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 H_2O_2 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 Ca^{2+} 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.

1. 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 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 **i)** increased eNOS/NO bioavailability in mitochondria, and consequently **ii)** modulated Ca^{2+}-dependent mitochondrial permeability transition and ROS production during IR.

2. MATERIALS AND METHODS

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

2.1. Animal model

All animal experiments were performed according to the European Parliament Directive 2010/63/EU (N° CEEA-00223) and approved by the French national research ethics committee (n°APAFIS#3487-2016010813443457). Male Wistar rats (12-week-old; weight = 225–275g, 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; $p < 0.05$).

2.2. Exercise protocol

Exercise training

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 25m/min (≈60% of their MAV). To avoid the acute effects of exercise, rats were sacrificed 24h after the last exercise training session.

2.3. 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 24h 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 minutes 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: $QTc = QT + 0.154 \times [1-RR]$. 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, 10µM, Caymal Chemical) for 25 min.

Measurements of ROS production using the DHE fluorescent dye

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

2.4. 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 (5µM, Ex: 510 nm; Em: 580 nm; Fisher Scientific, Invitrogen, M36008)[24], with or without L-NAME (100µM) or sodium L-ascorbate (1mM). Then, they were immediately transferred to a microplate reader (Synergy HT Microplate Spectrophotometer Biotek). Cells were stimulated with 10 µM of antimycin A (10 µM, 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; 5µM; Ex: 491nm, Em: 513nm, Abcam; ab145283) at 37°C for 15min. 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).

2.5. 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 Ca^{2+} indicator Calcium Green-5N (1µM, Invitrogen, C3737) as extramitochondrial calcium probe. Mitochondria were incubated or not with sodium L-ascorbate (1mM) to evaluate SNO impact [75], and with the NO donor GSNO (500µM). 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. C*alcium-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 $CaCl_2$ (250µM) to mitochondria, pre-incubated or not with ascorbate (1mM). Absorbance at 540 nm was recorded continuously for 25 min.

2.6. 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 10,000g, 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 *Figure 3A* and Suppl *Figure 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 twenty most abundant precursor ions with 10s 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-Exactive HF 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].

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.

2.7. Western blotting

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

2.8. Statistical analyses

Data were expressed as the mean ± SEM. Experimental conditions were compared with the 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).

3. RESULTS

3.1. 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 25m/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 (35min) (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-$P^{ser1177}$), we then evaluated the effect of exercise training on this phosphorylation. The level of eNOS-$P^{Ser1177}$ 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), 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.

3.2. 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 (5µM) to measure intracellular NO every 10s for 25 min in primary cardiomyocytes isolated from Ex-Tr and Sed rats before (basal conditions) and after addition or not of H_2O_2 (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 (100µM) 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 H_2O_2 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 H_2O_2 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 H_2O_2). Conversely, DAF2-DA fluorescence intensity increased in Sed cells (+80%; p=0.3) and particularly in Ex-Tr cells (+183%; $p<0.05$) after H_2O_2 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 H_2O_2, 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.

3.3. Exercise training modifies the S-nitrosylation profile of mitochondrial proteins.

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 nanoLC-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 β-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 (AMPKα1), and in the regulation of mPTP activation (cyclophilin D) explained the differences in protein SNO profile between Sed and Ex-Tr mitochondria.

3.4. Exercise training prevents 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 pathways, we next investigated whether exercise training also influenced mPTP activation in response to mitochondrial Ca^{2+} overload. For this, we performed a mitochondrial CRC test in isolated mitochondria. We first confirmed that our test was well designed to evaluate mPTP activation by showing that incubation with cyclosporine A, an inhibitor of mPTP activation, prevented the progressive increase in Ca^{2+} green-5N fluorescence in response to cumulative Ca^{2+} pulses compared with untreated mitochondria from Sed and Ex-Tr hearts (Fig. 4a). As previously published [45, 46], we found that the CRC was higher in isolated mitochondria from Ex-Tr than Sed hearts (Fig. 4a). Next, to evaluate SNO role in mitochondrial CRC, we pre-incubated isolated mitochondria with ascorbate (1mM), a reducing agent that decomposes SNO [31]. Ascorbate had only a moderate effect on CRC in mitochondria from Sed hearts, but strongly reduced it in mitochondria from Ex-Tr hearts (Fig. 4b-c). Consequently, the CRC differences between Sed and Ex-Tr mitochondria (Fig. 4a) were abolished following pre-incubation with ascorbate (Fig. 4c). We confirmed these results by assessing CRC in mitochondria isolated from hearts perfused or not with L-NAME (100 µM), a NOS inhibitor, for 25 min (Supplementary Fig. 4a-c), thus validating NO role in the increased CRC in mitochondria of Ex-Tr hearts. To further confirm protein SNO role in mitochondria, we evaluated mPTP opening by monitoring the rapid inflow of solutes in mitochondria and their subsequent swelling through absorbance measurement in response to exposure to a single high concentration of Ca^{2+} (250µM). Consistent with our previous results, mitochondrial swelling (i.e. the decrease of absorbance) was lower in mitochondria of Ex-Tr than Sed hearts (Fig. 4d). Pre-incubation with ascorbate further increased mitochondrial swelling in both Sed and Ex-Tr mitochondria and abolished the difference between these groups (Fig. 4d). To further confirm NO effect on mitochondrial CRC, we evaluated CRC in mitochondria incubated or not with the NO donor GSNO (500 µM). GSNO increased mitochondrial CRC (Fig. 4e-f). Altogether, these results confirm that exercise training has a beneficial impact on the mitochondrial response to increased Ca^{2+} load as observed during IR, and highlight protein SNO key role in this effect.

3.5. Exercise training reduces mitochondrial ROS production: a major role for S-nitrosylation.

The activation of mPTP during IR depends on a complex interplay between Ca^{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 20s for 15min (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 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 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 (50mg/kg i.p.) 24h 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 minutes of reperfusion. We did not observe any significant difference between Sed and Ex-Tr hearts (Suppl Fig. 8). However, we found that L-NAME treatment tended to reduce calcein fluorescence in Sed (-14.3%; $p=0.18$)

and also in Ex-Tr hearts (-20.1%; p=0.08 vs untreated), indicative of increased mitochondrial permeability transition (Suppl Figure 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.

4. 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 **i)** increases NO bioavailability in mitochondria, **ii)** results in SNO of key proteins involved in the mitochondrial response to stress, and **iii)** modulates Ca^{2+}-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 subfractions. 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 sarcolemma 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-NAME 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 oxidization, 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][10]. 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* the 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 minutes 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 minutes 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 minutes 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 contribute 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 oxidated 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 artefacts in mitochondrial protein quantification [36]. This can be particularly important for eNOS because this protein is docked to the outer mitochondrial membrane through a

proteinase 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 proteinase (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 (Figure 7). Additional studies are needed to better understand how each specific target contributes to limit mitochondrial ROS production and mPTP activation during IR.

5. DECLARATIONS

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5.2. Conflicts of interest/Competing interests

The authors have no conflict of interest.

5.3. Availability of data and material (data transparency)

Proteomic data are available via ProteomeXchange with the dataset identifier with identifier PXD023515.

5.5. Authors' contributions:

Conception, hypothesis definition, and study design, C.R., G.M., G.C., J.A., D.B.; data acquisition, analysis, and interpretation D.B., M.D., S.G., J.C.G., B.A-B., C.R.; article writing, D.B., C.R.

6. REFERENCES

1. Amanakis G, Sun J, Fergusson MM, McGinty S, Liu C, Molkentin JD, Murphy E Cysteine 202 of cyclophilin D is a site of multiple post-translational modifications and plays a role in cardioprotection. Cardiovasc Res. doi: 10.1093/cvr/cvaa053

2. Andersson DC, Fauconnier J, Yamada T, Lacampagne A, Zhang S-J, Katz A, Westerblad H (2011) Mitochondrial production of reactive oxygen species contributes to the β-adrenergic stimulation of mouse cardiomycytes. J Physiol 589:1791–1801. doi: 10.1113/jphysiol.2010.202838

3. Bassot A, Chauvin M-A, Bendridi N, Ji-Cao J, Vial G, Monnier L, Bartosch B, Alves A, Cottet-Rousselle C, Gouriou Y, Rieusset J, Morio B (2019) Regulation of Mitochondria-Associated Membranes (MAMs) by NO/sGC/PKG Participates in the Control of Hepatic Insulin Response. Cells 8:E1319. doi: 10.3390/cells8111319

4. Boengler K, Ruiz-Meana M, Gent S, Ungefug E, Soetkamp D, Miro-Casas E, Cabestrero A, Fernandez-Sanz C, Semenzato M, Lisa FD, Rohrbach S, Garcia-Dorado D, Heusch G, Schulz R, Mercola M (2012) Mitochondrial connexin 43 impacts on respiratory complex I activity and mitochondrial oxygen consumption. J Cell Mol Med 16:1649–1655. doi: 10.1111/j.1582-4934.2011.01516.x

5. Bøtker HE, Cabrera-Fuentes HA, Ruiz-Meana M, Heusch G, Ovize M (2020) Translational issues for mitoprotective agents as adjunct to reperfusion therapy in patients with ST-segment elevation myocardial infarction. J Cell Mol Med 24:2717–2729. doi: 10.1111/jcmm.14953

6. Boulghobra D, Coste F, Geny B, Reboul C (2020) Exercise training protects the heart against ischemia-reperfusion injury: A central role for mitochondria? Free Radic Biol Med 152:395–410. doi: 10.1016/j.freeradbiomed.2020.04.005

7. Boulghobra D, Grillet P-E, Laguerre M, Tenon M, Fauconnier J, Fança-Berthon P, Reboul C, Cazorla O (2020) Sinapine, but not sinapic acid, counteracts mitochondrial oxidative stress in cardiomyocytes. Redox Biol 34:101554. doi: 10.1016/j.redox.2020.101554

