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Exercise-induced cardioprotection: a role for eNOS uncoupling and NO metabolites

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Abstract Exercise is an efficient strategy for myocardial protection against ischemia–reperfusion (IR) injury. Although endothelial nitric oxide synthase (eNOS) is phosphorylated and activated during exercise, its role in exercise-induced cardioprotection remains unknown. This study investigated whether modulation of eNOS activation during IR could participate in the exercise-induced cardioprotection against IR injury. Hearts isolated from sedentary or exercised rats (5 weeks training) were perfused with a Langendorff apparatus and IR performed in the presence or absence of NOS inhibitors [*N*-nitro-L-arginine methyl ester, L-NAME or *N*5-(1-iminoethyl)-L-ornithine, L-NIO] or tetrahydrobiopterin (BH₄). Exercise training protected hearts against IR injury and this effect was abolished by L-NAME or by L-NIO treatment, indicating

that exercise-induced cardioprotection is eNOS dependent. However, a strong reduction of eNOS phosphorylation at Ser1177 (eNOS-P^{Ser1177}) and of eNOS coupling during early reperfusion was observed in hearts from exercised rats (which showed higher eNOS-P^{Ser1177} and eNOS dimerization at baseline) in comparison to sedentary rats. Despite eNOS uncoupling, exercised hearts had more S-nitrosylated proteins after early reperfusion and also less nitro-oxidative stress, indexed by lower malondialdehyde content and protein nitrotyrosination compared to sedentary hearts. Moreover, in exercised hearts, stabilization of eNOS dimers by BH₄ treatment increased nitro-oxidative stress and then abolished the exercise-induced cardioprotection, indicating that eNOS uncoupling during IR is required for exercise-induced myocardial cardioprotection. Based on these results, we hypothesize that in the hearts of exercised animals, eNOS uncoupling associated with the improved myocardial antioxidant capacity prevents excessive NO synthesis and limits the reaction between NO and O₂^{•-} to form peroxynitrite (ONOO⁻), which is cytotoxic.

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Introduction

Exercise training protects the heart against ischemia–reperfusion (IR) injuries [10, 17, 18, 39]. The mechanisms responsible for this cardioprotective effect remain unclear, although they could provide new targets to reduce heart vulnerability to IR injury. To date, exercise-induced cardioprotection to IR injury is mainly explained by improvement of the myocardial antioxidant status [23, 39,

41] and calcium homeostasis [13, 17, 18, 50]. Chronic exercise also increases nitric oxide (NO) bioavailability by enhancing endothelial nitric oxide synthase (eNOS or NOS3) expression [33, 43] and/or its phosphorylation [21, 53, 54]. Different strategies that improve NO bioavailability in the heart markedly reduced heart vulnerability to IR [20, 29, 36]. More particularly, the eNOS signaling pathway protects myocardium against IR injury [16, 24, 25, 42, 46]; however, the link between eNOS signaling and exercise-induced cardioprotection during IR is underexplored, although it may play an essential role as recently suggested [5].

eNOS is a critical homodimeric enzyme that, in physiological conditions, consumes NADPH to synthesize NO and L-citrulline from L-arginine and oxygen. eNOS dimers are stabilized by the cofactor tetrahydrobiopterin (BH₄). The activity of eNOS is also modulated by phosphorylation at multiple sites, particularly at Ser¹¹⁷⁷, which is considered to be the main activation site of this enzyme [15] and its phosphorylation is promoted by exercise [3, 5]. Depletion or oxidation of BH₄ can result in eNOS uncoupling, which generates superoxide anion (O₂⁻) instead of NO. In addition, O₂⁻ can react with local NO to produce peroxynitrite (ONOO⁻) that has strong cytotoxic activity [37]. Thus, eNOS uncoupling clearly may play a major role in myocardial IR injury by increasing reactive oxygen species (ROS) production and limiting NO availability [27]. In addition, many studies have demonstrated attenuation of heart sensitivity to IR when the eNOS–NO pathway is stimulated, for instance, through eNOS overexpression [4, 12], NO administration [47] or BH₄ supplementation [49, 52]. The pathway by which NO could modulate heart sensitivity to IR remains controversial. Indeed, depending on the model used to protect the heart, some reported that NO-dependent cardioprotection is mainly related to NO/cGMP [22], whereas others have shown that protein S-nitrosylation (SNO) is the key trigger of NO-dependent cardioprotection [45].

