Increased protein S-nitrosylation in mitochondria: a key mechanism of exercise-induced cardioprotection

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Abstract
Endothelial nitric oxide synthase (eNOS) activation in the heart plays a key role in exercise-induced cardioprotection during ischemia–reperfusion, but the underlying mechanisms remain unknown. We hypothesized that the cardioprotective effect of exercise training could be explained by the re-localization of eNOS-dependent nitric oxide (NO)/S-nitrosylation signaling to mitochondria. By comparing exercised (5 days/week for 5 weeks) and sedentary Wistar rats, we found that exercise training increased eNOS level and activation by phosphorylation (at serine 1177) in mitochondria, but not in the cytosolic subfraction of cardiomyocytes. Using confocal microscopy, we confirmed that NO production in mitochondria was increased in response to H2O2 exposure in cardiomyocytes from exercised but not sedentary rats. Moreover, by S-nitrosoproteomic analysis, we identified several key S-nitrosylated proteins involved in mitochondrial function and cardioprotection. In agreement, we also observed that the increase in Ca2+ retention capacity by mitochondria isolated from the heart of exercised rats was abolished by exposure to the NOS inhibitor L-NAME or to the reducing agent ascorbate, known to denitrosylate proteins. Pre-incubation with ascorbate or L-NAME also increased mitochondrial reactive oxygen species production in cardiomyocytes from exercised but not from sedentary animals. We confirmed these results using isolated hearts perfused with L-NAME before ischemia–reperfusion. Altogether, these results strongly support the hypothesis that exercise training increases eNOS/NO/S-nitrosylation signaling in mitochondria, which might represent a key mechanism of exercise-induced cardioprotection.

Keywords Nitric oxide · S-Nitrosylation · Ischemia–reperfusion · mPTP

Introduction
Ischemic heart disease remains one of the main causes of morbidity and mortality worldwide [66]. In animal models, many strategies, such ischemic pre- and post-conditioning and pharmacological conditioning, are used to efficiently protect the heart during ischemia–reperfusion (IR). Although, most of them remain poorly transferable or effective in humans, exercise training has beneficial effects in rodent [23, 33] and also human [21, 47] hearts during IR through not yet fully understood mechanisms. Recently, we [23] and others [10] reported that activation of the endothelial nitric oxide synthase/nitric oxide (eNOS/NO) pathway by exercise training strongly contributes to heart protection. This cardioprotective effect is lost when eNOS is pharmacologically inhibited [23] or the encoding gene is ablated [10]. Initially, much of NO cardioprotection was attributed

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to activation of guanylyl cyclase that results in the production of cyclic guanosine monophosphate (cGMP) and activation of protein kinase G (PKG) [17, 18]. In the last decade, protein S-nitrosylation (SNO), defined as the binding of one NO moiety to a thiol group of a cysteine residue [59], has emerged as an important modulator of cardiovascular functions [42, 69], and a possible key mechanism in NO-mediated cardioprotection [34, 35, 73].

Mitochondria are considered key triggers of post-ischemic reperfusion injuries. Indeed, post-ischemic reperfusion leads to mitochondria-driven injuries explained by excessive production of reactive oxygen species (ROS) and dysregulation of calcium handling. Both lead to mitochondrial permeability transition pore (mPTP) opening that results in abnormal permeation, swelling and disruption of mitochondrial membranes, and release of pro-apoptotic factors [41]. In the last decade, mitochondria have emerged as a key target of cardioprotective strategies (i.e., ischemic pre- and post-conditioning) [5, 30]. Interestingly, NO is a strong regulator of mitochondrial function [22] and the eNOS/NO pathway plays a key role in several cardioprotective pathways [8]. Recently, it has been reported that in the heart, 56% of all S-nitrosylated proteins are mitochondrial proteins and that 36% of the SNO proteome requires eNOS-derived NO [20]. Moreover, after ischemic pre-conditioning, mitochondrial proteins represent 52% of all S-nitrosylated proteins [51]. This could be explained by eNOS association with subsarcolemmal mitochondria (SSM) in response to ischemic pre-conditioning [75]. Indeed, incubation with a mitochondrial-specific NO-donor (mitoSNO) protects the heart during IR [16]. MitoSNO cardioprotection appears to be dependent on protein SNO and independent of cGMP-PKG activation [49], because it is not affected by ablation of the PKG gene in mouse cardiomyocytes. Thus, eNOS association with mitochondria and protein SNO are key elements to explain NO-dependent cardioprotection.

Despite the clear effect of physical exercise on mitochondria in healthy and diseased hearts, its role and the underlying mechanisms in exercise-induced cardiac pre-conditioning before IR are not clear [6]. Therefore, we investigated whether the cardioprotective effect of exercise pre-conditioning could be explained by eNOS association with mitochondria and the subsequent increase of NO bioavailability and protein SNO. Using western blotting, confocal microscopy, and SNO proteomic approaches, we found that exercise (1) increased eNOS/NO bioavailability in mitochondria, and, consequently, (2) modulated Ca^{2+}-dependent mitochondrial permeability transition and ROS production during IR.

**Materials and methods**

Detailed information on the methodology is available in the Supplemental Information file.

**Animal model**

All animal experiments were performed according to the European Parliament Directive 2010/63/EU (N° CEEA00223) and approved by the French national research ethics committee (no. APAFIS#3487-2016010813,43,57). Male Wistar rats (12-week-old; weight = 225–275 g, Janvier, France) were randomly distributed in two groups: sedentary control group (Sed), and 5-week treadmill exercise group (Ex-Tr). At the end of the study, the impact of exercise training in the Ex-Tr group was confirmed by their higher maximal aerobic velocity (MAV), measured as previously described [62], compared with sedentary rats (Sed = 36 ± 1.58 m/min; Ex-Tr: 46.25 ± 1.31 m/min; p < 0.05).