8. Burwell LS, Brookes PS (2007) Mitochondria as a Target for the Cardioprotective Effects of Nitric Oxide in Ischemia–Reperfusion Injury. Antioxid Redox Signal 10:579–600. doi: 10.1089/ars.2007.1845

9. Cadenas S (2018) ROS and redox signaling in myocardial ischemia-reperfusion injury and cardioprotection. Free Radic Biol Med 117:76–89. doi: 10.1016/j.freeradbiomed.2018.01.024

10. Calvert John W., Condit Marah E., Aragón Juan Pablo, Nicholson Chad K., Moody Bridgette F., Hood Rebecca L., Sindler Amy L., Gundewar Susheel, Seals Douglas R., Barouch Lili A., Lefer David J. (2011) Exercise Protects Against Myocardial Ischemia–Reperfusion Injury via Stimulation of β3-Adrenergic Receptors and Increased Nitric Oxide Signaling: Role of Nitrite and Nitrosothiols. Circ Res 108:1448–1458. doi: 10.1161/CIRCRESAHA.111.241117

11. Carvalho PC, Yates JR, Barbosa VC (2012) Improving the TFold test for differential shotgun proteomics. Bioinformatics 28:1652–1654. doi: 10.1093/bioinformatics/bts247

12. Casin KM, Fallica J, Mackowski N, Veenema RJ, Chan A, St. Paul A, Zhu G, Bedja D, Biswal S, Kohr MJ (2018) S-Nitrosoglutathione Reductase Is Essential for Protecting the Female Heart from Ischemia-Reperfusion Injury. Circ Res 123:1232–1243. doi: 10.1161/CIRCRESAHA.118.313956

13. Cazorla O, Szilagyi S, Le Guennec J-Y, Vassort G, Lacampagne A (2005) Transmural stretch-dependent regulation of contractile properties in rat heart and its alteration after myocardial infarction. FASEB J 19:88–90. doi: 10.1096/fj.04-2066fje

14. Chang AHK, Sancheti H, Garcia J, Kaplowitz N, Cadenas E, Han D (2014) Respiratory Substrates Regulate S-Nitrosylation of Mitochondrial Proteins through a Thiol-Dependent Pathway. Chem Res Toxicol 27:794–804. doi: 10.1021/tx400462r

15. Chouchani ET, James AM, Methner C, Pell VR, Prime TA, Erickson BK, Forkink M, Lau GY, Bright TP, Menger KE, Fearnley IM, Krieg T, Murphy MP (2017) Identification and quantification of protein S-nitrosation by nitrite in the mouse heart during ischemia. J Biol Chem 292:14486–14495. doi: 10.1074/jbc.M117.798744

16. Chouchani ET, Methner C, Nadtochiy SM, Logan A, Pell VR, Ding S, James AM, Cochemé HM, Reinhold J, Lilley KS, Partridge L, Fearnley IM, Robinson AJ, Hartley RC, Smith RAJ, Krieg T, Brookes PS, Murphy MP (2013) Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. Nat Med 19:753–759. doi: 10.1038/nm.3212

17. Costa ADT, Pierre SV, Cohen MV, Downey JM, Garlid KD (2008) cGMP signalling in pre- and post-conditioning: the role of mitochondria. Cardiovasc Res 77:344–352. doi: 10.1093/cvr/cvm050

18. Costa Alexandre D.T., Garlid Keith D., West Ian C., Lincoln Thomas M., Downey James M., Cohen Michael V., Critz Stuart D. (2005) Protein Kinase G Transmits the Cardioprotective Signal From Cytosol to Mitochondria. Circ Res 97:329–336. doi: 10.1161/01.RES.0000178451.08719.5b

19. Dedkova EN, Blatter LA (2009) Characteristics and function of cardiac mitochondrial nitric oxide synthase. J Physiol 587:851–872. doi: 10.1113/jphysiol.2008.165423

20. Doulias P-T, Tenopoulou M, Greene JL, Raju K, Ischiropoulos H (2013) Nitric Oxide Regulates Mitochondrial Fatty Acid Metabolism through Reversible Protein S-Nitrosylation **. Sci Signal 6:rs1. doi: 10.1126/scisignal.2003252

21. Ejlersen H, Andersen ZJ, von Euler-Chelpin MC, Johansen PP, Schnohr P, Prescott E (2017) Prognostic impact of physical activity prior to myocardial infarction: Case fatality and subsequent risk of heart failure and death. Eur J Prev Cardiol 24:1112–1119. doi: 10.1177/2047487317702046

22. Erusalimsky JD, Moncada S (2007) Nitric oxide and mitochondrial signaling: from physiology to pathophysiology. Arterioscler Thromb Vasc Biol 27:2524–2531. doi: 10.1161/ATVBAHA.107.151167

23. Farah C, Kleindienst A, Bolea G, Meyer G, Gayrard S, Geny B, Obert P, Cazorla O, Tanguy S, Reboul C (2013) Exercise-induced cardioprotection: a role for eNOS uncoupling and NO metabolites. Basic Res Cardiol 108:389. doi: 10.1007/s00395-013-0389-2

24. Fauconnier J, Andersson DC, Zhang S-J, Lanner JT, Wibom R, Katz A, Bruton JD, Westerblad H (2007) Effects of Palmitate on Ca2+ Handling in Adult Control and ob/ob Cardiomyocytes : Impact of Mitochondrial Reactive Oxygen Species. Diabetes 56:1136–1142. doi: 10.2337/db06-0739

25. Gao S, Chen J, Brodsky SV, Huang H, Adler S, Lee JH, Dhadwal N, Cohen-Gould L, Gross SS, Goligorsky MS (2004) Docking of Endothelial Nitric Oxide Synthase (eNOS) to the Mitochondrial

Outer Membrane A PENTABASIC AMINO ACID SEQUENCE IN THE AUTOINHIBITORY DOMAIN OF eNOS TARGETS A PROTEINASE K-CLEAVABLE PEPTIDE ON THE CYTOPLASMIC FACE OF MITOCHONDRIA. J Biol Chem 279:15968–15974. doi: 10.1074/jbc.M308504200

26. García-Niño WR, Correa F, Rodríguez-Barrena JI, León-Contreras JC, Buelna-Chontal M, Soria-Castro E, Hernández-Pando R, Pedraza-Chaverri J, Zazueta C (2017) Cardioprotective kinase signaling to subsarcolemmal and interfibrillar mitochondria is mediated by caveolar structures. Basic Res Cardiol 112:15. doi: 10.1007/s00395-017-0607-4

27. Görlach A, Bertram K, Hudecova S, Krizanova O (2015) Calcium and ROS: A mutual interplay. Redox Biol 6:260–271. doi: 10.1016/j.redox.2015.08.010

28. Guo Y, Li Q, Xuan Y-T, Wu W-J, Tan W, Slezak J, Zhu X, Tomlin A, Bolli R (2021) Exercise-induced late preconditioning in mice is triggered by eNOS-dependent generation of nitric oxide and activation of PKCε and is mediated by increased iNOS activity. Int J Cardiol 340:68–78. doi: 10.1016/j.ijcard.2021.08.021

29. Heinzel FR, Luo Y, Li X, Boengler K, Buechert A, García-Dorado D, Di Lisa F, Schulz R, Heusch G (2005) Impairment of diazoxide-induced formation of reactive oxygen species and loss of cardioprotection in connexin 43 deficient mice. Circ Res 97:583–586. doi: 10.1161/01.RES.0000181171.65293.65

30. Heusch G (2020) Myocardial ischaemia–reperfusion injury and cardioprotection in perspective. Nat Rev Cardiol 17(12):773-789. doi: 10.1038/s41569-020-0403-y

31. Jaffrey SR, Snyder SH (2001) The Biotin Switch Method for the Detection of S-Nitrosylated Proteins. Sci STKE 2001:pl1–pl1. doi: 10.1126/stke.2001.86.pl1

32. Klein G, Mathé C, Biola-Clier M, Devineau S, Drouineau E, Hatem E, Marichal L, Alonso B, Gaillard J, Lagniel G, Armengaud J, Carrière M, Chédin S, Boulard Y, Pin S, Renault J, Aude J, Labarre J (2016) RNA-binding proteins are a major target of silica nanoparticles in cell extracts. In: Nanotoxicology. https://pubmed.ncbi.nlm.nih.gov/27705051/. Accessed 2 Dec 2020

33. Kleindienst A, Battault S, Belaidi E, Tanguy S, Rosselin M, Boulghobra D, Meyer G, Gayrard S, Walther G, Geny B, Durand G, Cazorla O, Reboul C (2016) Exercise does not activate the β3 adrenergic receptor–eNOS pathway, but reduces inducible NOS expression to protect the heart of obese diabetic mice. Basic Res Cardiol 111:40. doi: 10.1007/s00395-016-0559-0

34. Kohr M, Aponte A, J S, Gucek M, Steenbergen C, E M (2012) Measurement of S-nitrosylation occupancy in the myocardium with cysteine-reactive tandem mass tags: short communication. In: Circ. Res. https://pubmed.ncbi.nlm.nih.gov/22865876/. Accessed 8 Nov 2020

35. Kohr MJ, Aponte AM, Sun J, Wang G, Murphy E, Gucek M, Steenbergen C (2011) Characterization of potential S-nitrosylation sites in the myocardium. Am J Physiol Heart Circ Physiol 300:H1327-1335. doi: 10.1152/ajpheart.00997.2010

36. Koncsos G, Varga ZV, Baranyai T, Ferdinandy P, Schulz R, Giricz Z, Boengler K (2018) Nagarse treatment of cardiac subsarcolemmal and interfibrillar mitochondria leads to artefacts in mitochondrial protein quantification. J Pharmacol Toxicol Methods 91:50–58. doi: 10.1016/j.vascn.2018.01.004

37. Kuznetsov AV, Margreiter R (2009) Heterogeneity of Mitochondria and Mitochondrial Function within Cells as Another Level of Mitochondrial Complexity. Int J Mol Sci 10:1911–1929. doi: 10.3390/ijms10041911

