations, as well as age-related differences of the 'slow' SR preparations.

Age-related posttranslational modifications of the SR Ca²⁺-ATPase

SR preparations from 5-months-old rats contained 93.2 and 114.8 nmol of free thiol/mg of SR protein for 'fast' and 'slow' preparations respectively (see Table 2). Following the reaction with [¹⁴C]NEM, and subsequent electrophoretic separation of the Ca²⁺-ATPase from the other SR proteins and liquid-scintillation counting, we calculate that approx. 73 and 60 % of the total thiols belong to the Ca²⁺-ATPase in 'fast' and 'slow' SR

Figure 2 Heat-inactivation of the Ca^{2+} -dependent ATPase in fast (■, □) and slow-twitch (●, ×, ○) skeletal-muscle SR preparations from 5-month-old (■, ●), 10-month-old (×) and 28-month-old (□, ○) rats

SR vesicles (4 mg/ml) were incubated at 40 °C in 20 mM Mops (pH 7.0)/5 mM MgCl_2 /0.4 mM CaCl_2 . At the indicated times, aliquots were withdrawn and assayed for ATPase activity as described in the Experimental section. Corresponding Ca^{2+} -dependent ATPase activity values of 100% are shown in Table 1.

preparations respectively. These results are in agreement with the lower total content of Ca^{2+} -ATPase in our 'slow' preparations, quantified by densitometry of Coomassie Blue-stained gels and Western immunoblots (Table 1). The difference in calculated thiol content between fibre-type-specific preparations also reflects the difference in cysteine residues between the two isoforms, where SERCA1 and SERCA2a contain 24 and 26 mol cysteine

Figure 3 Age-dependent accumulation of nitrotyrosine on the SERCA2a isoform of the SR Ca^{2+} -ATPase in 'slow' and 'fast' SR preparations, quantified by amino acid analysis (in mol/mol of protein)

residues/mol of protein respectively (and two or three intra-molecular disulphide bonds) [37–39].

Aging results in a progressive loss of SR protein thiols, ultimately 12–15%, for both 'fast' and 'slow' preparations over the whole investigated aging period, i.e. 5–28 months. Quantification of [^{14}C]NEM specifically associated with the Ca^{2+} -ATPase reveals an age-related loss of approx. 1.5 mol of free thiols/mol of Ca^{2+} -ATPase in 'fast' and 3.5 mol of thiols/mol of Ca^{2+} -ATPase in 'slow' SR preparations (Table 2). This specific loss of SR Ca^{2+} -ATPase thiols is complete at 10 months of age with no further age-related changes. However, the Ca^{2+} -dependent ATPase activity is *not* lost at ages less than 10 months, implying that the modified Ca^{2+} -ATPase Cys residues of both 'fast' and 'slow' preparations are not critically important for Ca^{2+} -ATPase activity. Especially for 'slow' SR preparations the decrease in the Ca^{2+} -dependent ATPase activity and Ca^{2+} -uptake rate observed between 10 and 16 months of age cannot be solely due to cysteine modification.

Western blotting using an anti-nitrotyrosine antibody (Figure 1D) demonstrates that only the SERCA2a, and not SERCA1, accumulates nitrotyrosine with increasing age. The exclusive nitration of SERCA2a was confirmed in additional experiments when especially NEM-labelled SERCA2a was well separated from residual phosphorylase *b*. The nitrotyrosine content of

Table 2 Determination of free thiol groups in rat skeletal-muscle SR preparations and Ca^{2+} -ATPase with [^{14}C]NEM

Age (months)	Muscle ...	Slow		Fast	
		SR vesicles*	Ca^{2+} -ATPase†	SR vesicles*	Ca^{2+} -ATPase†
5		114.8 ± 1.8 (100%)	25.2 ± 0.5 (26)	93.2 ± 1.9 (100%)	22.1 ± 0.1 (24)
10		110.4 ± 5.8 (96%)	21.5 ± 1.0§	78.5 ± 1.7 (84%)§	19.0 ± 0.1¶
16		108.3 ± 0.3 (94%)	22.6 ± 0.4	72.6 ± 1.1 (78%)	20.5 ± 0.1
28		106.2 ± 0.5 (93%)‡	21.7 ± 0.9§	72.5 ± 1.0 (78%)	20.6 ± 0.2§

* Values are means (± S.E.M.) (mol of NEM per mg of SR protein).

† Values are means (± S.E.M.) (mol of NEM per mol of Ca^{2+} -ATPase).