**Exercise protocol**

Moderate exercise training was performed as previously described [23]. Briefly, rats exercised on a motor-driven treadmill (Ugo Basile, Transforming ideas into instruments, 47300) 45 min/day, 5 days/week for 5 weeks at 25 m/min (≈ 60% of their MAV). To avoid the acute effects of exercise, rats were sacrificed 24 h after the last exercise training session.

**Measurement of ROS production following IR**

**In vivo IR**

In vivo IR was performed as previously described [33]. To evaluate the eNOS/NO/SNO pathway implication in heart sensitivity to IR injuries and ROS production, Sed and Ex-Tr rats received one intraperitoneal injection of vehicle (0.9% NaCl) or L-NAME (50 mg/kg in 0.9% NaCl; Abcam ab120136) at 24 h and 30 min before ligation. After anesthesia, Sed and Ex-Tr rats were ventilated, and a thoracotomy was performed to expose the interventricular coronary artery that was subsequently ligated for 30 min, followed by 10 min of reperfusion. The ECG signal was recorded with subcutaneous electrodes. Heart rate and QT intervals were measured at baseline, at the end of ischemia, and after 10 min of reperfusion. The corrected QT (QTc) was calculated according to the Framingham Heart Study method in which the QT interval is adjusted for heart rate: QTc = QT + 0.154 × [1 − RR]. The ischemic area was collected and embedded in optimal
cutting temperature (OCT from Tissue-Tek) and flash-frozen in liquid nitrogen.

**IR in isolated hearts**

IR in isolated hearts was performed as previously described [23]. Hearts were perfused or not with the NOS inhibitor L-NAME (Abcam, ab120136, 100 µM) or the S-nitrosoglutathione (GSNO) reductase inhibitor (N6022, 10 µM, Cayman Chemical) for 25 min.

**Measurements of ROS production using the DHE fluorescent dye**

Cardiac ROS production was measured as previously described [7].

**Measurement of mitochondrial ROS (mtROS) and NO production in isolated cardiomyocytes**

Ventricular cardiomyocytes were isolated as previously described [13].

**Measurement of mtROS production**

Adult cardiomyocytes (20,000 cell/mL) were distributed in a 96-well microplate and incubated (37 °C for 30 min) with MitoSOX Red, a fluorogenic probe sensitive to mtROS production (5 µM, Ex: 510 nm; Em: 580 nm; Fisher Scientific, Invitrogen, M36008)[24], with or without L-NAME (100 µM) or sodium l-ascorbate (1 mM). Then, they were immediately transferred to a microplate reader (Synergy HT Microplate Spectrophotometer Biotek). Cells were stimulated with 10 µM of antimycin A (10 mM, Sigma-Aldrich), a complex III blocker, to force mtROS production.

**Measurement of NO production**

NO production was measured as previously described [19]. Cardiomyocytes from Sed and Ex-Tr rats were loaded with the fluorescent NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF2-Da; 5 µM; Ex: 491 nm, Em: 513 nm, Abcam; ab145283) at 37 °C for 15 min. Then, cardiomyocytes were distributed in a 96-well microplate to evaluate intracellular NO production with a microplate reader (Synergy HT Microplate Spectrophotometer Biotek) or were used to determine NO production and colocalization with mitochondria (MitoTracker Orange CMXRos) by confocal microscopy (SP8 LEICA, platform 3A AU/INRAE microscopy facility).

**Mitochondrial calcium retention capacity and swelling assays**

Cardiac mitochondria were isolated as previously described [7] (Fig. 1a).

**Calcium retention capacity**

Calcium retention capacity (CRC) was examined as previously described [1, 57] using the fluorescent Ca²⁺ indicator Calcium Green-5N (1 µM, Invitrogen, C3737) as extramitochondrial calcium probe. Mitochondria were incubated or not with sodium l-ascorbate (1 mM) to evaluate SNO impact [75], and with the NO-donor GSNO (500 µM). Some mitochondria were obtained from isolated hearts perfused or not with L-NAME for 25 min. Calcium Green-5N fluorescence was measured throughout the experiments using excitation/emission wavelengths of 507/536 nm and a microplate reader (Synergy HT Microplate Spectrophotometer Biotek).

**Calcium swelling assay**

Calcium-induced mitochondrial swelling was measured spectrophotometrically as the decrease of the absorbance at 540 nm using a microplate reader (Epoch microplate reader, Biotek). Mitochondrial swelling was induced by adding a single dose of CaCl₂ (250 µM) to mitochondria, pre-incubated or not with ascorbate (1 mM). Absorbance at 540 nm was recorded continuously for 25 min.

