Proteomic analysis of protein nitration in aging skeletal muscle and identification of nitrotyrosine-containing sequences in vivo by nanoelectrospray ionization tandem mass spectrometry

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The nitration of protein tyrosine residues represents an important post-translational modification during development, oxidative stress and biological aging. In order to rationalize any physiological changes with such modifications, the actual protein targets of nitration must be identified by proteomic methods. While several studies have used proteomics to screen for 3-nitrotyrosine-containing proteins in vivo, most of these studies have failed to prove nitration unambiguously through the actual localization of 3-nitrotyrosine to specific sequences in proteins. In this paper we have applied sequential solution isoelectric focusing and SDS-PAGE for the proteomic characterization of specific 3-nitrotyrosine-containing sequences of nitrated target proteins in vivo using nanoelectrospray ionization-tandem mass spectrometry. Specifically, we analyzed proteins from the skeletal muscle of 34 months old Fisher 344/Brown Norway F1 hybrid rats, a well accepted animal model for biological aging. We identified the 3-nitrotyrosine-containing sequences of 11 proteins, including cytosolic creatine kinase, tropomyosin 1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), myosin light chain, aldolase A, pyruvate kinase, glycogen phosphorylase, actinin, gamma-actin, ryanodine receptor 3, and neurogenic locus notch homolog. For creatine kinase and neurogenic locus notch homolog, two 3-nitrotyrosine-containing sequences were identified, i.e. at positions 14 and 20 for creatine kinase, and at positions 1175 and 1205 for the neurogenic locus notch homolog. The selectivity of the in vivo nitration of creatine kinase at Tyr14 and Tyr20 does not correspond to the product selectivity in vitro, where exclusively Tyr82 was nitrated when creatine kinase was exposed to peroxynitrite. The latter experiments demonstrate that the in vitro exposure of an isolated protein to peroxynitrite may not always be a good model to mimic protein nitration in vivo.

The nitration of tyrosine to 3-nitrotyrosine (3-NT) represents a hallmark of post-translational protein modification associated with various pathologies (1,2) and the process of biological aging (3). Tyrosine nitration can compromise protein structure and function (4,5), partially due to the shift of the phenolic pKₐ value into the physiological range, where pKₐ ≈ 7.1 for 3-NT in several model peptides (6). That is, at physiological pH a significant fraction of 3-NT may exist in its deprotonated state, displaying a net negative charge. Hence, protein nitration may have biological consequences comparable to those of protein phosphorylation, i.e. may be involved in redox signaling. The potential reversibility of tyrosine nitration is indicated through the discovery of a putative denitrase, though a thorough characterization of this(these) enzyme(s) has not been achieved to date (7,8). Targeted purification and analysis has demonstrated the formation of 3-NT on several specific proteins in vivo such as, e.g., Mn superoxide dismutase (MnSOD) (9), prostacyclin synthase (10), the...
Biological aging leads to a significant formation of 3-NT on proteins in skeletal muscle (13) and heart (14). Of note is the age-dependent accumulation of ca. 4 mol 3-NT/mol of the SERCA2a isoform in skeletal muscle (11), and ca. 3 mol 3-NT/mol SERCA2a in heart (15), i.e. quantitative significant yields of nitration on individual proteins. Such high yields of 3-NT do not necessarily come as a surprise considering the close location of SERCA and nitric oxide synthase (NOS) (16), and the stimulation of muscle contraction and relaxation through oscillations of nitric oxide (NO) and superoxide formation (17). The combination of NO and superoxide yields peroxynitrite (ONOO$^-$), a strongly nitrating species especially in the presence of CO$_2$ (18).