‡ 28-month samples were significantly different from 5-month samples at the $P < 0.05$ level.

§ 10- and 28-month samples were significantly different from 5-month samples at the $P < 0.02$ level.

|| 28-month samples were significantly different from 5-month samples at the $P < 0.005$ level.

¶ 10-month samples were significantly different from 5-month samples at the $P < 0.001$ level.

Figure 4 Effect of NO/O₂ (A) and ONOO⁻ (B) on the SR vesicles isolated from fast-twitch skeletal muscles of 5-month-old rats

SR vesicles (2 mg/ml) were treated with DEA/NO (closed symbols) for 30 min or with ONOO⁻ (open symbols) for 10 min at room temperature in 10 mM potassium phosphate buffer (pH 7.4)/100 mM NaCl. Aliquots were withdrawn and assayed for Ca²⁺-dependent ATPase activity (■, □), Ca²⁺-ATPase free thiols (▲, △), Ca²⁺-ATPase monomers (◆, ◇) and the nitrotyrosine/SERCA2a ratio (●, ○) as described in the Experimental section. The results are the means for three independent experiments and are expressed as a percentage of control, or arbitrary units (a.u.) (for the nitrotyrosine/SERCA2a ratio). Corresponding parameters values of 100% are shown in Tables 1 and 2.

Figure 5 Effect of NO/O₂ (A) and ONOO⁻ (B) on the SR vesicles isolated from slow-twitch skeletal muscle of 5-month-old rats

SR vesicles (2 mg/ml) were treated with DEA/NO (closed symbols) for 30 min or with ONOO⁻ (open symbols) for 10 min at room temperature in 10 mM potassium phosphate buffer (pH 7.4)/100 mM NaCl. Aliquots were withdrawn and assayed for Ca²⁺-dependent ATPase activity (■, □), Ca²⁺-ATPase free thiols (▲, △), Ca²⁺-ATPase monomers (◆, ◇) and the nitrotyrosine/SERCA2a ratio (●, ○) as described in the Experimental section. Results are means for three independent experiments and are expressed as a percentage of control, or arbitrary units (a.u.) (for nitrotyrosine/SERCA2a ratio). Corresponding parameters values of 100% are shown in Tables 1 and 2.

purified SERCA2a of both 'slow' and 'fast' SR preparations was independently quantified by amino acid analysis following PITC derivatization (Figure 3). We observe a progressive age-dependent increase in the nitrotyrosine content for both preparations with maximum yields of $\approx 3.5 \pm 0.7$ mol of nitrotyrosine/mol of protein. This accumulation of nitrotyrosine is paralleled by the age-dependent loss of $\approx 3.3 \pm 1.0$ mol of tyrosine/mol of protein. Amino acid analysis showed no significant accumulation

of 3,5-dinitrotyrosine at all ages. Fluorescence measurements revealed no significant emission spectrum in the 400–500 nm region, documenting that bityrosine is not a product of tyrosine modification. The age-dependent accumulation of nitrotyrosine on the SERCA2a reached maximum levels earlier in 'fast' as compared with 'slow' SR preparations. For both preparations an average of $\approx 1.0 \pm 0.5$ mol of nitrotyrosine/mol of protein was already present in 5-month-old rats.

Table 3 Nitrotyrosine content in large tryptic and V8 fragments of the SERCA2a

Results were obtained by amino acid analysis of corresponding peptide band from slow-twitch muscle SR preparations, purified as described in the Experimental section. Values are means for two different analyses of one group of animals. Fragments A₁ and B are tryptic fragments [42], and p84, p53, and p28 are V8 fragments [43].

Peptide band	Age (months) ...	Nitrotyrosine content (mol/mol of protein)	
		5	28
A ₁ (199–505)		0.56	1.84
B (506–997)		0.90	1.01
p84 (232–997)		0.54	2.73
p53 (232–714)		0.00	2.09
p28 (749–997)		0.56	0.68

Figure 6 Immunodetection of nitrotyrosine in the V8 fragments of SERCA2a obtained by immunoprecipitation with monoclonal antibodies against nitrotyrosine

Peptides were separated with 10% (A)- or 16.5% (B)-polyacrylamide/Tricine gel and analysed by Western blot using anti-nitrotyrosine antibodies (see the Experimental section). Approximate sizes (kDa) of peptides were estimated using prestained molecular-mass markers (Bio-Rad) as shown on the left.