We thus investigated the role of eNOS activation in exercise-induced cardioprotection by evaluating the effects of exercise training on eNOS phosphorylation and dimerization during cardiac IR. Unexpectedly, we found that eNOS was markedly uncoupled during early reperfusion in hearts from exercised rats but without consequence on NO bioavailability. Especially, we reported here that pharmacological stabilization of eNOS dimers during early reperfusion was not associated with improved NO bioavailability, but increased the formation of ONOO⁻ and then abolished exercise-induced cardioprotection. The major result of the present work is that exercise-induced cardioprotection is mainly dependent on eNOS uncoupling during early reperfusion.

Methods

Detailed information on the methodology is available in the online data supplement.

Animals and myocardial IR protocol

All investigations complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and with the regulations of the French Ministry of Agriculture. All experiments were approved by the local research ethics committee (Comité Régional d’Ethique, no: 84.004).

Wistar male rats (Charles River Laboratories, France) (12-week old; 382 ± 38 g) were randomly assigned to the sedentary (Sed) or exercised (Ex) group. Rats in the exercised group were trained on a motor driven treadmill at 70 % of the maximal aerobic velocity (25 m/min; 45 min/day), 5 days/week for 5 weeks. Such a training program has previously been reported by our team to increase citrate synthase activity of the soleus muscle (35.2 ± 4.7 IU/g wet weight in sedentary rats and 62.4 ± 7.9 IU/g wet weight in exercised rats; $p < 0.01$) [19]. In addition, Doppler echocardiography was performed to evaluate the effects of exercise training on cardiac adaptations. Exercise training increase left ventricular end diastolic diameters (LVEDd, Sed 5.84 mm, Ex 6.36 mm, $p < 0.05$) and peak velocities of early left ventricular inflow (Peak E, Sed 59.5 cm/s, Ex 68.3 cm/s, $p < 0.05$), suggesting that such training program improves diastolic function. This corresponds to a common feature of adaptations to exercise. 24 h after the end of the training period, hearts were isolated and frozen in liquid nitrogen for biochemical measurements or mounted on a Langendorff apparatus as previously described [13]. Briefly, animals were anesthetized (sodium pentobarbital, 100 mg/kg, i.p.), a thoracotomy was performed and hearts were retrogradely perfused using a Langendorff apparatus. Hearts were paced at a rate of 300 beats/min (low voltage stimulator, BSL MP35 SS58L, 3 V) and a non-compliant balloon was inserted into the left ventricle (LV) to monitor the LV pressure. Hearts were stabilized for 30 min and then subjected to global no-flow ischemia for 30 min followed by 120 min of reperfusion. During the entire IR procedure, cardiac functional parameters, such as LV developed pressure (LVDP), were recorded (MP35, BioPac System Inc) and coronary effluents were collected to evaluate coronary blood flow. At the end of the experiments, the infarct size was assessed by triphenyltetrazolium chloride (TTC) staining. In protocol 1 (Fig. 1), to assess the involvement of eNOS in exercise-induced cardioprotection, hearts were perfused with 100 μM *N*-nitro-L-arginine methyl ester (L-NAME), a NOS

