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ORIGINAL ARTICLE

Tyrosine nitration of mitochondrial proteins during myocardial ischemia and reperfusion

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Abstract

Myocardial ischemia reperfusion is associated with mitochondrial dysfunction and increased formation of reactive oxygen/nitrogen species. The main purpose of this study was to assess the role of tyrosine nitration of mitochondrial proteins in postischemic contractile dysfunction known as myocardial stunning. Isolated Langendorff-perfused rat hearts were subjected to 20-min global ischemia followed by 30-min reperfusion. The reperfused hearts showed marked decline in left ventricular developed pressure, maximal rate of contraction (+dP/dt), and maximal rate of relaxation (−dP/dt). Immunofluorescence and ELISA assays demonstrated enhanced protein tyrosine nitration in reperfused hearts. Using two-dimensional gel electrophoresis and MALDI-TOF/TOF mass spectrometry, eight mitochondrial proteins were identified to be nitrated after ischemia reperfusion. These proteins are crucial in mitochondrial electron transport, fatty acid oxidation, tricarboxylic acid cycle, ATP synthesis, and control of high-energy phosphates. The proteome data also indicated reduced abundance in several of nitrated proteins. The results suggest that these changes may contribute to inhibition of aconitase activity but are unlikely to affect electron transport chain activity. Whether tyrosine nitration of mitochondrial proteins can be considered the contributing factor of postischemic contractile dysfunction remains to be explored.

Keywords Heart · Ischemia-reperfusion · Myocardial stunning · Mitochondria · Tyrosine nitration · Mass spectrometry

Introduction

Acute coronary artery disease leading to myocardial ischemia and infarction is among the major causes of morbidity and mortality worldwide. Although restoration of blood flow, i.e., reperfusion, is essential to salvage ischemic areas, it may also result in further negative effects such as ventricular arrhythmias, contractile dysfunction, and cell death. Ample evidence has suggested that ischemia and reperfusion (IR) is associated with increased generation of reactive oxygen/nitrogen species (ROS/RNS) and oxidative stress. Potential cellular sources of ROS/RNS include NADPH oxidase, xanthine oxidase, and mitochondrial electron transport chain (ETC). Current evidence suggests that mitochondrial dysfunction plays a critical role in ROS production and the pathogenesis of IR injury [28]. Superoxide radical ($^{\bullet}O_2^-$) is the primary form of ROS generated by complex I and complex III of electron transport chain. This radical is quickly dismutated to H_2O_2 by mitochondrial Mn-SOD (which in turn can be converted to the highly toxic hydroxyl radical ($OH^{\bullet}$)). Nitric oxide (NO) is a free radical generated by three distinct isoforms of NO synthase (NOS). There are conflicting reports concerning the existence of mitochondrial NOS, but despite this discrepancy, NO has been detected in mitochondria [for review see 9, 38]. Superoxide and NO are weak oxidants and at physiologic conditions, when produced at low levels, play important roles in redox signaling, regulation of mitochondrial function, and cardioprotection [16]. However, in the initial phase of reperfusion the generation of these two radical species increases greatly resulting in formation of peroxynitrite ($ONOO^-$). Both animal and human studies demonstrate that IR is associated with elevated production of $ONOO^-$ [12, 37].

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Peroxynitrite is relatively stable but may be decomposed to form two powerful oxidants and highly toxic species, nitrogen dioxide (NO_2) and hydroxyl radical ($OH^{\bullet}$). Moreover, peroxynitrite itself is a strong oxidant that can react with lipids, proteins, and DNA leading to cellular damage. Nitration of protein-bound tyrosine residues producing 3-nitrotyrosine (n-Tyr) is a well-established post-translational modification and is considered a relevant biomarker of protein oxidative/nitrative damage [25, 27]. Several studies have documented that protein tyrosine nitration increases in reperfused heart and contributes to oxidative cell damage and triggers apoptotic pathway [12, 17]. Proteomic analysis of n-Tyr after long-lasting ischemia revealed that mitochondria are the major target for $ONOO^-$ [18]. Selective nitration of proteins involved in the ETC and oxidative phosphorylation observed in their study suggests that $ONOO^-$ is an important factor contributing to dysfunction in mitochondrial energy metabolism. Long-lasting ischemia (30 min) leads to irreversible injury in the isolated perused heart [24]. On the other hand, a brief period of ischemia and subsequent reperfusion is insufficient to cause irreversible myocardial damage, but may result in a profound systolic and diastolic dysfunction termed myocardial stunning [3]. Several hypotheses concerning the mechanism underlying myocardial stunning have been suggested, including disturbance in intracellular Ca^{2+} handling, myofilament alterations, deficiency in energy supply, and cellular damage caused by ROS/RNS. However, the mechanism involved in the pathogenesis of stunning have not been completely elucidated [8]. Earlier reports have shown that partial proteolysis and oxidative damage to myofilament and cytoskeletal proteins may result in contractile dysfunction [26, 33]. Few studies have suggested that alterations to other myocardial proteins may also contribute to functional impairment [17]. It has been also shown that short-term ischemia, resulting in myocardial stunning, is associated with increased formation of $ONOO^-$ [22] and accumulation of protein nitrotyrosines [2, 13, 32, 39]. In these studies nitrotyrosine formation was demonstrated by immunohistochemistry or Western blot assays, but proteomic analyses identifying nitrated proteins in stunned heart are lacking.

Therefore, we apply a comparative proteomic approach to evaluate the formation of protein 3-nitrotyrosines in mitochondrial proteins from rat hearts subjected to short-term ischemia followed by reperfusion.