38. Lacza Z, Pankotai E, Busija DW (2009) Mitochondrial nitric oxide synthase: current concepts and controversies. Front Biosci Landmark Ed 14:4436–4443

39. Lee Y, Min K, Talbert EE, Kavazis AN, Smuder AJ, Willis WT, Powers SK (2012) Exercise protects cardiac mitochondria against ischemia-reperfusion injury. Med Sci Sports Exerc 44:397–405. doi: 10.1249/MSS.0b013e318231c037

40. Leite ACR, Oliveira HCF, Utino FL, Garcia R, Alberici LC, Fernandes MP, Castilho RF, Vercesi AE (2010) Mitochondria generated nitric oxide protects against permeability transition via formation of membrane protein S-nitrosothiols. Biochim Biophys Acta BBA - Bioenerg 1797:1210–1216. doi: 10.1016/j.bbabio.2010.01.034

41. Lemasters J, Theruvath T, Zhong Z, Al N (2009) Mitochondrial calcium and the permeability transition in cell death. In: Biochim. Biophys. Acta. https://pubmed.ncbi.nlm.nih.gov/19576166/. Accessed 8 Nov 2020

42. Lima B, Forrester MT, Hess DT, Stamler JS (2010) S-Nitrosylation in Cardiovascular Signaling. Circ Res 106:633–646. doi: 10.1161/CIRCRESAHA.109.207381

43. Lindenmayer G, Sordahl L, Schwartz A (1968) Reevaluation of oxidative phosphorylation in cardiac mitochondria from normal animals and animals in heart failure. In: Circ. Res. https://pubmed.ncbi.nlm.nih.gov/5676454/. Accessed 2 Dec 2020

44. Liu Y, Zhang Y (2015) CHCHD2 connects mitochondrial metabolism to apoptosis. Mol Cell Oncol 2. doi: 10.1080/23723556.2015.1004964

45. Magalhães J, Gonçalves IO, Lumini-Oliveira J, Marques-Aleixo I, Passos E, Rocha-Rodrigues S, Machado NG, Moreira AC, Rizo D, Viscor G, Oliveira PJ, Torrella JR, Ascensão A (2014) Modulation of cardiac mitochondrial permeability transition and apoptotic signaling by endurance training and intermittent hypobaric hypoxia. Int J Cardiol 173:40–45. doi: 10.1016/j.ijcard.2014.02.011

46. Marcil M, Bourduas K, Ascah A, Burelle Y (2006) Exercise training induces respiratory substrate-specific decrease in Ca2+-induced permeability transition pore opening in heart mitochondria. Am J Physiol Heart Circ Physiol 290:H1549-1557. doi: 10.1152/ajpheart.00913.2005

47. Marongiu E, Crisafulli A (2014) Cardioprotection Acquired Through Exercise: The Role of Ischemic Preconditioning. Curr Cardiol Rev 10:336–348. doi: 10.2174/1573403X10666140404110229

48. McMillin-Wood J, Wolkowicz PE, Chu A, Tate CA, Goldstein MA, Entman ML (1980) Calcium uptake by two preparations of mitochondria from heart. Biochim Biophys Acta BBA - Bioenerg 591:251–265. doi: 10.1016/0005-2728(80)90157-7

49. Methner C, Lukowski R, Grube K, Loga F, Smith RAJ, Murphy MP, Hofmann F, Krieg T (2013) Protection through postconditioning or a mitochondria-targeted S-nitrosothiol is unaffected by cardiomyocyte-selective ablation of protein kinase G. Basic Res Cardiol 108:337. doi: 10.1007/s00395-013-0337-1

50. Meziat C, Boulghobra D, Strock E, Battault S, Bornard I, Walther G, Reboul C (2019) Exercise training restores eNOS activation in the perivascular adipose tissue of obese rats: Impact on vascular function. Nitric Oxide 86:63–67. doi: 10.1016/j.niox.2019.02.009

51. Murphy E, Kohr M, Sun J, Nguyen T, Steenbergen C (2012) S-Nitrosylation: A Radical Way to Protect the Heart. J Mol Cell Cardiol 52:568–577. doi: 10.1016/j.yjmcc.2011.08.021

52. Murphy E, Steenbergen C (2007) Preconditioning: The Mitochondrial Connection. Annu Rev Physiol 69:51–67. doi: 10.1146/annurev.physiol.69.031905.163645

53. Nguyen TT, Stevens MV, Kohr M, Steenbergen C, Sack MN, Murphy E (2011) Cysteine 203 of cyclophilin D is critical for cyclophilin D activation of the mitochondrial permeability transition pore. J Biol Chem 286:40184–40192. doi: 10.1074/jbc.M111.243469

54. Olgar Y, Degirmenci S, Durak A, Billur D, Can B, Kayki-Mutlu G, Arioglu-Inan E E, Turan B (2018) Aging related functional and structural changes in the heart and aorta: MitoTEMPO improves aged-cardiovascular performance. Exp Gerontol 110:172–181. doi: 10.1016/j.exger.2018.06.012

55. Palmer JW, Tandler B, Hoppel CL (1977) Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. J Biol Chem 252:8731–8739

56. Palmer JW, Tandler B, Hoppel CL (1986) Heterogeneous response of subsarcolemmal heart mitochondria to calcium. Am J Physiol-Heart Circ Physiol 250:H741–H748. doi: 10.1152/ajpheart.1986.250.5.H741

57. Pan X, Liu J, Nguyen T, Liu C, Sun J, Teng Y, Fergusson MM, Rovira II, Allen M, Springer DA, Aponte AM, Gucek M, Balaban RS, Murphy E, Finkel T (2013) The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter (MCU). Nat Cell Biol 15:1464–1472. doi: 10.1038/ncb2868

58. Panel M, Ghaleb B, Morin D (2017) Ca2+ ionophores are not suitable for inducing mPTP opening in murine isolated adult cardiac myocytes. Sci Rep 7:4283. doi: 10.1038/s41598-017-04618-4

59. Penna C, Angotti C, Pagliaro P (2014) Protein S-nitrosylation in preconditioning and postconditioning. Exp Biol Med 239:647–662. doi: 10.1177/1535370214522935

60. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu D, Inuganti A, Griss J, Mayer G, Eisenacher M, Pérez E, Uszkoreit J, Pfeuffer J, Sachsenberg T, Yilmaz S, Tiwary S, Cox J, Audain E, Walzer M, Jarnuczak A, Ternent T, Brazma A, Vizcaíno J (2019) The PRIDE database and related tools and resources in 2019: improving support for quantification data. In: Nucleic Acids Res. https://pubmed.ncbi.nlm.nih.gov/30395289/. Accessed 22 Jan 2021

61. Rassaf T, Totzeck M, Hendgen-Cotta UB, Shiva S, Heusch G, Kelm M (2014) Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning. Circ Res 114:1601–1610. doi: 10.1161/CIRCRESAHA.114.303822

62. Reboul C, Tanguy S, Juan JM, Dauzat M, Obert P (2005) Cardiac remodeling and functional adaptations consecutive to altitude training in rats: implications for sea level aerobic performance. J Appl Physiol Bethesda Md 1985 98:83–92. doi: 10.1152/japplphysiol.00214.2004

63. Riva A, Tandler B, Loffredo F, Vazquez E, Hoppel C (2005) Structural differences in two biochemically defined populations of cardiac mitochondria. Am J Physiol-Heart Circ Physiol 289:H868–H872. doi: 10.1152/ajpheart.00866.2004

64. Rodríguez-Sinovas A, Ruiz-Meana M, Denuc A, García-Dorado D (2018) Mitochondrial Cx43, an important component of cardiac preconditioning. Biochim Biophys Acta BBA - Biomembr 1860:174–181. doi: 10.1016/j.bbamem.2017.06.011

65. Rohart F, Gautier B, Singh A, Lê Cao K-A (2017) mixOmics: An R package for 'omics feature selection and multiple data integration. PLoS Comput Biol 13. doi: 10.1371/journal.pcbi.1005752

66. Roth GA, Johnson C, Abajobir A, Abd-Allah F, Abera SF, Abyu G, Ahmed M, Aksut B, Alam T, Alam K, Alla F, Alvis-Guzman N, Amrock S, Ansari H, Ärnlöv J, Asayesh H, Atey TM, Avila-Burgos L, Awasthi A, Banerjee A, Barac A, Bärnighausen T, Barregard L, Bedi N, Belay Ketema E, Bennett D, Berhe G, Bhutta Z, Bitew S, Carapetis J, Carrero JJ, Malta DC, Castañeda-Orjuela CA, Castillo-Rivas J, Catalá-López F, Choi J-Y, Christensen H, Cirillo M, Cooper L, Criqui M, Cundiff D, Damasceno A, Dandona L, Dandona R, Davletov K, Dharmaratne S, Dorairaj P, Dubey M, Ehrenkranz R, El Sayed Zaki M, Faraon EJA, Esteghamati A, Farid T, Farvid M, Feigin V, Ding EL, Fowkes G, Gebrehiwot T, Gillum R, Gold A, Gona P, Gupta R, Habtewold TD, Hafezi-Nejad N, Hailu T, Hailu GB, Hankey G, Hassen HY, Abate KH, Havmoeller R, Hay SI, Horino M, Hotez PJ, Jacobsen K, James S, Javanbakht M, Jeemon P, John D, Jonas J, Kalkonde Y, Karimkhani C, Kasaeian A, Khader Y, Khan A, Khang Y-H, Khera S, Khoja AT, Khubchandani J, Kim D, Kolte D, Kosen S, Krohn KJ, Kumar GA, Kwan GF, Lal DK, Larsson A, Linn S, Lopez A, Lotufo PA, El Razek HMA, Malekzadeh R, Mazidi M, Meier T, Meles KG, Mensah G, Meretoja A, Mezgebe H, Miller T, Mirrakhimov E, Mohammed S, Moran AE, Musa KI, Narula J, Neal B, Ngalesoni F, Nguyen G, Obermeyer CM, Owolabi M, Patton G, Pedro J, Qato D, Qorbani M, Rahimi K, Rai RK, Rawaf S, Ribeiro A, Safiri S, Salomon JA, Santos I, Santric Milicevic M, Sartorius B, Schutte A, Sepanlou S, Shaikh MA, Shin M-J, Shishehbor M, Shore H, Silva DAS, Sobngwi E, Stranges S, Swaminathan S, Tabarés-Seisdedos R, Tadele Atnafu N, Tesfay F, Thakur JS, Thrift A, Topor-Madry R, Truelsen T, Tyrovolas S, Ukwaja KN, Uthman O, Vasankari T, Vlassov V, Vollset SE, Wakayo T, Watkins D, Weintraub R, Werdecker A, Westerman R, Wiysonge CS, Wolfe C, Workicho A, Xu G, Yano Y, Yip P, Yonemoto N, Younis M, Yu C, Vos T, Naghavi M, Murray C (2017) Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. J Am Coll Cardiol 70:1–25. doi: 10.1016/j.jacc.2017.04.052