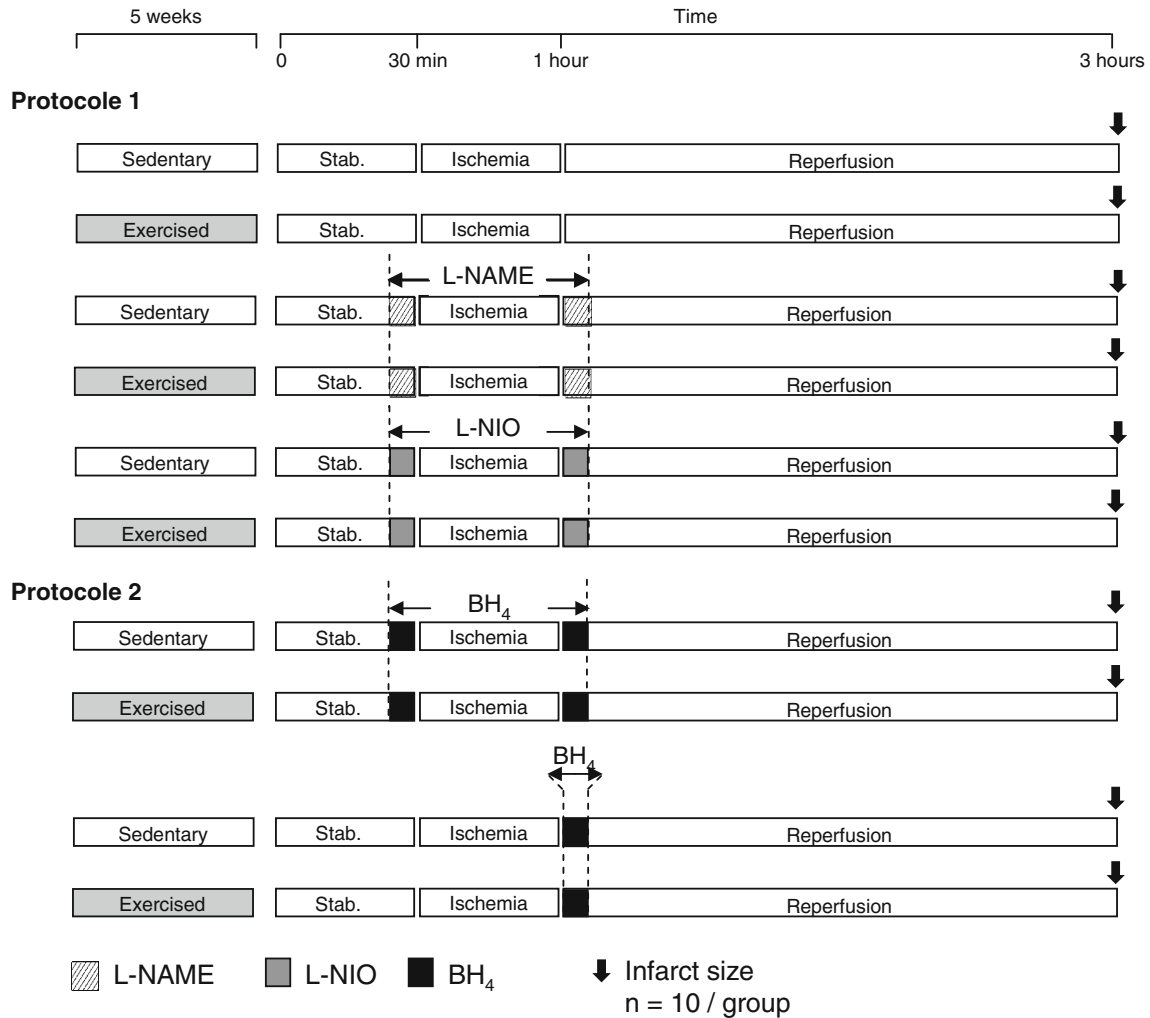


Fig. 1 Schematic illustration of the experimental protocol. L-NAME, *N*-nitro-*L*-arginine methyl ester; L-NIO, *N*5-(1-iminoethyl)-*L*-ornithine; BH₄, tetrahydrobiopterin. Hatched (L-NAME), gray (L-NIO)

inhibitor, or with 10 μ M *N*5-(1-iminoethyl)-*L*-ornithine (L-NIO), a more specific inhibitor of eNOS, 5 min before and after ischemia. In protocol 2 (Fig. 1), to evaluate the role of eNOS uncoupling in exercise-induced cardioprotection, hearts were perfused with 50 μ M BH₄, an eNOS coupling agent, 5 min before and after ischemia or only during the first 5 min of post-ischemic reperfusion. At the end of the ischemic period (ischemia) or after 10 min of post-ischemic reperfusion (reperfusion), hearts were quickly removed, the right ventricle cleaned and LV samples frozen in liquid nitrogen for biochemical analysis.

Biochemical assays

Measurement of nitrite

Quantification of nitrite in LV tissue lysates was carried out with the high-sensitivity nitrite assay kit (Measure-

or black (BH₄) boxes indicate when isolated hearts were perfused with L-NAME, L-NIO or BH₄ (for 5 min before and/or after the ischemic period) during ischemia-reperfusion using a Langendorff apparatus

iTTM High-Sensitivity Nitrite Assay Kit; InvitrogenTM) and was performed according to the manufacturer's instructions.