Materials and methods

Animals

Male Wistar rats (4-months-old, body weight 300–400 g), supplied by the Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Slovakia, were used for the experiments. Prior to experiments, animals were maintained for 1 week in an air-conditioned room (22 ± 2 °C, 12 h light/dark cycle, light on at 6.00 a.m.). Food and water were available ad libitum. Experiments were performed in accordance with the European Community guidelines and were approved by the Ethical Committee of the Jessenius Faculty of Medicine.

Preparation of isolated hearts and perfusion

The animals were decapitated after anesthetization by halothane. The hearts were rapidly excised, placed in ice-cold Krebs–Henseleit (K-H) solution, cannulated through the aorta, and perfused with the K-H buffer according to the Langendorff method using ML870B2 Langendorff system (ADInstruments, Spechbach, Germany) at a constant pressure of 73 mmHg. K-H buffer (pH 7.4) containing 135 mmol/l NaCl, 5.4 mmol/l KCl, 0.9 mmol/l $MgCl_2$, 24 mmol/l $NaHCO_3$, 1.2 mmol/l NaH_2PO_4, 1.8 mmol/l $CaCl_2$, and 10 mmol/l glucose was continuously saturated with 95% O_2 and 5% CO_2 and maintained at 37 °C. Isovolumetric left ventricular pressure (LVP) was measured with a latex balloon coupled to a pressure transducer. A balloon was inserted into the left ventricle through the left atrium and its volume was adjusted to maintain an initial left ventricular end-diastolic pressure at 8–12 mmHg. Temperature and hemodynamic parameters were continuously recorded and processed using PowerLab 8/30 Data Acquisition System with Chart Software (ADInstruments, Spechbach, Germany). After a 15-min equilibration period, the hearts were subjected to 20-min global ischemia, no-flow ischemia, or 20-min ischemia followed by 30-min of reperfusion. Subsequent to an equilibration period, control hearts were perfused for 50 min without ischemia. At the end of perfusion, the hearts were frozen and stored at − 80 °C until use for preparation of homogenates and mitochondria.

Immunohistochemical detection of nitrotyrosines

After the perfusion protocol was completed, the hearts were postfixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 24 h at 4 °C. The tissues were cryoprotected by infiltration using 30% sucrose for the next 24 h at 4 °C. The heart tissues were then frozen and sectioned with a cryostat at 30 μm and the sections mounted into Superfrost Plus glass (Thermo Scientific, USA). Sections were permeabilized with 0.1% Triton X-100, preblocked with 10% BSA for 60 min. Primary antibody, anti-3-Nitrotyrosine (39B6) mouse monoclonal antibody (Santa Cruz Biotechnology) was diluted 1:100 in the 0.1% Triton X-100 solution with 10% BSA. Detection was performed using Alexa Fluor 488 goat-anti-mouse IgG (1:100, Life Technologies). Finally, the sections were mounted in

Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA) and examined using OLYMPUS Fluoview FV10i confocal microscope. The images were analyzed using Image-Pro Plus 6.0 software.

Preparation of tissue homogenates and mitochondria

Frozen tissue of the whole heart (~ 1 g) was placed in 10 volumes of ice-cold homogenization buffer (pH 7.0) containing 30 mM KH_2PO_4, 5 mM EDTA, 0.3 M sucrose, and 0.3 mM phenylmethylsulfonyl fluoride, and homogenized for five 25-s periods with a Teflon pestle in Potter–Elvehjem homogenizer at 1200 rpm. Cardiac mitochondria were isolated from heart homogenates by differential centrifugation as described previously [1]. Briefly, homogenates were filtered through one layer of cheesecloth and centrifuged at 1000×*g* for 10 min. The supernatant was collected and centrifuged at 18000×*g* for 35 min. Then the mitochondrial pellets were resuspended in 30 mmol/l imidazole, 60 mmol/l KCl, 2 mmol/l $MgCl_2$ (pH 7.0), and stored in aliquots at − 80 °C for later use.

ELISA assay of protein nitrotyrosines

Protein nitrotyrosine content was determined using an ELISA kit (OxisResearch, Portland, USA). Briefly, after adsorption onto microtiter plate the tissue homogenates were incubated for 1 h at room temperature with nitrotyrosine antibody. After washing with Tween-20, the samples were incubated 1 h at room temperature with strepatavidin peroxidase. Following washing, samples were incubated with freshly prepared solution of tetramethylbenzidine substrate for 30 min in the dark. Reaction was then terminated by stop solution containing citric acid and the absorbance was measured at 450 nm using Bio-Tek plate reader. Protein nitrotyrosines were quantified using nitrotyrosine standard curve.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described previously [31]. Briefly, mitochondrial samples (corresponding to 200 μg of proteins) were precipitated with ice-cold acetone and rehydrated with rehydration buffer containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue. For the first dimension, the samples were separated according to their isoelectric point using ReadyStrip IPG strips pH 5–8 and the Protean IEF system (Bio-Rad Laboratories). Following reduction and alkylation, proteins were separated in the second dimension by SDS-PAGE on 12% polyacrylamide gels. Each sample was separated by 2-DE in duplicates. Proteins in one gel were stained with Bio-Safe Coomassie G-250 stain (Bio-Rad Laboratories), scanned by GS-800 Calibrated Densitometer (Bio-Rad Laboratories), and analyzed using PDQuest 8 software (Bio-Rad Laboratories). Proteins in another gel were transferred onto nitrocellulose membrane and probed with an anti-3-nitrotyrosine monoclonal antibody as described below. 3-NT content for each protein was calculated as the ratio of the 3-NT level on the membrane to the protein content on the gel.

