Supplementary Material: Tryptophan nitration physiologically regulates cardiac lactate dehydrogenase active site loop mobility and activity

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Supplementary Methods

Chemicals

Immunoblotting reagents and column materials were obtained from Amersham Pharmacia Biotech (Freiburg, Germany); membranes, from Bio-Rad Laboratories (Hercules, CA, USA). Primary antibodies against nitrotyrosine were generous gifts from L.O. Uttenthal and J.S. Beckman or purchased from HyCult Biotechnology (Uden, Netherlands), Calbiochem-Novabiochem GmbH/Merck Millipore (Bad Soden/Darmstadt, Germany), Alexis/Axxora GmbH (Grünberg/Lörrach, Germany) and Upstate Inc./Millipore (Lake Placid, NY, USA/Darmstadt, Germany). As secondary antibodies, we used horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins for polyclonal antibodies or anti-mouse immunoglobulins for monoclonal primary antibodies from DAKO Diagnostika GmbH/Agilent Technologies (Hamburg, Germany). For gel loading we used Rotiload buffer from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). The Oxyblot kit was supplied by Intergen Company/Millipore (Purchase, NY, USA/Darmstadt, Germany). Nonfat dry milk was obtained from Nestlé Inc. Carnation (Solon, Ohio, USA); Cell culture medium M199, from PAA Laboratories GmbH (Martinsried/Cölbe, Germany); Nitrating reagent, H_2O_2/NO_2^-, from Alexis/Axxora GmbH; Complete Protease Inhibitor Cocktail, from Roche Diagnostics GmbH (Mannheim, Germany). Porcine heart LDH was purchased from MyBiosource. All other chemicals were of the highest purity grade available and purchased from either Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) or Merck KG (Darmstadt, Germany).

Animals

Adult male Wistar Unilever rats (270-320 g) were purchased from Harlan-Winkelmann (Borchen, Germany). eNOS and MPO KO mice were generated and bred as described previously (19). Rats and mice were housed under standard or SPF conditions, respectively, at a temperature of 18-20°C and a day-night rhythm of 12 h, with access to water and food ad libitum. Porcine hearts were obtained from a local abattoir.

Nitration of proteins by peroxynitrite

If not stated otherwise, nitration was performed by continuous mixing protein and nitrating reagent (H_2O_2/NO_2^-) in a 1:5 molar ratio in phosphate buffer pH 7.3 (15 mM KH_2PO_4, 80 mM Na_2HPO_4) for 5 min at room temperature. Due to the strong alkaline storage conditions of H_2O_2/NO_2^-, the pH in the samples was immediately readjusted to neutral pH with HCl. No further purification or dialysis steps were conducted. For longer storage, samples were aliquoted and frozen at -20°C and used within 1 month or mixed with gel loading buffer and boiled for 10 min at 95°C for daily use. In this case, no change in signal characteristics or intensities was observed over time.

SDS-PAGE, western and dot-blot analysis

Protein samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 10% polyacrylamide gels. Alternatively, pre-cast gels (NuPAGE Novex 10% bis-Tris Midi Gel 1,0 mm x 26 well, Invitrogen) were used according to the manufacturer's protocol with MES running buffer XCell SureLock Midi-Cell running tanks. For mass spectrometry proteins were stained with colloidal Coomassie blue. Proteins were immobilized on nitrocellulose membranes using standard dot-blot or semi-dry blotting techniques (20). Efficiency of protein immobilization was evaluated by staining the membrane in 0.5 % (w/v) Ponceau S in 3 % (w/v) trichloroacetic acid (TCA). Membranes were treated with 5 % (w/v) nonfat dry milk in Tris-buffered saline (TBS, 20 mM Tris/HCl pH 7.5, 150 mM NaCl) containing 0.1 % (v/v) Tween 20 (TBS-T) for 1 hour to block nonspecific protein binding. Then the membrane was incubated overnight at 4°C with the first antibody in 5% (w/v) nonfat dry milk in TBS-T and afterwards washed 3 × 10 min with TBS-T. To test the specificity for NO_2Tyr, antibodies were pre-incubated with 3 mM NO_2Tyr [(footnote1)]. Alternatively, in order to reduce NO_2 groups of nitrated proteins to NH_2 groups, blots were first incubated with the reducing agent dithionite (1 mM) in 100 mM sodium borate pH 10 for 5 min at room temperature, washed 3

