

Proteomic Analysis of Peroxynitrite-Induced Protein Nitration in Isolated Beef Heart Mitochondria

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Summary

Mitochondria are exposed to reactive nitrogen species under physiological conditions and even more under several pathologic states. In order to reveal the mechanism of these processes we studied the effects of peroxynitrite on isolated beef heart mitochondria *in vitro*. Peroxynitrite has the potential to nitrate protein tyrosine moieties, break the peptide bond, and eventually release the membrane proteins into the solution. All these effects were found in our experiments. Mitochondrial proteins were resolved by 2D electrophoresis and the protein nitration was detected by immunochemical methods and by nano LC-MS/MS. Mass spectrometry confirmed nitration of ATP synthase subunit beta, pyruvate dehydrogenase E1 component subunit beta, citrate synthase and acetyl-CoA acetyltransferase. Immunoblot detection using chemiluminescence showed possible nitration of other proteins such as cytochrome b-c1 complex subunit 1, NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, elongation factor Tu, NADH dehydrogenase [ubiquinone] flavoprotein 2, heat shock protein beta-1 and NADH dehydrogenase [ubiquinone] iron-sulfur protein 8. ATP synthase beta subunit was nitrated both in membrane and in fraction prepared by osmotic lysis. The high sensitivity of proteins to nitration by peroxynitrite is of potential biological importance, as these enzymes are involved in various pathways associated with energy production in the heart.

Key words

Mitochondrial proteome • Peroxynitrite • 3-nitrotyrosine • 2D electrophoresis • Mass spectrometry

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Introduction

Mitochondrial metabolism is the major source of free radicals and oxidants in the cell, comprising both reactive oxygen and nitrogen species (RONS). They enter into reaction with carbohydrates, proteins, lipids and nucleic acids (Pfeiffer *et al.* 1999, Szabó *et al.* 2007). On the one hand, RONS overproduction leads to damage of cells and tissues; on the other hand they are essential part of signal transduction systems (Weidinger and Kozlov 2015). Maintaining the balance between pro-oxidative potential and antioxidant capacity of cells is an essential factor for maintaining cell survival.

Nitric oxide (NO) is produced from L-arginine by NO synthase (Habib and Ali 2011, Szabó *et al.* 2007). NO has important regulatory functions in the cardiovascular, immune and nervous system. The effect of NO as a signal molecule consists in its ability to activate or deactivate target enzyme molecules (Aktan 2004). The superoxide radical is a product of the one-electron reduction of the oxygen molecule. In mitochondria, superoxide is formed as a byproduct of

cellular respiration (Brand *et al.* 2004).

Reaction of nitric oxide with superoxide generates peroxynitrite (PeN) (Koppenol *et al.* 1996), which is a potent oxidizing and nitrating agent (Habib and Ali 2011, Kamat 2006). 3-nitrotyrosine (3-NT) is a product of post-translational modification occurring in the presence of nitrating agents, such as PeN. 3-NT is considered as a marker of oxidative stress caused by RONS (Herce-Pagliai *et al.* 1998). Nitration is not random and leads to a selective modification of protein molecules (Abello *et al.* 2009, Ischiropoulos 2003). Despite the fact that average tyrosine content in proteins is 3-4 mol%, only a few molecules of tyrosine are subjects of nitration process (Bartesaghi *et al.* 2007, Pacher *et al.* 2007, Radi 2004). The reasons for this selective nitration still remain unclear.

Mitochondria are a double membrane bound organelles with key function in the energy metabolism. NO regulates oxygen consumption and therefore ATP production in mitochondria (Ghafourifar and Cadenas 2005). As the mitochondria are rich in hydrophobic proteins localized in membranes, PeN can diffuse into the hydrophobic regions and initiate nitrosative stress (Brugiere *et al.* 2004). Stability of PeN is increased by the physiological alkali mitochondrial environment (Tien *et al.* 1999). Due to this fact, the effect of PeN takes a longer period of time and can lead to a more extensive modifications in protein molecules (Tien *et al.* 1999). PeN causes mitochondrial oxidative damage through direct oxidation, nitration or nitrosation of target molecules (Radi *et al.* 2002).

In vivo PeN modifies various proteins in the vicinity of its production (Pacher *et al.* 2007, Ren *et al.* 2008). Experimental results show, that high concentrations of PeN lead to significant changes in protein composition in studied systems (Di Mascio *et al.* 2000, Kohutiar *et al.* 2016). Oxidative damage and nitration affects physical and chemical properties of the proteins (Abello *et al.* 2009). Excessive protein nitration is associated with various pathological conditions, such as inflammation, ischemia, diabetes and neurodegenerative diseases (Pacher *et al.* 2007, Stadler 2011, Stavniichuk *et al.* 2014, Torreilles *et al.* 1999). Identification and characterization of nitrated proteins is important for understanding the mechanisms and roles of nitration process in cell metabolism.

In order to reveal the mechanism of protein nitration we exposed isolated beef heart mitochondria to PeN *in vitro* and analyzed nitrated proteins in water

soluble and lipophilic fraction using antibodies against nitrotyrosine and mass spectrometry.

As protein nitration is potentially harmful for their function the aim of our study was to identify proteins most endangered by this process.