Western blot analysis of nitrotyrosines

For Western blot analysis of protein nitrotyrosines, mitochondrial proteins separated by 2-DE were then transferred into nitrocellulose membranes using Mini Trans-Blot cell (Bio-Rad Laboratories). After blocking with 5% non-fat dry milk in 20 mM Tris/HCl and 0.05% Tween-20 (TBS-T), the blots were then incubated for 1 h with anti-nitrotyrosine rabbit polyclonal (1:1000, Santa Cruz Biotechnology) in Tris-buffered saline. After washing with TBS-T, the blots were incubated 1 h with secondary polyclonal goat anti-rabbit (1:1000, Santa Cruz Biotechnology) and then with SuperSignal West Pico Chemiluminescent Substrate (Genetica) solution for 5 min in dark. Immunoreactive proteins were visualized using Chemidoc XRS system (Bio-Rad Laboratories, CA) and quantified by Quantity One software (Bio-Rad Laboratories).

In-gel digestion and preparation of samples for MS analysis

Spots of interest were excised manually from the Coomassie-stained gels and gel pieces were trypsinized as described previously [31]. Briefly, gels pieces were destained with 50% acetonitrile (CH_3CN) in 25 mM ammonium bicarbonate at room temperature overnight. After washing with 100% CH_3CN and drying in a Speed Vac, the gels were reduced with dithiothreitol (DTT) and alkylated in iodoacetamide at dark. Dry gel pieces were then digested with trypsin (Promega) and resulting tryptic peptides were reconstituted in 10% trifluoroacetic acid.

Identification of proteins by mass spectrometry

Peptide samples (0.75 μl) were spotted onto AnchorChip™ target (Bruker Daltonics, Bremen, Germany) together with 1 μl of matrix (α-cyano-4 hydroxy cinnamic acid) and left to dry at room temperature. Samples were analyzed by MALDI-TOF/TOF using Ultraflex III mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in a positive ion reflectron mode, with an accelerating voltage of 20 kV and pulsed extraction. Spectra were externally calibrated using calibration standard mixture containing $[M + H]^+$ ions of bradykinin, angiotensin I, angiotensin II, substance P, bombesin, adrenocorticotropin fragments 1–17 and 18–39 and somatostatin. Peptide mass spectra were recorded in the m/z range

700–3000 by accumulation data from 2000 shots and processed with FlexAnalysis software (Bruker Daltonics, Bremen, Germany). The MS/MS fragmentation spectra were obtained by selecting two strongest peaks of each peptide mass map.

Combined MS and MS/MS spectra searched against Swiss-Prot or NCBI databases using the MASCOT (Matrix Science, London, UK) database search engine. The following parameter settings were used: enzyme: trypsin, taxonomy: rodents, one missed cleavage site allowed, fixed modification: carbamidomethylation, variable modification: methionine oxidation, modification of lysine/cysteine by HNE, mass tolerance for PMF 50 ppm, MS/MS tolerance 0.5 Da. Protein scores greater than 65 were accepted as significant.

Measurement of protein thiol groups

Total content of protein thiol groups was measured spectrophotometrically as described previously [15]. Mitochondrial samples (aliquots of 0.15 mg proteins) were incubated 10 min at room temperature in solution containing 30 mM imidazole (pH 7.4), 5 mM EDTA, 0.4 mM 2,2-dithiobisnitro-benzoic acid (DTNB) and then the sample absorbance at 412 nm was read. The sulfhydryl group content was calculated using molar absorption coefficient $\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$ after subtraction of blank absorbance from absorbance of sample.

Determination of enzyme activities

Enzyme activities of ETC complexes

Enzyme activities of ETC complexes I–IV were measured spectrophotometrically at 30 °C using Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan) as described previously [31].

Aconitase activity

Mitochondria (0.05 mg/ml protein) were added to the medium containing 25 mM KH_2PO_4 and 0.5 mM EDTA, pH 7.25, containing 0.01% Triton X-100, and placed in a sonicating water bath for 30 s. Aconitase activity was assayed as the rate of $NADP^+$ reduction (340 nm, $\varepsilon = 6200\ M^{-1}\ cm^{-1}$) by isocitrate dehydrogenase upon addition of 5.0 mM sodium citrate, 0.6 mM $MgCl_2$, 0.2 mM $NADP^+$, and 1.0 unit/ml isocitrate dehydrogenase to sonicated mitochondria [23].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences between groups were compared by two-tailed Student *t* test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Cardiac contractile function

Baseline values of contractile parameters of hearts from control and IR groups before ischemia were similar (Table 1). Ischemia resulted in a rapid decline in developed left ventricular pressure (devLVP) due to fall of systolic LVP. After 30-min reperfusion, devLVP was restored to 79.4 ± 4.1% of preischemic value or to 85.1 ± 4.4% when compared to control (Fig. 1, Table 1). Contractile function, expressed as rate-pressure product (product of heart rate and devLVP), was restored only to 63.6 ± 5.2% of preischemic value and was also significantly lower when compared to control. Maximum rate of pressure development (+LVdP/dt) was not altered, but maximum rate of relaxation (−LVdP/dt) depressed to 62.7 ± 3.3% of preischemic value or to 56.0 ± 3.0% when compared to control. Heart rate and coronary flow did not differ from preischemic values, slight decreases were observed when compared to control hearts.

Myocardial tissue from hearts subjected to IR showed normal morphology without signs of irreversible injury as suggested by hematoxylin and eosin staining (Fig. S1). These results are consistent with reversible postischemic contractile dysfunction, myocardial stunning.

