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Proteomic analysis of age dependent nitration of rat cardiac proteins by solution isoelectric focusing coupled to nano-HPLC tandem mass spectrometry

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Abstract

Protein nitration occurs as a result of oxidative stress induced by reactive oxygen (ROS) and reactive nitrogen species (RNS). Therefore, protein nitration serves as a hallmark for protein oxidation in vivo. We have previously reported on age dependent protein nitration in cardiac tissue of Fisher 344 BN-F1 rats analyzed by two-dimensional gel electrophoresis; however, only one specific nitration site was identified (Kanski et al., 2005a). In the present report, we used solution phase isoelectric focusing (IEF) followed by nanoHPLC-ESI-MS/MS that allowed us to obtain good MS/MS data to identify specific sites of protein nitration in cardiac tissue. As expected, more nitrated proteins were detected in cardiac tissue of old rats, including myosin heavy chain, neurofibromin, tropomyosin and nebulin-related anchoring protein. The post-translational modification of these cytoskeletal proteins may provide some rationale for the age-dependent functional decline of the heart.

Introduction

The post-translational modification of tyrosine to nitrotyrosine (3-NY) represents a marker for protein modifications associated with various pathologies (Turko and Murad, 2002; Turko et al., 2003) and the process of biological aging (Greenacre and Ischiropoulos, 2001; Ischiropoulos, 2003; Kanski et al., 2005b). Protein nitration occurs as a result of oxidative stress, which leads to the oxidative metabolism of nitric oxide (NO), resulting in the formation of reactive nitrogen species (RNS) (Beckman, 1996; Ischiropoulos, 2003). Reactive oxygen species (ROS) are also generated as normal byproducts of oxidative metabolism (Kozlov et al., 2005), where estimates show that 2-5% of the oxygen flux through the mitochondrial electron transport chain suffers conversion into superoxide anion radical ($O_2^{\cdot-}$) (Traverse et al., 2006). Superoxide reacts with nitrogen monoxide (NO) to form peroxynitrite ($ONOO^-$) (Kissner et al., 1997), a powerful oxidant of aromatic and organosulfur compounds (Szabo, 2003; Virag et al., 2003). In addition, $ONOO^-$ is able to nitrate Tyr via multiple reaction mechanisms, either via a direct reaction with Tyr (Beckman et al., 1992; Lehnig, 1999), via catalysis by transition metals (Beckman et al., 1992; Beckman, 1996; Virag et al., 2003), or through the proton or CO_2 -assisted formation of

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nitrogen dioxide ($\cdot\text{NO}_2$) (Prütz et al., 1985; Beckman et al., 1992; Lehnig, 1999; Radi et al., 2001).

Protein nitration may affect protein structure, function, and turnover. An illustrative example is the mitochondrial manganese superoxide dismutase (Mn-SOD), which catalyzes the disproportionation of superoxide to O_2 and H_2O_2 . Mn-SOD was found to undergo almost complete inhibition when nitrated at Tyr³⁴ (MacMillan-Crow and Thompson, 1999; Quint et al., 2006; Xu et al., 2006). The crystal structures of native Mn-SOD and nitrated Mn-SOD were found to be closely superimposable; however, the nitration of Tyr³⁴ disrupts the H-bonding network at the active site, which may be the reason for protein inactivation (Quint et al., 2006). A crystal structure was also obtained for nitrated glutathione reductase (GR) (Savvides et al., 2002). Here, the nitration of two Tyr residues, Tyr¹⁰⁶ and Tyr¹¹⁴, was found to be responsible for protein inactivation. Comparison of the crystal structures of both native and nitrated GR shows that specifically the hydroxy group of 3-NY¹¹⁴ appears to be rotated by $\sim 60^\circ$ due to the creation of a local negative charge that changes the electrostatics of the active site (Savvides et al., 2002).

There is a significant age-dependent accumulation of 3-NY on proteins in cardiac (Kanski et al., 2005a) and skeletal muscle (Kanski et al., 2005b). Cardiac proteins are highly susceptible to nitration due to the periodic formation of NO and superoxide, mediating myocardial contractility (Adeghate, 2004; Hare and Stamler, 2005; Saraiva and Hare, 2006). NO can regulate cardiac function through the S-nitrosation of effector molecules such as Ca^{2+} ion channels, in particular the plasmalemmal L-type calcium channel and the sarcoplasmic reticulum (SR) ryanodine receptor (RyR) (Hare, 2004; Saraiva and Hare, 2006). Through intermediary formation of peroxynitrite, NO also indirectly regulates the activity of another Ca^{2+} -transporting enzyme, the sarco/endoplasmic reticulum Ca-ATPase (SERCA) (Adachi et al., 2004).