Material and Methods

Isolation of mitochondria

In total, 5 beef hearts were obtained fresh from the local slaughter house. The wet weight of heart was 1.8-2.5 kg. Mitochondria from beef heart were isolated by the method of Haas and Elliott with some modifications (Haas and Elliott 1963). The final amount of mitochondrial suspension was 70-100 ml. Mitochondria were stored frozen in Tris buffer (0.05 mol l⁻¹ Tris, 0.15 mol l⁻¹ NaCl, 1 mmol l⁻¹ EDTA, pH 7.4 adjusted with HCl) and kept at -70 °C for up to 6 months. A small amount of mitochondria (100 µl) was taken for protein assay using Lowry's method (Lowry *et al.* 1951). The details of our modifications have been published in (Kohutiar *et al.* 2016).

Synthesis of peroxynitrite

Peroxyntirite was synthesized in a quenched-flow apparatus according to the procedure described by Koppenol *et al.* (1996). The resulting solution contained 50-80 mmol l⁻¹ of peroxynitrite determined by absorbance at 302 nm ($\epsilon = 1670 \text{ mol}^{-1} \text{ l cm}^{-1}$) (Koppenol *et al.* 1996).

In vitro treatment of mitochondria with peroxynitrite

The mitochondrial suspension was thawed at laboratory temperature and diluted with hypotonic phosphate buffer to protein concentration about 5 mg/ml and centrifuged at 16,000 g for 10 min at 4 °C. The sediment was considered as membrane fraction and the supernatant as soluble protein fraction (Bowman *et al.* 1976, Caplan and Greenawalt 1966).

Both fractions were buffered with 0.1 mol l⁻¹ phosphate buffer, at pH 7.8. Protein nitration was initiated by addition of PeN in final concentration of 0.5 mmol l⁻¹ to mitochondrial suspension. Samples for analysis were taken immediately after adding the oxidizing agent (labeled as time 0 h), and then only after 1 h of incubation at 37 °C. The concentration of oxidizing agent and time of incubation was chosen according to the results of our previous study (Kohutiar *et al.* 2016). Aliquots were stored at -70 °C.

Sample preparation for 2D electrophoresis (2-DE)

Frozen mitochondria sample was thawed and precipitated with 2 ml 10 % trichloroacetic acid in acetone with 20 mmol l⁻¹ 1,4-dithiothreitol (DTT). Sample was precipitated for 45 min at -20 °C. Sample was then centrifuged at 10,500 g for 10 min at 4 °C. The sediment was washed and resuspended in 1 ml of 20 mmol l⁻¹ DTT in acetone and centrifuged at 10,500 g for 10 min at 4 °C. This procedure was three times repeated.

The sediment was finally solubilized in the lysis buffer (6 mol l⁻¹ urea, 2 mol l⁻¹ thiourea, 4 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate hydrate, 3 % Triton X-100, 0.4 % DTT and 0.5 % carrier ampholytes). Solubilization was performed for 1-3 h at laboratory temperature.

Isoelectric focusing and 2-DE

The technique described by Rabilloud (2008) was used for isoelectric focusing and equilibration. Before focusing, undissolved samples were again centrifuged (10,500 g for 10 min at 4 °C) and the sediment was discarded.

For 2D electrophoresis, samples (60-300 µg of protein) were focused on 13 cm, pH 3-10 Immobiline DryStrip (GE Healthcare, Piscataway, NJ, USA) according to protocol: 1 h at 100 V, 2 h at 300 V, 1 h at 1,000 V and 16 h at 3,500 V. After focusing strips were incubated for 20 min in equilibration solution I (6 mol l⁻¹ urea, 0.375 mol l⁻¹ Tris-HCl, 2 % SDS, 20 % glycerol, 2 % DTT, pH 8.8) and for 20 min in equilibration solution II (6 mol l⁻¹ urea, 2 mol l⁻¹ thiourea, 2 % SDS, 20 % glycerol, 2.5 % iodoacetamide, pH 7.5). Strips were applied on 12 % separating gel. Separation was carried out for 6-8 h at 8 °C (180 V, 80 mA) in Tris-taurine system, until the blue dye front reached the end of the gel. The gels were then subjected for staining with Coomassie blue or transferring to the membrane.

Western blot analysis for 3-nitrotyrosine

After gel 2D electrophoresis proteins were blotted (transfer buffer: 0.015 mol l⁻¹ Na₂B₄O₇, 0.1 mol l⁻¹ H₃BO₃, 0.1 % SDS, pH 8.4) on a nitrocellulose membrane and probed with the anti-3-nitrotyrosine antibody prepared in our laboratory (Fišárková *et al.* 2004, Herget *et al.* 2000, Wilhelm *et al.* 2016, Zámečník *et al.* 2011). To verify protein transfer, nitrocellulose membranes were reversibly stained with 0.1 % Ponceau S (in 1 % acetic acid) for 30 min,

followed by destaining in distilled water. Membranes were blocked with 5 % non-fat dry milk in 0.1 % Tween/phosphate buffered saline. The secondary antibody (polyclonal rabbit anti-mouse immunoglobulins/HRP from Dako, Glostrup, Denmark; dilution 1:10,000) was labeled with peroxidase and the spots were visualized by enhanced chemiluminescence reaction (1.25 mmol l⁻¹ luminol, 0.05 mol l⁻¹ p-coumaric acid, 0.01 % H₂O₂). The figures are shown as negatives to enable better comparison with the 2-DE gels.

Mass spectrometry: in-gel digestion

Protein spots (from 2-DE: about 1-2 mm in diameter) were excised from the Coomassie-stained gels, and then processed as described in (Jágr *et al.* 2012). Briefly: the spots were first destained. After destaining, the gel pieces were shrunk by dehydration in 500 µl of acetonitrile. In further step, 100 µl of 10 mmol l⁻¹ DTT in 100 mmol l⁻¹ ammonium hydrogencarbonate was added, and the proteins were reduced for 1 h at 56 °C.