Table 1 Effect of ischemia and reperfusion on cardiac contractile function

	HR (%)	CF (%)	devLVP (%)	CO (%)	+dP/dt (%)	−dP/dt (%)
Control						
15 min	100 ± 9.5	100 ± 3.7	100 ± 4.4	100 ± 11.2	100 ± 15.2	100 ± 10.3
65 min	105.2 ± 6.9	97.5 ± 2.5	102.6 ± 3.8	107.7 ± 8.8	95.9 ± 12.9	83.1 ± 9.5
IR						
Before I	93.9 ± 7.8	91.4 ± 2.5	107.2 ± 4.1	101.1 ± 9.9	90.6 ± 9.9	89.3 ± 2.4
After R	84.4 ± 3.0$^{\#}$	85.3 ± 1.8$^{\#\#}$	85.1 ± 4.4**$^{\#}$	64.3 ± 5.2*$^{\#\#}$	78.9 ± 12.9	56.0 ± 3.0***$^{\#}$

HR, heart rate; *CF*, coronary flow; *devLVP*, developed LVP; *CO*, cardiac output; *+LV dP/dt*, maximum rate of pressure development; *-LV dP*, maximum rate of relaxation. Changes are expressed as percentages of 15 min control. Values represent mean±SEM of 5 hearts. * $p < 0.05$, *** $p < 0.001$, significantly different when compared to the preischemic value; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, significantly different when compared to control hearts

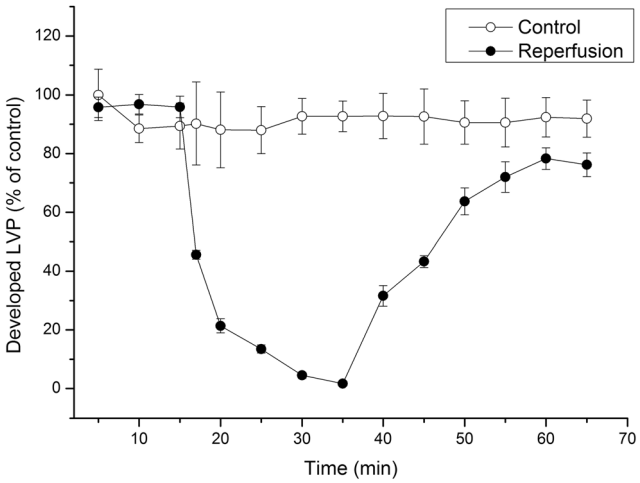

Fig. 1 Effect of ischemia and reperfusion on developed left ventricular pressure in rat heart. Values are given as mean ± SEM of five hearts

ELISA and immunohistochemical staining for protein nitrotyrosines

To determine whether depressed contractile function is associated with oxidative/nitrative damage the total protein 3-nitrotyrosine levels were estimated. As shown in Fig. 2, the 3-NT content in ischemic hearts showed small but nonsignificant increase when compared to control. In contrast, reperfusion resulted in nearly 12-fold increase in protein nitrotyrosines (0.94 ± 0.05 vs 11.44 ± 1.11 pmol/mg protein, in control and IR, respectively).

Alterations in protein tyrosine nitration were validated by immunofluorescence analyses. These studies confirmed slightly elevated nitrotyrosine staining (green) in the ischemic myocardium and massive increase in the intensity after ischemia followed by reperfusion. The results also indicate that number of cardiomyocytes positively stained for 3-NT increases after reperfusion (Fig. 3).

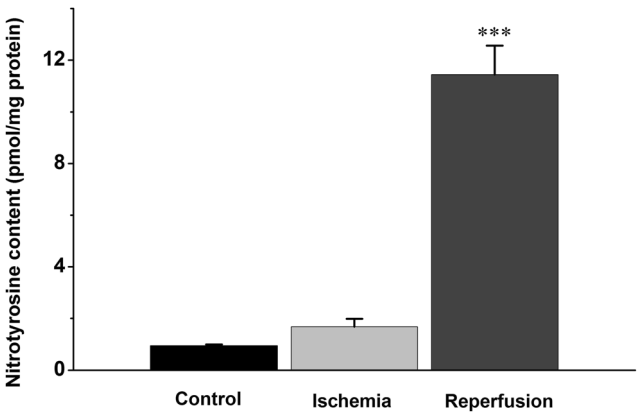

Fig. 2 Content of nitrated proteins in control, ischemic, and reperfused rat hearts. Values are given as mean ± SEM of five experiments. *** $p < 0.001$; significantly different as compared to control

Identification of nitrated proteins with mass spectrometry

To identify nitrated proteins in IR hearts the mitochondrial samples were analyzed by 2-DE followed by Western blot analysis with nitrotyrosine antibody and mass spectrometry. Figure 4 shows the representative 2-DE gel images of proteins stained with Coomassie for total proteins and the corresponding Western blot for nitrated proteins in mitochondria of control and IR hearts. Eight proteins were found to be significantly more nitrated after IR.

As shown in Table 2, protein with significantly increased tyrosine nitration were aconitase (Aco2), electron transfer flavoprotein-ubiquinone oxidoreductase (Etfdh), dihydrolipoyl dehydrogenase (Dld), ATP synthase subunit β (Atp5b), isovaleryl-CoA dehydrogenase (Ivd), long-chain specific acyl-CoA dehydrogenase (Acadl), β-enolase (Eno3), and creatine kinase (Ckmt2).

Interestingly, three of these proteins, Atp5b, Ivd, and Eno3, exhibited significantly decreased abundance in the stunned heart (Fig. 5). Also, other nitrated proteins show tendencies to decrease but the changes were not significant.

Oxidation of thiol groups during ischemia and reperfusion

Since peroxynitrite can react also with thiol groups of protein cysteines, we measured free thiol group content. As shown in Fig. 6, the –SH content was slightly decreased in ischemic hearts, but it was recovered after 30-min reperfusion.

Effect of ischemia and reperfusion on ETC and aconitase activities

To evaluate the possible effect of tyrosine nitration on mitochondrial function we measured the activities of ETC complexes I–IV. Figure 7 shows that both ischemia and IR similarly decrease activities of complexes I, III, and IV. In contrast, ischemia and IR did not significantly alter the activity of complex II.

Aconitase, the citric acid cycle enzyme, is frequently used as a biomarker of protein oxidative damage. As shown in Fig. 8, the catalytic activity of aconitase was not altered by ischemia, but the activity decreased to $58.1 \pm 3.5\%$ of control ($p < 0.001$) after reperfusion.