In vitro modification of the SR Ca²⁺-ATPase by NO and ONOO⁻

Activity and chemical characterization

One species potentially responsible for the *in vivo* nitration of proteins is ONOO⁻, formed according to reaction (1). Experimentally, the functional consequences of *in vitro* tyrosine nitration through ONOO⁻ [and other nitrating agents such as nitrogen dioxide (NO₂) or tetranitromethane] are difficult to

study as these agents also effectively modify thiols. Hence, we designed an approach for the evaluation of nitration-specific functional consequences of ONOO⁻ exposure by comparison with results with a weak thiol-modifying system, NO/O₂, as a reference system. Both 'slow' and 'fast' SR vesicles isolated from 5-month-old rats were allowed to react with 0–200 μM of either ONOO⁻ or the NO donor DEA/NO (DEA/NO yields 1.5 equivalents of NO per DEA/NO [19]). We shall first describe the results of the reference system. Figures 4A and 5A display the results of incubating 'fast' and 'slow' SR vesicles over 30 min at 25 °C in air-saturated solutions of DEA/NO. The exposure of both 'fast' and 'slow' SR vesicles to increasing concentrations of DEA/NO resulted in a gradual loss of the Ca²⁺-dependent ATPase activity (overall loss of up to ≈ 20 % for both SR preparations), accompanied by a small loss of the Ca²⁺-ATPase thiol groups (up to ≈ 15 %; monitored by NEM labelling of the Ca²⁺-ATPase). For 'fast' SR no significant loss of Ca²⁺-ATPase monomers was detected for concentrations of [DEA/NO] ≤ 100 μM (monitored by SDS/PAGE or Western immunoblots using anti-SERCA1 monoclonal antibodies), whereas 'slow' SR displayed a loss of Ca²⁺-ATPase monomers only for [DEA/NO] ≤ 50 μM. There was no significant accumulation of nitrotyrosine compared with controls for both preparations. These results suggest that the inactivation of the Ca²⁺-ATPase by NO/O₂ is predominantly caused by the modification of protein cysteine residues, corroborated by the strong correlation between Ca²⁺-ATPase inactivation and the loss of thiol groups. The fact that Ca²⁺-ATPase monomers of 'fast' and 'slow' SR are lost at different concentrations of DEA/NO suggests a different susceptibility of 'fast' and 'slow' SR to chemical modification, and likely a different susceptibility of SERCA1 and SERCA2a. Preliminary HPLC-ESI-MS data on SERCA1 indicate that NO/O₂-dependent thiol modification affects Cys³⁴⁴ and Cys³⁴⁹, most likely by disulphide formation (R. I. Viner, T. D. Williams and C. Schöneich, unpublished work).

The exposure of 'fast' and 'slow' SR vesicles to ONOO⁻ (Figures 4B and 5B), results in a more significant loss of Ca²⁺-ATPase activity (40–50 %). Both ONOO⁻-treated 'fast' and 'slow' SR vesicles demonstrate a significant loss of Ca²⁺-ATPase monomers for [ONOO⁻] ≤ 50 μM, ≈ 20 % for 'fast' SR and 10 % for 'slow' SR, where aggregation is predominantly caused by intermolecular disulfide formation [40]. The content of free thiol groups per Ca²⁺-ATPase monomer does not change for [ONOO⁻] ≤ 100 μM ('slow') and [ONOO⁻] ≤ 200 μM ('fast'). Both 'fast' and 'slow' SR vesicles selectively accumulate nitrotyrosine only on the SERCA2a isoform, with larger yields, ≈ 4-fold higher over controls, for 'slow' SR vesicles (containing ≈ 50 % SERCA2a) respective to 'fast' SR vesicles (containing ≈ 10 % SERCA2a). By comparison of the effects of ONOO⁻ and the reference system (NO/O₂) on 'slow' SR, functional consequences of nitrotyrosine formation may be appreciated. For both systems, the loss of Ca²⁺-ATPase monomers is essentially completed at oxidant concentrations ≤ 50 μM, and the ultimate loss of free Ca²⁺-ATPase thiols is comparable in particular for [oxidant] = 100–200 μM. In contrast, the exposure to ONOO⁻ results in a significantly larger (> 2-fold) decrease in the Ca²⁺-ATPase activity in the range of [ONOO⁻] = 100–200 μM as compared with [DEA/NO] = 100–200 μM, paralleled by the accumulation of nitrotyrosine on SERCA2a. Thus we cannot exclude that nitrotyrosine formation affects the activity of SERCA2a, either alone or in combination with thiol modifications. Supporting evidence is derived from preliminary HPLC-MS studies (R. I. Viner, T. D. Williams and Ch. Schöneich, unpublished work) showing that both NO/O₂ and ONOO⁻ modify the same Ca²⁺-ATPase cysteine residues, i.e. the