Measurement of cGMP

Quantification of total cGMP in LV tissue lysates (prepared in 0.1 M HCl) was carried out by enzyme immunoassay kit (Enzo Life Sciences) and was performed according to the manufacturer's instructions.

Western blot analysis

Proteins from LV homogenates were separated on polyacrylamide-SDS gels and transferred onto PVDF membranes. Membranes were incubated with primary antibodies at 4 °C in 10 % milk or 3 % bovine serum albumin in Tris-buffered saline containing 0.05 %

Tween-20 overnight. The primary antibodies used in this study were: anti-mouse eNOS (1:1,000; BD Transduction Laboratory), anti-mouse eNOS-P^{Ser1177} (1:1,000; BD Transduction Laboratory), anti-rabbit GAPDH (1:3,000; Santa Cruz) and anti-rabbit nitrotyrosine (1:20,000; Millipore Corporation). Immunodetection was carried out using ECL or ECL Plus system (SuperSignal[®] West Pico Chemiluminescence Substrate, Thermo Scientific; Luminata[™] Forte Western HRP substrate, Millipore Corporation, respectively) and membranes were then exposed to X-ray films for revelation. Protein content was expressed relative to the GAPDH content. To evaluate the eNOS dimer/monomer ratio, SDS-resistant eNOS dimers were detected using non-denaturing conditions and low-temperature SDS-PAGE.

Measurement of protein S-nitrosylation

S-nitrosylation was determined by the modified S-nitrosylation switch assay that uses a non-biological iodoTMT Reagent for labeling (Pierce S-nitrosylation Western blot kit, Thermo Fischer Scientific). Briefly, unmodified cysteines were first blocked with a sulfhydryl-reactive compound. S-nitrosylated cysteines were then selectively reduced with ascorbate in HENS Buffer for specific labeling with iodoTMTzero Reagents, which irreversibly bind to the cysteine thiol that has been S-nitrosylated. Proteins from LV homogenates were then separated on polyacrylamide-SDS gels and transferred onto PVDF membranes. Detection of the TMT reagent-modified proteins was then performed using an anti-TMT antibody (Thermo Fischer Scientific). Immunodetection was carried out using ECL Plus system (Thermo Scientific; Luminata[™] Forte Western HRP substrate, Millipore Corporation) and membranes were then exposed to X-ray films for revelation.

Measurement of total and NOS-dependent ROS generation

ROS production was measured by electron paramagnetic resonance (EPR) in fresh frozen LV homogenates as described [35]. Briefly, homogenates were treated with 1 mM 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine solution (1:1 v/v), put in the EPR glass capillary tube (Noxygen Science Transfer & Diagnostics, Germany), and were placed inside the e-scan spectrometer (Bruker, Germany) for data acquisition. The procedure was then repeated on the same samples but in the presence of L-NAME (3.10^{-4} M). The difference between the two conditions was the NOS-dependent ROS production. ROS production was normalized to the protein content of each sample and then expressed in $\mu\text{mol}/\text{min}/\text{mg}$.

Measurement of lipid peroxidation

Malondialdehyde (MDA), an end product of lipid peroxidation, was measured by dot blot analysis as previously described [1]. Briefly, homogenized tissues were spotted onto nitrocellulose membranes and incubated with the anti-rabbit MDA (1:500; Millipore Corporation) antibody at 4 °C overnight. Signal was revealed as described for Western blotting. MDA content was expressed relative to Ponceau S staining.

Measurement of myocardial total antioxidant capacity

To evaluate the heart total antioxidant capacity, tissues were homogenized in PBS (137 mM NaCl, 24 mM KCl, 19 mM Na₂HPO₄, 18 mM KH₂OP₄; pH 7) at 4 °C. Homogenates were centrifuged at 3,000g at 4 °C for 10 min and the total antioxidant capacity was determined with the Total Antioxidant Power Kit (TAO2, Oxford Biomedical Research) and was expressed in mM of Trolox.