Discussion

Myocardial stunning is characterized by reversible contractile dysfunction following short ischemic periods, occurring in the absence of cell necrosis. To date, molecular mechanism of myocardial stunning has not been fully elucidated. In this

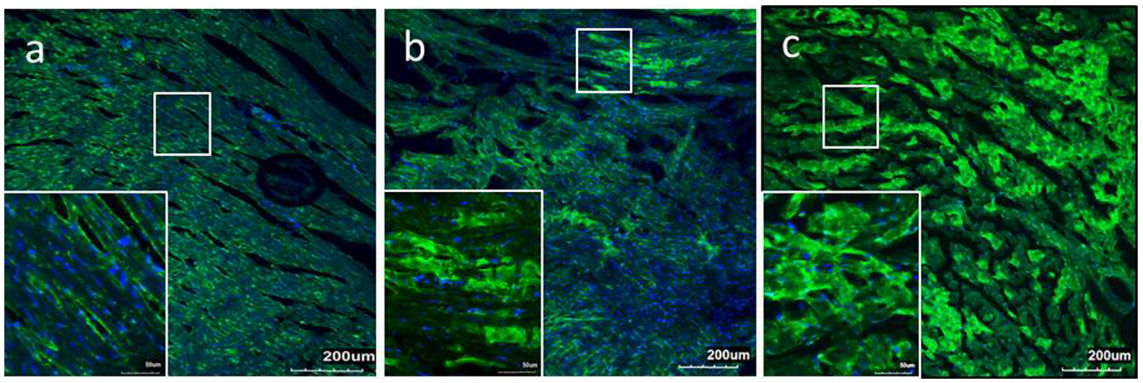

Fig. 3 Representative immunohistochemical staining with anti-3-nitrotyrosine monoclonal antibody in **a** control, **b** ischemic, and **c** stunned rat myocardium. Protein nitrotyrosines are identified by green fluorescence. Nuclei (blue fluorescence) are stained with DAPI (4′,6-diamidino-2-phenylindole)

study, we used proteomic approach to investigate the role of mitochondrial tyrosine nitration in stunning of isolated rat heart. Previous proteomic studies on myocardial stunning have investigated changes in protein expression [5, 35], but proteomic profiling of post-translational modifications was not reported.

Most of the latter studies used immunohistochemical staining with anti-tyrosine antibodies to demonstrate increased

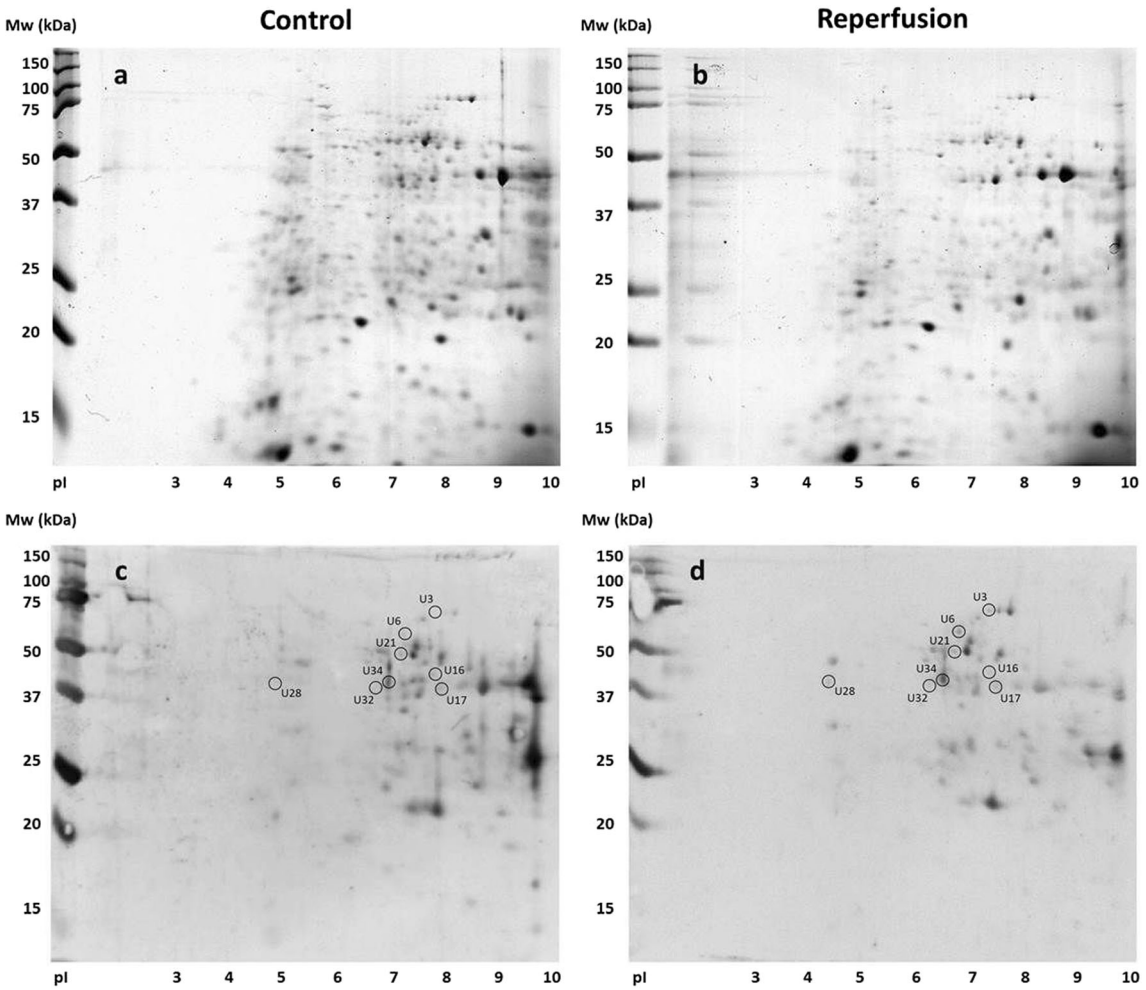

Fig. 4 Representative two-dimensional (2D) gel electrophoresis (top) and 2D immunoblots (bottom) indicating protein tyrosine nitration of mitochondrial proteins isolated from **a**, **c** control and **b**, **d** reperfused rat hearts. Spots showing significant changes in tyrosine nitration are labeled by numbers. Spot numbers correspond to those listed in Table 2