X 10 min with TBS-T and then incubated with anti-NO_2Tyr antibody (41). As second antibody, we used horseradish peroxidase (HRP)-coupled antibodies against rabbit immunoglobulins (Ig) for polyclonal first antibodies or against mouse Ig for monoclonal first antibodies. HRP-labeled conjugates against the first antibodies were dissolved in 5% (w/v) nonfat dry milk in TBS-T. After washing 3 X 10 min with TBS-T the immunosignals were developed using the ECL detection reagent by Amersham Biosciences and blots were analyzed using a Kodak chemiluminescence Imager (Scientific Imaging System, Eastman Kodak Company, New Haven, USA). The resolution of the imager was 752 x 582 pixels and the linear area of signal quantification was verified by repeated measurements using increasing protein amounts. Light signals were directly quantified employing the Kodak 1D Image analysis software (version 3.5.2).

Screening of tissues and determination of nitrated proteins

Several tissues from rat and pig were homogenized in 10 volumes (w/v) of lysis buffer, containing 25 mM triethanolamine HCl (TEA) pH 7, 1 mM EDTA, 5 mM dithiothreitol, 50 mM NaCl, 10 % (v/v) glycerol and Complete^{TM} Protease Inhibitor Cocktail (Roche, Mannheim, Germany). For long-term storage, the homogenates were shock frozen in liquid nitrogen and stored at –20 °C. For daily use, gel loading buffer was added to the samples and heated to 95°C for 10 min. Any remaining particles were pelleted by centrifugation in a desktop centrifuge (10000 x *g*, 10 min, 4°C). The samples were first screened by use of all antibodies listed in Tab. 1 by dot blot analysis. Tissues that yielded more than 4 hits by all antibodies were subjected to further analysis by SDS-PAGE and western blot as described above.

Characterization and isolation of a 38 kDa protein in pig heart tissue

A column packed with 2 ml of Blue Sepharose resin (Amersham Bioscience) was washed and pre-equilibrated with 10 mL lysis buffer. Pig heart supernatant (1 mL) was applied to the column. The flow-through was subsequently applied to 1 mL Q-Sepharose column that was also washed and equilibrated with lysis buffer. This column was then washed again with

lysis buffer (5 mL) and the bound proteins were eluted with 2 mL of lysis buffer containing 1 M NaCl. The partially purified 38-kDa protein was mixed with gel loading buffer, boiled at 95°C for 10 min and subjected to SDS-PAGE. It was stained with colloidal Coomassie blue (22). The stained protein band at 38 kDa was cut out, destained and enzymatically digested. Tryptic fragments were separated by high pressure liquid chromatography (HPLC). Fractions containing tryptic peptides were subjected to matrix-assisted laser-desorption/ionization mass spectrometry (MALDI-MS, Bruker Reflex III™, Bruker Daltonics, Bremen, Germany). Peptide sequences were compared with proteins in the NCBIprot database.

2D-PAGE analysis of rat heart tissue

2D-PAGE analysis was conducted using standard conditions (42). Briefly, 7 cm ReadyStrips™ IPG strips (BioRad) of pI 3-10 were subjected to active rehydration overnight with heart homogenate in Destreak™ IEF buffer, supplemented with 0.02% pI 3-10 ampholites (BioRad). Isoelectric focusing was performed in a Protean™ IEF cell (Bio-Rad) using a ramped voltage up to 4000 V, until a total of approximately 10,000 Vh had elapsed. Strips were subjected to reduction, alkylation, and equilibration for the second dimension, using Equilibration Buffers I & II (BioRad) for 30 min each, then SDS-PAGE, using 10% polyacrylamide NuPage™ Novex Bis-Tris gels (Invitrogen). Gels were subsequently Coomassie stained (Gelcode™ Blue, Pierce).