Statistical analysis

Data were expressed as the mean \pm SEM. For comparison of multiple experimental conditions, analysis of variance (ANOVA) or repeated ANOVA were used, followed by the Bonferroni adjusted *t* test. For assessing the difference between values, the Student's *t* test was used. A value of $p < 0.05$ was considered statistically significant.

Results

Implication of eNOS in exercise-induced cardioprotection against IR injury

Exercise training is classically reported to affect myocardial NO synthesis, mainly by increasing eNOS expression [28] or phosphorylation at Ser¹¹⁷⁷ [5, 21]. In our model, exercise training had no effect on eNOS expression, but significantly increased the baseline level of eNOS-P^{Ser1177} (Fig. 2a). In addition, exercise training increased the eNOS dimer/monomer ratio, indicating an increase of eNOS coupling in the hearts from exercised rats in comparison to sedentary animals (Fig. 2b). Chronic exercise did not affect the expression pattern of neuronal NOS and inducible NOS (online Fig. 1) in accordance with previous studies [5, 9]. In addition, exercise increased the level of NO metabolites storage as indicated by the higher protein S-nitrosylation forming S-nitrosothiols (SNO) (Fig. 2c) and the amount of nitrite (Fig. 2d) in exercised hearts. As expected, chronic exercise protected the heart against IR injury as indicated by the higher recovery of post-ischemic LVDP (Fig. 3a) and the smaller infarct size (Fig. 3b)

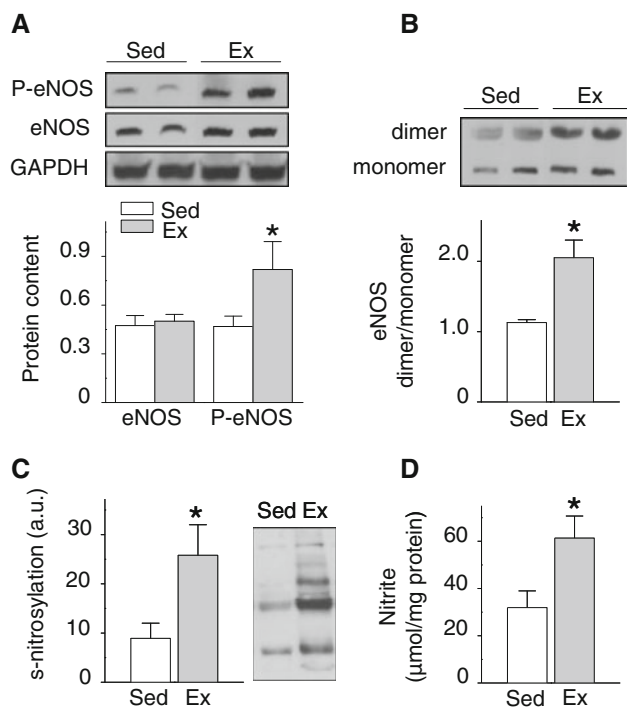


Fig. 2 Exercise training increases eNOS–NO pathway. **a** Effects of exercise on eNOS expression and eNOS phosphorylation at Ser¹¹⁷⁷ (P-eNOS) analyzed by Western blotting in hearts from sedentary (Sed) and exercised (Ex) rats. eNOS and P-eNOS levels were relative to the expression values of GAPDH and total eNOS, respectively. **b** Effect of exercise on eNOS dimer/monomer ratio. eNOS dimers were detected by SDS-PAGE at low temperature and in non-denaturing conditions. **c** Effects of exercise on left ventricle protein S-nitrosylation. **d** Effects of exercise on nitrite concentration in the left ventricle

in exercised than in sedentary rat hearts. To investigate the involvement of the NOS signaling pathway in the cardioprotective effects of exercise, hearts were perfused with either the NOS inhibitor L-NAME or the more specific eNOS inhibitor L-NIO before and after ischemia (Fig. 1, protocol 1). In the presence of L-NAME, post-ischemic LVDP recovery and infarct size in hearts from exercised animals were comparable to those observed in hearts from sedentary rats, which were not affected by exposure to L-NAME (Fig. 3a, b). Interestingly, the same results were obtained with the use of L-NIO (online Fig. 2). Thus, these results indicate that the cardioprotective effects of exercise against IR are NOS dependent and may be mediated by eNOS, whose activation was altered by exercise.

