

1 **Supplementary Material: Protein nitration is a physiological regulator of cardiac**  
2 **lactate dehydrogenase active site loop mobility and activity**

3 Cristian Nogales<sup>1,\*</sup>, Jeroen Frijhoff<sup>1</sup>, Detlef Thoenges, Richard B. Sessions, Albert  
4 Sickmann, Tatiana Nedvetskaya, Cesar Ibarra-Alvarado, Vera Schmitz, Peter Rösen, Axel  
5 Goedecke, Aleksandar Jovanović, David H. Perlman, Anna Klinke, Stephan Baldus, Ana I.  
6 Casas, Péter Bai, Csaba Szabo, Martin Feelisch & Harald H.H.W. Schmidt\*.

7 <sup>1</sup>Both first authors contributed equally to this work.

8 \*To whom correspondence may be addressed. Email: [h.schmidt@maastrichtuniversity.nl](mailto:h.schmidt@maastrichtuniversity.nl)  
9 [or c.nogales@maastrichtuniversity.nl](mailto:c.nogales@maastrichtuniversity.nl)

10 **Key words:** protein nitration, nitro-tryptophan, nitro-tyrosine, biomarker

## 1 **Supplementary Methods**

### 2 **Chemicals**

3 Immunoblotting reagents and column materials were obtained from Amersham Pharmacia  
4 Biotech (Freiburg, Germany); membranes, from Bio-Rad Laboratories (Hercules, CA, USA).  
5 Primary antibodies against nitrotyrosine were generous gifts from L.O. Uttenthal and J.S.  
6 Beckman or purchased from HyCult Biotechnology (Uden, Netherlands), Calbiochem-  
7 Novabiochem GmbH/Merck Millipore (Bad Soden/Darmstadt, Germany), Alexis/Axxora  
8 GmbH (Grünberg/Lörrach, Germany) and Upstate Inc./Millipore (Lake Placid, NY,  
9 USA/Darmstadt, Germany). As secondary antibodies, we used horseradish peroxidase  
10 (HRP)-conjugated goat anti-rabbit immunoglobulins for polyclonal antibodies or anti-mouse  
11 immunoglobulins for monoclonal primary antibodies from DAKO Diagnostika GmbH/Agilent  
12 Technologies (Hamburg, Germany). For gel loading we used Rotiload buffer from Carl Roth  
13 GmbH + Co. KG (Karlsruhe, Germany). The Oxyblot kit was supplied by Intergen  
14 Company/Millipore (Purchase, NY, USA/Darmstadt, Germany). Nonfat dry milk was  
15 obtained from Nestlé Inc. Carnation (Solon, Ohio, USA); Cell culture medium M199, from  
16 PAA Laboratories GmbH (Martinsried/Cölbe, Germany); Nitrating reagent,  $\text{H}_2\text{O}_2/\text{NO}_2^-$ , from  
17 Alexis/Axxora GmbH; Complete Protease Inhibitor Cocktail, from Roche Diagnostics GmbH  
18 (Mannheim, Germany). Porcine heart LDH was purchased from MyBiosource. All other  
19 chemicals were of the highest purity grade available and purchased from either Sigma-  
20 Aldrich Chemie GmbH (Taufkirchen, Germany) or Merck KG (Darmstadt, Germany).

### 21 **Animals**

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

## 1 Nitration of proteins by peroxyntirite

2 If not stated otherwise, nitration was performed by continuous mixing protein and nitrating  
3 reagent ( $\text{H}_2\text{O}_2/\text{NO}_2^-$ ) in a 1:5 molar ratio in phosphate buffer pH 7.3 (15 mM  $\text{KH}_2\text{PO}_4$ , 80 mM  
4  $\text{Na}_2\text{HPO}_4$ ) for 5 min at room temperature. Due to the strong alkaline storage conditions of  
5  $\text{H}_2\text{O}_2/\text{NO}_2^-$ , the pH in the samples was immediately readjusted to neutral pH with HCl. No  
6 further purification or dialysis steps were conducted. For longer storage, samples were  
7 aliquoted and frozen at  $-20^\circ\text{C}$  and used within 1 month or mixed with gel loading buffer and  
8 boiled for 10 min at  $95^\circ\text{C}$  for daily use. In this case, no change in signal characteristics or  
9 intensities was observed over time.