Figure 7 Structural diagram of the SERCA2a displaying the location of tyrosine residues [38]

This diagram is based on the predicted tertiary structure of the Ca²⁺-ATPase [69] that includes β -structure (arrows) and α -helices (rectangles) in the cytosolic part and 10 (M1–M10) transmembrane α -helices. Initial tryptic (T₁ at Arg⁵⁰⁵-Ala⁵⁰⁶; T₂ at Arg¹⁹⁸-Ala¹⁹⁹) [42] and V8 (V8₁ at Glu²³¹-Ile²³²; V8₂ at Glu⁷¹⁴-Ile⁷¹⁵; V8₃ at Glu⁷⁴⁸-Gly⁷⁴⁹) [43] cleavage sites as well as the enzyme's phosphorylation site P (Asp³⁵¹) are indicated by small arrows. At the bottom is shown a linear map for the main Ca²⁺-ATPase fragments after trypsin and V8 treatment; closed rectangles show nitrated areas of the protein.

difference in Ca²⁺-ATPase activity cannot be due to the selective modification of different cysteine residues.

Effect of *in vitro* modification on Ca²⁺ transport

In vitro, ONOO[−]-treated 'fast' SR vesicles show a $\approx$ 90–95 % decrease of the Ca²⁺-uptake rate (compared with reverse-order-of-addition experiments) already after exposure to 2 μ M of ONOO[−] (with no further change between 2 and 50 μ M ONOO[−]) (results not shown). This effect cannot necessarily be accounted for solely by Ca²⁺-ATPase modification, as it may also be the result of oxidant-induced Ca²⁺-release from the ryanodine receptor that is present in our SR preparations at levels of $\approx$ 2 % [7]. Preincubation of our SR vesicles with 5–20 μ M Ruthenium Red (a blocker of the ryanodine receptor) for up to 10 min at 25 °C before exposure to ONOO[−] did not inhibit the ONOO[−]-induced decrease in the Ca²⁺-uptake rate; however, this result is in line with previous reports documenting that low concentrations of ONOO[−] (0.78 μ M) induce Ca²⁺ release from the ryanodine receptor which could not be blocked by 20 μ M Ruthenium Red or reversed through subsequent exposure to a reducing agent

[41]. Thus ONOO[−] has a complex effect on skeletal-muscle SR vesicles and functional parameters such as the Ca²⁺-uptake rate cannot be rationalized by the modification of a single protein alone unless purification and reconstitution experiments allow the full characterization of a single protein independent from other SR components.

Localization of nitrotyrosine

For localizing the nitrotyrosine residues within the SERCA2a sequence we performed limited and exhaustive trypsin and V8 proteolysis.

Limited digestion of the SR Ca²⁺-ATPase with trypsin yields the major subfragments A, A₁, A₂ and B [42]. By Western-blot analysis we monitored the presence of nitrotyrosine on fragments A₁, A₂, and B. With the exception of one out of the six groups of old animals analysed we detected no nitration on the A₂ fragment, containing Tyr¹²² and Tyr¹³⁰. By amino acid analysis we quantified the extent of tyrosine nitration on the purified fragments A₁, A₂, and B (Table 3). Amino acid analysis of these purified fragments was representatively carried out for one group

Figure 8 Separation of SR Ca^{2+} -ATPase tryptic peptides after 24 h of digestion by reverse-phase HPLC on-line-coupled to ESI-MS

Separation was performed on a 1.00 mm $\times$ 150 mm Zorbax C_{18} column (Micro-Tech Scientific, Sunnyvale, CA, U.S.A.) at a flow rate of 50 $\mu\text{L}/\text{min}$, of which 8 $\mu\text{L}/\text{min}$ was diverted into the mass spectrometer by flow-splitting. In the peaks indicated by numbers, the following peptides of interest were identified: peak 1, Gly²⁹¹-Lys²⁹⁷; peak 2, Thr⁷²⁸-Lys⁷⁵⁷; peak 3, Val²⁶³-Lys²⁹⁷; peak 4, Ala⁷⁵¹-Arg⁷⁶¹. Peaks in which the identified peptides contained nitrated tyrosine are marked with an asterisk (*).