In-gel digestion of rat heart samples

In-gel digestion was conducted as previously described (43). Briefly, the well-resolved protein spot of interest was punch-excised using wide-orifice pipette tips. Gel pieces were destained with 100 mM ammonium bicarbonate, pH 9/50% acetonitrile, and were washed three times with wash solution 1 (100 mM ammonium bicarbonate, pH 9), wash solution 2 (100 mM ammonium bicarbonate, pH 9/50% acetonitrile), followed by wash solution 3 (100% acetonitrile). Gel pieces were swelled in 50 mM ammonium bicarbonate, pH 9/10%

acetonitrile, containing approximately 10 ng Trypsin Gold (Promega) and incubated overnight at 37°C. Peptides were extracted (each time for 20 min) once with 20 mM ammonium bicarbonate, pH 9, twice with 1% trifluoroacetic acid/50% acetonitrile, followed by once with 100% acetonitrile. The entire extraction process was performed in duplicate, and all extraction supernatants were pooled and spun to dryness in a SpeedVac™ (Thermo-Savant, Waltham, MA).

MALDI-TOF MS of rat heart samples

Peptides were desalted and prepared for MS using ZipTips™ (Millipore). Mass spectra were obtained after purified peptides were co-crystallized with the matrix 2,5-dihydroxybenzoic acid onto AnchorChip™ targets (Bruker Daltonics, Billerica, MA) using the dried-droplet technique (28, 44) and a Reflex IV™ MALDI-TOF MS instrument (Bruker-Daltonics) in positive ion, reflectron mode, over the range 400-8000. The laser intensity was adjusted to be ~3% above the threshold value for peptide ion signals. Signals from 100 to 200 laser shots were summed for each mass spectrum. External and internal calibration was achieved to within 50 ppm using the masses of known purified peptide standards and trypsin autolysis peptides.**MS data analysis and peptide mass fingerprinting**

Peak lists from MALDI-TOF mass spectra (derived using MoverZ™ software, Genomic Solutions, Ann Arbor, MI) were submitted on-line to the Mascot™ search engine (Matrix Science, London, UK) for peptide mass fingerprinting (PMF) analysis against the SwissProt or NCBI non-redundant protein databases, using the following restrictions: a) Rattus sp., b) trypsin digestion with up to 3 missed cleavages, c) +/- 50 ppm error, and d) cysteine carbamidomethylation and methionine oxidation as fixed and variable modifications, respectively. LDH was unambiguously identified by PMF, with a Mascot™ Score of 150 (corresponding to an E value of 6×10^{-12}, the likelihood of a false assignment) and a sequence coverage of 65% from 35 matched peptide ions, given search parameters.

Additional peptide ion assignments (as well as validation of Mascot™ assignments) were made by manual inspection of spectra using theoretical digest and post-translational modification mass values for LDH.

LDH activity, oxidation and nitration by H_2O_2/NO_2^-

Commercial LDH was treated with different amounts of peroxynitrite as described above. Aliquots of H_2O_2/NO_2^- were diluted with 2 M NaOH to reach the desired concentrations indicated in **Fig. 2**. LDH activity and kinetic parameters of exogenously nitrated LDH were measured using a fluorescence-based assay, using excitation/emission setting of 340/470 nm, respectively. The assay used uses the fluorescent properties of NADH which can be excited at 340 nm and emission is measured at 440 nm. The oxidized form of NADH, NAD^+, does not have these properties. Measurements were collected every 20 seconds during 5 minutes. The final concentrations in the reaction were LDH, 2 mU/mL, NADH 0.12 mM and Sodium pyruvate 1.13 mM. Nitration of LDH was measured as the intensity of the NO_2Tyr immunosignal in arbitrary units.