In biological systems, NO and superoxide coexist in a delicate balance, where even slight variations in the concentrations of these species dictate whether oxidation or nitrosation pathways will be followed (Wink et al., 1997). The relative levels of superoxide have an effect on the levels of nitric oxide due to the diffusion-controlled reaction between NO and superoxide to form ONOO⁻ (Kissner et al., 1997; Nauser and Koppenol, 2002). Superoxide dismutase (SOD) regulates the levels of superoxide and, therefore, has the potential to regulate redox-dependent signaling pathways through modulation of the effective levels of NO, superoxide, H_2O_2 and ONOO⁻. The relative amounts of these species, in turn, control the levels of nitrosating species, such as N_2O_3 , or oxidizing/nitrating species, such as ONOO⁻ (Patel et al., 2000). Disruption of the delicate balance between NO and superoxide leads to a so-called “nitroso-redox imbalance”, which may cause pathological conditions such as heart failure (Hare and Stamler, 2005).

While protein nitrosation can be reversed chemically, protein nitration leads to a chemically stable protein modification. Hence, the accumulation of nitrated proteins in tissue may define the phenotype of biological aging or of any pathology. The knowledge of specific protein nitration sites represents the ultimate goal for a correlation between protein modification and protein structure and function. This can be illustrated by the targeted purification and analysis of specific nitrated proteins from tissue, such as, for example, SERCA. Here, biological aging leads to the selective nitration of the slow-twitch skeletal and cardiac muscle isoform SERCA2a nitrated at Tyr²⁹⁴ and Tyr²⁹⁵, which can be linked to the loss of enzyme activity (Schöneich et al., 1999; Viner et al., 1999; Knyushko et al., 2005; Xu et al., 2006). Other proteins studied to similar functional detail include manganese superoxide dismutase (MnSOD) (Guo et al., 2003; Quint et al., 2006), prostacyclin synthase (Zou et al., 1999), and actin (Aslan et al., 2003).

Earlier, we had communicated two-dimensional gel electrophoresis (2DE) experiments targeted at the identification of cardiac proteins susceptible to nitration, and a list of 48 putatively nitrated proteins was assembled (Kanski et al., 2005a). However, due to the known limitations of the 2DE method (limited sample load, discrimination against membrane proteins and proteins of very low and very high molecular weight and pI), only one nitration site was actually located by MS/MS (for the electron transfer flavoprotein). In order to overcome the limitations of traditional 2DE, we have now applied a combination of solution isoelectric focusing, 1D SDS-PAGE and reversed-phase chromatography coupled to nanoelectrospray ionization-MS/MS analysis to identify cardiac proteins subject to age-dependent nitration. We will demonstrate here that biological aging results in the accumulation of 3-NY on distinct protein sequences, including a zinc-finger and nebulin-related anchoring protein (N-RAP). Of particular interest is also the accumulation of 3-NY on Tyr⁴⁹¹ of neurofibromin, observed for cardiac tissue from both young and old rats.

Materials and Methods

General

The research protocol was approved by the University of Kansas Animal Care Facility; we used 17 Fisher 344/BN F1 rats (ten 34 months old and seven 5 months old). These rats were housed in a 12 hour light/dark cycle with food and water provided ad libitum. The animals were decapitated, the hearts were removed and frozen immediately at -80°C and stored until needed.

For 1D gel electrophoresis experiments (1DE), Tris-glycine sample and running buffers and precast 4–20% gels were obtained from Invitrogen (Carlsbad, CA). For Western blotting experiments, TBST buffer was prepared by adjusting a solution of 150mM NaCl and 20mM Tris, pH 7.4, prior to the addition of 0.05% (v/v) Tween 20. The anti-3-nitrotyrosine (anti-3-NY) monoclonal antibody, clone 1A6, was purchased from Upstate, Inc. (Charlottesville, VA), and the anti-mouse IgG Fc-peroxide-conjugated antibody from Pierce Biotechnology (Rockford, IL). Protein concentration was quantified with the reducing-agent compatible BCA Protein Assay Kit, purchased from Pierce Biotechnology (Rockford, IL). Complete EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN). Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), dithiothreitol (DTT) and iodoacetamide were obtained from Sigma Chemicals (St. Louis, MO), and the 3/10 ampholite solution for solution isoelectric focusing was provided by Bio-Rad, Inc. (Hercules, CA).