More recently, a higher throughput characterization of protein targets for tyrosine nitration in cells and several tissues, including aging tissues, has been attempted using proteomic methodologies (13,14,19-28). These studies predominantly employed 2-D gel electrophoresis (2DE) to separate the proteins of interest, followed by Western blot analysis using anti-3-NT antibodies. Mass spectrometry served to identify the proteins present in the gel spots. However, in most of these studies no mass spectrometric identification of actual nitrated peptides was given. That is, the proposed protein identifications would truly require the discrete presence of a single protein per gel spot. In reality, often gel spots contain multiple proteins or protein isoforms (29) so that a positive identification of nitrated proteins in these spots would require tandem MS sequencing of the peptide sequences containing the 3-NT modification. Especially for tissue samples, few reports show the tandem MS analysis of nitrated peptides following 2DE separation. Examples are 3-NT at position 105 within the $\alpha$-chain of the electron transfer flavoprotein in mitochondria of aged heart (14), and five proteins, including actin, in the human pituitary (30). The failure of many 2DE approaches to characterize such peptides is likely due to multiple causes such as (i) the low steady-state levels of 3-NT on specific proteins, (ii) the low abundance of some of the 3-NT-containing proteins, (iii) the solubility, size and/or extreme pI values of proteins, which may compromise the isoelectrofocusing in the first step of the 2DE separation, and (iv) the recovery of 3-NT-containing peptides from the gels and/or HPLC columns during subsequent LC-MS analysis.

In order to correlate protein nitration with potential functional and/or structural alterations, it is absolutely mandatory that proteomic studies identify the location of 3-NT residues in the target proteins. Such studies are described in the present paper. In order to achieve the characterization of 3-NT-containing protein sequences in tissue, we have now substituted in-gel isoelectrofocusing by solution isoelectrofocusing in the first dimension, which allows the application of higher sample loads and does not discriminate against membrane proteins (31). In the second dimension, proteins were separated by SDS-PAGE, and subsequently analyzed by electrospray ionization (ESI) tandem mass spectrometry following in-gel digestion. Earlier we had used 2DE and Western blotting to demonstrate an age-dependent accumulation of 3-NT on rat skeletal muscle proteins (13). Therefore, the present paper only focuses on the characterization of 3-NT-containing protein sequences of skeletal muscle of 34 months old Fisher 344/BN F1 rats, a well-accepted model of biological aging (32).

**Materials and Methods:**

*General*

The research protocol outlined in this manuscript has been approved by the University of Kansas Animal Care Facility. Fisher 344/BN F1 rats (34 months old, $n = 3$) have been housed in a 12 hour light/dark cycle and have been provided with water and food *ad libitum*. The animals have been sacrificed by decapitation, and the muscles have been rapidly removed and immediately frozen at $-80^\circ$C. The specimen (2.5 g of tissue) was then ground and exposed to 25 ml of the lysis solution (6 M urea, 2% CHAPS, 10 mM Tris, 0.5 mM PMSF, 10 $\mu$g/ml of leupeptin, 10 $\mu$g/ml aprotinin, 20 mM DTT, pH=7.5), and homogenized with an ultra-turrax T8 homogenizer (Fisher, Pittsburgh, PA). The solution was centrifuged at 5,500 g for 30
minutes, and supernatant was collected for further processing.

**Solution isoelectrofocusing**

To 18 ml of the protein solution, 400 µl of pH 3-10 carrier ampholyte (Bio-Rad, Hercules, CA) was added, and the mixture was applied to solution isoelectrofocusing on a Bio-Rad ROTOFOR mini-focusing chamber according to the protocol of the manufacturer. Briefly, the anode and cathode were conditioned overnight in 0.1 M \( \text{H}_3\text{PO}_4 \) and 0.1 M NaOH respectively. 18 ml of the sample were applied to the cell, and the separation was achieved at a constant power setting (12 W) at 4°C. Initially, the voltage reading was 524 V. The separation was concluded after 4 hours when the voltage stabilized at 2400V. The samples were then collected into 20 vials, each representing a discrete pH region, and processed immediately.

**SDS-PAGE and transfer to the membrane**

The fractions were treated with 2 volumes of SDS-PAGE running buffer, containing 100 mM DTT, and boiled for 5 minutes. They were applied to 4%-20% acrylamide gels in Tris-Glycine running buffer (Invitrogen, Carlsband, CA). Protein separation (40 µl per line) was conducted for 45 minutes at 100 V and 12°C. SDS-PAGE gels were submitted to transfer to the 0.45 µm polyvinyl difluoride (PVDF) membrane (Millipore, Billerica, MA) (412 mA, 2 hours, 4°C) and Western blot analysis.