After cooling to room temperature, the DTT solution was replaced by roughly the same volume of 55 mmol l⁻¹ iodoacetamide in 100 mmol l⁻¹ ammonium hydrogencarbonate, and the gels were incubated at ambient temperature for 45 min in the dark. Then the gel pieces were washed with 100 µl of 100 mmol l⁻¹ ammonium hydrogencarbonate, and dehydrated. Subsequently, the liquid phase was removed and the gel pieces were dried in a vacuum centrifuge.

Gel pieces were swollen in a 100 µl of digestion buffer containing trypsin (20 µg/ml) in 50 mmol l⁻¹ ammonium hydrogencarbonate, sonicated (5 min), placed to the air circulation thermostat, and incubated overnight at 37 °C. The supernatant of each spot was then transferred to a new vial. The in-gel digestion was performed once more the same way.

The resulting tryptic peptides were extracted with sonication (15 min) by 150 µl of extraction buffer (5 % formic acid/acetonitrile, 1:2, v/v). Then the solution was spun, the supernatants were transferred, pooled and concentrated to dryness by lyophilization. Dried extracts were stored at -80 °C before analysis.

Mass spectrometry: analysis of tryptic digests with nLC-MS/MS

Dried protein digests were dissolved in 25 µl of 1 % formic acid, centrifuged at 16,000 g for 5 min at 4 °C and the supernatant was transferred to vials.

The nano-HPLC apparatus used for protein

digests analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a maXis Q-TOF (quadrupole – time of flight) mass spectrometer with ultrahigh resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and micrOTOF-control 3.0. The data were collected and manipulated with the software packages ProteinScape 2.0 and DataAnalysis 4.0 (Bruker Daltonics).

3 μl of the peptide mixture were injected into a NS-AC-11-C18 Biosphere C18 column (particle size: 5 μm , pore size: 12 nm, length: 150 mm, inner diameter: 75 μm), with a NS-MP-10 Biosphere C18 precolumn (particle size: 5 μm , pore size: 12 nm, length: 20 mm, inner diameter: 100 μm), both manufactured by NanoSeparations (Nieuwkoop, Netherlands).

The separation of peptides was achieved *via* a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1 % (v/v) formic acid. Separation was started by running the system with 5 % mobile phase B, followed by gradient elution to 30 % B at 70 min. In the next step, gradient elution to 50 % B in 10 min and then a gradient to 100 % B in 8 min was used. Finally, the column was eluted with 100 % B for 2 min. Equilibration before the next run was achieved by washing the column with 5 % mobile phase B for 10 min. The flow rate was 0.25 $\mu\text{l min}^{-1}$, and the column was held at ambient temperature (25 °C).

On-line nano-electrospray ionization (easy nano-ESI) in positive mode was used. The ESI voltage was set at +4.5 kV, scan time 1.3 Hz. Operating conditions: drying gas (N_2) 1 l min^{-1} ; drying gas temperature 160 °C; nebulizer pressure 0.04 MPa. Experiments were performed by scanning from 100 to 2,200 m/z . The reference ion used (internal mass lock) was a monocharged ion of $\text{C}_{24}\text{H}_{19}\text{F}_{36}\text{N}_3\text{O}_6\text{P}_3$ (m/z 1221.9906). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. All nLC-MS/MS analyses were done in duplicates.

Protein identification with MS and database searching

Data were processed using ProteinScape software as described in Eckhardt *et al.* (2014). Briefly: proteins were identified by correlating tandem mass spectra to the IPI and SwissProt databases, using the MASCOT searching engine <http://www.matrixscience.com>

(Mascot search engine 2016). The taxonomy was restricted to *Bos taurus* to remove protein identification redundancy. Trypsin was chosen as the enzyme parameter. One missed cleavage was allowed, and an initial peptide mass tolerance of ± 10.0 ppm was used for MS and ± 0.05 Da for MS/MS analysis. Cysteines were assumed to be carbamidomethylated, proline and lysine to be hydroxylated, tyrosine to be nitrated, and methionine was allowed to be oxidated. All these possible modifications were set to be variable. Monoisotopic peptide charge was set to 1+, 2+ and 3+. The Peptide Decoy option was selected during the data search process to remove false-positive results. Only significant hits (MASCOT score ≥ 80 for proteins and MASCOT score ≥ 40 for peptides) were accepted.

Results

The proteins from the whole mitochondrial preparation before separation of the membrane and soluble fraction have been resolved by 2-DE. The results of protein identification with MS are summarized in Table 1. The typical fragmentation pattern of the control mitochondria shows Figure 1A, and mitochondria exposed to 0.5 mmol l^{-1} PeN for 1 h Figure 1C. The immunoblot of control is illustrated in Figure 1B. The gel illustrated in Figure 1C was blotted, and the nitrated proteins were visualized on the blot with antibodies labeled with chemiluminescence probe. The set of nitrated proteins is shown in Figure 1D. The most intensively stained spots in Figure 1D have been labeled by numbers 1-12, and their position was traced back on the gel. These spots that were indicated by circles with corresponding number have been subjected to nLC-MS/MS. Nine proteins that did not stain with nitrotyrosine antibodies, indicated by rectangles and capital letters A-I were chosen as a control for nLC-MS/MS analysis. All protein spots indicated with numbers and letters are consistent across all Figures.