Sample Preparation—For each analysis proteins were pooled from either 2 or 3 hearts from 34 month or 5 month old rats, respectively. The hearts were cut into small pieces, and the tissue rinsed twice in tissue wash buffer (50mM Tris, 150mM NaCl, 10 $\mu\text{g}/\text{ml}$ protease inhibitor cocktail mixture) to remove as much hemoglobin as possible. Subsequently, the tissue was homogenized in 25 ml of lysis solution (6M urea, 2% CHAPS, 10mM Tris, 0.5mM PMSF, 10 $\mu\text{g}/\text{ml}$ protease inhibitor cocktail mixture, 20 mM DTT, pH 7.5) using an ultra-turrax T8 homogenizer (Fisher, Pittsburgh, PA). Finally, the homogenate was centrifuged at 5,500 g at 4°C for 30 minutes. The supernatant was collected for analysis, taking care that the pellet was not disturbed during sampling of the supernatant.

1DE and Western Blot Analysis—Either total cardiac protein or proteins obtained in the individual fractions after solution isoelectric focusing were precipitated (see below). The precipitated proteins were redissolved in 100 μl of 50mM NH_4HCO_3 buffer, pH 7.4, containing 0.02% SDS. Protein concentrations were measured with the BCA Protein Assay Kit according to the manufacturer's protocol. Protein samples were mixed with equal

volumes of sample buffer, boiled for 5 minutes in the presence of 20mM DTT, and cooled for another 5 minutes prior to separation on precast 4-20% gels for 75 minutes at 5°C, at a constant voltage of 200V.

For Western blot analysis, proteins were transferred from the gels onto 0.45µm polyvinylidene difluoride (PVDF) membranes at a constant voltage of 100V for 2hrs. The PVDF membranes were blocked with 5% (w/v) milk-TBST buffer for 1hr at room temperature. The membranes were incubated overnight at 3°C with the primary anti-3-NY antibody (1:4000 dilution in 5% (w/v) milk-TBST buffer). Subsequently, the membranes were washed with 5% (w/v) milk-TBST buffer twice for 15 minutes prior to incubation with the anti-mouse IgG Fc-peroxide-conjugated secondary antibody (1:10,000 dilution in 5% (w/v) milk-TBST buffer) for 1hr at room temperature. The membranes were thoroughly washed with 2 short washes of 5% (w/v) milk-TBST buffer, followed by three 30 minute washes with TBST buffer alone. The immunoblots were visualized by chemiluminescence using the ECL kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol. The images were captured on a Kodak X-ray film using a Kodak developer/fixer kit.

The densitometry analysis for both the SDS-PAGE and Western blot was achieved with the Image Quant software (Amersham Biosciences; Pittsburgh, PA).

Solution Phase Isoelectric Focusing (IEF)—To the supernatant of the homogenized and centrifuged cardiac tissue, 500µl of 3/10 carrier ampholite solution were added. The solution was slowly mixed for a few minutes and was loaded into a Bio-Rad Rotofor mini-focusing chamber according to the manufacturer's protocol. Briefly, the anode membrane was conditioned in 0.1 M H₃PO₄ and the cathode membrane was conditioned in 0.1 M NaOH overnight. The apparatus was assembled according to the guidelines provided by the manufacturer. A total of 18 ml containing between 10-15mg/ml protein sample was applied to the Rotofor focusing chamber and fractionated at a constant power setting (12 W) at 4°C. The voltage stabilized at 524 V after about 30-40 minutes and increased to 2400 V after 3 hours when protein fractionation was stopped. The samples were then collected into 20 test tubes, each representing a discrete pH region. These samples were processed further immediately.

Reduction, Alkylation and Protein Precipitation—50µl of each Rotofor fraction were adjusted to a volume of 100µl with 50mM NH₄HCO₃ buffer, pH 7.4. The fractions were treated with 2mM DTT for 30 min at 37°C to reduce disulfide bonds, followed by alkylation with 5mM iodoacetamide for 30 min at room temperature. Subsequently, proteins were precipitated overnight at -20°C by addition of 1 ml of pure ethanol to the reaction mixtures. The precipitated proteins were collected by centrifugation at 13,000g for 5 minutes. The supernatants were discarded and the solid protein pellets were saved for further processing.

Solution Digestion—Precipitated proteins were collected and re-dissolved in 100-200µl of 50mM NH₄HCO₃, pH7.4. To each sample, 2µg of trypsin were added and digestion was carried out overnight at 37°C. The digests were then analyzed by nanoHPLC-electrospray ionization (NSI)-tandem mass spectrometry (MS/MS).