67. Ruiz-Meana M, Núñez E, Miro-Casas E, Martínez-Acedo P, Barba I, Rodriguez-Sinovas A, Inserte J, Fernandez-Sanz C, Hernando V, Vázquez J, Garcia-Dorado D (2014) Ischemic preconditioning protects cardiomyocyte mitochondria through mechanisms independent of cytosol. J Mol Cell Cardiol 68:79–88. doi: 10.1016/j.yjmcc.2014.01.001

68. Sartoretto JL, Kalwa H, Pluth MD, Lippard SJ, Michel T (2011) Hydrogen peroxide differentially modulates cardiac myocyte nitric oxide synthesis. Proc Natl Acad Sci 108:15792–15797. doi: 10.1073/pnas.1111331108

69. Schulman IH, Hare JM (2012) Regulation of cardiovascular cellular processes by S-nitrosylation. Biochim Biophys Acta 1820:752–762. doi: 10.1016/j.bbagen.2011.04.002

70. Soetkamp D, Nguyen TT, Menazza S, Hirschhäuser C, Hendgen-Cotta UB, Rassaf T, Schlüter KD, Boengler K, Murphy E, Schulz R (2014) S-nitrosation of mitochondrial connexin 43 regulates mitochondrial function. Basic Res Cardiol 109:433. doi: 10.1007/s00395-014-0433-x

71. Srisakuldee W, Makazan Z, Nickel BE, Zhang F, Thliveris JA, Pasumarthi KBS, Kardami E (2014) The FGF-2-triggered protection of cardiac subsarcolemmal mitochondria from calcium overload is mitochondrial connexin 43-dependent. Cardiovasc Res 103:72–80. doi: 10.1093/cvr/cvu066

72. Sun J, Kohr MJ, Nguyen T, Aponte AM, Connelly PS, Esfahani SG, Gucek M, Daniels MP, Steenbergen C, Murphy E (2012) Disruption of Caveolae Blocks Ischemic Preconditioning-Mediated S-Nitrosylation of Mitochondrial Proteins. Antioxid Redox Signal 16:45–56. doi: 10.1089/ars.2010.3844

73. Sun J, Morgan M, Shen R-F, Steenbergen C, Murphy E (2007) Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. Circ Res 101:1155–1163. doi: 10.1161/CIRCRESAHA.107.155879

74. Sun J, Murphy E (2010) Protein S-Nitrosylation and Cardioprotection. Circ Res 106:285–296. doi: 10.1161/CIRCRESAHA.109.209452

75. Sun J, Nguyen T, Aponte AM, Menazza S, Kohr MJ, Roth DM, Patel HH, Murphy E, Steenbergen C (2015) Ischaemic preconditioning preferentially increases protein S-nitrosylation in subsarcolemmal mitochondria. Cardiovasc Res 106:227–236. doi: 10.1093/cvr/cvv044

Figures Legends:

Figure 1: 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, anti-VDAC1 antibodies. ***(C, D)***- Effect of exercise training on eNOS level in the cytosolic and mitochondrial subfractions. ***(C)*** 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 mitochondria samples. ***(E, F)***- Effect of exercise training on eNOS phosphorylation at serine 1177 (eNOS-$P^{ser1177}$) in the cytosolic and mitochondrial subfractions. ***(E)***-Representative western blots of eNOS-$P^{ser1177}$ in the cytosolic and mitochondrial subfractions. ***(F)-*** Quantification of eNOS-$P^{ser1177}$ 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-$P^{ser1177}$ 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 mitochondria 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.

Figure 2: Effect of exercise training on NO production in isolated cardiomyocytes. ***(A)-*** Cardiomyocytes (100,000 cells/ml) from Sed and Ex-Tr hearts (n=5 hearts per group) were loaded with a fluorescent dye sensitive to NO production (DAF2; 5µM), pre-incubated or not with L-NAME (100µM) and stimulated or not with H_2O_2 (100µM). Fluorescence was measured every 10s for 30min and was normalized to the baseline fluorescence (F_0). Changes in NO production are expressed as F/F_0. (*$p<0.05$ Sed vs Ex-Tr; $^{\$}p<0.05$ H_2O_2 effect; repeated two-way ANOVA followed by Turkey's multiple comparisons test). ***(B)-*** Confocal images of left ventricle cardiomyocytes from Sed and Ex-Tr rats loaded simultaneously with DAF-2 (green channel, emission 491/513 nm; 5µM) and MitoTracker Orange (red channel, emission/excitation 554/576 nm; 200nM). Colocalization is shown in the merged images. ***(C)-*** Quantification of DAF2 fluorescence intensity in cardiomyocytes (n=3 hearts per group, each point corresponds to the mean of DAF2 fluorescence intensity measured in at least 8 cells/heart); $^{\$}p<0.05$ H_2O_2 effect (parametric one-way ANOVA followed by Turkey's multiple comparisons test). ***(D)-***Quantification of mitochondrial DAF2 fluorescence intensity (n=3 hearts per group, each point corresponds to the mean of DAF2 fluorescence intensity measured in at least at least 8 cells/heart); *$p<0.05$ Sed vs Ex-Tr (parametric t test). Data are the mean ± SEM.

Figure 3. Effect of exercise training on SSM protein S-nitrosylation profile. ***(A)***- Schematic illustration of the site-specific S-nitrosoproteomic analysis in the mitochondrial subfractions and total left ventricle (LV) homogenates from Sed (n=3) and Ex-Tr (n=3) hearts. **(1)** Protein extraction, **(2)** SNO occupancy was determined

by Iodoacetyl Tandem Mass Tag™ (iodoTMT™) labeling and **(3)** TMT-peptide enrichment, **(4)** Liquid chromatography-tandem mass spectrometry analysis followed by data interpretation using the Mascot Daemon software. ***(B,C)*** Volcano plot showing the − log 10 p-value vs. the log2 expression levels representing the SNO changes of individual proteins in response to exercise training versus sedentary condition. Red dots, mitochondrial proteins; black dots, non-mitochondrial proteins. Blue and green squares represent the cutoff of fold change >1.5, p<0.1. 296 proteins with at least one TMT-labeled modification were identified in the total LV homogenates ***(B),*** and 345 proteins in the enriched mitochondrial subfractions ***(C)***. ***(D)***-Supervised partial least squares discriminant analysis (sPLS-DA). Component 1 versus Component 2, using 95% confidence ellipses. TH, total left ventricle lysates. ***(E)-*** Component 1 versus Component 3, using 95% confidence ellipses obtained by supervised partial least squares discriminant analysis. TH, total LV homogenates. ***(F)-*** Spectral counts of key proteins that explain the differences in the Sed and Ex-Tr S-nitrosoproteome profiles obtained in mitochondrial samples; *p<0.05 Sed vs Ex-Tr (t-test with Welch's correction).

Figure 4: Effect of exercise training on mitochondrial calcium retention capacity and mPTP activation: a key role for S-nitrosylation. ***(A,B,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: 507nm; Ex: 536nm). ***(A, B)-*** Mean traces of the CRC tests. Each peak corresponds to calcium addition (1µM, 5µM and 50µM pulses of $CaCl_2$; changes in concentration are represented by arrows). Black curve, representative trace in the presence of 2µM cyclosporine A (CsA; 2µmol/L). CRC was tested in the absence ***(A)-*** or presence ***(B)-*** of sodium L-ascorbate (1mmol/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 the Sidak's multiple comparisons test). ***(C)*** Quantification of CRC (nmol Ca^{2+} 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 Ca^{2+} (250µM) 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 30s. (*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.

Figure 5: Effect of exercise training on mitochondrial ROS production: a major role for protein S-nitrosylation. ***(A, B)-*** Sed and Ex-Tr cardiomyocytes (100,000 cells/mL) isolated from 3-6 independent hearts/group were loaded with MitoSOX Red, a fluorescent dye sensitive to mitochondrial ROS production (Ex: 510 nm; Em: 580 nm; 5µM) that can detect superoxide anion production in mitochondria. Fluorescence was measured every 20s and normalized to baseline fluorescence (F/F_0). Cardiomyocytes were stimulated with antimycin A (AA, 10µM), a mitochondrial electron transport chain complex III blocker, to force ROS production by mitochondria. Some myocytes were incubated with sodium L-ascorbate (Asc; 1mM) or L-NAME (LN; 100µM) for 30 min before AA addition. ***(B)-*** Mean fluorescence level after 15 min ($^{£}$p<0.05 ascorbate effect; $^{\#}$p<0.05 L-

NAME effect, non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test). ***(C)-*** Schematic illustration of the experimental protocol. Sed (n=4) and Ex-Tr (n=4) hearts were stabilized for 20 min and then perfused or not with L-NAME (LN; 100 µM) for 45min using a Langendorff apparatus. Hearts were subject to global no-flow ischemia for 20 min followed by 10 min of reperfusion. ***(D)-*** Determination of ROS production by DHE staining after 10 min of post-ischemic reperfusion, in hearts of Sed and Ex-Tr animals perfused or not with L-NAME (LN). Scale bar=80 µm. DHE fluorescence intensity was quantified in 20 images from 3-4 hearts in each group; *p<0.05 Ex-Tr *vs* Sed, $^{\#}$p<0.05 with *vs* without L-NAME effect (parametric t-test). ***(E)-*** Schematic illustration of the experimental protocol (only sedentary animals were used). Hearts were stabilized for 20 min and then perfused (n=4) or not (n=4) with the GSNO reductase inhibitor N6022 (10 µM) for 45 min. Then, hearts underwent global no-flow ischemia for 20 min followed by 10 min of reperfusion. ***(F)-*** ROS production in hearts of control animals pretreated or not with N6022 was monitored by quantifying DHE fluorescence at 10 min after ischemia reperfusion. DHE fluorescence intensity was quantified in 20 images from 4 hearts in each group (§p<0.05 with *vs* without N6022; parametric t test). Data are the mean ± SEM.