It is apparent by comparison of Figure 1A and Figure 1C, that spots C, E and F are not pronounced on the gel from the control mitochondria. The major proteins in these spots were characterized by mass spectrometry as myosin light chain 3 (spot C), NADH dehydrogenase iron-sulfur protein 3 (spot E) and myosin regulatory light chain 2 (spot F). Evidently, these proteins were modified by PeN without nitration.

Table 1. List of proteins identified in beef heart mitochondria resolved by 2-DE.

Spot number	Accession number ^a	Protein	NT position ^b	Change by radicals	Protein score	Sequence coverage [%]
1	P02769	Serum albumin	355	yes	1582.6	35.9
2	P00829	ATP synthase subunit beta, mitochondrial	395	yes	2292.9	67.2
3	P31800	Cytochrome b-c1 complex subunit 1, mitochondrial		possible	839.2	25.2
4	P31800	Cytochrome b-c1 complex subunit 1, mitochondrial		possible	649.5	19.2
5	P17694	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial		possible	1141.8	47.3
6	P49410	Elongation factor Tu, mitochondrial		possible	1002.5	34.1
7	P60712	Actin, cytoplasmic 1		possible	1073.4	8.8
8	P04394	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial		possible	996.8	47.8
9	Q3T149	Heat shock protein beta-1		possible	884.6	66.2
10	P31800	Cytochrome b-c1 complex subunit 1, mitochondrial		possible	515.8	19.4
11	P42028	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial		possible	527.8	31.6
12	P11966	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	63; 67	yes	1039.6	35.7
13	Q29RK1	Citrate synthase, mitochondrial	381	yes	646.9	24.7
14	Q29RZ0	Acetyl-CoA acetyltransferase, mitochondrial	326	yes	174.8	15.6
A	P15690	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial		no	1627.3	41.0
B	P31081	60 kDa heat shock protein, mitochondrial		no	2460.4	58.3
C	P85100	Myosin light chain 3		yes	753.0	57.8
D	P23709	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial		yes	1144.2	60.3
E	P23709	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial		yes	277.0	21.5
F	Q3SZE5	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform		yes	573.8	52.4
G	P00426	Cytochrome c oxidase subunit 5A, mitochondrial		no	359.7	26.3
H	P00428	Cytochrome c oxidase subunit 5B, mitochondrial		no	328.6	35.7
I	P13620	ATP synthase subunit d, mitochondrial		no	979.5	68.9

^aAccession number – specific protein number in database: <http://www.uniprot.org> (The UniProt Consortium 2017), ^bNT position – position of nitration in protein sequence.

Not all the nitroproteins detected by antibodies were confirmed by nLC-MS/MS analysis. Actually, only three proteins were found to be nitrated: spot 1 – albumin (position of nitration in protein amino acid sequence p.n. 355; peptide: K.DAFLGSFLYEYSR.R), spot 2 – ATP synthase subunit beta (p.n. 395; R.AIAELGIYPAVDPLDSTSR.I), and spot 12 – pyruvate dehydrogenase E1 component subunit beta (p.n. 63 and 67; K.VFLLGEEVAQYDGAYK.V). The bold **Y**

indicates the nitrated tyrosine. It is apparent that pyruvate dehydrogenase contains two nitrotyrosine moieties.

As the next step we incubated membrane and soluble protein fraction with PeN after separation to individual fractions. Figure 2A shows 2-DE gel of the soluble fraction. The spots 1, 2, 3, 4, 6, 7, 8, 11 and 12 were found on the gel, and on immunoblot nitrotyrosine was detected in the spots 1, 2, 3, 4, 6, 7, 8, 11 and 12 (Fig. 2B). To the right of spot 6 newly appeared positive