Nanoelectrospray ionization-tandem mass spectrometry (NSI-MS/MS) analysis—Solution digests were analyzed by nanoHPLC-ESI-MS/MS on either a Thermoelectron LCQ Duo or a Thermoelectron Classic ion trap mass spectrometer, both equipped with a nanoelectrospray source (Thermoelectron). Separation of the tryptic peptides was achieved prior to MS/MS analysis on an in-house packed C18 Biobasic stationary phase

(Thermoelectron, CA) nanoflow column (300 A, 10 cm $\times$ 75 μ m, 15 μ m tip size) (New Objective, Woburn, MA). The following chromatographic conditions were applied: mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in acetonitrile. The flow rate was adjusted to 0.5 μ l/min with the chromatographic flow delivered by either an Ultraplus 2 pump (after 1:20 split; Microtech Inc, CA) or directly by an Xtreme simple nanoflow pump (Microtech Inc., CA). The gradient profile applied to the separation of peptides from in-gel digests included the following steps for mobile phase B: 0 to 5 minutes held at 15%, then linearly increased to 70% between 5 and 35 minutes; held at 70% until 40 minutes before being readjusted to the initial equilibration conditions. For peptides recovered from solution digests, the increase in composition of B from 15 to 70% was instead accomplished over a much slower time period of 95 minutes such that the total run time was extended to 2 hours for each sample. The following instrument conditions were used for the mass spectrometer: number of microscans = 3, length of microscans = 200 ms, capillary temperature = 160 $^{\circ}$ C, spray voltage = 41 V, tube lens offset = -10V. The tuning for both mass spectrometers was performed using a static nanospray setup with a 5 μ M solution of angiotensin 1 (MW = 1296.5 Da) infused by a picotip emitter. The data acquisition was performed in a data-dependent manner such that an MS scan was followed by 3 or 4 MS/MS scans of the 3 or 4 most intense peaks with the normalized collision energy for MS/MS set at 35% and the isolation width of 2.0 m/z. A minimal signal threshold of 2×10^6 was set for acquiring the MS/MS data. Additionally, the dynamic exclusion option was enabled with the following parameters: repeat count = 3, repeat duration = 5 ms, exclusion list size = 25, exclusion mass width = 3.

Protein Identification—The MS/MS data were subjected to a protein database search using the Bioworks 3.1 software (Thermoelectron). The most recently updated non-redundant NCBI protein database was used for the searches when downloaded from <ftp.ncbi.nlm.nih.gov/blast/db> with the following amino acid modifications included in the search options: oxidation of Met (+16 amu; amu = atomic mass units), alkylation of cysteine (+57 amu) and nitration of tyrosine (+45 amu). Additionally, the observed MS/MS data of proteins were manually examined for the presence of 3-NY-containing peptides.

Database Search and data interpretation—The following criteria were set for positive identification of nitrated peptides:

1. For singly, doubly, and triply charged peptides, we set Xcorr values (the cross-correlation value from the search for well matched peptides (MacCoss et al., 2002)) of ≥ 1.0 , ≥ 2.0 , ≥ 2.5 , respectively.
2. The major peaks in the MS/MS spectra needed to be unambiguously assigned to peptide fragments of the nitrated protein.
3. Positive identification of nitrated proteins is contingent upon identification of at least one non-nitrated, native peptide from the respective protein besides the 3-NY containing peptide.

Once a list of proteins of interest was compiled, Protein Prospector v 4.0.6 MS-product (<http://prospector.ucsf.edu/ucsfhtml4.0/msprod.htm>) was used to analyze the data manually. During this procedure, we were able to find peaks corresponding to internal fragments, and neutral losses of H₂O, NH₃ and CO. The spectra with all major peaks labeled unambiguously identified the nitrated peptides found during the analysis.

Results

1D SDS-PAGE and Western blot analysis (Figure 1) confirmed our earlier data (Kanski et al., 2005a) that nitrated proteins are present in cardiac tissue, and that their abundance

increases with age. Based on densitometry analysis, ca. 1.5 to 2 fold increase in protein nitration, depending on the animal set, when 34 months old animals were compared to 5 months old animals. The data were normalized to account for some differences of protein load between the individual lanes.