eNOS role in exercise-induced cardioprotection

Time course of eNOS phosphorylation and coupling during IR

We next evaluated eNOS activation during IR in sedentary and exercised animals. Ischemia (30 min) reduced the level

of eNOS-P^{Ser1177} in both exercised (–88 %) and sedentary (–57 %) hearts (Fig. 4a). However, after 10 min of reperfusion, eNOS-P^{Ser1177} was restored to the baseline level in sedentary animals, but not in exercised hearts. Thus, while the level of eNOS-P^{Ser1177} was higher in exercised hearts before ischemia, it was strongly reduced during IR in comparison to sedentary animals (Fig. 4a). No modification of eNOS expression was observed in both sedentary and exercised hearts (Fig. 4a).

Many studies have suggested that eNOS uncoupling has a major role in IR injuries because uncoupled eNOS produces O₂^{•-} instead of NO [8, 31]. We thus investigated whether exercise could protect myocardium during IR by limiting eNOS uncoupling. To this aim, we evaluated eNOS coupling by measuring the eNOS dimer/monomer ratio during IR. Before ischemia, the eNOS dimer/monomer ratio was higher in exercised hearts than in sedentary hearts (Fig. 4b). Ischemia decreased the eNOS dimer/monomer ratio in both groups by about 51 %, although the ratio values remained higher in exercised than in sedentary hearts (Fig. 4b). After 10 min of reperfusion, eNOS dimer/monomer ratio was restored to baseline level in sedentary hearts, whereas it was further reduced in exercised hearts and then was significantly lower than that in sedentary hearts (Fig. 4b).

Altogether, these findings indicate that exercise-induced cardioprotection is associated with reduced eNOS-P^{Ser1177} and increased eNOS uncoupling and consequently reduced eNOS activation during early reperfusion. This may affect the amount of NO and ROS produced.

NO and nitro-oxidative stress during reperfusion

As eNOS uncoupling increases ROS production [51], we first tested whether this phenomenon can modulate oxidative stress in exercised hearts during early reperfusion. After 10 min of reperfusion, despite no difference of total ROS production between sedentary and exercised hearts (Fig. 5a), the contribution of NOS-dependent ROS production was increased in exercised hearts (Fig. 5a, b). This result further suggests eNOS uncoupling after early reperfusion in exercised hearts. Potential oxidative stress damages were assessed by measuring the content of MDA, an end product of lipid peroxidation. Despite eNOS uncoupling, exercised hearts had lower MDA content than sedentary hearts after reperfusion (Fig. 5c). This lower myocardial oxidative stress was associated with increased myocardial total antioxidant power (TAP) in exercised hearts (Fig. 5d).

Considering that eNOS uncoupling is also associated with reduced NO production, we next evaluated the bio-availability of NO during IR in the presence of a recoupling agent (BH₄). For this purpose, some hearts were perfused