**Western blot analysis**

The membranes were incubated overnight at 4°C in 5% dry milk T-TBS. Following blocking, the membrane was rinsed with copious amounts of T-TBS and exposed to the IA6 mouse anti-3-NT primary antibody solution (Upstate, Waltham MA) (1 : 5,000 dilution in 1% BSA T-TBS) for 1 hour at room temperature. After incubation, the membrane was washed with T-TBS and subjected to anti-mouse IgG Fc-peroxide conjugate secondary antibody (Pierce, Rockford, IL) (1 : 10,000 dilution in T-TBS) for 1 hour at room temperature. The spots were visualized by the ECL-plus detection kit (Amersham Pharmacia, Piscataway, NJ)) according to the manufacturer’s procedure, and the images were captured on a KODAK (Rochester, NY) X-ray film using a KODAK-developer/fixer kit.

**Protein visualization**

After completion of electrophoresis the gels were stained with Coomassie Blue according to a well-established protocol (33). Visualized gels were processed immediately or stored in the dry state in Saran-wrap until further processing.

**In gel digestion**

Coomassie Blue-stained gel spots of interest were excised from the gels, ground to small pieces, and extensively washed with two changes of a 1:1 (v/v) mixture of 200 mM \( \text{NH}_4\text{HCO}_3 \) and acetonitrile (MeCN) for 45 minutes at 37°C. Gel pieces were then shrunk in 50 µl of MeCN for 10 minutes. After the removal of residual solvent, the pieces were rehydrated in 40 mM \( \text{NH}_4\text{HCO}_3 \), pH 7.8, containing 0.5 µg of modified trypsin (Promega, Madison, WI). Exhaustive digestion was carried out overnight at 37°C. Formic acid was added to yield a final content of 0.1% (v/v), and aliquots were removed and analyzed by mass spectrometry.

**Solution digestion**

ROTOFOR fractions (20 µl) were diluted to 100 µl and treated with 2 mM DTT for 30 min at 37°C, and then alkylated with 5 mM iodoacetic acid for 30 min at room temperature in the dark. Proteins were then precipitated in 1 ml of ethanol for 4 hours at 20°C, collected and redissolved in 100 µl of 50 mM \( \text{NH}_4\text{HCO}_3 \). Trypsin (2 µg) was added and an exhaustive digestion was carried out overnight at 37°C. The digests were then analyzed by nanoelectrospray ionization (NSI)-tandem mass spectrometry (MS/MS).

**Nanoelectrospray ionization-tandem mass spectrometry**

In-gel tryptic digests (2 µl) were submitted to NSI-MS/MS analysis on either a ThermoElectron LCQ Duo or a ThermoElectron Classic (San Jose, CA), equipped with a nanoelectrospray source (ThermoElectron). Separation of tryptic peptides was achieved online prior to MS/MS analysis on in-house packed BioBasic C18 stationary phase (Thermo
Electron) nanoflow columns (300Å, 10cm x 75µm, 15 µm tip size) (New Objective, Woburn, MA) with the following chromatographic conditions: mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeCN. The flow rate was 0.5 µL/min, delivered by a MicroTech Scientific Ultra Plus II pump (after 1:20 split), or by a MicroTech Xtreme Simple nano-flow pump (direct flow). The following gradient profile was used to increase mobile phase B linearly to the following fractions: from 0 to 5 min gradient held at 10% B, then increased to 60% B within 40 minutes, and continued at 60% B for additional 5 minutes. After each run, the column was allowed to re-equilibrate to the initial conditions for 15 minutes. For solution digestion, the gradient was extended to 105 minutes for 60% B. The following instrumental conditions were used for mass spectrometric analysis: number of microscans = 3, length of microscans = 200 ms, capillary temperature = 160°C, spray voltage = 1.9 kV, capillary voltage = 35V, tube lens offset = -14V. The mass spectrometer was tuned using the static nanospray setup with a 5 µM solution of Angiotensin I (MW 1296.5) infused by a picotip emitter (New Objective). Data acquisition was performed in the data-dependent fashion, i.e. an MS scan 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 for MS/MS acquisition was set to 2 x 10^6. Additionally, the dynamic exclusion option was enabled and set with the following parameters: repeat count = 3, repeat duration = 5 min, exclusion list size = 25, exclusion duration = 5, and exclusion mass width = 3.