**Proteomics and SNO proteomics**

**Determination of SNO occupancy with cysteine-reactive tandem mass tags**

Intact heart mitochondria (1 mg/mL) and total LV homogenates were isolated as previously described [43, 55]. Proteins were extracted in HENS buffer, followed for mitochondria by three freeze/thaw cycles in liquid nitrogen and vigorous vortexing. Lipid fragments were collected by centrifugation at 10,000 g, 4 °C, for 10 min. SNO occupancy was determined using the iodoacetyl Tandem Mass Tag (iodoTMT) labeling and enrichment kit (Thermo Fisher Scientific, Waltham/MA); see Fig. 3a and Suppl Fig. 7A for a depiction of the labeling protocol. All procedures were performed according to the manufacturer’s instructions. Labeled peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) that was performed using an Ultimate 3000 nano-LC system coupled to a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific) operated as described previously [32]. The Q-Exactive HF mass spectrometer was operated in a data-dependent mode with a Top20 strategy (i.e., one MS1 high-resolution [60,000]
scan for precursor ions followed by data-dependent MS2 scans for the 20 most abundant precursor ions with 10 s dynamic exclusion time of previously selected ions), with a scan range of MS acquisition from 350 to 1800 m/z. Secondary ions were isolated with a window of 2.0 m/z. The raw files generated by the Q-ExactHF mass spectrometer were analyzed with the Proteome Discoverer v1.3 software (Thermo Fisher Scientific). Peak lists were generated with the Mascot Daemon software (version 2.6.1 Matrix Science) using the extract_msn.exe data import filter (Thermo Fisher Scientific) from the Xcalibur FT package (version 2.0.7; Thermo Fisher Scientific). Protein abundance between groups was compared with the T fold test [11]. The mixOmics package for R (https://www.bioconductor.org/packages/release/bioc/html/mixOmics.html) was used for the PLS-DA and sPLS-DA analyses [65].
Exercise training increases eNOS level and phosphorylation, but does not modify nNOS, in the mitochondrial subfraction. a Schematic representation of the SSM isolation protocol. Heart lysates were fractionated by differential centrifugation. Centrifugation, homogenization, and enzymatic reactions were performed at 4 °C. b Representative western blots of the cytosolic and mitochondrial subfractions obtained with anti-GAPDH and anti-VDAC1 antibodies. c, d Effect of exercise training on eNOS level in the cytosolic and mitochondrial subfractions. e Representative western blots of eNOS level in the cytosolic and mitochondrial subfractions. d Quantification of eNOS level in the cytosolic (left panel) and mitochondrial (right panel) subfractions analyzed by western blotting in hearts from sedentary (Sed; n = 7 hearts in duplicate) and exercise trained (Ex-Tr; n = 6 hearts in duplicate) rats. eNOS level was expressed relative to GAPDH level in cytosolic samples and to VDAC1 level in mitochondrial samples. e, f Effect of exercise training on eNOS phosphorylation at serine 1177 (eNOS-Pser1177) in the cytosolic and mitochondrial subfractions. e Representative western blots of eNOS-Pser1177 in the cytosolic and mitochondrial subfractions. f Quantification of eNOS-Pser1177 level in the cytosolic (left panel) and mitochondrial (right panel) subfractions from Sed (n = 4 hearts in duplicate) and Ex-Tr (n = 4 hearts in duplicate) rats. eNOS-Pser1177 level was expressed relative to eNOS level. g, h Effect of exercise training on nNOS level in the cytosolic and mitochondrial subfractions. g Representative western blots of nNOS level in the cytosolic and mitochondrial subfractions. h Quantification of nNOS level in the cytosolic (left panel) and mitochondrial (right panel) subfractions from Sed (n = 4 hearts in duplicate) and Ex-Tr (n = 4 hearts in duplicate) rats. nNOS level was expressed relative to GAPDH level for cytosolic fractions and to VDAC1 level for mitochondrial fractions (*p < 0.05 Sed vs Ex-Tr, parametric t test). Data are the mean ± SEM.

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE [60] partner repository under the dataset identifiers PXD023515 and 10. 6019/PXD023515 [reviewers may access this private dataset using reviewer_pxd023515@ebi.ac.uk as Username and vmykdlpb as password] for the S-nitrosoproteomic analysis.

Western blotting

Immunoblotting was performed using standard techniques, as previously described [50].

Statistical analyses

Data were expressed as the mean ± SEM. Experimental conditions were compared with Student’s t test, analysis of variance (ANOVA), or repeated-measures ANOVA followed by the Turkey’s multiple comparisons test when data were normally distributed (confirmed with the Shapiro–Wilk normality test). When necessary, non-parametric tests were used: Mann–Whitney test for two groups, or Kruskal–Wallis test followed by the Dunn’s multiple comparisons test. A value of p < 0.05 was considered as statistically significant. Statistical analyses were done with the GraphPad Prism software (8.4.3).

Results

Exercise training increases eNOS level and activation in the mitochondrial subfraction

It is acknowledged that exercise training activates eNOS in the heart, a key trigger of exercise-induced cardioprotection [10, 23]. As cardiac ischemic pre-conditioning induces eNOS re-localization from the cytosol to mitochondria [75], we first asked whether exercise training had the same effect. To this aim, we isolated by differential centrifugation the cytosolic and mitochondrial subfractions from the heart of Sed and Ex-Tr (treadmill running for 45 min/day, 5 days/week for 5 weeks at 25 m/min, ≈ 60% of their MAV) rats (Fig. 1a), and confirmed their separation by western blotting with antibodies against VDAC1 (mitochondria marker) and GAPDH (cytosolic marker) (Fig. 1b). In the cytosolic fraction, eNOS level was comparable in Sed and Ex-Tr samples (Fig. 1c, d), as we previously reported using total heart homogenates [10, 23]. Conversely, eNOS level was significantly higher in Ex-Tr than Sed mitochondrial samples (Fig. 1c, d). Similarly, eNOS level was significantly increased only in the mitochondrial subfraction after a single bout of exercise (35 min) (mitochondrial eNOS normalized to cytosolic eNOS; Supplementary Fig. 1a), suggesting that eNOS re-localizes from the cytosol to mitochondria in response to exercise. In cardiomyocytes, there are two populations of mitochondria: subsarcolemmal (SSM) and intermyofibrillar (IFM) mitochondria. However, our protocol mainly allows the isolation of SSM. Therefore, we next evaluated whether exercise training also influenced eNOS level in the IFM-enriched fraction (containing both myofilaments and IFM). In this fraction, eNOS level was increased in response to exercise training (Supplementary Fig. 1c, d). This strengthened the hypothesis that in response to exercise, eNOS can re-localize from the cytosol to specific microdomains that contain mitochondria. As eNOS is activated by phosphorylation on serine 1177 (eNOS-Pser1177), we then evaluated the effect of exercise training on this phosphorylation. The level of eNOS-Pser1177 was significantly increased in the mitochondrial fraction of Ex-Tr compared with Sed rats, but not in the cytosolic fraction (Fig. 1e, f). We [23] and others [10] previously reported that exercise training does not have any impact on the level of neuronal NOS (nNOS), and another NOS isoform also expressed in heart. We confirmed this observation in the cardiac cytosolic and mitochondrial fractions (Fig. 1g, h). Altogether, these results indicate that eNOS association...
with mitochondria is increased in response to exercise training.