In the present work we applied solution IEF to prefractionate proteins prior to tryptic digestion and analysis of the resulting peptides with nanoHPLC-NSI-MS/MS. Solution IEF permits higher protein loads compared to gel IEF, the first step in a traditional 2DE, enhancing the likelihood for positive identification of nitrated peptides. The initial protein amount applied to solution IEF was calculated based on the total amount of protein present in the homogenate. After IEF, aliquots from each of the collected fractions were used for pH measurements, and others were applied to 1DE analysis and recovered proteins were quantified by densitometry. Every fraction contained a different protein load, varying from ~1.4 mg/ml to ~56 mg/ml. The pH of the recovered fractions changed gradually from 3 to 10, where the pH changed by 0.5 or 1 unit every two or three fractions, as was expected based on the separation conditions used. The pIs of the identified nitrated proteins did not necessarily match the pH of the rotofor fraction from which they were recovered. The latter may have various reasons such as additional post-translational modifications, or in vivo proteolysis yielding protein fragments rather than intact protein. Our 2 hour nanoHPLC separation prior to MS/MS analysis (Materials and Methods section) routinely yielded about 2500 identified peptides per fraction. Table 1 summarizes our MS/MS analysis of cardiac proteins from 5 and 34 months old rats. A total of 10 nitrated proteins were identified. Two of these, myosin heavy chain polypeptide 7 and neurofibromin were observed in cardiac homogenates from both ages. For each protein in the list, we were able to detect at least one 3-NY-containing peptide along with a number of non-nitrated peptides. Hence, identification of some native peptides in addition to the nitrated peptides confirmed the presence of the identified protein in the respective IEF fraction, improving the reliability of the protein identification. We note that the molecular weights of the nitrated proteins identified by MS/MS analysis (Table 1), and those of the immunoresponsive bands in the Western blot analysis (Figure 1B) do not correlate. There are several possible reasons for this: (i) in vivo proteolysis of nitrated proteins (see also above); (ii) not all nitrated proteins detected by MS/MS will display immunoresponsive spots on the Western blot due to the fact that the anti-3-NY antibody can discriminate against selected nitrated proteins (Giasson et al., 2000); (iii) not all immunoresponsive proteins on the Western blot will contain sufficient yields of 3-NY for the positive MS/MS identification of nitrated peptides. It is also important to note that the table of identified proteins by MS/MS in this paper is very different from the list that was generated by traditional 2DE analysis in our previous study (Kanski et al., 2005a). The only protein that was found in common in both studies was Tropomyosin 1, α chain. This may again be due to the inherent differences in the methods that were applied to each study.

Representative MS/MS spectra are shown for the nitrated peptide Glu⁵⁹⁶⁸-Arg⁵⁹⁷⁸ (Figure 2) from nebulin-related anchoring protein (N-RAP) isoform C (GenInfo accession number 109468170), nitrated at Tyr⁵⁹⁶⁹, for the nitrated peptide Glu¹⁰⁶-Lys¹²⁸ (Figure 3) from myosin heavy chain polypeptide 6 (GenInfo accession number 8393804), nitrated at both Tyr¹¹⁴ and Tyr¹¹⁶, for the nitrated peptide Tyr²²¹-Lys²²⁶ (Figure 4) from tropomyosin 1, α isoform e (GenInfo accession number 78000196), nitrated at Tyr²²¹, and, finally for the nitrated peptide Ser⁴⁸⁸-Lys⁴⁹⁸ (Figure 5) from neurofibromatosis 1 (neurofibromin) (GenInfo accession number 6981264), nitrated at Tyr⁴⁹¹. It is interesting to note that several of the nitrated proteins detected after solution IEF are located in the cytoskeleton, whereas our previous 2DE experiments (Kanski et al., 2005a), localized many nitrated cardiac proteins to the mitochondria. This difference between the nature of identified proteins via two independent methods reflects, again a phenomenon commonly observed during

proteomic experiments, where different techniques lead to the identification of different subsets of proteins (Arab et al., 2006).

Significantly fewer (a total of three) nitrated proteins were detected in the analysis of cardiac tissue from young (5 months old) rats (Table 1). Here, only NADH dehydrogenase was not detected in the heart of old rats, whereas both myosin heavy chain polypeptide 7 and neurofibromin were also detected in the aging heart. Figure 6 displays a representative MS/MS spectrum for the nitrated peptide Thr¹⁰¹-Lys¹¹² from NADH dehydrogenase (GenInfo accession number 9506913), nitrated at Tyr¹⁰⁶.