## 10 SDS-PAGE, western and dot-blot analysis

11 Protein samples were separated by sodium dodecylsulfate-polyacrylamide gel  
12 electrophoresis (SDS-PAGE) under reducing conditions using 10% polyacrylamide gels.  
13 Alternatively, pre-cast gels (NuPAGE Novex 10% bis-Tris Midi Gel 1,0 mm x 26 well,  
14 Invitrogen) were used according to the manufacturer's protocol with MES running buffer  
15 XCell SureLock Midi-Cell running tanks. For mass spectrometry proteins were stained with  
16 colloidal Coomassie blue. Proteins were immobilized on nitrocellulose membranes using  
17 standard dot-blot or semi-dry blotting techniques (20). Efficiency of protein immobilization  
18 was evaluated by staining the membrane in 0.5 % (w/v) Ponceau S in 3 % (w/v)  
19 trichloroacetic acid (TCA). Membranes were treated with 5 % (w/v) nonfat dry milk in Tris-  
20 buffered saline (TBS, 20 mM Tris/HCl pH 7.5, 150 mM NaCl) containing 0.1 % (v/v) Tween  
21 20 (TBS-T) for 1 hour to block nonspecific protein binding. Then the membrane was  
22 incubated overnight at  $4^\circ\text{C}$  with the first antibody in 5% (w/v) nonfat dry milk in TBS-T and  
23 afterwards washed  $3 \times 10$  min with TBS-T. To test the specificity for  $\text{NO}_2\text{Tyr}$ , antibodies  
24 were pre-incubated with 3 mM  $\text{NO}_2\text{Tyr}$  <sup>(footnote1)</sup>. Alternatively, in order to reduce  $\text{NO}_2$  groups  
25 of nitrated proteins to  $\text{NH}_2$  groups, blots were first incubated with the reducing agent  
26 dithionite (1 mM) in 100 mM sodium borate pH 10 for 5 min at room temperature, washed 3

1 X 10 min with TBS-T and then incubated with anti-NO<sub>2</sub>Tyr antibody (41). As second  
2 antibody, we used horseradish peroxidase (HRP)-coupled antibodies against rabbit  
3 immunoglobulins (Ig) for polyclonal first antibodies or against mouse Ig for monoclonal first  
4 antibodies. HRP-labeled conjugates against the first antibodies were dissolved in 5% (w/v)  
5 nonfat dry milk in TBS-T. After washing 3 X 10 min with TBS-T the immunosignals were  
6 developed using the ECL detection reagent by Amersham Biosciences and blots were  
7 analyzed using a Kodak chemiluminescence Imager (Scientific Imaging System, Eastman  
8 Kodak Company, New Haven, USA). The resolution of the imager was 752 x 582 pixels and  
9 the linear area of signal quantification was verified by repeated measurements using  
10 increasing protein amounts. Light signals were directly quantified employing the Kodak 1D  
11 Image analysis software (version 3.5.2).

## 12 **Screening of tissues and determination of nitrated proteins**

13 Several tissues from rat and pig were homogenized in 10 volumes (w/v) of lysis buffer,  
14 containing 25 mM triethanolamine HCl (TEA) pH 7, 1 mM EDTA, 5 mM dithiothreitol, 50 mM  
15 NaCl, 10 % (v/v) glycerol and Complete™ Protease Inhibitor Cocktail (Roche, Mannheim,  
16 Germany). For long-term storage, the homogenates were shock frozen in liquid nitrogen and  
17 stored at -20 °C. For daily use, gel loading buffer was added to the samples and heated to  
18 95°C for 10 min. Any remaining particles were pelleted by centrifugation in a desktop  
19 centrifuge (10000 x g, 10 min, 4°C). The samples were first screened by use of all antibodies  
20 listed in Tab. 1 by dot blot analysis. Tissues that yielded more than 4 hits by all antibodies  
21 were subjected to further analysis by SDS-PAGE and western blot as described above.