Protein identification was achieved with the ThermoElectron Bioworks 3.1 software package with the most current non-redundant NCBI protein database downloaded from ftp.ncbi.nlm.nih.gov/blast/db (34). The following modifications were accounted for during the search: oxidation of Met (+16 amu; amu = atomic mass units), alkylation of Cys (+58 amu), and nitration of Tyr (+45 amu). Additionally, MS/MS spectra of interest were examined manually for the presence 3-NT-containing peptides.

**In vitro nitration of creatine kinase**
Aqueous solutions of human creatine kinase (Sigma) (1mg/ml in 200 mM NH_4HCO_3) were submitted to various concentrations of peroxynitrite (ONOO^-) (0-1000 µM). The protein was then submitted to SDS-PAGE analysis for protein visualization, Western blot analysis, and in-gel trypsin digestion, as described above. In addition, the activity of the nitrated protein was recorded according to established methods (35).

**Results**

**Identification of 3-NT-containing protein sequences**

The goal of this study was the unambiguous location of 3-NT to specific protein sequences in skeletal muscle of aged rats. Earlier, our 2DE separation and Western blot analysis have clearly demonstrated an age-dependent accumulation of 3-NT on rat skeletal muscle proteins. Several of these proteins were identified by MALDI-TOF and NSI-MS/MS analysis, but the shortcomings of the 2DE technique did not allow for localization of 3-NT to specific protein sequences (13). These shortcomings have now been overcome in this paper by application of a two-dimensional separation consisting of solution isoelectrofocusing, followed by SDS-PAGE. This approach permitted protein loads of several mg in the first dimension, i.e. an increase of sample loads by a factor of ca. 10, when compared to in-gel isoelectrofocusing, where protein loads were generally on the order of ≤ 0.5 mg (13).

The solution isoelectrofocusing procedure yields a number of distinct fractions corresponding to different pH regions. Proteins present in these fractions were separated by SDS-PAGE and subjected to Western blot analysis with the anti-3-NT antibody. Figure 1 displays a representative Western blot analysis, showing the bulk of the nitrated proteins present in the neutral to alkaline pH region and in the apparent molecular weight (MW) region between 25 and 75 kDa. The specificity of the antibody was confirmed earlier when 3-NT on the blots was reduced to 3-aminotyrosine with Na_2S_2O_3 prior to incubation with the antibody, resulting in a loss of immunostaining (13).
large fraction of the nitrated proteins display apparent molecular weights between 37 and 50 kDa. These results are in excellent agreement with our previous 2DE analysis using in-gel isoelectrofocusing in the first dimension, followed by SDS-PAGE (13). In a parallel experiment, the second dimension SDS-PAGE was subjected to Coomassie Blue staining (instead of Western blot), in order to prepare samples for in-gel digestion and NSI-MS/MS analysis (see below). Moreover, several fractions obtained from solution isoelectrofocusing were digested directly without any further SDS-PAGE separation, in order to screen more globally for 3-NT-containing peptides by the so-called multidimensional protein identification technique (MudPIT) (36) using HPLC-NSI-MS/MS. Figure 2 shows a representative chromatogram, where the total ion current is plotted versus the chromatographic run time.

Table 1 summarizes our results. For each of the displayed proteins, at least one 3-NT-containing fragment was identified by NSI-MS/MS analysis, together with a number of non-nitrated peptides, confirming the presence of the respective protein in our samples. A representative MS/MS spectrum for the peptide Thr^{95}-Arg^{139} from glycogen phosphorylase, displaying nitration of Tyr^{113}, is shown in Figure 3. Interestingly, most of the nitrated proteins are derived from the cytosol, confirming our earlier data obtained by 2DE (13). This is in stark contrast to aging heart, where a significant fraction of the nitrated proteins is located in the mitochondria (14). Two proteins in Table 1 are present in more than one isoelectric focusing fraction, creatine kinase and aldolase A. This phenomenon may be caused by additional post-translational modifications present on the protein, such as phosphorylation and/or Cys oxidation (to sulfinic and sulfonic acid), and has been realized quite frequently in the 2DE analysis of tissues. For two proteins, we recorded more than one 3-NT residue, i.e. 3-NT at positions 14 and 20 for creatine kinase (GenInfo 6978861), and 3-NT at positions 1175 and 1206 for the neurogenic locus notch homolog (GenInfo 6093542).