**Exercise training increases NO production in response to oxidative stress**

As exercise training increased eNOS activation and level in mitochondria, we next evaluated whether exercise training also promoted NO production in response to a stress that mimics IR in primary cardiomyocytes isolated from Ex-Tr and Sed rats. We first confirmed that eNOS re-localizes from the cytosol to mitochondria in Ex-Tr cardiomyocytes, but not in Sed cardiomyocytes (Supplementary Fig. 2a, b). Next, we used the NO-sensitive DAF2-DA fluorescent dye (5 µM) to measure intracellular NO every 10 s for 25 min in primary cardiomyocytes isolated from Ex-Tr and Sed rats before (basal conditions) and after addition or not of H₂O₂ (100 µM) to promote eNOS-dependent NO production [68] and to mimic IR-induced oxidative stress. We pre-incubated some cardiomyocytes with the NO synthase inhibitor L-NAME (100 µM) for 30 min before NO measurement to evaluate the contribution of NOS-dependent NO production. In basal conditions, DAF2-DA fluorescence intensity remained unchanged over time (Fig. 2a). Addition of H₂O₂ resulted in a progressive increase of the fluorescence signal in Ex-Tr and, to a lesser extent, also in Sed cardiomyocytes (Fig. 2a). In cardiomyocytes pre-incubated with L-NAME, this increase was reduced compared with cells without L-NAME, and consequently, fluorescence intensity was similar in Sed and Ex-Tr cardiomyocytes (Fig. 2a and Supplementary Fig. 3a). To determine whether NO production by cardiomyocytes upon exposure to H₂O₂ was localized in mitochondria, we used confocal microscopy to analyze cardiomyocytes stained with DAF2-DA (NO production marker) and with MitoTracker (mitochondrial stain). As before, we did not observe any difference between Ex-Tr and Sed cardiomyocytes in basal conditions (no H₂O₂). Conversely, DAF2-DA fluorescence intensity increased in Sed cells (+80%; p = 0.3) and particularly in Ex-Tr cells (+183%; p < 0.05) after H₂O₂ addition (Fig. 2b, c). Pre-incubation with L-NAME reduced DAF2-DA fluorescence intensity in both Sed and Ex-Tr cardiomyocytes (Supplementary Fig. 3b-c). It has been reported that in cardiomyocytes, NO-dependent DAF2-DA fluorescence signal is localized in mitochondria [19]. By merging the DAF2-DA and MitoTracker signals, we observed their colocalization in Sed and Ex-Tr cardiomyocytes (Fig. 2b, Supplementary Fig. 3d, e), with a good Pearson’s correlation coefficient (r² = 0.85 for Sed; r² = 0.89 for Ex-Tr). We next quantified DAF2-DA fluorescence intensity specifically in mitochondria (based on the MitoTracker signal). Upon exposure to H₂O₂, DAF2-DA fluorescence intensity (indicative of NO production) was higher in mitochondria of Ex-Tr than Sed cardiomyocytes (Fig. 2d). Altogether, these findings suggest that exercise training increases NO bioavailability in mitochondria of cardiomyocytes.

**Exercise training modifies the S-nitrosylation profile of mitochondrial protein.**

As eNOS/NO-dependent cardioprotection has been mostly attributed to protein SNO [74], we next used a proteomic approach to evaluate the impact of exercise training on the protein SNO profiles of total left ventricle (LV) homogenates and mitochondrial samples from Sed and Ex-Tr hearts. In this approach, the iodoTMT switch assay was followed by enrichment of TMT-labeled peptides using an anti-TMT resin, and shotgun proteomics by nano-LC–MS/MS (Fig. 3a). Using this redox proteomic approach, we identified 296 and 345 proteins with at least one TMT-labeled modification in total LV homogenates and mitochondrial samples, respectively. Among the 345 proteins identified in mitochondrial samples, 125 were mitochondrial proteins. Next, we compared the SNO profile of individual proteins in total LV homogenates and mitochondrial samples from Sed and Ex-Tr hearts. Volcano plots showed no major effect of exercise training on protein SNO in total LV homogenates (Fig. 3b). Conversely, the extent of protein SNO tended to be higher in mitochondrial subfractions from Ex-Tr rats, particularly for mitochondrial proteins (red dots) (Fig. 3c). We did not observe any clear separation between groups and cell subfractions by unsupervised partial least-squares discriminant analysis (PLS-DA). Therefore, we used a supervised PLS-DA and all proteins (345 proteins from 12 samples; 3 samples/subfraction/condition) to determine the separation between subfractions/conditions and identify the proteins the abundance of which contributed to separate the groups. This approach separated total LV homogenates and mitochondrial fractions along component 1, and Sed and Ex-Tr samples as well as total LV homogenates (TH) and mitochondria along component 2 (Fig. 3d). Moreover, the Sed and Ex-Tr mitochondrial fractions were separated along component 3 (Fig. 3e). We identified 20 proteins that contributed to explain the separation of the Sed and Ex-Tr mitochondrial subfractions along component 3 (Fig. 3e). This subgroup of proteins included eleven mitochondrial proteins and nine proteins that can directly interact with mitochondria. We then used another approach based on the S-nitrosylated proteins that were more abundant in mitochondria in response to exercise training. Using an optimized cut-off (fold change > 1.5, p value < 0.05), we identified five mitochondrial proteins, including two proteins already identified with the first method (connexin 43 and coiled-coil-helix-coiled-coil-helix domain containing 2, CHCHD2) (spectral counts in Fig. 3f). On the basis of its biological importance during IR and its trend to be more abundant in the Ex-Tr group...
(fold change: 2.33; \( p = 0.0572 \)), we also added cyclophilin D (PPIf) to this list of proteins. Proteins involved in the electron transport chain (ETC) regulation (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex 10-like1, NDUFA10; electron transfer flavoprotein-ubiquinone oxidoreductase, ETFDH; CHCHD2, and connexin 43), in mitochondrial \( \beta \)-oxidation (acyl-CoA dehydrogenase, long chain), in the TCA cycle (malate dehydrogenase 2), in calcium homeostasis (ryanodine receptor 2 and connexin 43), in the regulation of energetic metabolism (AMPK\( \alpha \)1), and in the regulation of mPTP activation (cyclophilin D) explained the differences in protein SNO profile between Sed and Ex-Tr mitochondria.