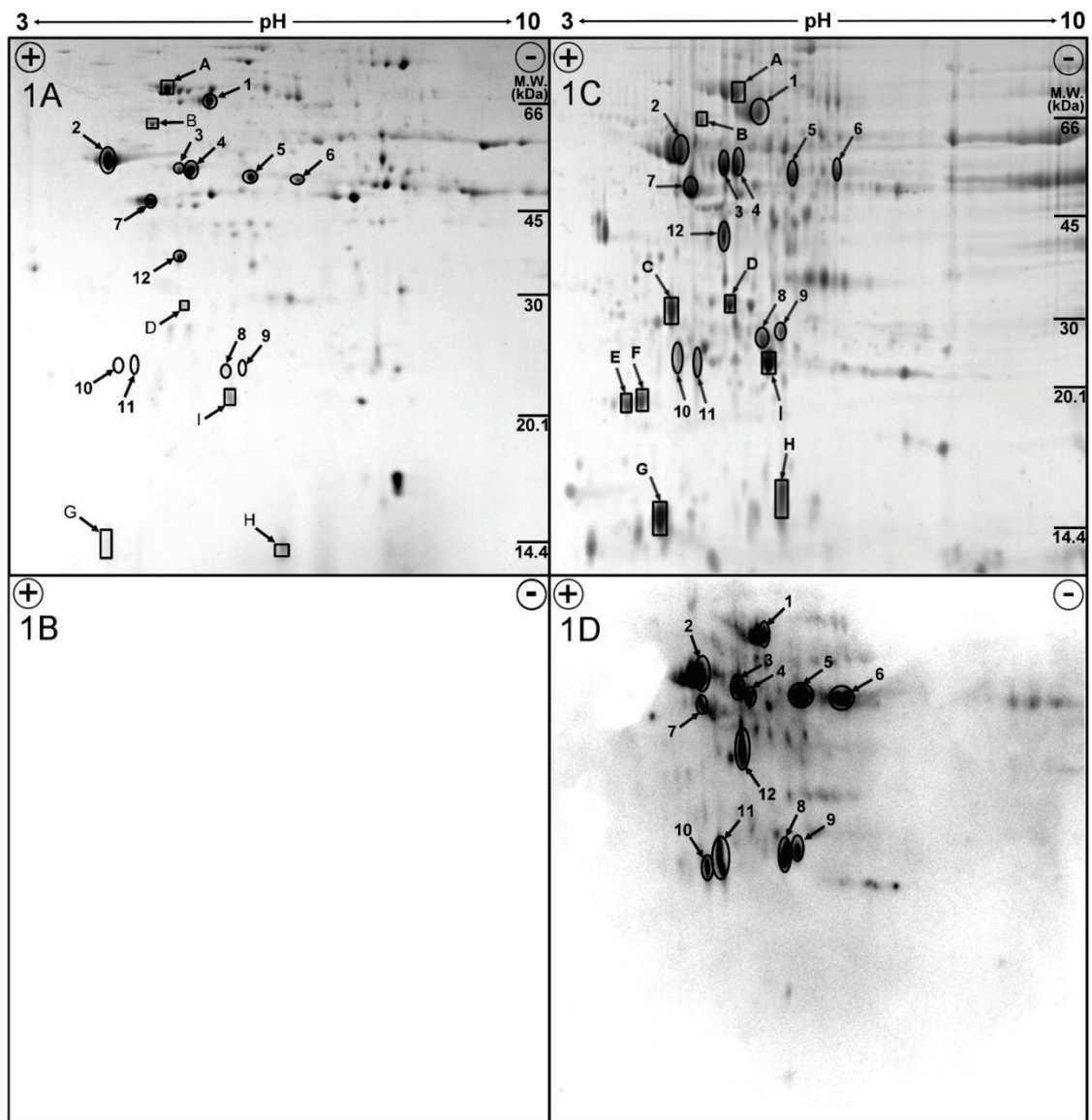


Fig. 1. 2-DE of mitochondrial proteins isolated from beef heart. Mitochondria were exposed to 0.5 mmol l^{-1} PeN for 1 h. Spots indicated with circles have been subjected to nLC-MS/MS. Spots indicated by rectangles were chosen as a control for nLC-MS/MS. **A.** control, **B.** immunoblot of control, **C.** 0.5 mmol l^{-1} PeN for 1 h, **D.** immunoblot of nitrated proteins.

spots labeled as 13 and 14. These spots were traced back on the 2-DE gel and their nitration was confirmed by nLC-MS/MS analysis. They were identified as spot 13 – citrate synthase (p.n. 381; K.LVAQLYK.I) and spot 14 – acetyl-CoA acetyltransferase (p.n. 326; R.IAAFADAAVEPIDFPLAPAYAVPK.V).

The membrane fraction was incubated and analyzed under identical conditions as the soluble fraction. 2-DE gel contains all the positive proteins except albumin and spot 9 (Fig. 3A). On immunoblot the spots 2, 3, 4, 5, 7, and 12 were detected (Fig. 3B).

The mitochondrial suspension exposed to PeN was separated to soluble and membrane fraction and each

fraction was analyzed independently.

The results of 2-DE and immunoblot of the soluble mitochondrial fraction are shown in Figure 4A and 4B. The protein spots 5, 6, 8, 9, 12 were not found on 2-DE gel. Although other nitrotyrosine-positive spots were present, on immunoblot only spot 2 was detected (Fig. 4B).

The membrane fraction was analyzed in a similar manner. In the 2-DE gel only spot 1 (albumin) was not found, other nitrotyrosine-positive spots were present (Fig. 5A). Immunoblot showed nitration of spots 2, 3, 5, 7, 12 and also slight positivity of spots 8 and 11 (Fig. 5B).