Molecular Dynamics Methods

System setup: Two simulation systems were setup based on the crystal structure of the ternary complex of human-heart LDH (pdb 1I0Z). One being the wild type tetramer (WT) and the other being the tetramer with a nitro group substituted at each Trp-324 residue (T4N). The oxamate molecules were converted to pyruvate and acpype (45) used to generate Amber GAFF parameters for the substrate and cofactor molecules. A combination of GAFF parameters for nitrotryptophan and analogy was used to generate a residue library entry for a nitrotryptophan in the Gromacs AmberSB99-ildn forcefield (46). Gromacs tools were used to add hydrogen atoms to all residues consistent with pH 7 and to protonate the catalytic histidine (His-193). The complexes were solvated in boxes of TIP3P water containing 0.15 M sodium chloride ions. Calculations were performed at NTP under period boundary conditions and the PME method used for long range electrostatics. The pressure was 1 Barr

and the temperature 300 K for all molecular dynamics (MD) simulations. Both systems were relaxed by 2000 steps of steepest descent energy minimisation followed by 0.2 ns MD while restraining the protein atoms to their initial positions.

Another pair of simulation systems were prepared in the same fashion, with the pyruvate removed. This gave a total of four simulation systems, referred to as WT-ternary, WT-binary, T4N-ternary and T4N-binary.

Trajectory development and analysis: Three 200 ns simulations (initialized with different velocities) were performed for each of the four simulation systems. These simulations were performed on the University of Bristol HPC machines BlueCrystal and BlueGem. Structures were extracted every 5 ns from each trajectory and energy minimized. This gave 120 starting structures for each of the four systems for further parallel trajectory development. Each new trajectory was initialized with a fresh set of random velocities and run for 28-31 ns on the UK National HPC machine Archer, giving around 3.5 μs combined simulation data for each system. All simulations were run and analyzed using the Gromacs 5.1.2 suite of software (47) and visualizations performed with VMD 1.9.2 (48) and Chimera 1.11 (49).

Molecular Dynamics Analysis

The active site loop (residues 98-110) was found to be less mobile in T4N than WT in the binary complex (LDH+NADH), while the C-terminal helix (α-H) and the juxtaposed loop (residues 277-287) were more mobile in T4N. The lower mobility of the active site loop is also detectable in the ternary complex (LDH+NADH+pyruvate) albeit greatly attenuated, while the C-terminal helix mobility is similar to the WT. The other main features in these plots are the differences in mobility of the 205-225 loop. However, as this is one of the most flexible parts of the protein and is not located near the active site we do not consider these differences to impact the activity of the two proteins. By contrast, the C-terminal side of the active site loop juxtaposes the C-terminal end of the final helix in the protein, α-H (residues 309-332). A crucial contact between these two structural elements is made by Leu-107 and

Ile-326. Hence, we define one metric reporting the association of the active site loop with α-H via the distance between CB atoms of these residues, as m:LH. Likewise, two residues spanning the mouth of the active site Gly-103 and Lys-243 were chosen to monitor openness of the active site loop, or m:LO. Distance histograms show the distribution of these distances over each of the four 3.5 μs combined trajectories. They indicate an increased mobility in the binary complexes compared with the ternary, expressed as a broadening of the histograms.

The distance between the active site loop and α-H (m:LH) is slightly shorter on average for WT than T4N in the ternary complex and this difference is amplified in the binary complex where about half of the WT loops interact more tightly with α-H than T4N. Comparison with the second metric monitoring active site loop closure (m:LO) in the binary complex shows that the T4N loop spends more time at a distance comparable with the ternary complex than the WT and also is an “over-closed” state. The results of the ternary simulations are again more similar, but a tendency towards and “over-closed” state persists.