<u>Figure 6</u>: Effect of exercise training on the electrocardiography profile and cardiac ROS production during *in vivo* ischemia-reperfusion in rats: a major role for NO. ***(A)-*** Representative ECG recording before (baseline) and during ischemia (30 min) and reperfusion (10 min) in sedentary (Sed) and exercised (Ex-Tr) rats pre-treated by intraperitoneal injection of vehicle (NaCl 0.9%) or L-NAME (LN; 50mg/kg in 0.9% NaCl) 24h and 30min before coronary artery ligation. ***(B)-*** QTc values recorded at baseline in Sed and Ex-Tr animals pre-treated with NaCl (0.9%) or L-NAME (LN; 50mg/kg in 0.9% NaCl). ***(C)-*** QTc values recorded after 10 min of reperfusion in Sed and Ex-Tr animals pre-treated with NaCl (0.9%) or L-NAME (LN; 50mg/kg in 0.9% NaCl). Dotted line represents the mean QTc interval in Sed rats at baseline. QTc was measured in 3-4 animals/group. ***(D)-*** Determination of ROS production by DHE staining after 10 min of post-ischemic reperfusion *in vivo*, in hearts of Sed and Ex-Tr animals pre-treated or not with L-NAME (LN; 50mg/kg in 0.9% NaCl) 24h and 30min before coronary artery ligation. Scale bar=100 µm. The ischemic area was collected and embedded in OCT and flash-frozen in liquid nitrogen. DHE fluorescence intensity was quantified in 20 images from 3-4 hearts/group. *p<0.05 Ex-Tr *vs* Sed, $^{\#}$p<0.05 with *vs* without L-NAME (parametric one-way ANOVA followed by Turkey's multiple comparisons test). Data are the mean ± SEM.

<u>Figure 7</u>: 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 Ca^{2+} overload results in a strong rise in ROS production in mitochondria. A complex interplay between ROS production and Ca^{2+} 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 Ca^{2+} 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.

Figure 1

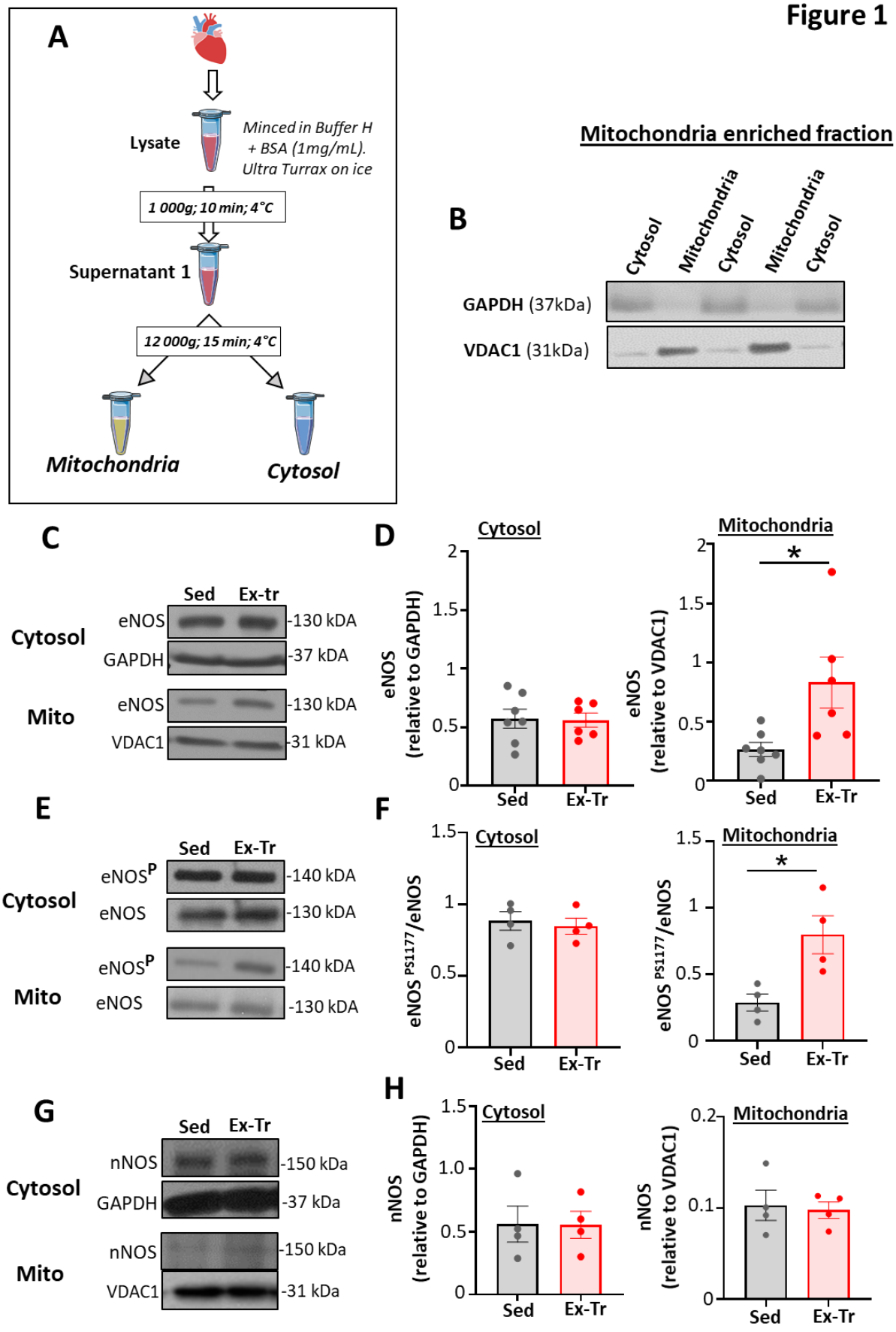

A
Lysate
Minced in Buffer H + BSA (1mg/mL). Ultra Turrax on ice
1 000g; 10 min; 4°C
Supernatant 1
12 000g; 15 min; 4°C
Mitochondria
Cytosol
Mitochondria enriched fraction
B
Cytosol
Mitochondria
Cytosol
Mitochondria
Cytosol
GAPDH (37kDa)
VDAC1 (31kDa)
C
Sed
Ex-tr
Cytosol
eNOS
GAPDH
Mito
eNOS
VDAC1
-130 kDA
-37 kDA
-130 kDA
-31 kDA
D
Cytosol
eNOS (relative to GAPDH)
Mitochondria
eNOS (relative to VDAC1)
*
Sed
Ex-Tr
E
Sed
Ex-Tr
Cytosol
eNOSP
eNOS
Mito
eNOSP
eNOS
-140 kDA
-130 kDA
-140 kDA
-130 kDA
F
Cytosol
eNOS PS1177/eNOS
Mitochondria
*
Sed
Ex-Tr
G
Sed
Ex-Tr
Cytosol
nNOS
GAPDH
Mito
nNOS
VDAC1
-150 kDa
-37 kDa
-150 kDa
-31 kDa
H
Cytosol
nNOS (relative to GAPDH)
Mitochondria
nNOS (relative to VDAC1)
Sed
Ex-Tr