**Exercise training prevents \( \text{Ca}^{2+} \)-induced mPTP opening: a major role for \( S \)-nitrosylation**

As exercise training affected the SNO profile of key proteins involved in ETC and in cardioprotective signaling...
A. S-nitrosoproteome

Cardiac left ventricle (LV)

1. Protein extraction

2. Differential centrifugation

3. Mitochondrial enriched fraction

4. Total LV homogenate

B. LV total homogenates

C. LV mitochondria

D. sPLS-DA on TMT-TH&Mito, comp 1 & 2

E. sPLS-DA on TMT-TH&Mito, comp 1 & 3

F. Spectral counts
Exercise training reduces mitochondrial ROS production: a major role for S-nitrosylation

The activation of mPTP during IR depends on a complex interplay betweenCa^{2+} overload and mtROS production [27]. According to a previous study, SNO of mitochondrial proteins contributes to limit mtROS production during IR [16]. Thus, we asked whether exercise training modulated mtROS production and whether SNO had a role. For this, we loaded cardiomyocytes with MitoSOX Red (5 µM), a sensitive mtROS probe [2], and incubated them or not with ascorbate or L-NAME (to evaluate the eNOS/NO/SNO pathway implication in mtROS production) for 30 min. Then, we stimulated cells with antimycin A (10 µM), a mitochondrial electron transport chain complex III blocker, to force mtROS production before determining the fluorescence intensity every 20 s for 15 min (Fig. 5a) [7]. In both Sed and Ex-Tr cardiomyocytes, MitoSOX Red fluorescence intensity increased progressively for 15 min after antimycin A addition, and mtROS production tended to be higher in Ex-Tr than Sed cardiomyocytes (not significant) (Fig. 5a, b). Incubation with ascorbate or L-NAME significantly increased mtROS production in Ex-Tr cardiomyocytes, but not in Sed cardiomyocytes (Fig. 5a, b). This suggested that the increased NO bioavailability in cardiomyocytes in response to exercise training contributes to modulate mtROS production during stress. Next, we performed global IR in perfused isolated hearts to evaluate the impact of exercise training on ROS production (Fig. 5c). As mitochondrial oxidative stress is a key trigger of cardiac injuries and dysfunction [9], we measured, using the DHE fluorescent dye, ROS production during early reperfusion in hearts from Sed and Ex-Tr rats. In line with the previous studies [23], post-IR ROS production was lower in Ex-Tr than Sed hearts (Fig. 5d). When we perfused hearts with MitoTEMPO (0.1 µM), a specific mtROS scavenger, at a concentration previously used to limit cardiac mtROS production [7, 54], we found that the difference between Sed and Ex-Tr hearts was abolished (Supplementary Fig. 6). This suggests that mtROS contributes mainly to the difference observed between Sed and Ex-Tr hearts. To evaluate the role of the eNOS/NO/SNO pathway in ROS production during early reperfusion, we perfused Sed and Ex-Tr hearts with L-NAME (100 µM) for
Fig. 4 Effect of exercise training on mitochondrial calcium retention capacity and mPTP activation: a key role for S-nitrosylation. 

a–c Calcium retention capacity (CRC) in mitochondria from Sed (15 experiments from n = 5 hearts) and Ex-Tr (12 experiments from n = 4 hearts) hearts was monitored using the Calcium Green-5N probe (Em: 507 nm; Ex: 536 nm). a, b Mean traces of the CRC tests. Each peak corresponds to calcium addition (1 µM, 5 µM, and 50 µM pulses of CaCl₂; changes in concentration are represented by arrows). Black curve, representative trace in the presence of 2 µM cyclosporine A (CsA; 2 µmol/L). CRC was tested in the absence (a) or presence (b) of sodium L-ascorbate (1 mmol/L) in mitochondria obtained from Sed (9 experiments from n = 3 hearts) and Ex-Tr (12 experiments form n = 4 hearts) hearts (*p < 0.05 Sed vs Ex-Tr, repeated two-way ANOVA followed by Sidak’s multiple comparisons test). c Quantification of CRC (nmol Ca²⁺ per mg protein); *p < 0.05, Sed vs Ex-Tr (non-parametric Kruskal–Wallis test followed by Dunn’s multiple comparisons test). d Mitochondrial swelling assay in mitochondria from Sed and Ex-Tr hearts. Mitochondrial swelling was induced by adding Ca²⁺ (250 µM) to mitochondria isolated from Sed (n = 6) and Ex-Tr (n = 5) hearts incubated or not with sodium L-ascorbate (1 mmol/L). Absorbance was monitored at 540 nm every 30 s (*p < 0.05 Sed vs Ex-Tr; £p < 0.05 with ascorbate vs without ascorbate; repeated two-way ANOVA followed by Turkey’s multiple comparisons test). e Representative CRC traces in mitochondria pre-treated or not with 500 µM of GSNO. f Quantification of CRC in the presence (12 experiments, n = 4 hearts) or absence (9 experiments, n = 3 hearts) of GSNO (*p < 0.05 GSNO (+) vs GSNO (−); non-parametric Mann–Whitney test). Data are the mean ± SEM.
20 min before ischemia and during early reperfusion. We previously reported that L-NAME perfusion results in the loss of exercise-induced cardioprotection in rats [23]. Here, we first confirmed, using the iodoTMT switch assay (Supplementary Fig. 7a) and western blot analysis, that L-NAME resulted in lower protein SNO levels in SSM isolated from Sed and Ex-Tr hearts (Supplementary Fig. 7b, c), as previously described [40]. In these conditions, we then measured ROS production by monitoring DHE fluorescence. L-NAME markedly increased (+48%) ROS production in Ex-Tr hearts during early reperfusion, but not in Sed hearts (−3%) (Fig. 5d), indicating that NO is essential to blunt ROS production during post-ischemic reperfusion in exercised hearts.