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

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

1 lysis buffer (5 mL) and the bound proteins were eluted with 2 mL of lysis buffer containing 1  
2 M NaCl. The partially purified 38-kDa protein was mixed with gel loading buffer, boiled at  
3 95°C for 10 min and subjected to SDS-PAGE. It was stained with colloidal Coomassie blue  
4 (22). The stained protein band at 38 kDa was cut out, destained and enzymatically digested.  
5 Tryptic fragments were separated by high pressure liquid chromatography (HPLC).  
6 Fractions containing tryptic peptides were subjected to matrix-assisted laser-  
7 desorption/ionization mass spectrometry (MALDI-MS, Bruker Reflex III™, Bruker Daltonics,  
8 Bremen, Germany). Peptide sequences were compared with proteins in the NCBIprot  
9 database.

## 10 **2D-PAGE analysis of rat heart tissue**

11 2D-PAGE analysis was conducted using standard conditions (42). Briefly, 7 cm  
12 ReadyStrips™ IPG strips (BioRad) of pI 3-10 were subjected to active rehydration overnight  
13 with heart homogenate in Destreak™ IEF buffer, supplemented with 0.02% pI 3-10  
14 ampholites (BioRad). Isoelectric focusing was performed in a Protean™ IEF cell (Bio-Rad)  
15 using a ramped voltage up to 4000 V, until a total of approximately 10,000 Vh had elapsed.  
16 Strips were subjected to reduction, alkylation, and equilibration for the second dimension,  
17 using Equilibration Buffers I & II (BioRad) for 30 min each, then SDS-PAGE, using 10%  
18 polyacrylamide NuPage™ Novex Bis-Tris gels (Invitrogen). Gels were subsequently  
19 Coomassie stained (Gelcode™ Blue, Pierce).

## 20 **In-gel digestion of rat heart samples**

21 In-gel digestion was conducted as previously described (43). Briefly, the well-resolved  
22 protein spot of interest was punch-excised using wide-orifice pipette tips. Gel pieces were  
23 destained with 100 mM ammonium bicarbonate, pH 9/50% acetonitrile, and were washed  
24 three times with wash solution 1 (100 mM ammonium bicarbonate, pH 9), wash solution 2  
25 (100 mM ammonium bicarbonate, pH 9/50% acetonitrile), followed by wash solution 3 (100%  
26 acetonitrile). Gel pieces were swelled in 50 mM ammonium bicarbonate, pH 9/10%

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

## 7 **MALDI-TOF MS of rat heart samples**

8 Peptides were desalted and prepared for MS using ZipTips™ (Millipore). Mass spectra  
9 were obtained after purified peptides were co-crystallized with the matrix 2,5-  
10 dihydroxybenzoic acid onto AnchorChip™ targets (Bruker Daltonics, Billerica, MA) using  
11 the dried-droplet technique (28, 44) and a Reflex IV™ MALDI-TOF MS instrument (Bruker-  
12 Daltonics) in positive ion, reflectron mode, over the range 400-8000. The laser intensity  
13 was adjusted to be ~3% above the threshold value for peptide ion signals. Signals from  
14 100 to 200 laser shots were summed for each mass spectrum. External and internal  
15 calibration was achieved to within 50 ppm using the masses of known purified peptide  
16 standards and trypsin autolysis peptides.