Table 2 Identification of cardiac proteins nitrated during reperfusion

Spot	Protein name	UniProt access. no.	Gene ID	Mw (kDa)	pI	Fold change	MS score	Matchedpeptides	Sequence coverage (%)
U3	Aconitate hydratase	Q9ER34	*Aco2*	86.121	7.9	3.3**	290	20	23
U6	Electron transfer flavoprotein-ubiquinone oxidoreductase	Q6UPE1	*Etfdh*	69.010	7.3	3.2***	86	5	13
U16	β-enolase	P15429	*Eno3*	47.326	7.1	2.7*	179	12	29
U17	Creatine kinase S-type	P09605	*Ckmt2*	47.811	8.8	1.8*	113	10	28
U21	Dihydrolipoyl dehydrogenase	Q6P6R2	*Dld*	54.574	8.0	2.2**	140	8	21
U28	ATP synthase subunit β	P10719	*Atp5b*	56.318	5.2	3.1**	297	22	41
U32	Isovaleryl-CoA dehydrogenase	P12007	*Ivd*	46.862	8.0	3.1**	95	6	18
U34	Long-chain specific acyl-CoA dehydrogenase	P15650	*Acadl*	48.242	7.6	2.7***	325	23	43

Spot numbers refer to numbers indicated in Fig. 4. Proteins were identified by 2-DE followed by combined MS and MS/MS analysis. The mass and pI values were obtained from the MASCOT database. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; significantly different as compared to control

tyrosine nitration in canine model of myocardial stunning [22, 39]. Subsequent Western blot study on isolated rabbit heart showed that nitration of mitochondrial proteins increases after 15-min ischemia and 30-min reperfusion but not after ischemia without reperfusion [32]. Using isolated rat heart model of stunning and immunofluorescence staining, we confirmed increased nitrotyrosine levels in cardiomyocytes.

To further confirm that stunning is associated with tyrosine nitration, we apply ELISA method. In accordance with previous report [32], protein tyrosine nitration was not significantly altered after 20-min ischemia, but dramatic increase was observed after 30-min reperfusion.

To identify mitochondrial proteins which show increased tyrosine nitration, we used 2DE-MS-based proteomic approach. Analysis revealed that nitration of eight proteins is significantly increased after ischemia and reperfusion. It is worth mentioning that nitrotyrosine labeling in reperfused hearts was higher when compared to control despite the lower contents of nitrated proteins. This finding suggests extensive nitration of mitochondrial

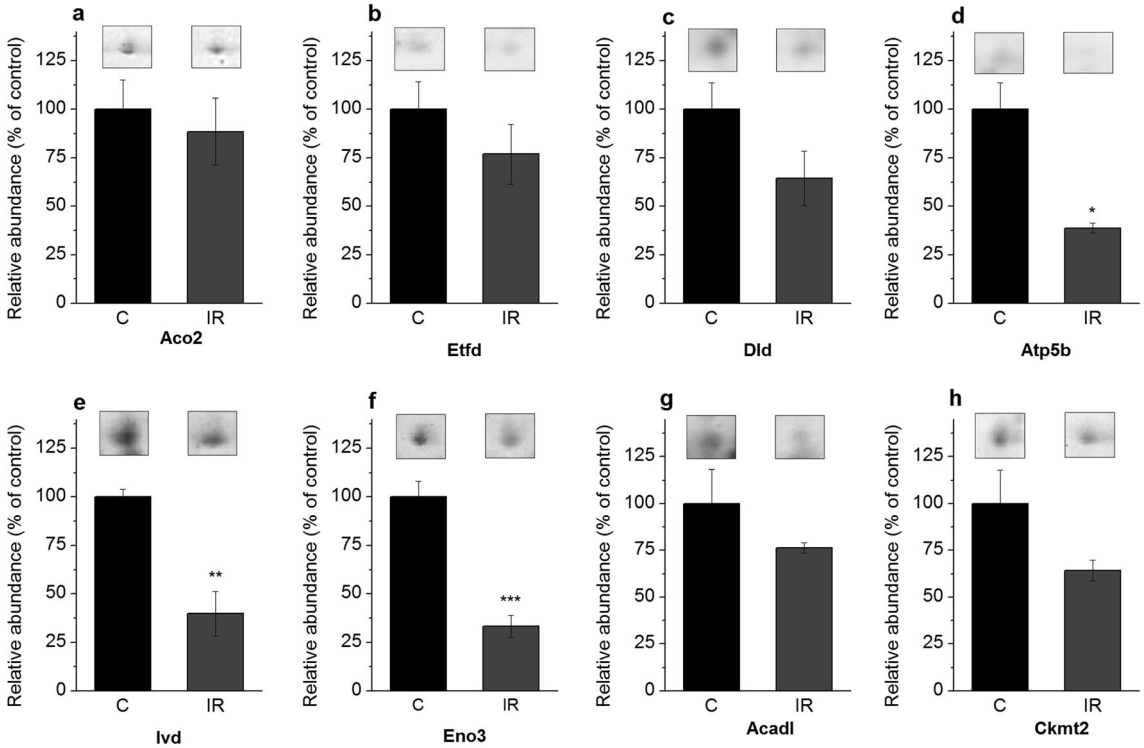

Fig. 5 Relative abundances of nitrated proteins in control (C) and reperfused (IR) rat hearts. **a**–**h** Bars represent spot volumes given as mean ± SEM of five experiments. * $p < 0.05$, ** $p < 0.01$***, $p < 0.001$; significantly different as compared to control. Representative spots are also shown, left spot corresponds to control and the right to IR heart