Given the protective role of protein SNO during early reperfusion, we next evaluated whether N6022 (10 μM), a specific and potent GSNO reductase inhibitor that increases protein SNO level [12], could limit ROS production during early reperfusion (Fig. 5e). In line with results obtained after exercise training in rats, N6022 significantly reduced the cardiac DHE fluorescence signal after 10 min of post-ischemic reperfusion (Fig. 5f), indicating lower post-ischemic ROS production.

Finally, to confirm in vivo our in vitro and ex vivo results, we performed cardiac IR in Sed and Ex-Tr rats treated or not with L-NAME (50 mg/kg i.p.) 24 h and 30 min before IR, because it has been reported that L-NAME decreases NO bioavailability [3] and mitochondrial protein SNO [40]. We then compared their ECG profiles throughout IR. Before ischemia, we did not observe any ECG difference between groups (Fig. 6a, b). Ischemia resulted in an elevation of the ST segment and prolongation of the QTc interval, compared with baseline, in all groups (Fig. 6a). After 10 min of reperfusion, the QTc interval was significantly prolonged by 19% in Sed animals (p < 0.05; t test) compared with baseline, and also in Sed animals treated with L-NAME (by 17.8%; not significant; p = 0.1). We did not observe any difference between Sed animals treated or not with L-NAME (Fig. 6c). In Ex-Tr animals, the QTc interval was only prolonged by 3%, and after 10 min of reperfusion, and was lower than in Sed animals (Fig. 6a–c). On the other hand, in Ex-Tr animals treated with L-NAME, the QTc interval was prolonged by 11% after 10 min of reperfusion (p = 0.06 compared with baseline), and was significantly higher than in untreated Ex-Tr animals (Fig. 6a–c). We next evaluated whether our experimental conditions influenced mitochondrial permeability transition during reperfusion. To this aim, we stained cardiac tissues with calcein-cobalt after 10 min of reperfusion, the QTc interval was only prolonged by 11% after 10 min of reperfusion (p = 0.06 compared with baseline), and was significantly higher than in untreated Ex-Tr animals (p = 0.08 vs untreated), indicative of increased mitochondrial permeability transition (Suppl Fig. 8). This suggests that NO could contribute to modulate mPTP activation during early reperfusion and that exercise training contributes, although moderately, to this effect. As in isolated hearts eNOS–NO–SNO signaling influenced ROS production during early reperfusion only in Ex-Tr hearts, we then measured ROS production in the ischemic area using the DHE fluorescent dye. In line with results obtained in isolated hearts, we found that L-NAME treatment did not influence ROS production in Sed animals, but strongly increased its production in Ex-Tr rat hearts (Fig. 6d). Altogether, these results show that in exercised hearts, NO–SNO signaling plays a key role in limiting excessive mtROS production during early reperfusion.

**Discussion**

Our results clearly support the hypothesis that the eNOS/NO/SNO pathway plays a key role in the exercise ability to protect mitochondria during IR. Specifically, we found that exercise training (1) increases NO bioavailability in mitochondria, (2) results in SNO of key proteins involved in the mitochondrial response to stress, and (3) modulates Ca2+-dependent mPTP opening and ROS production in conditions that mimic IR. Collectively, these results provide additional insights to better understand eNOS role in exercise-induced cardioprotection.

There is now considerable evidence, indicating that eNOS/NO signaling plays a central role in exercise-induced cardioprotection during IR [10, 23]. This eNOS-dependent cardioprotection is mediated through the increase of NO metabolite storage (i.e., nitrite and S-nitrosothiols) before ischemia and higher protein SNO during early reperfusion [23, 61]. However, the underlying mechanisms and the identification of specific SNO sites remain limited. Here, we showed that mitochondria constitute a privileged target of NO-dependent cardioprotective signaling in response to exercise training. Mitochondria are end-effectors of cell death, and many cardioprotective signaling mechanisms converge to mitochondria [52]. In line with our results, a previous study showed that in response to ischemic pre-conditioning, eNOS can translocate from the cytosol to mitochondria and increase the level of protein SNO [75]. However, the underlying mechanisms were not elucidated. The interactions between caveolae, caveolin-3, and eNOS could play a key role. Indeed, in a model of cardiac ischemic pre-conditioning, disruption of caveolae with a cholesterol sequestering agent [72] or caveolin-3 gene deletion [75] abolished eNOS translocation to mitochondria and the subsequent increase in protein SNO. Interestingly, both caveolae and caveolin-3 are considered stretch-sensitive elements in cardiomyocytes. As exercise increases the mechanical workload...
and results in myocardial stretch, it could constitute a key trigger of eNOS association with mitochondria. Nevertheless, additional studies are necessary to better understand this effect. Adrenergic stress also could trigger eNOS activation and translocation. Indeed, in mice in which the gene encoding β3-adrenergic receptor was ablated, exercise training does not activate eNOS and the exercise cardioprotective effect is lost [10]. Upon re-localization to mitochondria, eNOS certainly does not enter mitochondria, because it does not harbor the transport sequence required for passing through the inner mitochondrial membrane [38]. However, eNOS can dock on the outer mitochondrial membrane, where it constitutes a NO source to regulate mitochondrial respiration [25].