## 17 **MS data analysis and peptide mass fingerprinting**

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

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

#### 4 **LDH activity, oxidation and nitration by H<sub>2</sub>O<sub>2</sub>/NO<sub>2</sub><sup>-</sup>**

5 Commercial LDH was treated with different amounts of peroxyxynitrite as described above.  
6 Aliquots of H<sub>2</sub>O<sub>2</sub>/NO<sub>2</sub><sup>-</sup> were diluted with 2 M NaOH to reach the desired concentrations  
7 indicated in **Fig. 2**. LDH activity and kinetic parameters of exogenously nitrated LDH were  
8 measured using a fluorescence-based assay, using excitation/emission setting of 340/470  
9 nm, respectively. The assay used uses the fluorescent properties of NADH which can be  
10 excited at 340 nm and emission is measured at 440 nm. The oxidized form of NADH, NAD<sup>+</sup>,  
11 does not have these properties. Measurements were collected every 20 seconds during 5  
12 minutes. The final concentrations in the reaction were LDH, 2 mU/mL, NADH 0.12 mM and  
13 Sodium pyruvate 1.13 mM. Nitration of LDH was measured as the intensity of the NO<sub>2</sub>Tyr  
14 immunosignal in arbitrary units.

#### 15 **Molecular Dynamics Methods**

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

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

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

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

## 16 **Molecular Dynamics Analysis**

17 The active site loop (residues 98-110) was found to be less mobile in T4N than WT in the  
18 binary complex (LDH+NADH), while the C-terminal helix ( $\alpha$ -H) and the juxtaposed loop  
19 (residues 277-287) were more mobile in T4N. The lower mobility of the active site loop is  
20 also detectable in the ternary complex (LDH+NADH+pyruvate) albeit greatly attenuated,  
21 while the C-terminal helix mobility is similar to the WT. The other main features in these plots  
22 are the differences in mobility of the 205-225 loop. However, as this is one of the most  
23 flexible parts of the protein and is not located near the active site we do not consider these  
24 differences to impact the activity of the two proteins. By contrast, the C-terminal side of the  
25 active site loop juxtaposes the C-terminal end of the final helix in the protein,  $\alpha$ -H (residues  
26 309-332). A crucial contact between these two structural elements is made by Leu-107 and



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

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

### 15 **Synthesis and Characterization of Nitrotryptophan**

16 Nitrotryptophan (NO<sub>2</sub>Trp) was synthesized according to Moriya, et al. (15). 20 g L-Trp and  
17 0.25 g urea were suspended in 350 mL glacial acetic acid. Under vigorous stirring at 4°C,  
18 10 mL 24 M nitric acid in 30 mL glacial acetic acid were added. After the suspension changed  
19 to a yellow clear solution, it was stirred at 4°C until it changed again to a suspension. Then  
20 an additional 10 mL fuming nitric acid in 30 mL glacial acetic acid were added drop by drop  
21 at 15°C. After stirring overnight at room temperature, the resulting yellow precipitate was  
22 collected by filtration and washed with glacial acetic acid. The crude product was  
23 recrystallized in 100 mL 2 M nitric acid at temperatures below 50°C and converted to the  
24 free amino acid in 100 mL hot water by the addition of sodium carbonate until the pH was  
25 neutral. As analyzed by its infrared (IR) and nuclear magnetic resonance (NMR) spectra, no  
26 4-NO<sub>2</sub>Trp was detectable. Because 7-NO<sub>2</sub>Trp cannot be synthesized by the action of 24 M