Synthesis and Characterization of Nitrotryptophan

Nitrotryptophan (NO_2Trp) was synthesized according to Moriya, et al. (15). 20 g L-Trp and 0.25 g urea were suspended in 350 mL glacial acetic acid. Under vigorous stirring at 4°C, 10 mL 24 M nitric acid in 30 mL glacial acetic acid were added. After the suspension changed to a yellow clean solution, it was stirred at 4°C until it changed again to a suspension. Then an additional 10 mL fuming nitric acid in 30 mL glacial acetic acid were added drop by drop at 15°C. After stirring overnight at room temperature, the resulting yellow precipitate was collected by filtration and washed with glacial acetic acid. The crude product was recrystallized in 100 mL 2 M nitric acid at temperatures below 50°C and converted to the free amino acid in 100 mL hot water by the addition of sodium carbonate until the pH was neutral. As analyzed by its infrared (IR) and nuclear magnetic resonance (NMR) spectra, no 4-NO_2Trp was detectable. Because 7-NO_2Trp cannot be synthesized by the action of 24 M

nitric acid on the tryptophan ring (16), the products of the synthesis with respect to UV spectrum are a mixture of 5/6-NO_2Trp. NO_2Trp crystallized as yellow needles with a melting point at 265°C (decomp.). The compound was characterized according to: UV (in 1 N NaOH) 330 nm, 380 nm; IR (KBr) ν(-NH_3^+) 3190 cm^{-1}, ν(C=C) and amide I 1600-1650 cm^{-1}, amide II and δ(-NH_3^+) 1550-1600 cm^{-1}, ν_{as}(-NO_2) 1495 cm^{-1}, ν_s(-NO_2) 1330 cm^{-1}, predominantly 5/6-NO_2 substitution at 818 cm^{-1} instead of 4-NO_2 substitution at 780 cm^{-1}; NMR (D_2O-NaOH, δ 4.7 HDO) δ 2.9-3.1 (2H, m), 3.4-3.6 (1H, m), 7.35 (1H,s), 7.5 (1H, d, J= 9 Hz), 7.7 (1H, dd, J= 2 and 9 Hz), 7.95 (1H, d, J= 2 Hz).

Animal models of disease

To examine LDH nitration under pathophysiological conditions, two *in vivo* models involving nitrative stress were assessed, including a short- and longer-term streptozotocin diabetes model in the rat, and a murine model of doxorubicin-induced cardiotoxicity.

Streptozotocin model of diabetes mellitus

At an age of 10 weeks, rats (n=12) received a single intraperitoneal injection of 60 mg/kg BW of streptozotocin (STZ) dissolved in citrate buffer 50mM pH 4,5. Age matched control rats (n=12) received vehicle injections with citrate buffer. Hyperglycemia was defined as a positive test of glucose in urine (Haemo-Glukotest, Boehringer Mannheim, Germany) and a non-fasting blood glucose level higher than 11 mmol/L (hexokinase method) one week after STZ application. Rats were sacrificed 3 (n=12) and 16 weeks (n=12) after diabetes induction by CO_2-inhalation. After thoracotomy, hearts were excised and freed of connective tissue while immersed in modified Krebs-Hepes buffer pH 7.35 containing 99 mM NaCl; 4.7 mM KCl; 1.9 mM $CaCl_2$; 1.2 mM $MgSO_4$; 25 mM $NaHCO_3$; 1 mM K_2HPO_4; 20 mM Na-Hepes; 11.1 mM D-glucose. Hearts were shock frozen in liquid nitrogen, pulverized, and homogenized in lysis buffer. For SDS-PAGE and western blot, gel loading buffer was added and protein concentrations were determined after TCA precipitation by the method of Lowry.

Doxorubicin cardiotoxicity model

Doxorubicin (DOX, Adriamycin) is a broad-spectrum anthracycline antibiotic that is used to treat a variety of cancers. However, the clinical use of DOX is limited because of its serious cardiotoxicity, possibly involving increased oxidative stress, alteration of cardiac energetic status and direct effects on DNA. Hearts from DOX-treated mice were obtained as described elsewhere (50), and homogenized and shock-frozen as described above.