Insert: enlarged part of the chromatogram.

of animals. Old (28 months) animals contain ≈ 1.8 mol of nitrotyrosine/mol on fragment A₁ which carries seven of 18 total tyrosine residues of the SERCA2a [38] and ≈ 1.0 mol of nitrotyrosine/mol of peptide on fragment B (nine out of 18 tyrosine residues [38]). A significant age-dependent increase of nitrotyrosine accumulation was only observed for fragment A₁.

Subsequently, SR proteins were digested by V8 protease in the presence of a non-ionic detergent, C_{12}E_9 , followed by immunoprecipitation with a monoclonal anti-nitrotyrosine antibody. Western-blot analysis of immunoprecipitated peptides fractionated by a 10%- or a 16.5%-acrylamide/3%-methylene-bisacrylamide/Tricine gel was performed using the same anti-nitrotyrosine antibody to permit detection of nitrated SERCA2a peptides. Figure 6 shows the Western-blot analysis of the V8-derived peptides, labelled as described by Le Maire et al. [43], who first reported the V8-digestion of native SR Ca^{2+} -ATPase. The V8 fragments were characterized by their migration on the gel, by amino acid analysis and, in the case of p28, by N-terminal sequencing (N-terminal sequence of p28 is GRAIYNNM). We were unable to retrieve any lower-molecular-mass nitrated peptides from the original V8 digestion by immunoprecipitation, probably due to an insufficient affinity of the anti-nitrotyrosine antibodies to smaller peptides. Both p84 and p53 are well separated from the IgG heavy chain on a 10% gel (Figure 6A), whereas the clean separation of p28 from IgG light chain was achieved on a 16.5% gel, representatively shown for a sample from 28-month-old rats in Figure 6(B). Amino acid analysis of the purified V8 fragments (Table 3) revealed an additional age-

dependent accumulation of ≈ 2 mol of nitrotyrosine/mol of peptide on fragment p53 (Ile²³²-Glu⁷¹⁴) and small additional yields on p28. Western-blot analysis of 'slow' SR from 5-month-old rats exposed to 200 μM ONOO⁻ demonstrates accumulation of nitrotyrosine on p53 (Figure 6A), supporting a potential role for ONOO⁻ in the age-dependent formation of nitrotyrosine.

Figure 7 illustrates the location of all primary V8 and trypsin proteolysis sites in the SERCA2a sequence, important for a discussion of the results. The results shown in Table 3 exclude *in vivo* nitration of tyrosine at positions 122, 130 and 586.

In order to further identify the exact location of nitrated tyrosine residues, purified and delipidated SERCA2a of 28-month-old rats was subjected to exhaustive tryptic digestion, followed by HPLC-ESI-MS analysis. Figure 8 displays the tryptic-peptide map obtained by reversed-phase HPLC on a microbore Zorbax C_{18} column using a binary gradient of water and acetonitrile in 0.1% TFA. These conditions provided satisfactory separation and MS resolution of tryptic peptides corresponding to $\approx 84\%$ of the Ca^{2+} -ATPase sequence (only a few mostly transmembrane fragments of the protein could not be resolved). As an internal control, for some randomly selected peptides, fragmentation patterns obtained via tandem MS analysis verified the correct assignment of detected masses to the respective Ca^{2+} -ATPase peptides (results not shown).

HPLC-ESI-MS analysis revealed only non-nitrated peptides containing Tyr¹²² (Glu¹²¹-Lys¹²⁸), Tyr¹³⁰ (Val¹²⁹-Arg¹³¹) and Tyr⁵⁸⁶ (Arg⁵⁷²-Arg⁶⁰³), confirming our initial result from limited proteolytic digest and amino acid analysis. Further, we only

Table 4 Tyrosine and nitrotyrosine in tryptic peptides of the SERCA2a, identified by HPLC–ESI MS

The asterisk (*) indicates a nitrated tyr residue in the respective fragment.