1 nitric acid on the tryptophan ring (16), the products of the synthesis with respect to UV  
2 spectrum are a mixture of 5/6-NO<sub>2</sub>Trp. NO<sub>2</sub>Trp crystallized as yellow needles with a melting  
3 point at 265°C (decomp.). The compound was characterized according to: UV (in 1 N NaOH)  
4 330 nm, 380 nm; IR (KBr)  $\nu(-NH_3^+)$  3190 cm<sup>-1</sup>,  $\nu(C=C)$  and amide I 1600-1650 cm<sup>-1</sup>, amide  
5 II and  $\delta(-NH_3^+)$  1550-1600 cm<sup>-1</sup>,  $\nu_{as}(-NO_2)$  1495 cm<sup>-1</sup>,  $\nu_s(-NO_2)$  1330 cm<sup>-1</sup>, predominantly  
6 5/6-NO<sub>2</sub> substitution at 818 cm<sup>-1</sup> instead of 4-NO<sub>2</sub> substitution at 780 cm<sup>-1</sup>; NMR (D<sub>2</sub>O-  
7 NaOH,  $\delta$  4.7 HDO)  $\delta$  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  
8 (1H, dd, J= 2 and 9 Hz), 7.95 (1H, d, J= 2 Hz).

### 9 **Animal models of disease**

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

#### 13 **Streptozotocin model of diabetes mellitus**

14 At an age of 10 weeks, rats (n=12) received a single intraperitoneal injection of 60 mg/kg  
15 BW of streptozotocin (STZ) dissolved in citrate buffer 50mM pH 4,5. Age matched control  
16 rats (n=12) received vehicle injections with citrate buffer. Hyperglycemia was defined as a  
17 positive test of glucose in urine (Haemo-Glukotest, Boehringer Mannheim, Germany) and a  
18 non-fasting blood glucose level higher than 11 mmol/L (hexokinase method) one week after  
19 STZ application. Rats were sacrificed 3 (n=12) and 16 weeks (n=12) after diabetes induction  
20 by CO<sub>2</sub>-inhalation. After thoracotomy, hearts were excised and freed of connective tissue  
21 while immersed in modified Krebs-Hepes buffer pH 7.35 containing 99 mM NaCl; 4.7 mM  
22 KCl; 1.9 mM CaCl<sub>2</sub>; 1.2 mM MgSO<sub>4</sub>; 25 mM NaHCO<sub>3</sub>; 1 mM K<sub>2</sub>HPO<sub>4</sub>; 20 mM Na-Hepes;  
23 11.1 mM D-glucose. Hearts were shock frozen in liquid nitrogen, pulverized, and  
24 homogenized in lysis buffer. For SDS-PAGE and western blot, gel loading buffer was added  
25 and protein concentrations were determined after TCA precipitation by the method of Lowry.

#### 26 **Doxorubicin cardiotoxicity model**

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

#### 6 **Endogenous LDH nitration in eNOS-KO and MPO-KO mice**

7 Hearts from eNOS-KO and MPO-KO mice were excised, washed in perfusion buffer, blotted  
8 dry on filter paper, weighed, and homogenized immediately in ice-cold 50 mM PBS, pH=  
9 7.4, containing 2.5 mM EDTA, 1 mM DTT, 1% NP-40, phosphatase inhibitors (10 mM  
10 sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, and 50 mM  
11  $\beta$ -glycerophosphate), and Complete™ protease inhibitor cocktail (Roche Diagnostics,  
12 Indianapolis, IN). Homogenates were incubated on ice for 30 min to facilitate solubilization,  
13 after which they were clarified by centrifugation at 12000 x g for 15 min at 4°C, aliquoted  
14 and stored at -80°C. Prior to use, samples were thawed on ice and assayed for protein  
15 concentration using the BCA method.