Figure 2

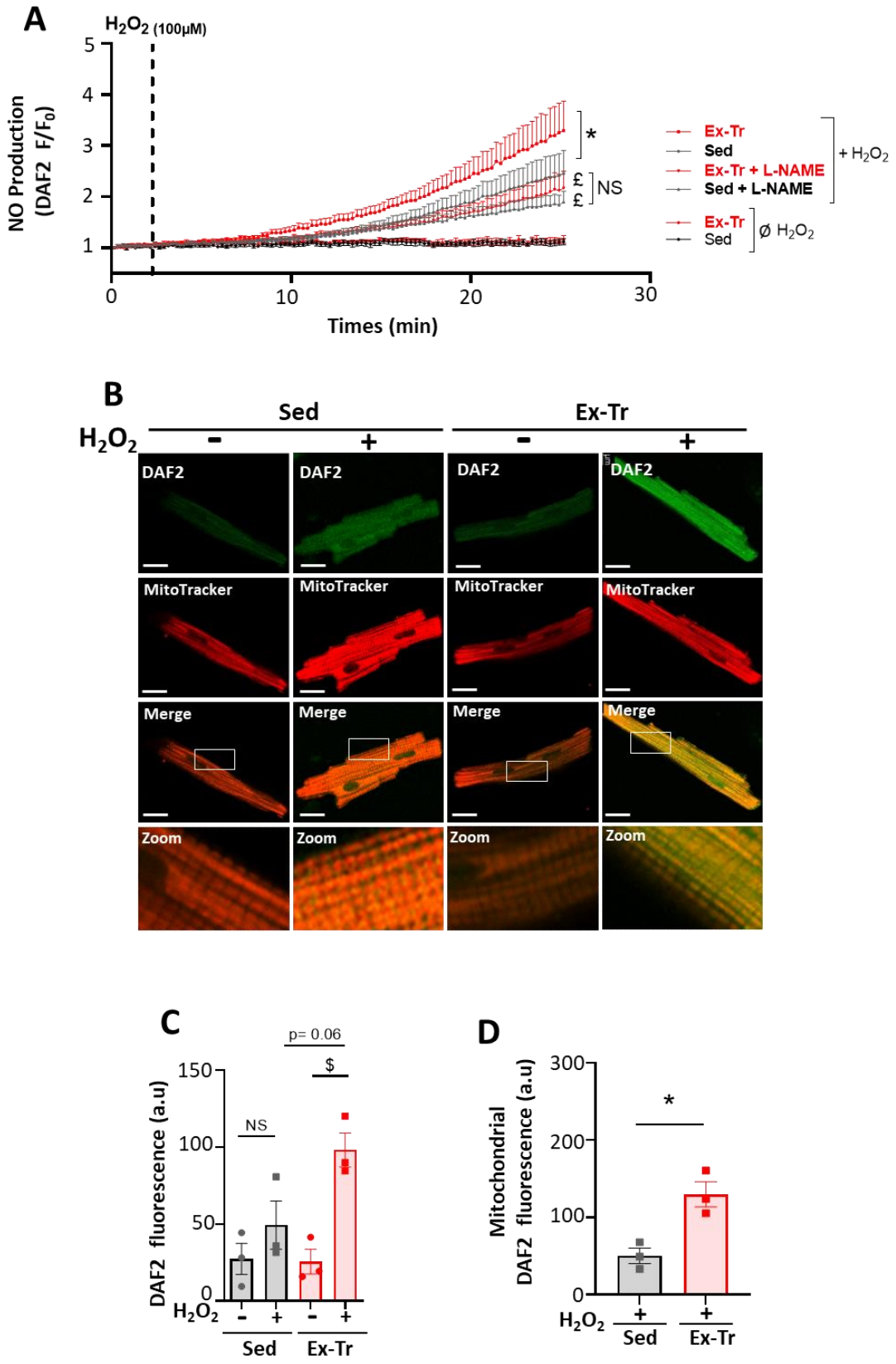
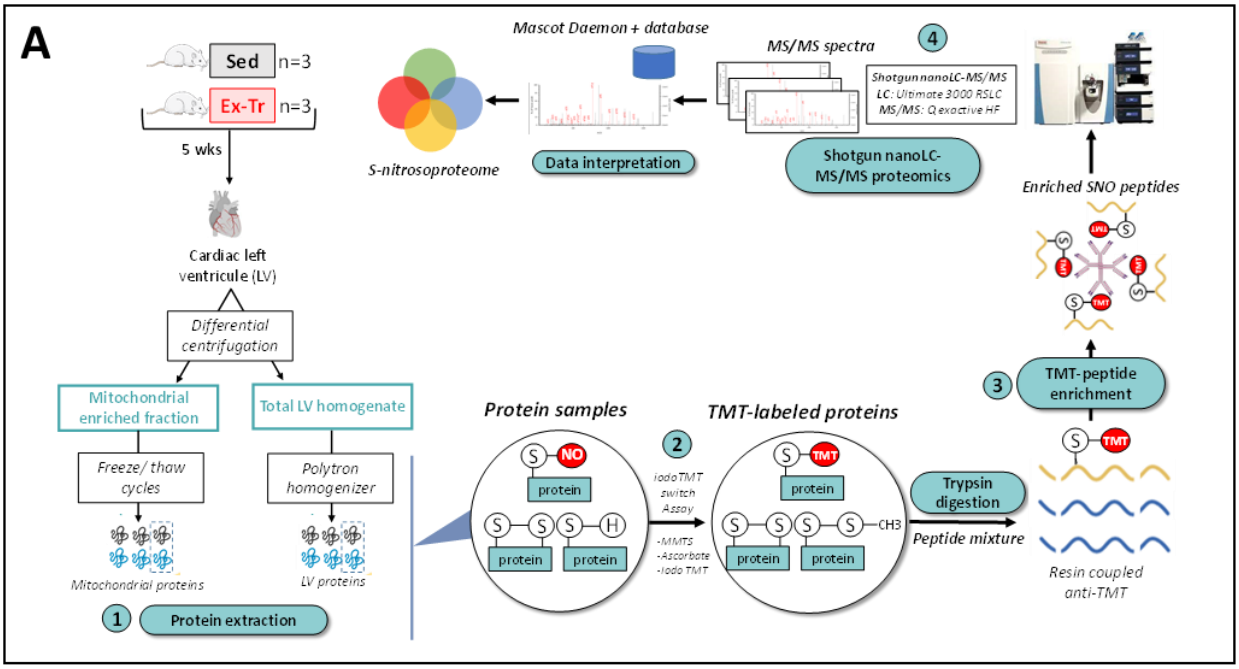
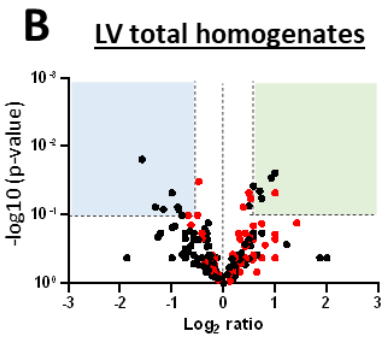
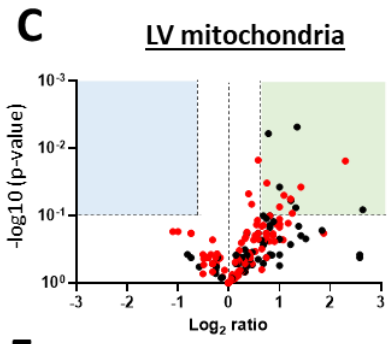
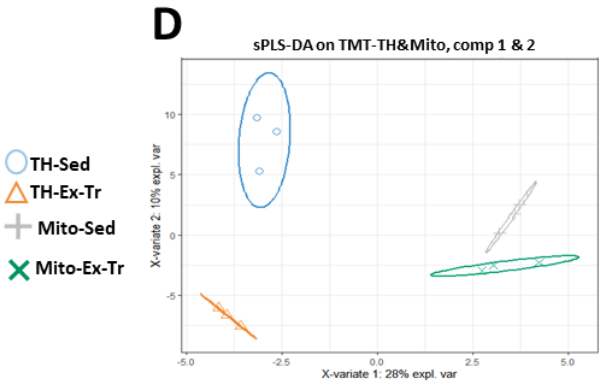

A
H2O2 (100µM)
NO Production (DAF2 F/F0)
Times (min)
Ex-Tr
Sed
Ex-Tr + L-NAME
Sed + L-NAME
+ H2O2
NS
Ø H2O2
B
Sed
Ex-Tr
H2O2
DAF2
MitoTracker
Merge
Zoom
C
DAF2 fluorescence (a.u)
p= 0.06
NS
Sed
Ex-Tr
D
Mitochondrial DAF2 fluorescence (a.u)

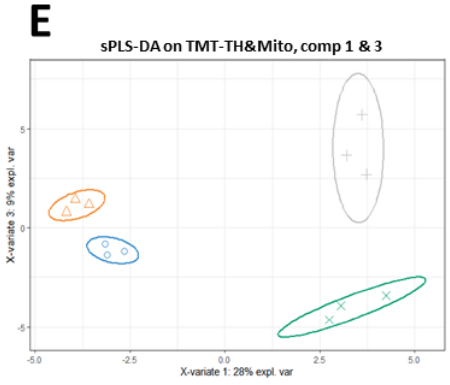
Figure 3


A
Sed n=3
Ex-Tr n=3
5 wks
Cardiac left ventricule (LV)
Differential centrifugation
Mitochondrial enriched fraction
Total LV homogenate
Freeze/ thaw cycles
Polytron homogenizer
Mitochondrial proteins
LV proteins
1 Protein extraction
Protein samples
TMT-labeled proteins
2
Iodo TMT switch Assay
-MMTS
-Ascorbate
-Iodo TMT
Trypsin digestion
Peptide mixture
3 TMT-peptide enrichment
Resin coupled anti-TMT
Enriched SNO peptides
Shotgun nanoLC-MS/MS proteomics
Shotgun nanoLC-MS/MS
LC: Ultimate 3000 RSLC
MS/MS: Q exactive HF
MS/MS spectra
4
Mascot Daemon + database
Data interpretation
S-nitrosoproteome


B
LV total homogenates
-log10 (p-value)
Log2 ratio


C
LV mitochondria
-log10 (p-value)
Log2 ratio


D
sPLS-DA on TMT-TH&Mito, comp 1 & 2
TH-Sed
TH-Ex-Tr
Mito-Sed
Mito-Ex-Tr
X-variate 2: 10% expl. var
X-variate 1: 28% expl. var


E
sPLS-DA on TMT-TH&Mito, comp 1 & 3
X-variate 3: 9% expl. var
X-variate 1: 28% expl. var