Discussion

NO plays an important role in signal transduction to regulate proper cardiac muscle function (Balligand et al., 1993; Gong et al., 2004). NO specifically integrates cell system responses by controlling oxygen supply and demand, especially during an ischemic attack (Gong et al., 2004). NO regulates proper hemoglobin function and cardiac smooth muscle contractility to coordinate the respiratory cycle in order to ensure proper oxygen delivery to target tissues (Wolin et al., 1997; Gong et al., 2004; Davidson and Duchen, 2006). Therefore, cardiac muscles are periodically exposed to fluxes of endogenous NO (synthesized by local NOS) (Gong et al., 2004), and superoxide (Reid et al., 1993). This creates an environment amenable for the formation of peroxynitrite (ONOO⁻), a major nitrating species (Beckman, 1996), through the diffusion controlled reaction of NO and superoxide (Nauser and Koppenol, 2002). Therefore, the formation of nitrated proteins in cardiac muscle is not unexpected. Importantly, aging leads to an accumulation of nitrated proteins, which may affect cardiac function (Kanski et al., 2005a). This accumulation may be, in part, an indication of an age-dependent slower proteasomal degradation of nitrated proteins (Conconi and Friguet, 1997), higher concentrations of nitrating species (Kanski et al., 2005a), and/or slower repair by the putative protein “denitrase” (Kamisaki et al., 1998; Irie et al., 2003) (or a combination of all three parameters). We note that peroxynitrite is not the only reagent which can nitrate proteins in vivo, but that other nitrating species, such as nitrogen dioxide (NO₂), may be involved as well.

Tyrosine nitration can affect protein structure and function. The magnitude of such effect will primarily depend on the location of 3-NY within the protein and its quantity. Densitometry measurements quantify a ca. 1.5-2.0 fold overall age-dependent increase in protein nitration. The location of 3-NY within the detected proteins has been addressed in this study through the MS/MS analysis of individual nitrated peptides. Quantitation of 3-NY within each of the individual target proteins by our mass spectrometric method was not possible. Such quantitation and experimental proof of potential functional and structural consequences of nitration will be part of future studies focusing on the targeted purification of the proteins identified in the present study. In the following we will provide a discussion of the potential effects of tyrosine nitration within these peptide sequences.

Some of the more interesting nitrated proteins detected in this work are located in the cytoskeleton, namely myosin, N-RAP (isoform C), tropomyosin, and neurofibromin. For myosin, we detected nitration at Tyr¹¹⁴ and Tyr¹¹⁶ for polypeptide 6, and nitration at Tyr¹³⁴ and Tyr¹⁴² for polypeptide 7. It has been observed that an age-dependent slower muscle movement is due to functional and structural changes of myosin, in particular the heavy chain (Höök et al., 2001). This is important to note, especially in view of the slow turnover rate, and decreased rate of synthesis and degradation of this protein (Höök et al., 2001). The myosin heavy chain converts chemical energy, present in the form of ATP, into mechanical energy (Lompre et al., 1984). Therefore, structural changes that can affect the normal function of these proteins may explain the age dependent loss of muscle force and

contractile speed (Höök et al., 2001). Figure 7 displays a cartoon locating the 3-NY residue within these protein sequences of myosin polypeptides.

To date, relatively little information is available about the functional roles of N-RAP (isoform C). It has been proposed that N-RAP anchors the terminal actin filaments in the myofibril to the membrane as well as transmits the tension from the myofibrils to the extracellular matrix. N-RAP is specifically expressed in skeletal and cardiac muscles (Luo et al., 1997; Luo et al., 1999). The N-RAP super repeats have been shown to bind actin very tightly analogous to nebulin. This suggest that N-RAP binding to actin filaments is a highly cooperative process, which leads to the transmission of the tension created during myofibril contraction and relaxation. N-RAP displays a sequence homology to the C-terminal half of nebulin, as well as the LIM domain (a Cys rich domain, that can bind to two Zn ions) at the N-terminus, responsible for mediating protein-protein interactions (Luo et al., 1999). Figure 8 shows a cartoon indicating that the nitration of N-RAP targets specifically the C-terminal domain involved in actin binding.

Tropomyosin regulates the interactions between actin and actin-binding proteins (Clarkson et al., 2004). Tropomyosin 1 represents the predominant isoform present in the cardiac muscle and fast skeletal muscle fibers. Mutations of the TPM1 gene (which encodes for tropomyosin 1), result in different types of myopathies causing abnormal muscle function (Clarkson et al., 2004; Ilkovski et al., 2004). Figure 9 shows a cartoon locating the 3-NY residue within the protein sequence of tropomyosin.