16

## 1 **Supplementary Tables**

| Abbreviation       | Specificity              | Antigen   | Species | Source               | Reference |
|--------------------|--------------------------|-----------|---------|----------------------|-----------|
| pAb1               | anti-NO <sub>2</sub> Tyr | ONOO-KLH  | rabbit  | L.O. Uttenthal       | (33)      |
| pAb2               |                          |           |         | J.S. Beckman         | (34)      |
| mAb1               |                          |           | mouse   |                      | (34)      |
| Identical to mAb1: |                          |           |         |                      |           |
| mAb2               | anti-NO <sub>2</sub> Tyr | ONOO-KLH  | mouse   | Alexis               | (34)      |
| mAb3               |                          |           |         | Upstate              | (34)      |
| Rejected:          |                          |           |         |                      |           |
| mAb4               | anti-NO <sub>2</sub> Tyr | ONOO-KLH  | mouse   | Calbiochem           | (51)      |
| mAb5               |                          |           |         | HyCult biotechnology | (37)      |
| pAb3               | anti-NOTrp               | NOTrp-KLH | rabbit  | Calbiochem           | None      |
| mAb6               | anti-NOCys               | NOCys     | mouse   | Biolabs              | None      |

## 2 **Supplementary Table 1. Commercial and non-commercial antibodies detecting**

3 **reactive nitrogen species-induced protein modifications.** Poly (pAb)- and monoclonal  
4 (mAb) antibodies from the indicated species were obtained from either commercial sources  
5 or kindly provided by the listed researchers. All antibodies, except pAb3 and mAb6, were  
6 raised against keyhole limpet hemocyanin (KLH) treated with 'nitration reagent', i.e.  
7 H<sub>2</sub>O<sub>2</sub>/NO<sub>2</sub><sup>-</sup>. For pAb3, the hapten was nitrosocysteine (NOCys); for mAb6, nitrosotryptophan  
8 (NOTrp), and in both cases glutaraldehyde-coupled to KLH. Antibodies mAb1, mAb2 and  
9 mAb3 behaved identically and referred to the same reference (34) , and were thus  
10 considered identical. Antibodies mAb4, mAb5 and pAb3 were later omitted from the study  
11 because of low titer and/or low affinity, which allowed to detect only highly abundant antigens  
12 such as H<sub>2</sub>O<sub>2</sub>/NO<sub>2</sub><sup>-</sup>-treated bovine serum albumin (BSA), but not lower abundance  
13 endogenously nitrated proteins in native tissues. Antibody mAb6 was also excluded from  
14 further use because it reacted similarly with untreated and H<sub>2</sub>O<sub>2</sub>/NO<sub>2</sub><sup>-</sup>-treated BSA.

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

| Antigen \ Antibody            | pAb1<br>1:3000 | pAb2<br>1:3000 | mAb1<br>1:3000 |
|-------------------------------|----------------|----------------|----------------|
| NO <sub>2</sub> Tyr-Block     | +              | +              | +              |
| NO <sub>2</sub> BSA (Control) |                |                |                |
| Pul. Artery (pig)             |                |                |                |
| Heart (pig)                   |                |                |                |
| Lung (rat)                    |                |                |                |
| Kidney (rat)                  |                |                |                |
| Cerebellum (rat)              |                |                |                |
| Spleen (rat)                  |                |                |                |
| Cortex (rat)                  |                |                |                |

1

2 **Supplementary Figure 1. Anti-NO<sub>2</sub>-Tyr antibodies greatly vary in sensitivity and**

3 **specificity in native tissues.** Dot blot screen of tissue homogenates of porcine pulmonary

4 artery and heart, and rat heart, lung, kidney, cerebellum, spleen and cortex. For each dot,