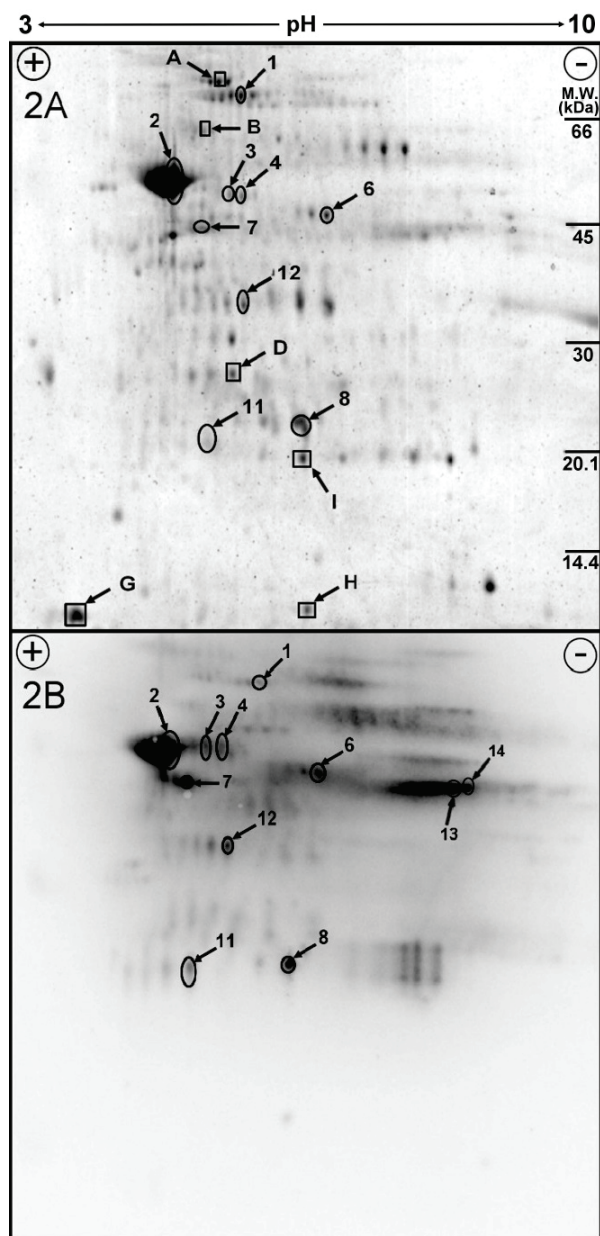


Fig. 2. 2-DE of mitochondrial fraction prepared by osmotic lysis. Separation was carried out before incubation with 0.5 mmol l^{-1} PeN. **A.** soluble fraction, **B.** immunoblot of soluble fraction.

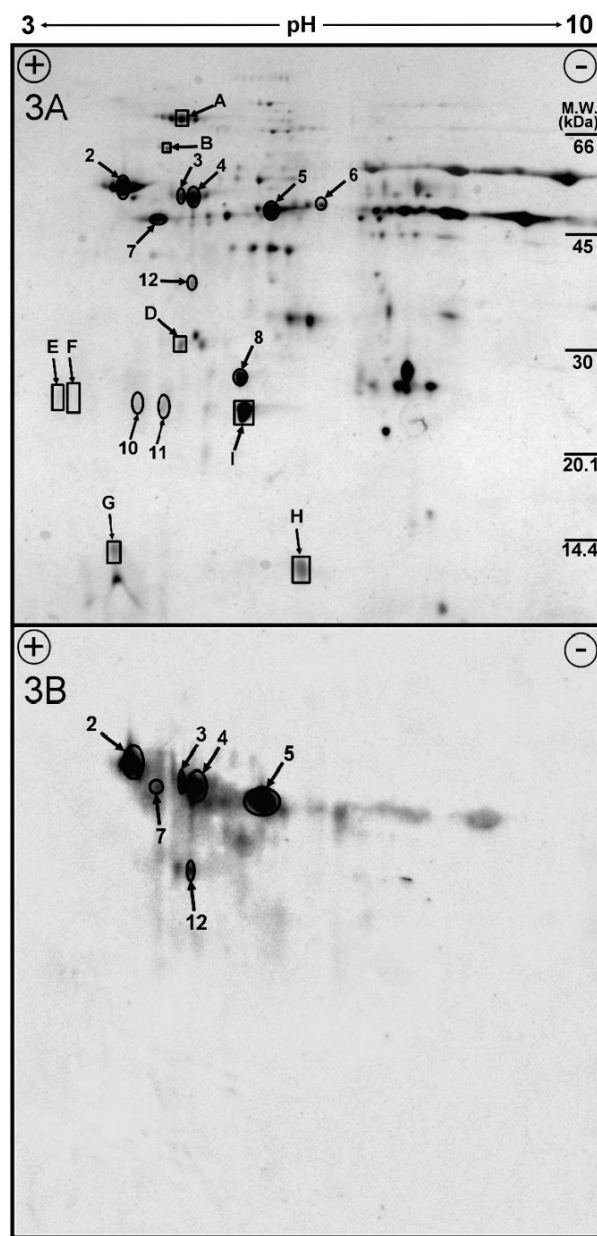


Fig. 3. 2-DE of mitochondrial membrane fraction. Separation was carried out before incubation with 0.5 mmol l^{-1} PeN. **A.** membrane fraction, **B.** immunoblot of membrane fraction.

Discussion

Peroxynitrite acts upon proteins in several ways. Besides nitration of tyrosine moieties it can break the protein backbone by a free radical mechanism (Hawkins and Davies 2001), eventually it can release membrane-bound proteins into the solution. We have observed all these effects in the present study.

Mitochondria are the source of both ROS and NO, so peroxynitrite is a probable factor causing nitration of mitochondrial proteins. Peroxynitrite can also diffuse from other cell areas into the mitochondria due to

a relatively longer lifetime (Brugiere *et al.* 2004, Tien *et al.* 1999). In our previous work we have intensively studied the effect of tert-butyl hydroperoxide and peroxynitrite on oxidative damage to mitochondria. We observed the changes in dynamics of various oxidative stress markers: malondialdehyde, protein carbonyls, lipofuscin-like pigment and 3-nitrotyrosine. The amount of 3-nitrotyrosine formed after exposure of mitochondria to peroxynitrite ($0.05\text{--}3 \text{ mmol l}^{-1}$) was quantified by ELISA. Electrophoretic and Western blotting techniques were also used in the study of protein nitration (Kohutiar *et al.* 2016).