Endogenous LDH nitration in eNOS-KO and MPO-KO mice

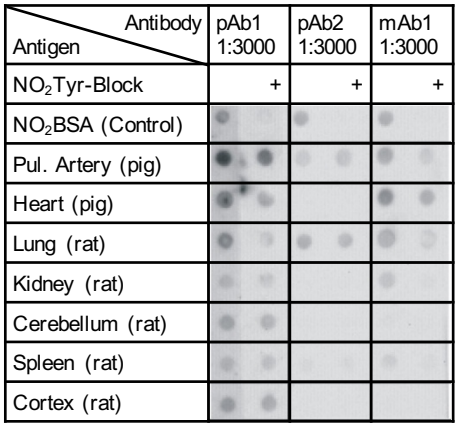
Hearts from eNOS-KO and MPO-KO mice were excised, washed in perfusion buffer, blotted dry on filter paper, weighed, and homogenized immediately in ice-cold 50 mM PBS, pH= 7.4, containing 2.5 mM EDTA, 1 mM DTT, 1% NP-40, phosphatase inhibitors (10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, and 50 mM β-glycerophosphate), and Complete™ protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Homogenates were incubated on ice for 30 min to facilitate solubilization, after which they were clarified by centrifugation at 12000 x g for 15 min at 4°C, aliquoted and stored at -80°C. Prior to use, samples were thawed on ice and assayed for protein concentration using the BCA method.

Supplementary Tables

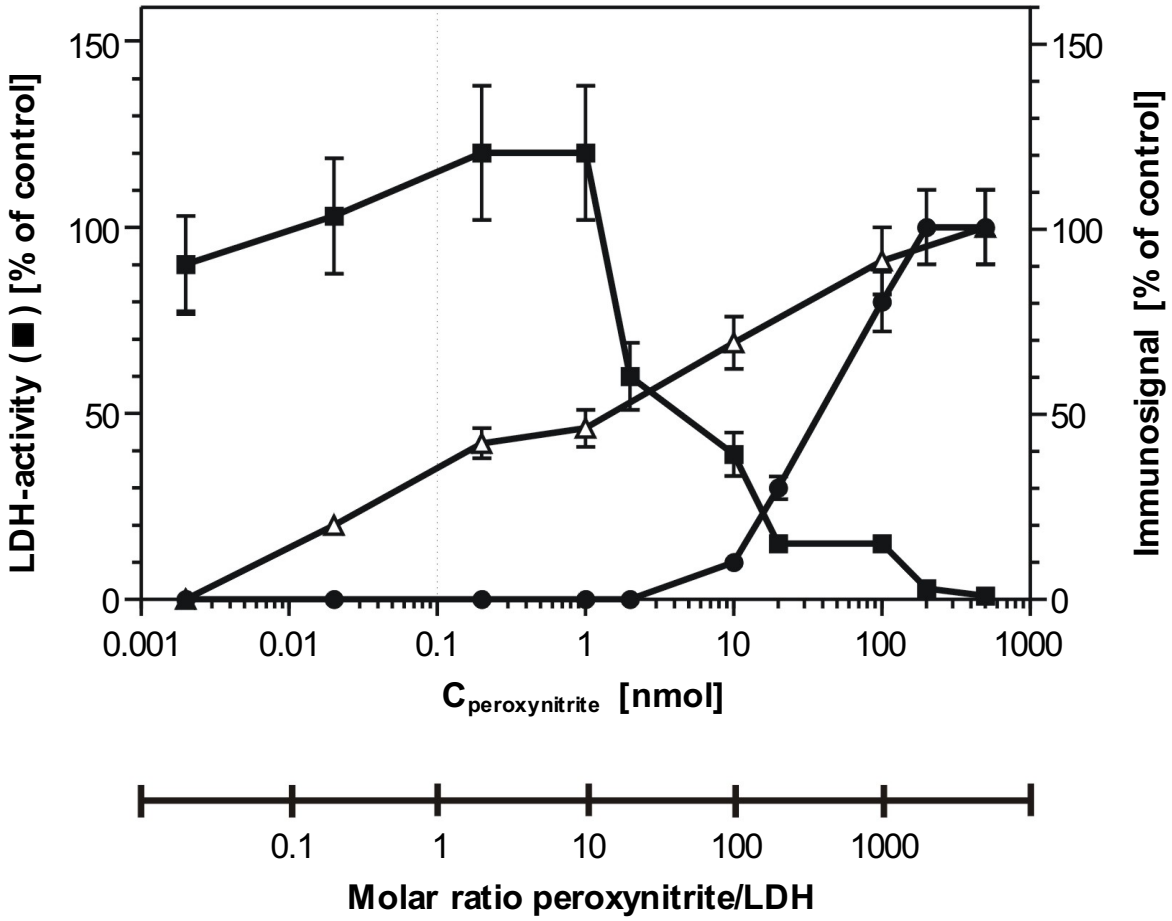
Abbreviation	Specificity	Antigen	Species	Source	Reference
pAb1	anti-NO_2Tyr	ONOO-KLH	rabbit	L.O. Uttenthal	(33)
pAb2				J.S. Beckman	(34)
mAb1			mouse		(34)
Identical to mAb1:					
mAb2	anti-NO_2Tyr	ONOO-KLH	mouse	Alexis	(34)
mAb3				Upstate	(34)
Rejected:					
mAb4	anti-NO_2Tyr	ONOO-KLH	mouse	Calbiochem	(51)
mAb5				HyCult biotechnology	(37)
pAb3	anti-NOTrp	NOTrp-KLH	rabbit	Calbiochem	None
mAb6	anti-NOCys	NOCys	mouse	Biolabs	None