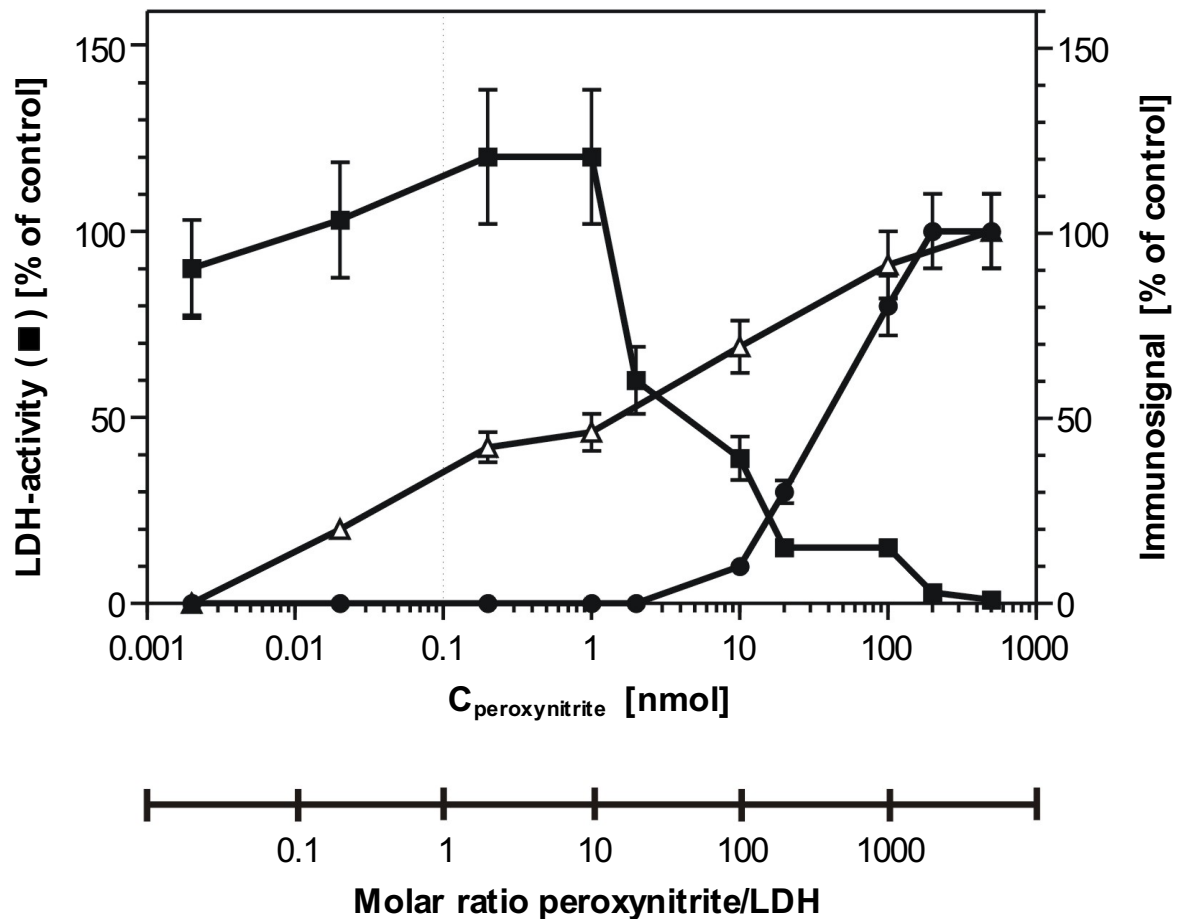
5 50 µg or, in the case of pulmonary artery, 100 µg of protein were blotted onto a nitrocellulose

6 membrane. NO<sub>2</sub>Tyr-immuno-positive proteins were then detected using antibodies pAb1,