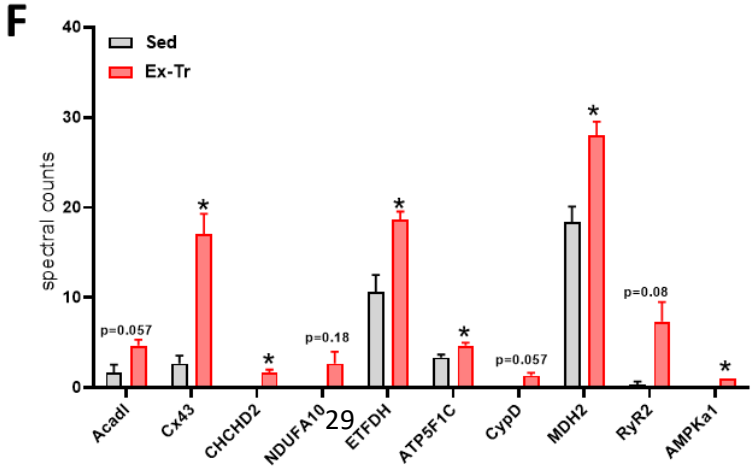
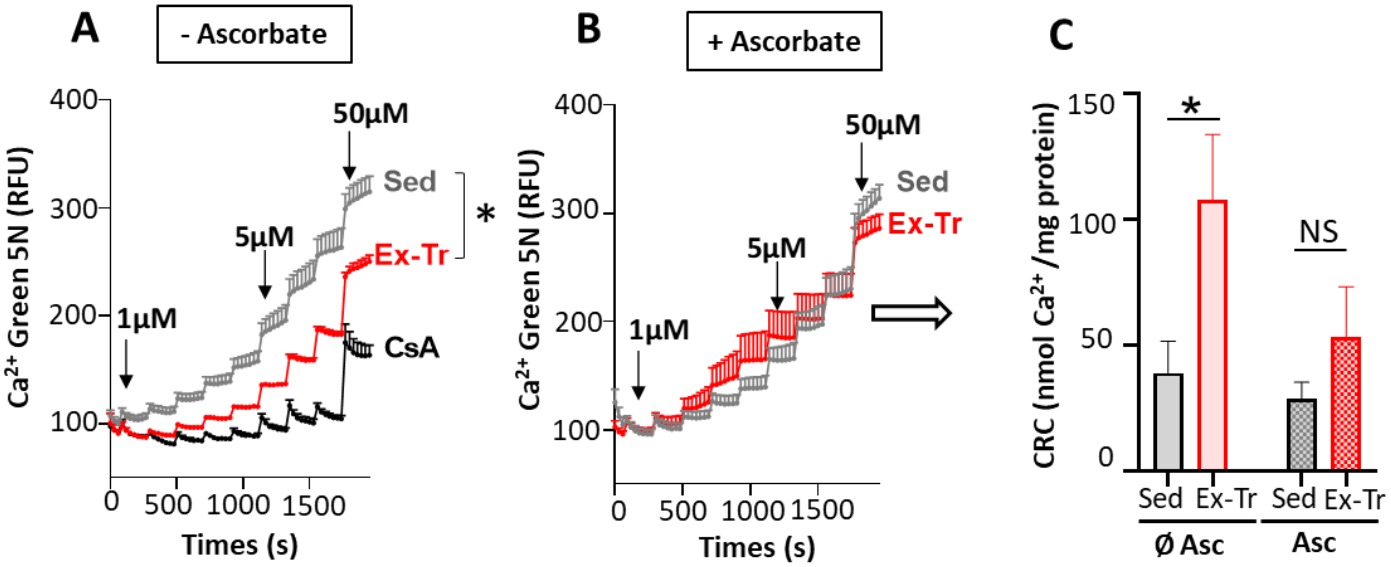
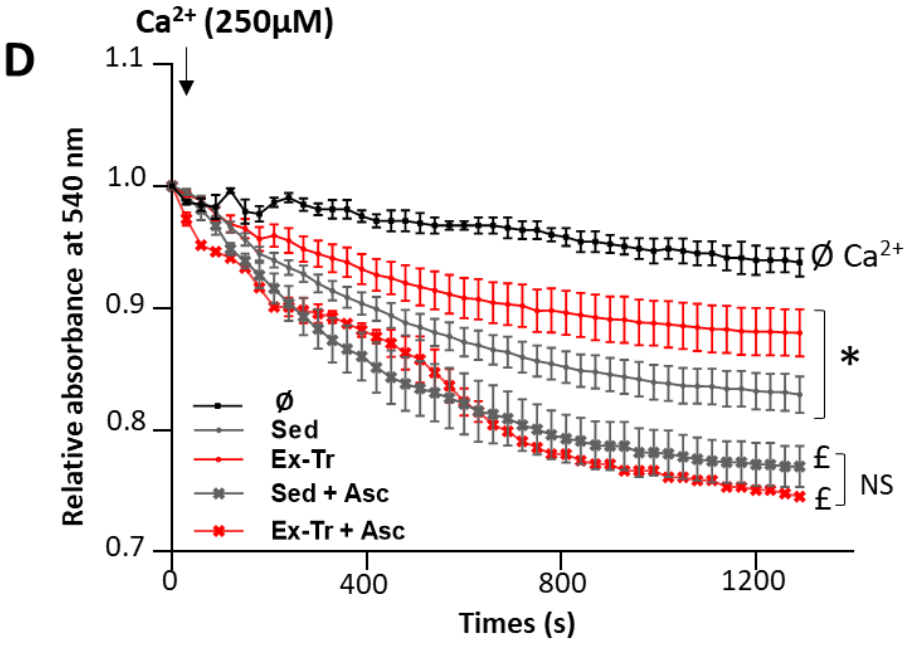

F
Sed
Ex-Tr
spectral counts
p=0.057
p=0.18
p=0.057
p=0.08
AcadI
Cx43
CHCHD2
NDUFA10
ETFDH
ATP5F1C
CypD
MDH2
RyR2
AMPKa1

Figure 4


A
- Ascorbate
B
+ Ascorbate
C
Ca2+ Green 5N (RFU)
Times (s)
1µM
5µM
50µM
Sed
Ex-Tr
CsA
*
CRC (nmol Ca2+ /mg protein)
NS
Sed
Ex-Tr
Ø Asc
Asc


D
Ca2+ (250µM)
Relative absorbance at 540 nm
Times (s)
Ø Ca2+
Ø
Sed
Ex-Tr
Sed + Asc
Ex-Tr + Asc
*
£
£
NS

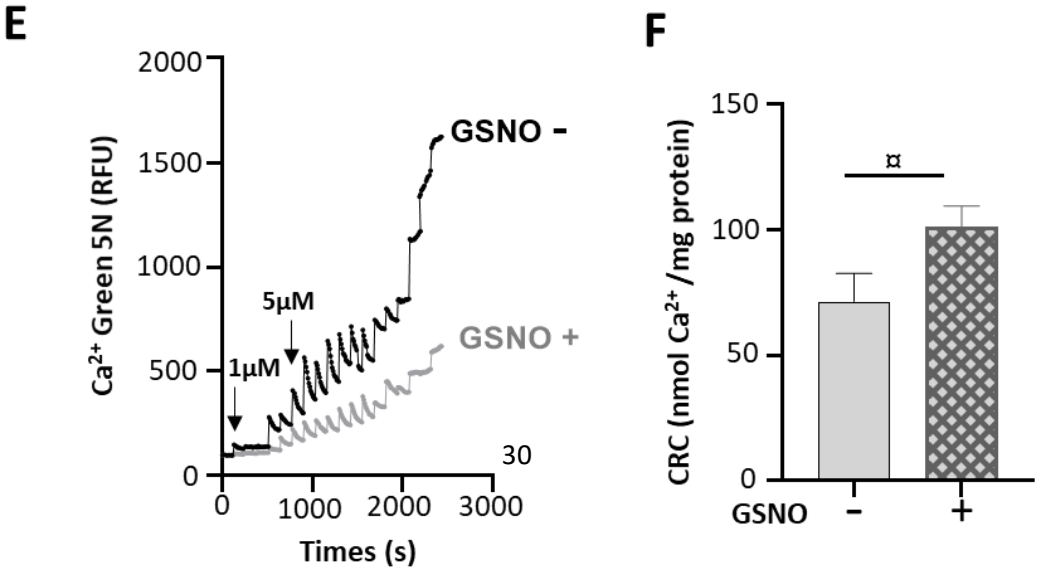

E
F
Ca2+ Green 5N (RFU)
Times (s)
GSNO -
GSNO +
1µM
5µM
30
CRC (nmol Ca2+ /mg protein)
¤
GSNO
-
+