**Correlation of in vivo with in vitro nitration**

Frequently, proteins have been exposed to nitrating agents in vitro in order to determine their potential sensitivity towards nitration in vivo and to obtain standard nitrated peptides for the screening of biological samples. However, in some instances these two approaches have yielded quite different selectivities. For example, for SERCA2a, biological aging leads predominantly to nitration of Tyr^{294} and Tyr^{295} in vivo (11), while peroxynitrite treatment in vitro preferentially targets Tyr^{122} (37). From the listed proteins in Table 1, we have representatively selected creatine kinase for a comparison of the chemical selectivity of in vitro and in vivo nitration. This protein shows a good sequence coverage in our in vivo analysis (65%), where all the Tyr-containing peptides are accounted for, rendering it amenable for such comparison. The exposure of creatine kinase to increasing concentrations of peroxynitrite leads to a successive inactivation of the protein (Figure 4A), accompanied by the accumulation of 3-NT, monitored by Western blotting (Figure 4B). To control for the specificity of the anti-3-NT antibody, we reduced the blots with Na_{2}S_{2}O_{4} prior to incubation with the antibody, which resulted in no staining (data not shown). The NSI-MS/MS analysis of creatine kinase exposed to peroxynitrite in vitro yielded an exclusive nitration of Tyr^{82} with no further nitration of any of the other 8 Tyr residues in the protein. Figure 4C displays the sequence of creatine kinase with all the peptides covered by MS/MS analysis in bold (80% sequence coverage). Importantly, Tyr^{82} was not found nitrated in vivo even though our 65% sequence coverage in vivo included the region of Tyr^{82}. Hence, there is a significant difference in the product pattern of creatine kinase nitration in vivo and by peroxynitrite in vitro. These differences will be analyzed further in the Discussion section.

**Discussion:**

Skeletal muscle is constantly exposed to periodic fluxes of NO (17), associated with proper muscle contraction and relaxation. Moreover, oxygen consumption, associated with respiration and motor function of muscle, creates the possibility of formation of superoxide anion (38) as a consequence of “electron leakage” from the mitochondrial respiratory chain. The
combination of NO and superoxide results in the formation of peroxynitrite (ONOO\(^-\)), a major nitrating species \textit{in vivo} (18), especially in the presence of CO\(_2\). Hence, the nitration of skeletal muscle proteins is not unexpected. Of note is the \textit{selectivity} of protein nitration in skeletal muscle, demonstrated earlier by proteomic screening on the basis of 2DE analysis (13), and confirmed by tandem MS analysis in this paper. This selectivity may be caused by a combination of several factors such as (i) a chemical selectivity of the nitrating agent, (ii) the relative abundance of the target proteins, (iii) the accelerated turnover of some of the nitrated proteins, and (iv) the “repair” of nitrated proteins by a putative enzyme called “denitrase” (7,8).

The present paper provides the first detailed \textit{in vivo} proteomic identification of specific 3-NT-containing sequences of the “nitroproteome” in aging skeletal muscle (see Table 1). Such localization of 3-NT sites is of fundamental importance for a potential correlation of protein nitration with functional and/or structural modifications. Several of these proteins are involved in energy metabolism, an important result in view of the recent findings that biological aging is associated with a metabolic shift (39,40). The nitration of the ryanodine receptor 3, at position Tyr\(^{2812}\), may hold important consequences for cellular Ca\(^{2+}\) homeostasis. Generally, biological aging is associated with a loss of Ca\(^{2+}\) homeostasis and higher cytosolic levels of Ca\(^{2+}\) (41,42). Our earlier results demonstrate significant levels of 3-NT on another SR/ER protein, SERCA2a, which cause partial inactivation and may be responsible for a reduced efficiency of the SR to sequester cytosolic Ca\(^{2+}\) (11). It remains to be shown whether the nitration of the ryanodine receptor is functionally important and may contribute to lower SR/ER Ca\(^{2+}\) levels.