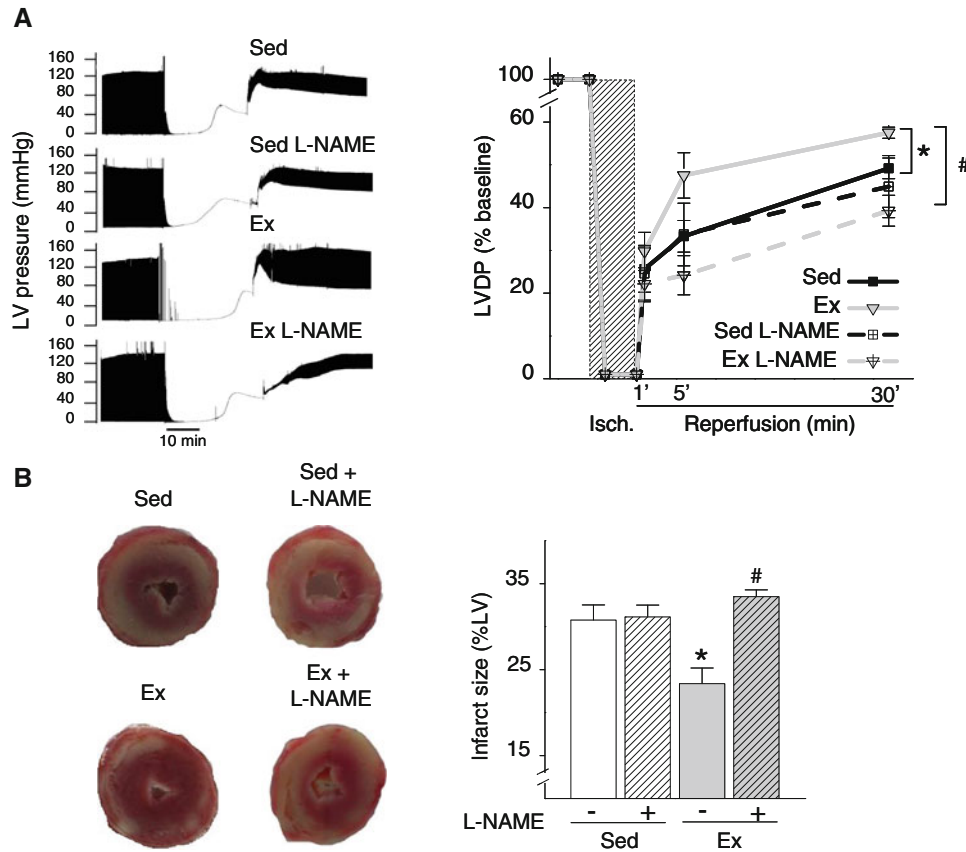


Fig. 3 Implication of eNOS in exercise-induced cardioprotection. **a** *Left panel* Left ventricular (LV) pressure traces in hearts mounted on a Langendorff apparatus, perfused or not with L-NAME during the last 5 min of stabilization and the first 5 min of reperfusion after 30 min of total global ischemia. *Right panel* Left ventricular developed pressure (LVDP) in hearts from sedentary and exercised rats, perfused or not with L-NAME. LVDP is expressed as percentage of the baseline value. **b** *Left panel* Heart sections stained with

triphenyltetrazolium chloride (TTC) to show the infarct size after 30 min of total global ischemia and 120 min of reperfusion. *Right panel* Infarct size in hearts from exercised and sedentary rats, treated or not with L-NAME as described in **a**, following ischemia-reperfusion. Infarct size is expressed as percentage of the left ventricular section area. Values are the mean \pm SEM. * $p < 0.05$ vs. sedentary group; # $p < 0.05$ vs. homolog group treated with L-NAME

with BH₄ before ischemia and/or during early reperfusion (protocol 2, Fig. 1). To ensure that this pharmacological strategy could not be considered as a preconditioning strategy, we freeze clamped control rat hearts at the end of the BH₄ perfusion procedure (5 min, 50 μ M) and measured nitrite concentration in LV homogenates. No effect of BH₄ perfusion on nitrite concentration was observed (online Fig. 3). However, this treatment induced eNOS coupling as indicated by the higher eNOS dimer/monomer ratio in treated exercised hearts at the end of the ischemic period (Fig. 6a) and during early reperfusion (Fig. 6b). In addition, the eNOS dimer/monomer ratio in BH₄-treated exercised hearts was restored to the level of sedentary hearts during early reperfusion (Fig. 6b). We next tested whether eNOS uncoupling and recoupling modulate NO availability during early reperfusion. In the heart, NO acts either by activation of soluble guanylyl cyclase, to increase the conversion of GTP to cGMP, or directly by protein

S-nitrosylation to generate SNO. We measured the level of cGMP, nitrite and SNO in heart homogenates frozen after 10 min of reperfusion. Despite eNOS uncoupling, cGMP (Fig. 6c) was not altered and nitrite tended to be lower ($p = 0.075$, Fig. 6d) in exercised hearts when compared to sedentary hearts. However, SNO remained significantly higher when compared to sedentary hearts (Fig. 6e). In addition, we observed that L-NAME infusion during ischemia-reperfusion markedly reduced nitrite and nitrate (NO_x) in coronary effluents of only sedentary hearts (online Fig. 4). The result strongly suggests that despite eNOS uncoupling, NO availability is not altered and that NO synthesis during early reperfusion in exercised hearts is mainly eNOS independent. Then, we tested whether the recoupling agent BH₄ could improve NO availability in exercised hearts during early reperfusion. Interestingly, in exercised hearts, the levels of cGMP (Fig. 6c), nitrite (Fig. 6d) and SNO (Fig. 6e) during early reperfusion were