Figure 5

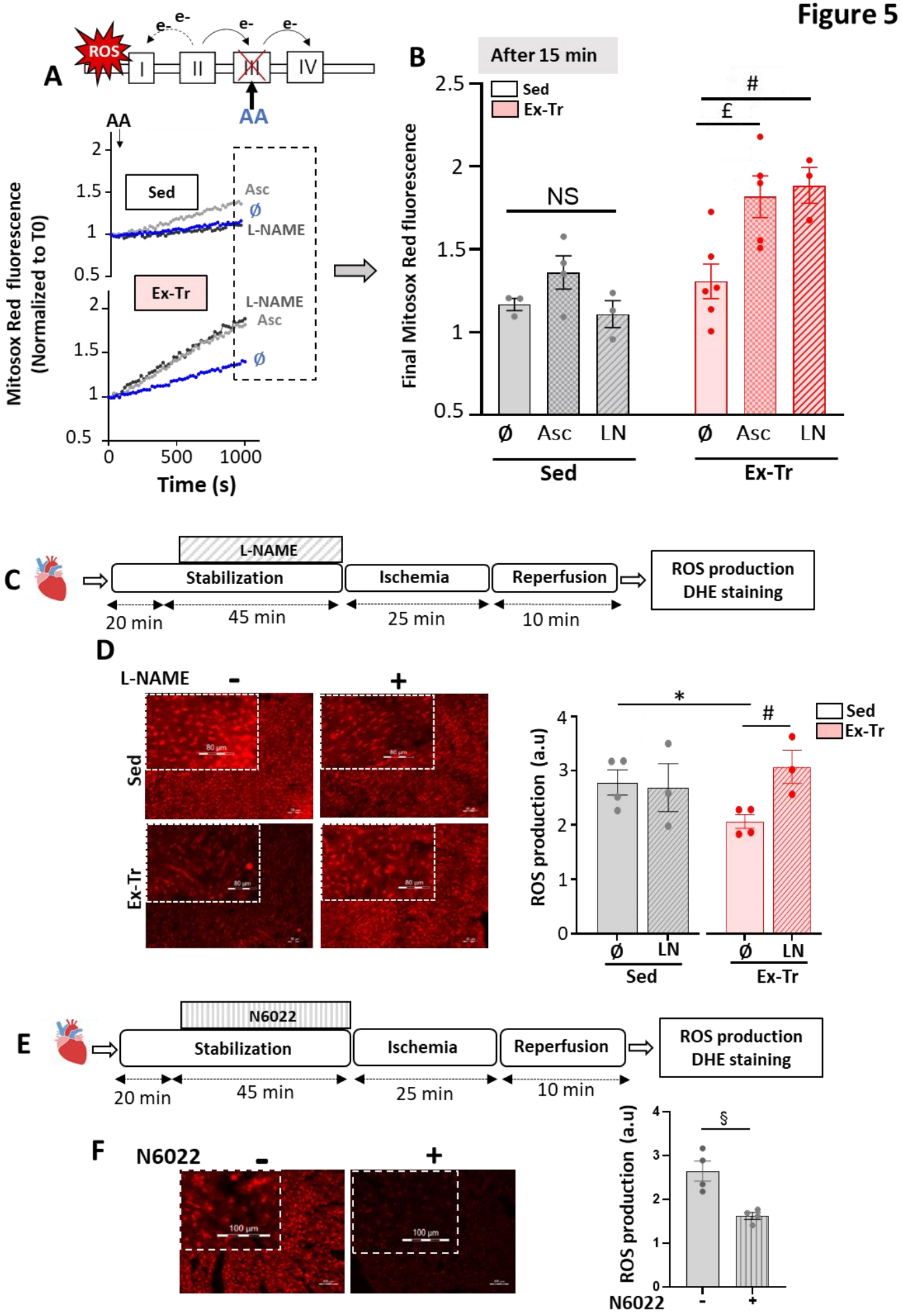

A
ROS
e-
e-
e-
e-
I
II
III
IV
AA
AA
Mitosox Red fluorescence (Normalized to T0)
Sed
Asc
Ø
L-NAME
Ex-Tr
L-NAME
Asc
Ø
Time (s)
B
After 15 min
Sed
Ex-Tr
Final Mitosox Red fluorescence
NS
#
£
Ø
Asc
LN
Sed
Ex-Tr
C
L-NAME
Stabilization
Ischemia
Reperfusion
ROS production
DHE staining
20 min
45 min
25 min
10 min
D
L-NAME
-
+
Sed
Ex-Tr
ROS production (a.u)
*
#
Sed
Ex-Tr
Ø
LN
E
N6022
Stabilization
Ischemia
Reperfusion
ROS production
DHE staining
20 min
45 min
25 min
10 min
F
N6022
-
+
ROS production (a.u)
§
N6022

Figure 6

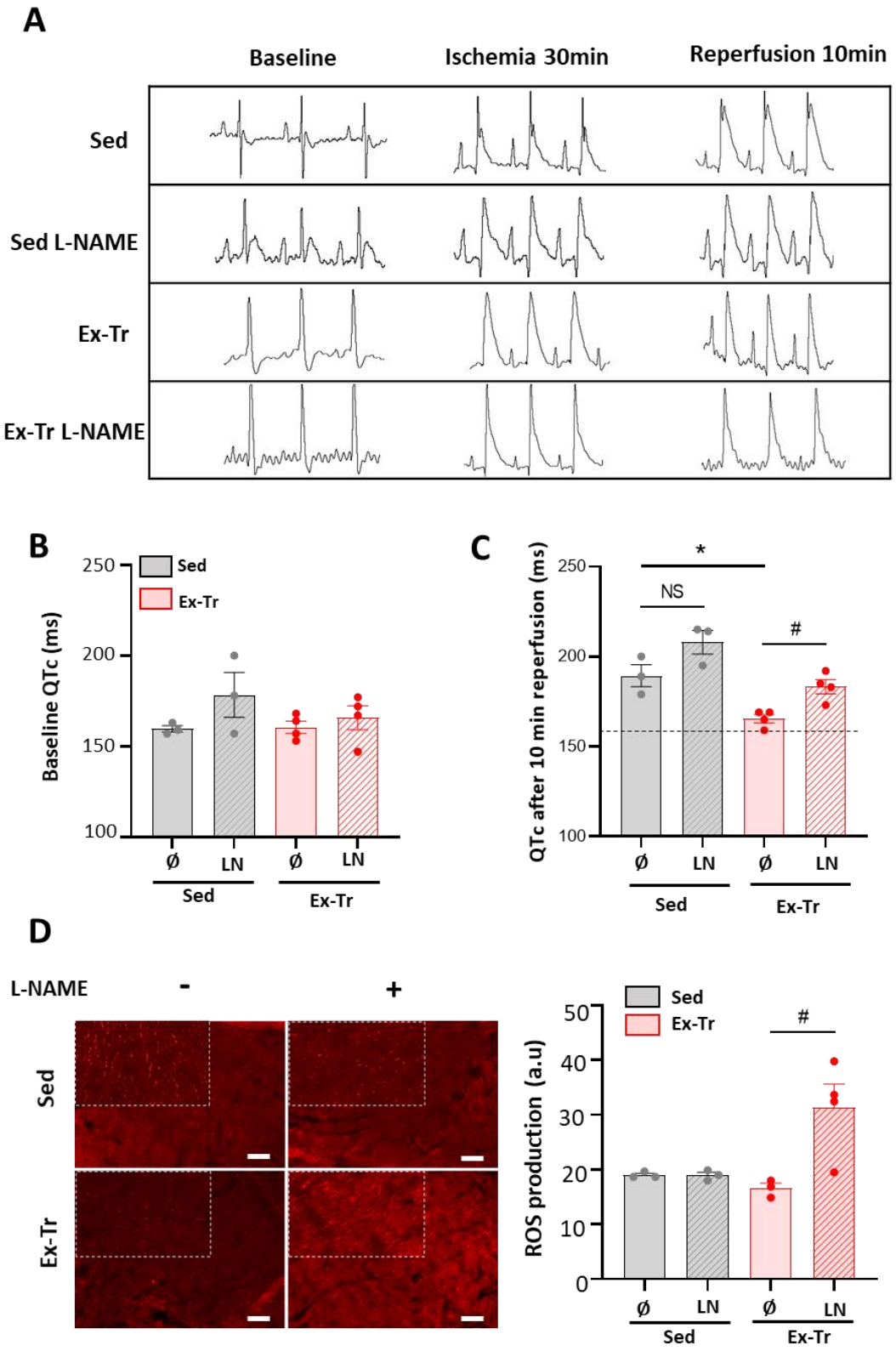
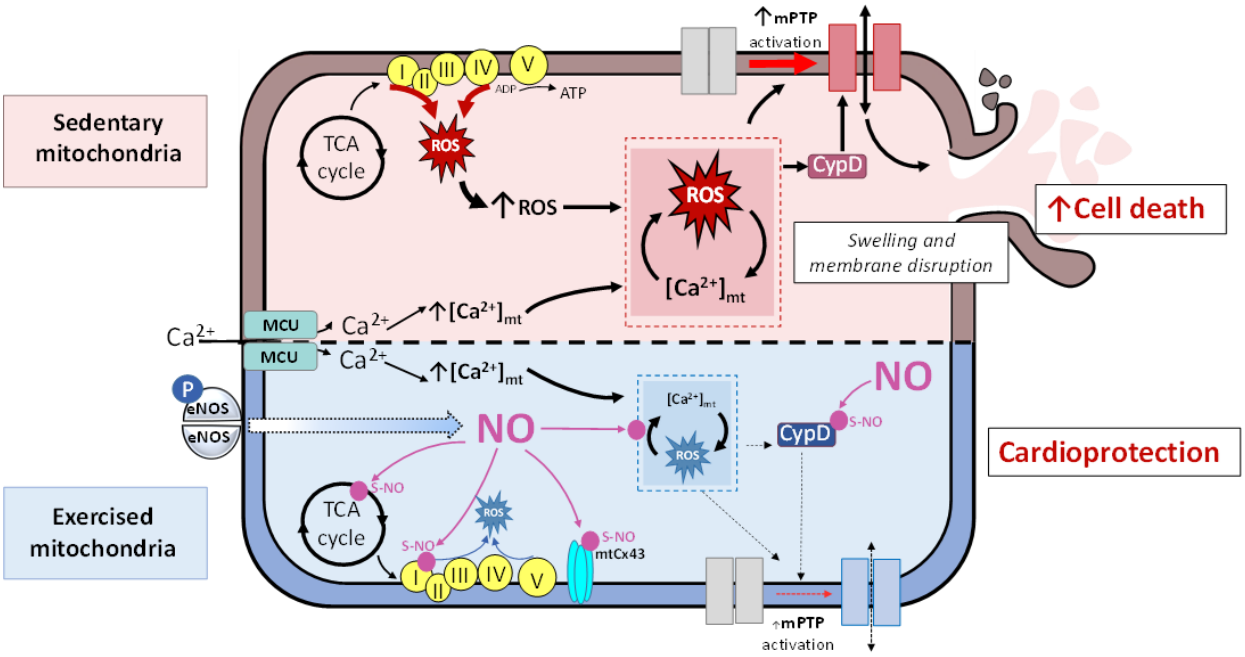

A
Baseline
Ischemia 30min
Reperfusion 10min
Sed
Sed L-NAME
Ex-Tr
Ex-Tr L-NAME
B
Baseline QTc (ms)
250
200
150
100
Sed
Ex-Tr
Ø
LN
Ø
LN
Sed
Ex-Tr
C
QTc after 10 min reperfusion (ms)
*
NS
#
250
200
150
100
Ø
LN
Ø
LN
Sed
Ex-Tr
D
L-NAME
-
+
Sed
Ex-Tr
ROS production (a.u)
50
40
30
20
10
0
Sed
Ex-Tr
#
Ø
LN
Ø
LN
Sed
Ex-Tr

Figure 7


Sedentary mitochondria
TCA cycle
ROS
ADP
ATP
↑mPTP activation
CypD
↑ROS
ROS
[Ca2+]mt
Swelling and membrane disruption
↑Cell death
Ca2+
MCU
MCU
Ca2+
↑[Ca2+]mt
Ca2+
↑[Ca2+]mt
P
eNOS
eNOS
NO
[Ca2+]mt
ROS
CypD
S-NO
NO
Cardioprotection
Exercised mitochondria
TCA cycle
S-NO
ROS
S-NO
mtCx43
↑mPTP activation