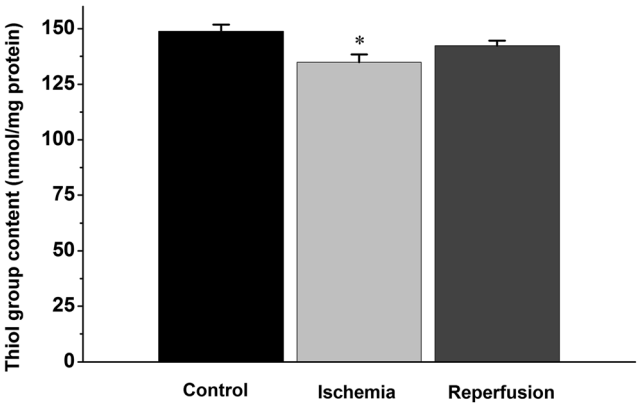

Fig. 6 Total thiol group content in mitochondria isolated from control, ischemic, and reperfused rat hearts. Values are given as mean ± SEM of five experiments. * $p < 0.05$; significantly different as compared to control

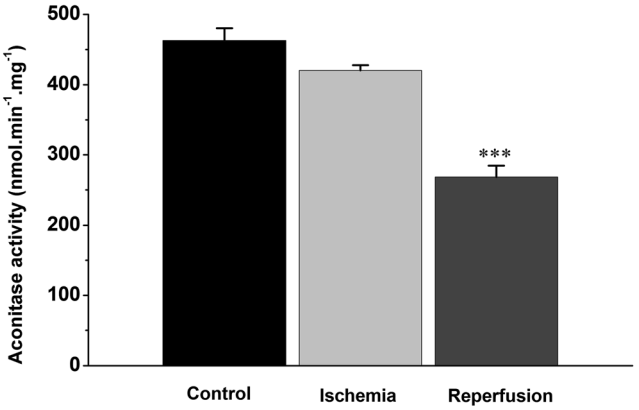

Fig. 8 Aconitase activity in mitochondria isolated from control, ischemic, and reperfused rat hearts. Values are given as mean ± SEM of 5 experiments. *** p < 0.001; significantly different as compared to control

proteins after ischemia and reperfusion. The functions of nitrated proteins are mainly related to cellular energy metabolism. There are little proteomic analyses of protein nitration after myocardial ischemia-reperfusion. Liu et al. [18] identified 23 proteins in tissue homogenate, 10 of them were from mitochondria that were nitrated after 60-min ischemia followed by 60-min reperfusion. Their study suggests that the mitochondria are the major target of nitration injury. Yang et al. [36] have used model of isolated guinea pig heart to investigate mitochondrial protein nitration after 30-min ischemia followed by 10-, 30-, or 60-min reperfusion. Thirteen proteins showed enhanced nitration. They were mostly involved in ETC and tricarboxylic acid cycle (TCA), but subset of proteins was different than that observed after 60-min ischemia and 60-min reperfusion [18]. Among the proteins which were identified in the present study, only two

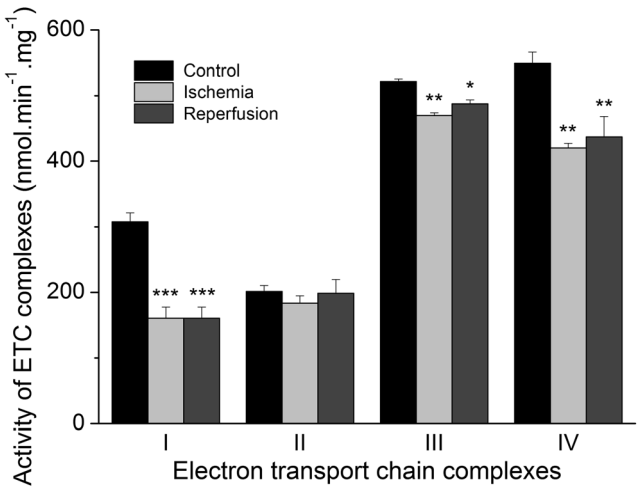

Fig. 7 Activities of ETC complexes in mitochondria isolated from control, ischemic, and reperfused rat hearts. Values are given as mean ± SEM of five experiments. * $p < 0.05$, ** $p < 0.01$***, $p < 0.001$; significantly different as compared to control

(dihydrolipoyl dehydrogenase and ATP synthase subunit β) were reported by Liu et al. [18] or Yang et al. [36]. The possible reasons for these differences might be (1) the different animal models of IR injury or (2) different duration of ischemia. As shown by Palmer et al. [24] duration of global ischemia is a major determinant of contractile and energetic dysfunction. Persistent depression of high-energy phosphates, increased release of creatine kinase, and significant mitochondrial edema were observed after 30-min ischemia followed by 30-min reperfusion, but not after shorter ischemia-reperfusion periods.

The principal finding of this study is that in stunned heart, several key enzymes of TCA cycle, fatty acid β-oxidation, and ATP synthesis are subjected to enhanced tyrosine nitration which may result in premature proteolytic degradation.