Supplementary Table 1. Commercial and non-commercial antibodies detecting reactive nitrogen species-induced protein modifications. Poly (pAb)- and monoclonal (mAb) antibodies from the indicated species were obtained from either commercial sources or kindly provided by the listed researchers. All antibodies, except pAb3 and mAb6, were raised against keyhole limpet hemocyanin (KLH) treated with 'nitration reagent', i.e. H_2O_2/NO_2^-. For pAb3, the hapten was nitrosocysteine (NOCys); for mAb6, nitrosotryptophan (NOTrp), and in both cases glutaraldehyde-coupled to KLH. Antibodies mAb1, mAb2 and mAb3 behaved identically and referred to the same reference (34) , and were thus considered identical. Antibodies mAb4, mAb5 and pAb3 were later omitted from the study because of low titer and/or low affinity, which allowed to detect only highly abundant antigens such as H_2O_2/NO_2^--treated bovine serum albumin (BSA), but not lower abundance endogenously nitrated proteins in native tissues. Antibody mAb6 was also excluded from further use because it reacted similarly with untreated and H_2O_2/NO_2^--treated BSA.

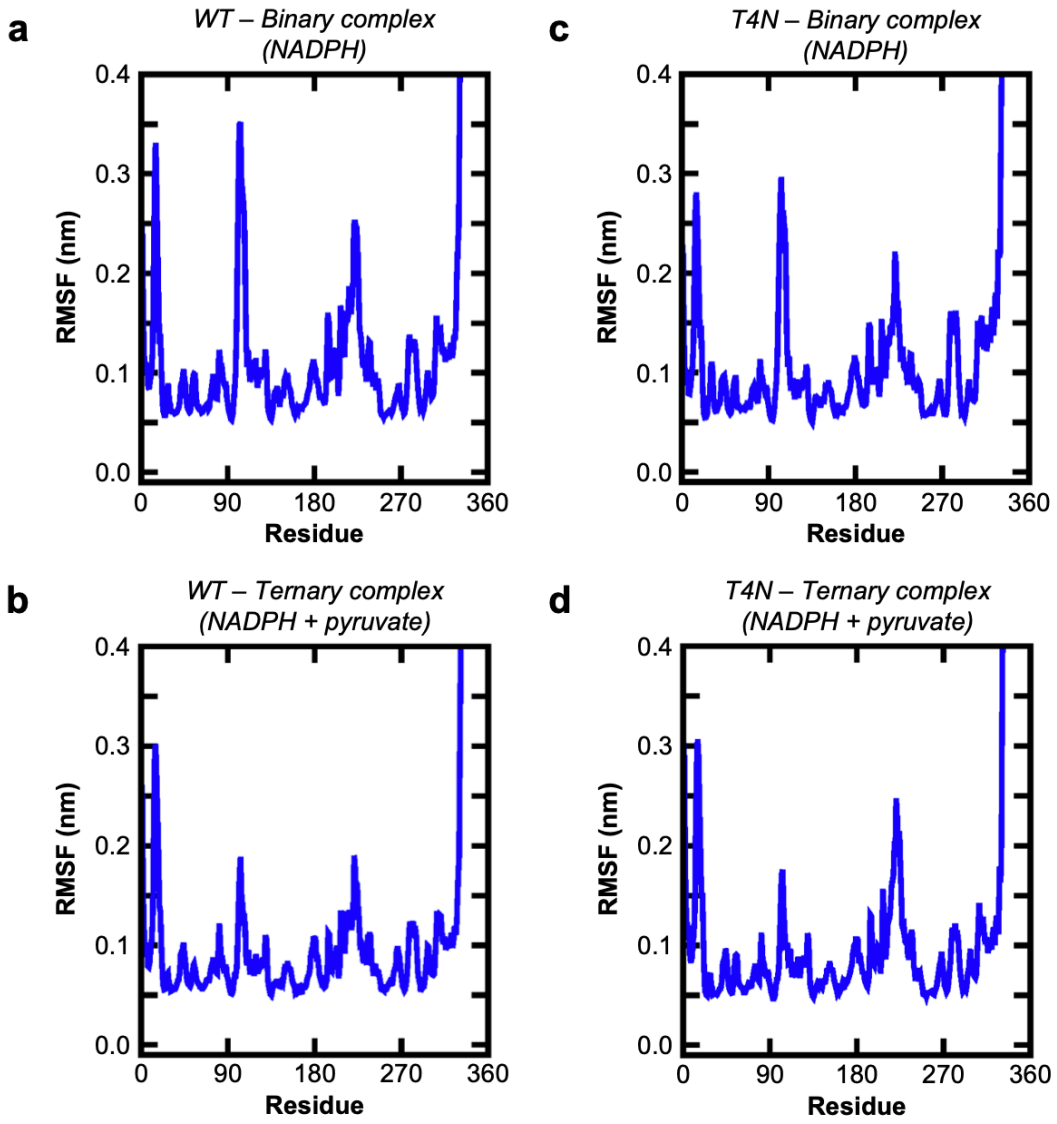
Consequently, we pursued the further use of the first three antibodies, pAb1, pAb2 and mAb1, for the present study (see **Fig. 2**).