Identified peptide	Tyrosine residue
Glu ¹²¹ –Lys ¹²⁸	Tyr ¹²²
Val ¹²⁹ –Arg ¹³¹	Tyr ¹³⁰
Val ²⁶³ –Lys ²⁹⁷	*Tyr ²⁹⁴ , *Tyr ²⁹⁵
Gly ²⁹¹ –Lys ²⁹⁷	*Tyr ²⁹⁴ , *Tyr ²⁹⁵
Gly ²⁹¹ –Arg ³²⁴	*Tyr ²⁹⁴ , *Tyr ²⁹⁵
Gly ⁴³² –Arg ⁴⁶⁷	Tyr ⁴³⁴
Gly ⁴³² –Lys ⁴³⁶	Tyr ⁴³⁴
Lys ⁴⁸¹ –Lys ⁵¹⁰	Tyr ⁴⁹⁷
Ser ⁴⁹³ –Lys ⁵¹⁴	Tyr ⁴⁹⁷
Ser ⁴⁹³ –Arg ⁵²⁸	Tyr ⁴⁹⁷
Arg ⁵⁷² –Arg ⁶⁰³	Tyr ⁵⁸⁶
Thr ⁷²⁸ –Lys ⁷⁵⁷	*Tyr ⁷⁵³
Ala ⁷⁵¹ –Arg ⁷⁶¹	*Tyr ⁷⁵³
Tyr ⁷⁶² –Arg ⁸²¹	Tyr ⁷⁶²
Val ⁸⁶⁴ –Lys ⁸⁷⁶	Tyr ⁸⁶⁷
Asn ⁹⁸⁹ –Glu ⁹⁹⁷	Tyr ⁹⁹⁰

detected non-nitrated peptides containing tyrosine at positions 434, 497, 762, 867 and 990, summarized in Table 4. On the other hand, we only detected molecular masses 90 a.m.u. higher than those expected for the peptides Gly²⁶³–Lys²⁹⁷ and Gly²⁹¹–Lys²⁹⁷ (Table 4), each containing the sequence Tyr²⁹⁴–Tyr²⁹⁵. For example, the monoisotopic mass of native [Gly²⁹¹–Lys²⁹⁷ + H] is 861.4 and the detected mass was 951.4 a.m.u. The detected mass of [M (the molecular ion) + H] = 951.4 suggests simultaneous mononitration of both Tyr²⁹⁴ and Tyr²⁹⁵ or dinitration of either Tyr²⁹⁴ or Tyr²⁹⁵ in the sequences Gly²⁶³–Lys²⁹⁷ and Gly²⁹¹–Lys²⁹⁷. However, 3,5-dinitrotyrosine was not detected by amino acid analysis. A small quantity of nitrotyrosine (peak area of the mass spectrometry signal of the modified peak was < 10% relative to the peak area of the native unmodified peak) was also located to the sequences Thr⁷²⁸–Lys⁷⁵⁷ and Ala⁷⁵¹–Arg⁷⁶¹, both containing Tyr⁷⁵³ (Tyr⁷⁵³ belongs to the V8 fragment p28; that Tyr⁷⁵³ cannot be the only nitrated tyrosine residue of p28 is realized from the fact that nitrated p28 was immunoprecipitated with the anti-nitrotyrosine antibody and subsequently identified by N-terminal sequencing, yielding the sequence GRAIYNNM). Sample quantities of the potentially nitrated peptides were too low in order to obtain satisfactory MS/MS results. However, analysis of the corresponding HPLC fractions by UV spectroscopy at pH 12 showed a detectable peak with a maximum around 420–428 nm, diagnostic for nitrotyrosine in these fractions [30]. MS analysis did not show any evidence for the age-dependent accumulation of nitrosothiols or nitrosoamines.

DISCUSSION

Isoform-specific nitration of the SR Ca²⁺-ATPase

Recently, Narayanan et al. reported that the effects of aging on skeletal-muscle SR function are muscle-specific, with a significant age-dependent change in ATP-supported Ca²⁺-uptake activity for slow-twitch (soleus), but no change for fast-twitch (gastrocnemius), muscle [35]. Our results may provide a molecular-chemical rationale for these observations. The skeletal-muscle SR Ca²⁺-ATPase, primarily responsible for the transport of cytosolic Ca²⁺ into the lumen of SR, is selectively nitrated *in vivo* on the slow-twitch muscle isoform SERCA2a. *In vitro*, the exposure of SR membranes to ONOO[−] also results in the