In cardiomyocytes, there are two populations of mitochondria (SSM and IFM). It was previously reported that
in response to ischemic pre-conditioning, eNOS translocates preferentially to SSM [75]. Here, we found that in response to exercise training, eNOS re-localizes both to the SSM and IFM + myofilament fractions. These results are supported by the increased NO production in mitochondria of isolated Ex-Tr cardiomyocytes. We also showed that both SSM (Fig. 4a–c) and IFM (Supplementary Fig. 5c–e) were protected by exercise training and were sensitive to denitrosylation by ascorbate. This highlights the key role of eNOS/NO/SNO signaling in this effect. However, in line with previous work [75], SNO role seemed to be more obvious in SSM than IFM. Although SSM represent only about 10% of all cardiac mitochondria, they are more vulnerable to IR and are the first to be affected in IR injuries [56, 75]. Specifically, SSM might be more sensitive to calcium overload and alterations of oxidative phosphorylation than IFM [48, 63]. In addition, the enzymatic antioxidant system is altered by IR in cardiac SSM but not in IFM [26, 37]. The fact that the junction protein connexin 43 is only present in SSM and is markedly targeted by SNO in Ex-Tr hearts might contribute to explain eNOS/NO key role. Connexin 43 has been identified in the sarclemma and also in mitochondria of cardiomyocytes, and is mainly located in the inner mitochondrial membrane of SSM. Its role in mitochondria remains controversial. Mitochondrial connexin 43 influences the mitochondrial respiratory function, ETC [4], ATP synthesis, and ROS production [64]. Connexin 43 also plays a key role in the modulation of cardiac IR injuries [64]. Indeed, mitochondrial CRC is reduced by blocking connexin 43 with the specific inhibitor Gap27 [71]. Moreover, protection by ischemic pre-conditioning is lost in cardiomyocytes and hearts of heterozygous connexin 43-deficient mice [29]. It has been proposed that SNO of mitochondrial connexin 43 upon ischemic pre-conditioning might explain the link between NO and connexin 43 in the signal transduction cascade of cardioprotective interventions [70]. This in line with our results, showing that in response to exercise training, increased CRC of mitochondria is associated with increased SNO of connexin 43. However, the underlying mechanisms are not clear. Indeed, Soetkamp et al. [70] demonstrated in isolated mitochondria that increased SNO of connexin 43 by a NO-donor increases mtROS production. This is in line with our results, showing that mtROS production in response to antimycin A tended to be higher in Ex-Tr cardiomyocytes. However, in our study, the use of L-NNAME to block NO production or of ascorbate to decompose SNO, unmasked higher mtROS production in Ex-Tr cells compared with Sed cardiomyocytes, showing that in Ex-Tr cells NO rather contributes to limit mtROS production. Interestingly, IR is associated with increased connexin 43 oxidation that is attenuated by ischemic pre-conditioning [67]. As we did not investigate whether SNO of connexin 43 contributes to prevent its irreversible oxidation, additional studies are needed to better understand the link between SNO of connexin 43, mtROS, and cardioprotection.

These results associated with the finding that IFM also were altered in the Ex-Tr group may suggest that SNO of connexin 43 alone is not sufficient to explain the cardioprotective effect of exercise training. Indeed, in line with previous studies using ischemic pre-conditioning or a NO-donor to protect the myocardium during IR [15, 16, 75], we found that several key proteins involved in cardiac injury during IR are S-nitrosylated in response to exercise training. Among the biological processes sensitive to NO level during reperfusion, excessive ROS production by the mitochondrial ETC is attenuated by ischemic pre-conditioning and NO donors [16]. Interestingly, it has been suggested that SNO of the NADH dehydrogenase 3 (ND3) subunit of mitochondrial complex I, which is considered as the key trigger of ROS overproduction during IR, could explain the cardioprotective effect of mitoSNO, a NO-donor that targets mitochondria [16]. Here, we did not identify the ND3 subunit among the proteins S-nitrosylated in response to exercise training, but we observed that several ETC proteins were S-nitrosylated, including NDUFA10 (a complex I subunit), ETFDH, and CHCHD2 that connects metabolism to...
apoptosis [44]. In agreement, pre-incubation with ascorbate or with L-NAME increased mtROS production in Ex-Tr but not in Sed cardiomyocytes. Similarly, perfusion of isolated hearts or treatment of animals with L-NAME before IR increased cardiac ROS production during early reperfusion, suggesting that NO–SNO-mediated regulation of key ETC proteins can contribute to limit ROS overproduction during IR. Among the other proteins that could explain exercise training cardioprotection, we also identified SNO of cyclophilin D. This protein is a key regulator of mPTP and highly redox-sensitive. During ischemia, the rise in mitochondrial calcium leads to cyclophilin D deacylation, its oxidation, and mPTP activation [1, 53]. SNO of cyclophilin D might contribute to cardioprotection by inhibiting its oxidation on cysteine 202 [1, 53]. In agreement, we found that exercise training increased cyclophilin D SNO on this cysteine residue, which was associated with the delayed activation of mPTP in response to Ca\(^{2+}\).