Supplementary Figure 1. Anti-NO2-Tyr antibodies greatly vary in sensitivity and specificity in native tissues. Dot blot screen of tissue homogenates of porcine pulmonary artery and heart, and rat heart, lung, kidney, cerebellum, spleen and cortex. For each dot, 50 µg or, in the case of pulmonary artery, 100 µg of protein were blotted onto a nitrocellulose membrane. NO_2Tyr-immuno-positive proteins were then detected using antibodies pAb1, pAb2 or mAb1. NO_2BSA was used as a positive control; pre-incubation with 3 mM free NO_2Tyr, as a negative control (+).



Supplementary Figure 2. Nitration and oxidation of LDH *in vitro* inversely correlates with LDH activity. As LDH (0.1 nmol) was treated with increasing amounts of peroxynitrite, total LDH activity (■, left axis) was determined by the NADH-coupled photometric assay of Warburg, as described, and all LDH samples were tested under the same conditions for the occurrence of nitration (●) using quantitative Western analysis with the NO_2Tyr antibody pAb1 and oxidation (△) using the oxyblot detection kit (both, right axes).



Supplementary Figure 3. Plots of the backbone RMSF per residue averaged over all subunits of all 120 simulations. (**a**) WT-binary. (**b**) WT-ternary. (**c**) T4N-binary. (**d**) T4N-ternary.