selective nitration of the SERCA2a, even in the presence of nearly equal or excess amounts of SERCA1, suggesting that ONOO[−] could be the source of the nitrating species *in vivo*. Several rationales may be forwarded for this remarkable selectivity for SERCA2a. The highly (84%) similar [37,38] SERCA1 and SERCA2a isoforms may exist in different conformations in the SR membrane. For example, SERCA2a associates with the regulatory protein phospholamban [44,45] which is present in slow-twitch, but absent from fast-twitch, muscle fibres. We note that different reactivities towards selected reactive oxygen species were also observed for the SERCA2b and SERCA3 isoforms of the SR Ca²⁺-ATPase [46] and different isoforms of the Na,K-ATPase [47]. An alternative possibility for the selective accumulation of nitrotyrosine on SERCA2a could be that post-translationally modified SERCA1 and SERCA2a are turned over at different rates (so far turnover rates have only been measured for SERCA1 [7]). However, it is unlikely that such different turnover rates would only apply to nitrated and not to thiol-modified isoforms; both SERCA1 and SERCA2a show an age-dependent accumulation of thiol modifications. This, and the fact that the *in vitro* exposure of SR membranes to ONOO[−] results in the exclusive nitration of the SERCA2a, suggests that the specific nitration of SERCA2a is caused by a chemical selectivity of the nitrating species. Recently, Kamisaki et al. described a 'nitrotyrosine denitrant' activity located especially in spleen and lung [48]. This activity, likely a protein, targets several, but not all, nitrotyrosine-containing proteins. At present this activity has not been localized to skeletal muscle. However, the presence of significant levels of nitrated SERCA2a, even in young tissue, would suggest that SERCA2a is not a substrate for the denitrant activity, if present.

Localization of nitrotyrosine and functional effects

On the basis of the amino acid analysis of large proteolytic fragments, most of the age-dependent increase of ≈ 2.0 mol of nitrotyrosine/mol of protein affects the sequence Ile²³²–Arg⁵⁰⁵. This is in accord with HPLC–MS analysis, which suggests a relatively complete age-dependent bisnitration of the sequence Tyr²⁹⁴–Tyr²⁹⁵, consistent with an incorporation of 2 mol of nitrotyrosine/mol of protein between Ile²³² and Arg⁵⁰⁵. SERCA2a isolated from 5-month-old animals contains a small initial amount of nitrotyrosine, part of which is located on the V8 fragment p28, which contains several tyrosine residues within the luminal and transmembrane domain (see Figure 7) [37,38].

Our *in vitro* experiments demonstrate that tyrosine nitration may affect the Ca²⁺-ATPase activity. This is also suggested by the *in vivo* data on 'slow' SR. During the first 10 months of *in vivo* aging, the Ca²⁺-ATPase accumulates ≈ 2 mol of nitrotyrosine/mol of protein (on the SERCA2a) and loses a maximum amount of thiols. However, there is no effect of these modifications on enzyme activity, demonstrating that the affected residues are not critically important for activity. Between 10 and 16 months of age, the Ca²⁺-ATPase loses ≈ 26% of its activity (Table 1, entry 8), but the only additional chemical modification observed is a further accumulation of ≈ 0.8 mol of nitrotyrosine/mol of protein (Figure 3). However, whether nitrotyrosine formation alone, or nitrotyrosine in combination with already modified cysteine residues causes this loss of activity cannot be specified.

Mechanisms of nitration, and formation and reaction of ONOO[−] in skeletal muscle

Our *in vitro* experiments demonstrate the selective nitration of SERCA2a by ONOO[−], suggesting that ONOO[−] may be one possible source of the nitrating agent *in vivo*. Several potential

mechanisms for tyrosine nitration have been discussed, including free-radical reactions, involving tyrosyl radicals and $^{\circ}\text{NO}_2$ [49–51], and ionic reactions, involving $^{+}\text{NO}_2$ [52]. The latter species served to rationalize the fact that nitration-sensitive sequences of superoxide dismutase [52,53] and neurofilament-L [54] contain the Tyr-Glu motif where the carboxylic acid side chain of Glu was proposed to stabilize an intermediary nitronium ion. We note, however, that the identified sequences nitrated in SERCA2a do not contain proximal glutamic acid residues C-terminal to the Tyr targets; only the three-dimensional structure of SERCA2a may provide information whether glutamic acid residues may be located close to the nitration-sensitive tyrosine residues. However, at present only an 0.8 nm (8 Å)-resolution three-dimensional structure of the rabbit SERCA1 is known [55].