Neurofibromin, encoded by the gene NF1, represents a tumor suppressor protein which acts as a negative regulator of Ras. Mutations to NF1 or the absence of the NF1 gene can cause congenital heart disease associated with neurofibromatosis type 1, e.g. benign neurofibromas, leukemias and multiple cardiac abnormalities (Yutzey et al., 2005). These disorders likely depend on an unregulated Ras signaling pathway, which may stimulate cell growth or inhibit apoptosis. The 3-NY⁴⁹¹ residue in this protein is located very close to a mutation site, Tyr⁴⁸⁹, commonly found in genetic studies of patients suffering neurofibromatosis type 1 (Messiaen et al., 2000). Nitration of Tyr⁴⁹¹ may have a similar negative effect on the protein as mutation to Tyr⁴⁸⁹ within the same protein domain. Figure 10 displays a cartoon locating the 3-NY residue within the protein sequence of neurofibromin.

Some of the other nitrated proteins identified in this work (Table 1) play important roles in cellular function, such as for example, organelle transport by myosin 5C, cell membrane adhesion regulation by cadherin EGF-LAG seven pass G-type receptor 2, and transcriptional regulation in the nucleus by the Cys/His Zn-finger protein. The modifications to these proteins together with the cytoskeletal proteins already discussed, may offer some rationale for cardiac dysfunction, though it can be expected that additional nitrated proteins may be detected in the heart as more sensitive proteomic methods become available.

Some oxidized and nitrated proteins are susceptible to accelerated turnover (Grune et al., 1997; Souza et al., 2000), while others tend to accumulate (Harman, 1956; Greenacre and Ischiropoulos, 2001). Our earlier (Kanski et al., 2005a) results suggest a rather selective accumulation of a few nitrated cardiac proteins. In addition to selective turnover, other parameters such as cellular location, protein structure, and protein-protein interactions, will define the susceptibility to nitration (Greenacre and Ischiropoulos, 2001). The target proteins that we have identified in this work serve as evidence for age dependent protein nitration in vivo. Future studies must now focus on a targeted isolation of identified proteins to quantitate age-dependent nitration, the possible accumulation of additional modifications, and to correlate these modifications with protein function.

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List of abbreviations used inside the text

ROS	Reactive oxygen species
RNS	Reactive nitrogen species
BN-F1	Fisher 344 Brown Norway F1 rats
IEF	Isoelectric focusing
HPLC-ESI-MS/MS	High performance liquid chromatography-electrospray ionization tandem mass spectrometry
3-NY	3-nitrotyrosine
NO	nitrogen monoxide
ONOO⁻	peroxynitrite
Mn-SOD	manganese superoxide dismutase
GR	glutathione reductase
SR	sarcoplasmic reticulum
RyR	ryanodine receptor
SERCA	sarco/endoplasmic reticulum Ca-ATPase
2DE	two-dimensional gel electrophoresis
1D SDS-PAGE/1DE	one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
N-RAP	nebulin-related anchoring protein
BSA	bovine serum albumin
DTT	dithiothreitol
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
PVDF	polyvinylidene difluoride
TBST	tris-buffered saline with tween
RP nano LC MS	Reverse phase nano liquid chromatography mass spectrometry

Figure 1.

SDS-PAGE and Western blot analysis of whole heart homogenates displaying (A) total protein (by coomassie blue staining) and (B) the levels of 3-NY (using a anti-3-NY antibody). Four animals are represented in this figure, two 5 months old rat hearts (Y1, Y2) and two 34 months old rat hearts (O1, O2). The protein amounts in each lane were determined by densitometry analysis and normalized to conclude that there is a 1.5 to 2.0 fold increase in nitration with aging.

Figure 2.

Representative MS/MS spectrum of the nitrated peptide sequence Glu⁵⁹⁶⁸-Arg⁵⁹⁷⁸ detected for nebulin-related anchoring protein (N-RAP), in cardiac tissue of 34 months old Fisher 344 BN-F1 rats. Here we have highlighted the y-ions covered in the series, y₂-y₁₀.

Figure 3.

Representative MS/MS spectrum of the nitrated peptide sequence Glu¹⁰⁶-Lys¹²⁸ detected for myosin heavy chain polypeptide 6, in cardiac tissue of 34 months old Fisher 344 BN-F1 rats. Here we have highlighted the b-ions covered in the series, b₃-b₁₄.

Figure 4.

Representative MS/MS spectrum of the nitrated peptide sequence Tyr²²¹-Lys²²⁶ detected for tropomyosin, in cardiac tissue of 34 months old Fisher 344 BN-F1 rats. Here we have highlighted the b-ions covered in the series, b₂-b₆.

Figure 5.