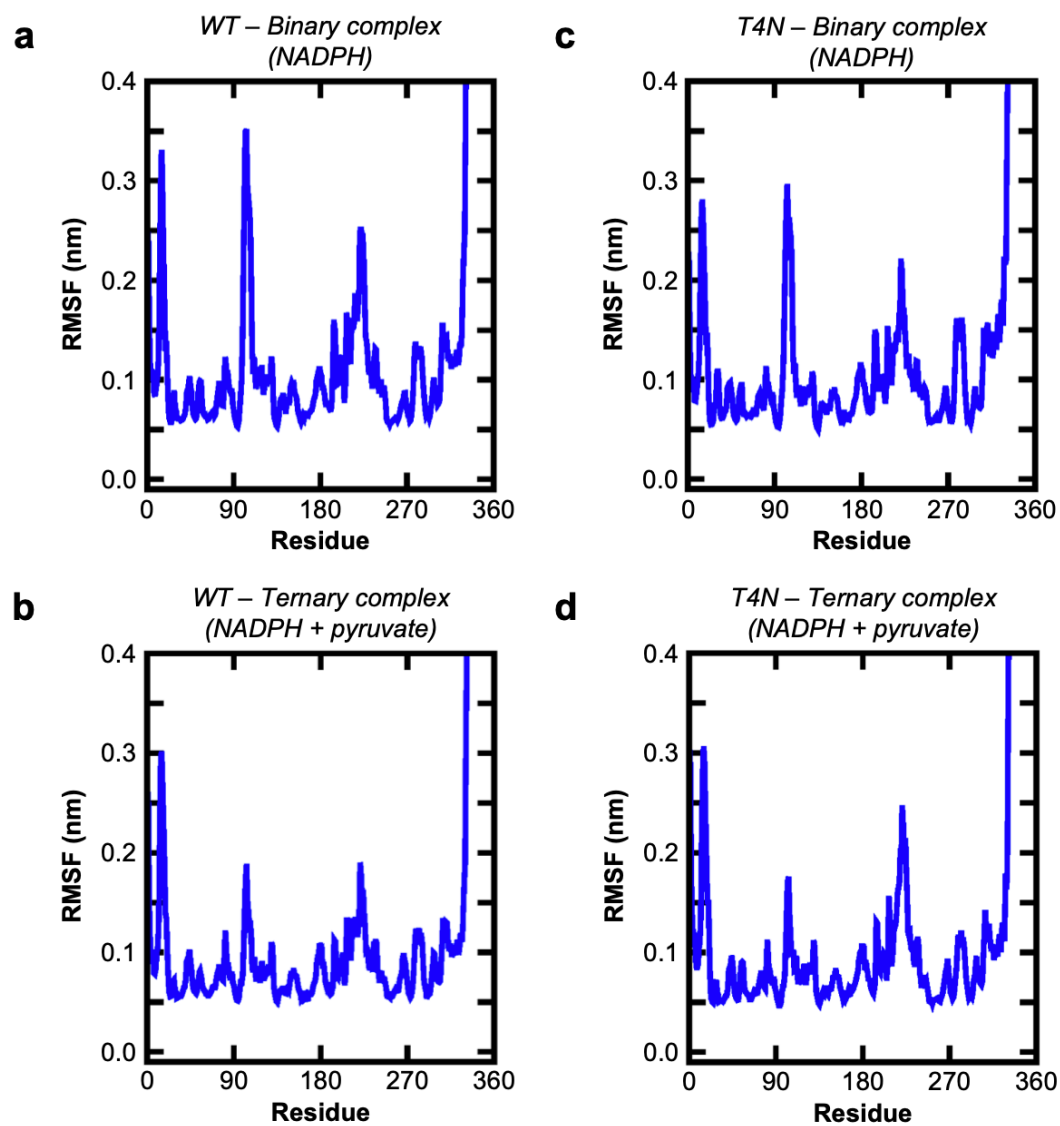
7 pAb2 or mAb1. NO<sub>2</sub>BSA was used as a positive control; pre-incubation with 3 mM free

8 NO<sub>2</sub>Tyr, as a negative control (+).

9



1 **Supplementary Figure 2. Nitration and oxidation of LDH *in vitro* inversely correlates**  
 2 **with LDH activity.** As LDH (0.1 nmol) was treated with increasing amounts of peroxynitrite,  
 3 total LDH activity (■, left axis) was determined by the NADH-coupled photometric assay of  
 4 Warburg, as described, and all LDH samples were tested under the same conditions for the  
 5 occurrence of nitration (●) using quantitative Western analysis with the NO<sub>2</sub>Tyr antibody  
 6 pAb1 and oxidation (△) using the oxyblot detection kit (both, right axes).



1

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