Importantly, the nitration of tyrosine by ONOO^- can be catalysed by CO_2 through formation of an intermediary nitrosoperoxycarbonate anion [50]. At present we cannot specify whether any CO_2 -catalysed nitration plays a role for nitration during the biological aging of skeletal muscle. However, in one set of *in vitro* nitration experiments of SR membranes we deliberately added a final concentration of 25 mM bicarbonate to the reaction mixtures (at pH 7.4). Under these conditions, several SR proteins were nitrated, as monitored by Western-blot using an anti-nitrotyrosine antibody. This is in contrast with our *in vitro* experiments without added bicarbonate, where only SERCA2a was selectively nitrated, comparable with the results obtained *in vivo*. Thus it seems unlikely that formation of nitrosoperoxycarbonate causes the selective nitration of the SERCA2a *in vivo*. The selective nitration of SERCA2a was also observed when SR vesicles (2 mg/ml of protein) were exposed to 0.2–2.0 mM SIN-1 (a compound which simultaneously releases NO and $\text{O}_2^{\cdot -}$) or to 0.5 mM of each H_2O_2 and NO_2^- in the presence (but not in the absence) of 1.0 μM horseradish peroxidase (for the peroxidase-catalysed nitration of proteins, see also [56,57]). Thus, besides ONOO^- , other, nitrite-derived nitrating species have the potential for the selective nitration of the SERCA2a.

Immunostaining has demonstrated the presence of nitric oxide synthase (NOS) I and II immunoreactivity in rat [15,58–60] and human [61] skeletal muscle, mainly localized at the sarcolemma and enriched at the neuromuscular endplates [59,62,63]. More recently, the protein recognized by the anti-NOS I antibody has been characterized as a specific isoform, nNOS μ , which is 34 amino acids longer than NOS I and present only in skeletal and cardiac muscle [60]. Capanni et al. demonstrated an age-dependent increase of the levels of immunoreactive NOS I protein in rat skeletal muscle as well as the fact that, only in aged rats, is NOS I also localized to the cytoplasm [64]. Interestingly, NOS II immunoreactivity was localized to intracellular structures compatible with the SR and/or the transverse tubular system [59]. On the basis of these findings the formation of NO and NO-derived nitrating species can be expected in both fast- and slow-twitch muscle. The absolute amount of nitrating species would then depend on the rate of formation and the presence of antioxidants which competitively scavenge the nitrating species. In general, the antioxidant capacity of skeletal muscle increases with age, but more so for slow-twitch muscle. For example, age-dependent increased activities of glutathione peroxidase, catalase and Mn-SOD (normalized to citrate synthase) were only found for the soleus (mostly slow-twitch fibres), but not the EDL (predominantly fast-twitch) muscle, whereas levels and activity of Cu,Zn-SOD were elevated in both muscles [65]. In addition, the content of GSH was significantly higher in the soleus of aged rats [66]. Thus it appears that the slow-twitch (soleus) muscle is

better protected against the formation and reaction of nitrating species. Consistent with this fibre-type difference in antioxidant capacity we find that maximum nitration yields on the SERCA2a were attained at earlier age in 'fast' SR as compared with 'slow' SR. Importantly, ONOO^- readily diffuses across cell membranes [67] and does not necessarily have to react close to the location of its formation. A higher abundance of fast-twitch fibres in a particular muscle may lead to higher steady-state levels of ONOO^- available for nitration of SERCA2a in the residual slow-twitch fibres.

Recently, Leeuwenburgh et al. have reported that homogenate of skeletal muscle did not show a significant age-dependent increase of the total levels of nitrotyrosine in female Long-Evans/Wistar hybrid rats [68]. This is an important result, demonstrating that molecular-chemical changes associated with or responsible for age-dependent physiological alterations may not be detectable through the analysis of whole-tissue homogenates or extracts. The reason for this may be too low levels of individual modified proteins or offset of potentially higher levels of nitrotyrosine through the nitration of one protein by, for example, age-dependent lower expression of other potentially nitrated proteins. Thus for a molecular understanding of age-dependent alterations and the potential role of reactive oxygen and/or nitrogen species it is important to utilize enriched preparations and to characterize individual biomolecules, as pursued in the present study.

This work was supported by the National Institutes of Health (grant P01AG12993). We thank Professor J. S. Beckman for many helpful discussions and his gift of monoclonal antinitrotyrosine antibodies, Dr. R. L. Levine for preliminary experiments on simultaneous sequencing of the SERCA2a peptides, Dr. A. F. R. Hühmer for the preparation of ONOO^- , and Ms. J. L. Jensen for her assistance in SR vesicle preparation and amino acid analysis.

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Received 3 December 1998/1 March 1999; accepted 23 March 1999