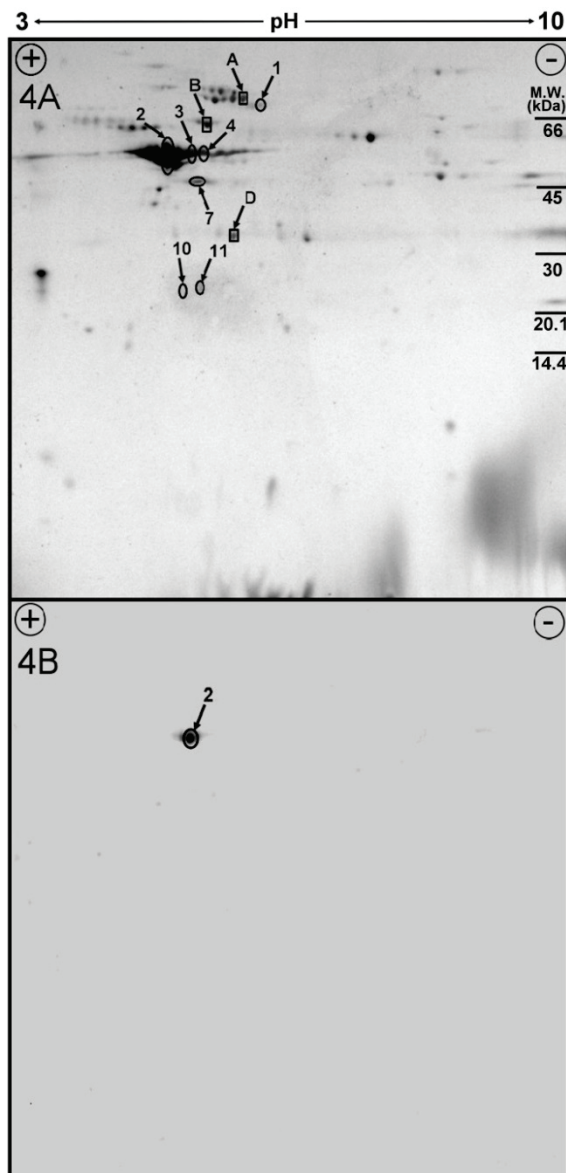


Fig. 4. 2-DE of mitochondrial soluble fraction prepared by osmotic lysis. Separation was carried out after incubation with 0.5 mmol l^{-1} PeN. **A.** soluble fraction, **B.** immunoblot of soluble fraction.

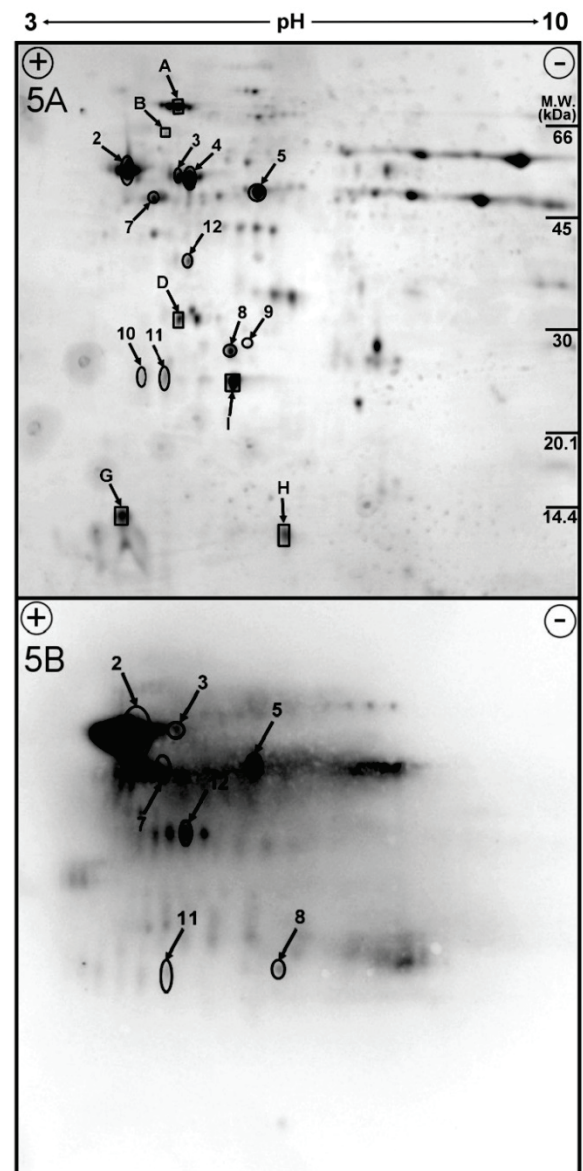


Fig. 5. 2-DE of mitochondrial membrane fraction. Separation carried out after incubation with 0.5 mmol l^{-1} PeN. **A.** membrane fraction, **B.** immunoblot of membrane fraction.

Immunoblot detection of nitrotyrosine using chemiluminescence is more sensitive than nLC-MS/MS analysis. This fact explains why we found more proteins to be nitrated by immunoblot detection. In spots 1, 2, 12, 13 and 14 nitration was confirmed by immunoblot and also by nLC-MS.

In spots 3-11 nitrotyrosine was confirmed only by immunoblot. Cytochrome b-c1 complex subunit 1 (spot 3, 4 and 10), NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (spot 5), elongation factor Tu (spot 6), actin (spot 7), NADH dehydrogenase [ubiquinone] flavoprotein 2 (spot 8), heat shock protein beta-1 (spot 9) and NADH dehydrogenase [ubiquinone]

iron-sulfur protein 8 (spot 11) were also probably nitrated, but their nitration was not confirmed by nLC-MS/MS. This can be explained by fact, that maximum protein sequence coverage was about 50 % (what is usual for this type of detection). However, it cannot be excluded that some nonspecific interactions of the antibodies took place and therefore we consider nitration only in those proteins confirmed by nLC-MS/MS. No nitrotyrosine-positive spots appeared in control immunoblot. If some none-specific interactions took place, they must be caused by oxidative modifications. Proteins found in spots 3, 4, 5, 8, 10 and 11 are involved in respiratory chain. Change of their

activity by nitrosative stress and its impact on cellular respiration is well documented (Brown and Borutaite 2004, Cassina and Radi 1996, Murray *et al.* 2003). Elongation factor Tu (spot 6) participates in mitochondrial protein synthesis. The effect of damage on its biological role was also described (Suzuki *et al.* 2006, Yutthanasirikul *et al.* 2016, Zhang *et al.* 2012).