Aconitase (aconitate hydratase, Aco2) catalyzes the conversion of citrate to isocitrate in TCA cycle. The enzyme is highly vulnerable to oxidative damage and its activity is widely used as a biomarker of oxidative stress [20]. Using in vivo model of IR, Bulteau et al. [4] showed that aconitase activity is not affected by 30-min coronary occlusion, but decreases at 5 min of reperfusion. Extended periods of reperfusion resulted in restoration of activity. Our results only partially agree with these findings since we observed significant decrease in activity at 30 min of reperfusion. Still, these results support the view that aconitase inactivation is a reperfusion injury. Molecular mechanism of enzyme inactivation is well elucidated. Peroxynitrite was shown to inactivate aconitase in a dose-dependent manner via loss of cysteine thiol groups and nitration of tyrosines [11]. Thiol proteome was not investigated in the present study but data showing recovery of thiol group content during reperfusion may suggest that not thiol modification, but tyrosine nitration might be a dominant mechanism of aconitase inactivation in stunned heart.

Dihydrolipoyl dehydrogenase (Dld) is the functional component of the TCA enzyme 2-oxoglutarate dehydrogenase (OGDH) complex and pyruvate dehydrogenase complex. Increased nitration of Dld has been reported after 60-min ischemia followed by 60-min reperfusion [18]. Yang et al. [36] observed nitration of another subunit of OGDH complex, dihydrolipoyl succinyltransferase. Our observation of increased Dld nitration in stunned heart supports the view of its high vulnerability to oxidative damage and suggests alterations of TCA cycle and pyruvate oxidation in reperfused myocardium.

Electron transfer flavoprotein dehydrogenase (Etfdh) also known as electron transfer flavoprotein:ubiqionone oxidoreductase (ETF-QO) is located in the inner mitochondrial membrane and catalyzes transfer of electrons from electron transfer flavoprotein (ETF) to ubiquinone. This system enables electron transfer from 11 different flavoprotein dehydrogenases, which are involved in oxidation of fatty acids and some amino acids [34]. Interestingly, we found that two of these dehydrogenases, long-chain specific acyl-CoA dehydrogenase (Acadl) and isovaleryl-CoA dehydrogenase (Ivd) are also the targets of tyrosine nitration in the stunned heart. Moreover, the relative abundance of Ivd was significantly reduced in the stunned heart. It can be reasoned that decrease in Ivd abundance results from accelerated proteolytic degradation since it is unlikely that significant changes in protein expression can occur during short reperfusion period. Literature data on tyrosine nitration of Etfdh, Acadl, and Ivd during cardiovascular injuries are lacking. Increased nitration of ETF was observed in the aged rat heart [14]. Our findings suggest that mitochondrial oxidation of long-chain fatty acids and amino acid catabolism are impaired and these changes can contribute to reperfusion damage of the heart.

Another remarkable protein showing increased nitration is ATP synthase subunit β (Atp5b). Moreover, we showed that Atp5b content decreases in postischemic heart, suggesting elevated protein degradation due to nitrative damage. Our finding that Atp5b content decreases to 38.8 ± 2.6% after 30-min reperfusion is consistent with data of Elfering et al. [7] who showed that half-life of nitrated Atp5b is 30 min. ATP synthase synthesizes ATP from ADP and phosphate during electron transport in ETC and Atp5b is one of the five different subunits of its F_1 domain located above the membrane.

Homeostasis of ATP and other high-energy phosphates is controlled also by mitochondrial creatine kinase (Ckmt). The nitration of Ckmt was reported in the aging heart [14]. Myofibrillar isoform of creatine kinase was shown to be nitrated and inactivated in the failing rat heart [21]. This is the first study to show that tyrosine nitration of Ckmt increases after short-term ischemia reperfusion. These findings are consistent with the view that Ckmt is a sensitive and early target for peroxynitrite reactions in mitochondria [29].

One of peroxynitrite targets in the stunned heart, β-enolase, is a non-mitochondrial protein. It is a glycolytic enzyme that catalyzes conversion of 2-phosphoglycerate to phosphoenolpyruvate associated with formation of macroergic bond. Enolase is located mainly in cytosol, but recent study has shown that it can bind to cardiac mitochondria and stabilize mitochondrial membrane [10]. Eno3 is one of three enolase isoforms and in the heart, the predominant isoform is Eno1 (α-enolase). Nitrative and/or oxidative modifications of this isoform was reported in the diabetic [19] and aged [14] rat hearts. Increased carbonylation of cardiac Eno3 in adriamycin-treated mice suggests that this isoform is also vulnerable to oxidative damage [6]. Our finding of increased nitration of Eno3 is consistent with this view and suggests altered glucose metabolism in reperfused heart.

Taken together, increased tyrosine nitration of proteins involved in cellular energy metabolism was observed in the stunned heart. Non-enzymatic post-translational modifications (PTM) such as tyrosine nitration are frequently associated with inhibition of protein function, e.g., by inducing structural changes or preventing tyrosine phosphorylation. Our data suggest that tyrosine nitration may contribute to inhibition of aconitase activity but not to ETC dysfunction since ischemia alone had similar impact on ETC activities as IR. Nitrated proteins are usually the targets of accelerated proteolytic degradation [30]. Under conditions of sustained NO production, several mitochondrial proteins, including those identified in the present study (Atp5b, Acadl, Dld, Ivd, and Etf), were nitrated and prematurely degraded as indicated by decreased half-lives [7]. Differences in protein abundance which appeared to be the result of chemical modifications and proteolytic fragmentation were reported in stunned rabbit myocardium [35], but nature of modifications was not investigated. Results of our study suggest that nitration of tyrosines may result in premature proteolytic degradation and reduced contents of mitochondrial enzymes. Additional studies, however, are necessary to evaluate their roles in mitochondrial energy metabolism and temporal contractile dysfunction in myocardial stunning.

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Compliance with ethical standards

All experiments were conducted in accordance with the European Community guidelines and were approved by the Ethical Committee of the Jessenius Faculty of Medicine.

Conflict of interest The authors declare that they have no conflict of interest.

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