\textit{In vivo}, the skeletal muscle creatine kinase suffers nitration of both Tyr\(^{14}\) and Tyr\(^{20}\), but of none of the other seven Tyr residues at positions 39, 82, 125, 140, 173, 174, and 279. All tryptic peptides containing these remaining Tyr residues were covered in the tandem mass spectrometric analysis of the skeletal muscle creatine kinase. In contrast, the \textit{in vitro} exposure of creatine kinase to peroxynitrile nitrates exclusively Tyr\(^{82}\). Tryptic peptides containing all of the other eight Tyr residues were covered in the tandem mass spectrometric analysis. Hence, these significant differences in nitration patterns between \textit{in vivo} and \textit{in vitro} samples are not caused by any analytical discrimination against specific Tyr- and/or 3-NT-containing peptides. The distinctly different patterns are likely caused by one or more of the following factors. (i) Specific nitrated isoforms of creatine kinase may be degraded \textit{in vivo} through the proteasome, which is known for its ability to degrade peroxynitrite-exposed proteins (43). A nitrating agent may generate 3-NT predominantly at one specific location in the protein, and negligible yields of 3-NT at other positions. Nevertheless, protein isoforms containing the 3-NT at these other positions may accumulate with age if they are resistant to degradation and, at the same time, the protein isoforms containing 3-NT at the predominant target Tyr residue are susceptible to degradation. (ii) Peroxynitrite may not be the (only) nitrating agent \textit{in vivo}. Some evidence for protein nitration through \(^{\cdot}\)NO\(_2\) has been provided (44) and to date it is not known to which extent protein nitration \textit{in vivo} is carried out by peroxynitrite, the peroxynitrite-CO\(_2\) adduct, \(^{\cdot}\)NO\(_2\) and/or potentially other hitherto unknown nitrating species. (iii) Creatine kinase may exist \textit{in vivo} in complexes with other proteins or small molecular weight compounds (phosphocreatine, ATP), which affect the chemical selectivity of nitration. Our previous experiments with calmodulin have demonstrated such an effect of protein-protein interaction, where complexation with calmodulin-binding peptides and proteins, melittin and nitric oxide synthase 2 (NOS2), have changed the susceptibility of calmodulin towards oxidation by peroxynitrite (45). In addition, Mihm et al. have recognized a protective effect of phosphocreatine and ATP on creatine kinase modification by peroxynitrite (46). (iv) Our \textit{in vitro} reaction conditions of 200 mM NH\(_4\)HCO\(_3\) buffer, pH 7.8, are not exactly physiologic. These conditions could cause some conformational transition of creatine kinase compared to \textit{in vivo} conditions, potentially associated with an altered susceptibility of specific tyrosine residues to nitration.

Interestingly, an earlier study focused on the susceptibility of \textit{mitochondrial} creatine
kinase towards oxidation by peroxynitrite, where analysis by matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) mass spectrometry gave evidence for the nitration of Trp$^{264}$ and Trp$^{268}$ but no Tyr residue (47). This is in contrast to our results with cytosolic creatine kinase, displaying tyrosine nitration both in vivo and in vitro. An important difference between the cytosolic and mitochondrial isoforms of creatine kinase is their aggregation state, where the cytosolic protein exists exclusively in dimers whereas the mitochondrial protein forms octamers. Of note is that both isoforms appear to suffer at least part of their oxidative inactivation through oxidation of a critical Cys residue, Cys$^{278}$ (mitochondrial) (47) and Cys$^{283}$ (cytosolic) (48). However, the differential sensitivity of the individual isoforms towards the nitration of Tyr vs. Trp, and the differences in the cytosolic isoforms towards nitration in vivo and in vitro, highlights the importance of protein structure and environment on product formation during oxidative stress.

Acknowledgement
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1Abbreviations
2DE, two-dimensional gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; NSI-MS/MS, nanoelectrospray ionization-tandem mass spectrometry; 3-NT, 3-nitrotyrosine; SERCA, sarco/endoplasmic reticulum Ca-ATPase.