ATP synthase subunit beta was extensively nitrated both in membrane fraction and in solution. Actually, it was the only nitrated protein released from the membrane. More proteins were released into soluble fraction after osmotic lysis of mitochondria including ATP synthase. When exposed to peroxynitrite, other proteins except ATP synthase subunit beta were nitrated in the soluble fraction. It implies that the change of protein conformation associated with protein release enables tyrosine nitration. On the other hand, the nitrated tyrosines are not located in any specific environment. We have compared the 3D structures of the nitrated proteins to those that were not nitrated and have not found any difference in spatial organization of the tyrosine neighborhood. Also we have compared amino acid sequences in the vicinity of nitrotyrosine in nitrated proteins. It is apparent, that nitrotyrosine area is rich in the hydrophobic amino acids, such as L, M, V, I, P, A, F as described in Abello *et al.* (2009) and Elfering *et al.* (2004).

We have found several non-mitochondrial proteins in our mitochondria preparation. They could have been adhered to mitochondria during sedimentation. The best example is albumin (spot 1), that was also intensively nitrated. Possible nitration, not confirmed by nLC-MS/MS, was also found in actin (spot 7) and heat shock protein beta-1 (spot 9). Both of them are involved in cellular contractile apparatus (Mymrikov *et al.* 2011). Another contamination could have been produced by proteins of muscle fibers. These proteins were not discerned in the control mitochondria preparation as they might have been localized among the high molecular components not entering the gel. However, upon exposure to peroxynitrite some their parts were probably split without nitration and we found myosin light chain 3 (spot C), and myosin regulatory light chain 2 (spot F) in the positions not corresponding to the control mitochondria. Peroxynitrite might affect in a similar way also mitochondrial proteins. It appears, that NADH dehydrogenase iron-sulfur protein 3 (spot E) was also split without nitration.

Other effects of peroxynitrite action were

observed: soluble proteins citrate synthase (spot 13) and acetyl-CoA acetyltransferase (spot 14) were broken and nitrated (Fig. 2B). Both enzymes participate in various pathways involving acetyl-CoA. The preferred energy sources for working heart muscle are fatty acids (Ventura-Clapier *et al.* 2004). During beta-oxidation acetyl-CoA is produced followed by ATP synthesis in further pathways. Acetyl-CoA acetyltransferase play a key role in ketone bodies formation. Citrate synthase is essential for integrating of acetyl-CoA molecule into the energetic metabolism. Oxidative decarboxylation catalyzed by pyruvate dehydrogenase connects carbohydrate metabolism with citric acid cycle (Cotter *et al.* 2013). These pathways are the main sources of carbon dioxide in the cell. PeN can readily react with carbon dioxide producing carbonate radicals and promote further tyrosine nitration (Radi *et al.* 2002). Nitration of molecules is the process occurring under various physiological and pathological conditions. Several works documented the reversibility of nitration and its potential role in the same way as phosphorylation (Aulak *et al.* 2004, Koeck *et al.* 2004). From this point of view, the mitochondrial components are more exposed to nitration, and this modification may play an important role in regulation of mitochondrial metabolism.

The high sensitivity of ATP synthase to nitration by peroxynitrite is of potential biological importance, as this enzyme is essential for energy production in the heart. As increased peroxynitrite production was documented under several pathologic states (Pacher *et al.* 2007, Szabó *et al.* 2007) we intend to investigate the levels of protein nitration in the samples obtained from the operations on human heart in the future studies.

In our study we did not find nitration in manganese superoxide dismutase (MnSOD) molecule. MnSOD plays a critical role in maintaining of balance between pro-oxidants and antioxidants (Miriayala *et al.* 2011). Also it is a target of nitration mediated by peroxynitrite *in vitro* and *in vivo* (Macmillan-Crow *et al.* 1996, Macmillan-Crow *et al.* 1998, Surmeli *et al.* 2010). Immunoprecipitation and proteomic techniques were used in above mentioned studies (Ischiropoulos 2003, Surmeli *et al.* 2010). Whereas the content of MnSOD is too low (Marklund 1984), it is highly probable, that the level in our samples was under detection limit of our methods. In further studies, it is possible to enrich mitochondrial preparation for MnSOD by immunoprecipitation from a larger mitochondrial sample and take this for 2-DE followed by blotting and staining for 3-NT.

Conclusion

Protein nitration is not random process. Peroxynitrite can break the protein backbone by free radical mechanism and it can release membrane-bound proteins into the solution. Immunoblot detection of nitrotyrosine using chemiluminescence is more sensitive than nLC-MS/MS analysis, however mass spectrometry surely confirmed nitration of several proteins and revealed the exact location of this modification. MS confirmed nitration of ATP synthase subunit beta, pyruvate dehydrogenase E1 component subunit beta, citrate synthase and acetyl-CoA acetyltransferase. We found nitration after peroxynitrite exposure in proteins

essential for heart muscle metabolism; they play a key role in various pathways and nitration may be an important factor, which affects their biological role. The protein nitration by PeN appeared to be non-random, showing that some proteins are more sensitive to nitration than the others.

Conflict of Interest

There is no conflict of interest.

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