Representative MS/MS spectrum of the nitrated peptide sequence Ser⁴⁸⁸-Lys⁴⁹⁸ detected for neurofibromin, in cardiac tissue of 34 months old Fisher 344 BN-F1 rats. Here we have highlighted the b-ions covered in the series, b₂-b₁₀.

Figure 6.

Representative MS/MS spectrum of the nitrated peptide sequence Thr¹⁰¹-Lys¹¹² detected for NADH dehydrogenase (ubiquinone) Fe-S, in cardiac tissue of 5 months old Fisher 344 BN-F1 rats. Here we have highlighted the y-ions covered in the series, y₂-y₉.

Figure 7.

Cartoon displaying the relative location for the nitrated peptides of myosin heavy chain that were found in the present study. In this cartoon, we display the whole myosin complex, composed of two heavy chains, and two light chains. The two heavy chains are shown in gray, and the light chains that wrap around the neck of the two myosin light chains are shown in black. The 3-NY modification is located in the head region of the myosin heavy chain.

Figure 8.

Cartoon displaying the relative location for the nitrated peptide of nebulin-related anchoring protein (N-RAP) found in the present study, which is composed of three distinct regions.

The LIM-domain, nebulin like simple repeats domain and the actin binding domain. The 3-NY modification is located at the actin-binding domain of N-RAP.

Figure 9.

Cartoon displaying the reported shape of this protein and the relative location for the nitrated peptide of tropomyosin found in the present study. The 3-NY modification in this protein is located towards the C-terminus of its amino acid sequence.

Figure 10.

Cartoon displaying the relative location for the nitrated peptide of neurofibromin found in the present study. This protein is being shown in its denatured form. The 3-NY modification in this protein is located towards the N-terminus of its amino acid sequence.

Table 1

List of nitrated proteins identified by solution IEF and NSI-LC-MS/MS in cardiac tissue of both 5 months old and 34 months old Fisher 344 BN-F1 rats. The specific nitrated sequences for each protein are shown here.

Sample	Protein	GI no. ^a	Molecular mass (KDa)	% Coverage	No. of identified peptides	Nitrated peptide	Sequence	Cellular location and function
5 months old rat cardiac tissue	Myosin heavy chain polypeptide 7	8393807	222.9	5.27	9	WLPVYNAQVVAA ^Y R	130-143	Cytoskeleton, muscle contraction
	Neurofibromatosis 1	6981264	316.9	1.84	3	SYKYLLLSMVK	488-498	Cytoskeleton, tumor suppressor protein
	NADH dehydrogenase (Ubiquinone) Fe-S	9506913	12.8	29.31	4	TGTCAYCGLQFK	101-112	Mitochondria, NADH reduction and oxydation
34 months old rat cardiac tissue	Myosin-5C	109483639	202.2	3.24	3	FAESKLIITYSGILVAMNPYK	91-111	Cytoplasmic, organelle transport
	Myosin heavy chain polypeptide 6	8393804	223.4	5.68	7	ERYAAWMIITYSGLFCVTNPYK	106-128	Cytoskeleton, converts chemical energy into mechanical energy through the hydrolysis of ATP
	Tropomyosin 1, α isoform e	78000196	32.7	44.72	16	YEEEEK	221-226	Cytoskeleton, muscle contraction through the tropomyosin-tropoinin- Ca^{2+} system.
	Neurofibromatosis 1 (Neurofibromin)	6981264	316.9	1.17	6	SYKYLLLSMVK	488-498	Cytoskeleton, tumor suppressor protein
	Cadherin EGF-LAG seven pass G-type receptor 2	22095545	220.0	1.88	2	EPENYMRCSVLR	460-473	Cell membrane, cell adhesion mediator
	Dynein, cytoplasmic, heavy chain 1	31377489	531.9	0.34	3	QLTAYMK	665-671	Cytoplasm, cytoplasmic motor transport protein that propels membrane organelles towards (-) ends of polarized microtubules through ATP hydrolysis
	Nebulin-related anchoring protein isoform C (N-RAP)	109468170	771.7	0.57	9	EYRKDLEESIR	5968-5978	Cytoskeleton, thin filament length and assembly regulation
Cys2/His2 Zn finger protein (rKr1)		21426789	73.8	6.00	5	LLIEGGQYK	282-290	Nucleus, transcriptional regulator
	Myosin heavy chain polypeptide 7	8393807	222.9	16.33	17	WLPVYNAQVVAA ^Y R	130-143	Cytoskeleton, muscle contraction

^a Gen Info accession number.