References:
Figure Legends:

**Figure 1:** SDS-PAGE and Western blot analysis (anti-3-nitrotyrosine antibody) of the individual fractions collected after solution isoelectrofocusing. Protein fractions (20 µg) were loaded on 4-20% PAGE gels and submitted to electrophoresis and electrophoretic transfer as described in the text. The numbers of the fractions are indicated at the top; from left to right represents an increase in the pl value of the individual proteins. In a parallel experiment, the proteins of fraction 10 were not separated by SDS-PAGE but directly subjected to trypsin digestion and analyzed by HPLC-nanoelectrosprayionization tandem mass spectrometry. A representative chromatogram is shown in Figure 2.

**Figure 2:** Plot of total ion current vs. run time of a representative HPLC-nanoelectrospray ionization tandem mass spectrometry analysis. Proteins of fraction 10 (Figure 1) were not separated by SDS-PAGE but directly subjected to trypsin digestion and analyzed by HPLC-nanoelectrospray ionization tandem mass spectrometry.

**Figure 3:** Representative MS/MS spectrum of the 3-nitrotyrosine-containing tryptic peptide Thr⁹⁵-Arg¹³⁹ from glycogen phosphorylase of aged skeletal muscle, showing nitration at position Tyr¹¹³. For experimental conditions, see text.

**Figure 4:** In vitro nitration of human creatine kinase by peroxynitrite. (A) Enzyme activity as a function of exposure to various concentrations of peroxynitrite, (B) Western Blot analysis with the anti-3-nitrotyrosine antibody showing a peroxynitrite-dependent increase in protein nitration, and (C) sequence coverage obtained by tandem mass spectrometric analysis, showing all detected peptides in bold and the position of the nitrated Tyr⁸⁲ underlined.
Figure 1
Figure 3

Glycogen phosphorylase

T<sup>95</sup>LQNTMVNLALENACDEAT(3-NT)<sup>113</sup>QLGLDMELEEIEEDAGLGNGGLGR<sup>139</sup>

m/z

Relative Abundance

<table>
<thead>
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<th>ONOO- concentration (µM)</th>
<th>Activity (units/mg)</th>
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<tr>
<td>0</td>
<td>0.725(3)</td>
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<tr>
<td>100</td>
<td>0.615(5)</td>
</tr>
<tr>
<td>200</td>
<td>0.354(2)</td>
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<tr>
<td>500</td>
<td>0.067(8)</td>
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[Figure 4]
Table 1: Summary of 3-nitrotyrosine (3-NT)-containing proteins detected in skeletal muscle from 34 months old Fisher 344/Brown Norway F1 rats. Tissue of a total of three individual animals was analyzed. The nitrated tyrosine residues in the sequences are displayed in underlined, bold font.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession # (GenInfo)</th>
<th>MW, kDa</th>
<th>% Coverage (AA)</th>
<th># of identified peptides</th>
<th>Nitrated peptide Sequence</th>
<th># of times, the 3-NT peptide was detected</th>
<th>Isoelectric fraction # (refers to Figure 1)</th>
<th>Function and cellular location</th>
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<tr>
<td>Creatine Kinase</td>
<td>6978661</td>
<td>42.9</td>
<td>65</td>
<td>18</td>
<td>LNYKSQEYYPDL and SLEAQAEEKYSDK</td>
<td>12-25</td>
<td>3, 9, 10, 11</td>
<td>Cytoplasmic, Energy metabolism</td>
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<tr>
<td>Tropomyosin 1</td>
<td>1351289</td>
<td>32.6</td>
<td>64</td>
<td>27</td>
<td>SLEAQAEEKYSDK</td>
<td>214-226</td>
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<td>31</td>
<td>7</td>
<td>WGDAGAEYYVESTGVFTMEKAG AHLK</td>
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<td>Cytoplasmic, Energy metabolism</td>
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<td>Myosin light chain</td>
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<td>9</td>
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<td>15</td>
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Proteomic analysis of protein nitration in aging skeletal muscle and identification of nitrotyrosine-containing sequences in vivo by nanoelectrospray ionization tandem mass spectrometry

Jaroslaw Kanski, Sung J Hong and Christian Schoneich

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