Our in vitro and ex vivo results support the hypothesis that eNOS re-localization to mitochondria explains the effect of exercise training on mtROS and mPTP opening during IR. In line, the eNOS-NO pathway plays a key role...
in exercise-induced cardioprotection [23]. Indeed, in animals that lack the gene encoding eNOS [10, 28] or the gene encoding β3-adrenergic receptor [10] and in which exercise-induced phosphorylation (and thus activation) of eNOS is abolished, exercise training does not reduce myocardial infarct size. This is also observed when eNOS is pharmacologically inhibited [23]. Using the QTc interval as an index of heart sensitivity to IR, we confirmed in vivo that exercise training is associated with cardioprotection and that its beneficial effect is abolished by pre-treatment with L-NAME to reduce NO production and SNO level [40]. We also confirmed in vivo results we obtained in cardiac cells and isolated hearts showing that the eNOS-NO pathway plays a key role in ROS modulation during IR. Our results on mPTP activation are less clear-cut. Indeed, exercise delayed mPTP activity in isolated mitochondria. Conversely, in vivo we did not observe any difference of mitochondrial permeability after 10 min of reperfusion between Sed and Ex-Tr hearts. The effect of exercise training on mPTP was previously described in isolated mitochondria [46] and cardiac tissue after 45 min of reperfusion of isolated hearts [39]. Such discrepancy could be explained by the time when we measured mitochondrial permeability. Indeed, Panel et al. [58], using the same method (i.e., calcein-cobalt staining) in isolated cardiomyocytes subjected to anoxia reoxygenation, found that approximately 25 min are necessary to observe marked alterations of mitochondrial permeability. In addition, considering the key role of ROS on mPTP activation, we can also hypothesize that the effects of exercise on mPTP are secondary to the modulation of ROS production and, consequently, are observed later. More studies are necessary to precisely understand the impact of exercise training on mPTP activation during IR. Nevertheless, our in vivo results support the key role of NO in the modulation of mPTP activation during reperfusion, because L-NAME pre-treatment tended to reduce calcein fluorescence in Sed and slightly more in Ex-Tr hearts. This is in line with previous studies, showing that incubation of isolated mouse fibroblasts with GSNO improves mitochondrial CRC in conditions mimicking reperfusion [53] and treatment with the mitochondrial NO-donor mitoSNO can protect the mouse heart during IR [16]. Altogether, these results reinforce the hypothesis that the NO–SNO pathway plays a key role in protecting mitochondria during IR, and help to understand that the re-localization of this pathway to mitochondria following exercise training contributes to the exercise-induced cardioprotection.

**Study limitations**

This study has some limitations. The first limitation concerns the purity of our mitochondrial preparations. As SNO is a very labile post-translational protein modification [75], we did not isolate mitochondrial subfractions using Percoll gradient ultracentrifugation. Indeed, mitochondrial proteins can become oxidized during isolation of mitochondria using a discontinuous Percoll gradient [14]. Therefore, we cannot rule out the possibility that the SNO of other proteins also contribute to modulate mPTP activation and ROS production in mitochondria. Indeed, we also observed the SNO of key proteins involved in calcium homeostasis (RyR2) and in the cell metabolic response (AMPKα1). The list of all proteins in which SNO is influenced by exercise training is available in the online Suppl Table 1. We quantitatively evaluated the purity of our mitochondrial preparations after shotgun proteomics by comparing the summed abundance of all mitochondrial proteins, identified using MitoMiner (mitominer.mrc-mbu/cam.ac.uk), to the total protein abundance. We found a mitochondrial protein enrichment of 81.8 ± 0.021%, with a variation coefficient of 2.7% between samples. The second limitation concerns the isolation of IFM that requires the use of proteases, leading to artifacts in mitochondrial protein quantification [36]. This can be particularly important for eNOS, because this protein is docked to the outer mitochondrial membrane through a protease cleavable peptide [25]. Therefore, we chose to evaluate eNOS level in the IFM + myofilament fraction to avoid the use of proteases. The results we obtained by western blotting were supported by the increased NO production, measured by DAF2-DA fluorescence, in the IFM from isolated Ex-Tr cardiomyocytes. However, when IFM were isolated using protease (i.e., nargase) to evaluate the CRC, we observed that NO–SNO signaling appeared to be less evident compared with SSM. Thus, more studies are needed to thoroughly investigate eNOS translocation to IFM and its functional consequences.

Overall, our findings suggest that several key mitochondrial proteins involved in cardiac injuries during IR are S-nitrosylated in response to exercise training. Collectively, these results provide additional insights to better understand eNOS role in exercise-induced cardioprotection and bring evidence to support the hypothesis that cardioprotection by exercise training is explained by the re-localization of eNOS/NO/SNO cardioprotective signaling to mitochondria (Fig. 7). Additional studies are needed to better understand how each specific target contributes to limit mitochondrial ROS production and mPTP activation during IR.
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**Author contributions** Conception, hypothesis definition, and study design: CR, GM, GC, JA, and DB; data acquisition, analysis, and interpretation: DB, MD, SG, JCG, BA-B, and CR; article writing, DB and CR.

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**Availability of data and materials (data transparency)** Proteomic data are available via ProteomeXchange with the dataset identifier PXD023515.

**Declarations**

**Conflict of interest** The authors have no conflict of interest.

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**Fig. 7** Increased protein S-nitrosylation in mitochondria: a key mechanism of exercise-induced cardioprotection. During reperfusion, in sedentary hearts, the rapid re-establishment of OX-PHOS and mitochondrial membrane potential associated with mitochondrial Ca²⁺ overload results in a strong rise in ROS production in mitochondria. A complex interplay between ROS production and Ca²⁺ overload triggers mPTP opening, leading to mitochondrial swelling and disruption, and cell death. Exercise training increases eNOS association with mitochondria, leading to increased mitochondrial NO bioavailability. This results in SNO of key proteins involved in the mitochondrial respiratory function, electron transport, and regulation of mPTP opening. This contributes to blunt the interplay between ROS production and Ca²⁺ overload that triggers mPTP opening during ischemia–reperfusion. Altogether, these results support the hypothesis that cardioprotection by exercise training is explained by the re-localization of eNOS/NO/SNO cardioprotective signaling to mitochondria.
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