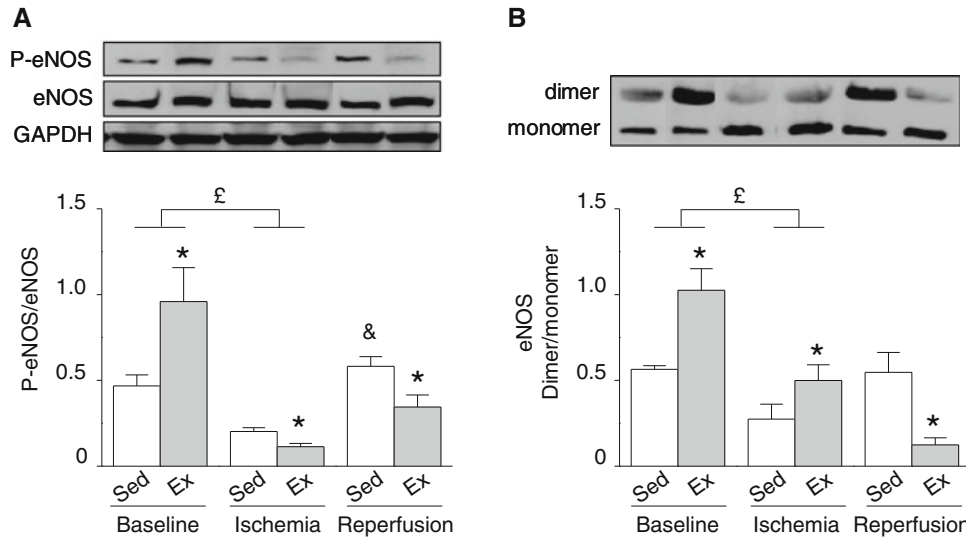


Fig. 4 Reduced eNOS phosphorylation at Ser¹¹⁷⁷ and eNOS dimerization during ischemia–reperfusion in exercised hearts. **a** Time course of eNOS phosphorylation at Ser¹¹⁷⁷ (P-eNOS) in hearts from sedentary (Sed) and exercised (Ex) rats during ischemia–reperfusion (baseline = stabilization, ischemia = after 30 min of ischemia, reperfusion = after 10 min of post-ischemic reperfusion) analyzed by Western blotting. eNOS is expressed relative to GAPDH content

and P-eNOS relative to eNOS total content. **b** Time course of eNOS dimer/monomer ratio in hearts from sedentary and exercised rats during ischemia–reperfusion analyzed by detecting SDS-resistant eNOS dimers using low-temperature SDS-PAGE. Values are the mean \pm SEM. * $p < 0.05$ vs. sedentary group. $^{\xi}p < 0.05$ vs. baseline, and $p < 0.05$ sedentary ischemia vs. sedentary reperfusion

not altered in the presence of BH₄, indicative of unchanged NO availability. In pro-oxidant conditions such as ischemia–reperfusion, NO can react with oxidants to form potent nitroso-oxidant species such as peroxynitrite (ONOO⁻). Then, we measured the amount of protein nitrotyrosination, which is an index of peroxynitrite formation, derived from the reaction between NO and O₂⁻, and is considered an index of nitro-oxidative stress. The amount of protein nitrotyrosination at early reperfusion was lower in exercised than in sedentary hearts (Fig. 6f), suggesting that in exercised hearts, the formation of peroxynitrite is reduced during early reperfusion. In the presence of BH₄, protein nitrotyrosination was significantly increased in exercised hearts and was then normalized to sedentary hearts level (Fig. 6f).

Role of eNOS uncoupling in exercise-induced cardioprotection

Finally, we tested whether eNOS uncoupling was required for the cardioprotective effects of chronic exercise. Maintenance of eNOS coupling during reperfusion in BH₄-treated exercised hearts caused the total loss of the cardioprotective effects of exercise, as indicated by their reduced post-ischemia LVDP recovery (Fig. 7a) and increased infarct size (by 60 %) (Fig. 7b). Similar results were obtained when hearts were treated with BH₄ only during reperfusion (online Fig. 6). Conversely, the infarct size tended to be lower in BH₄-treated than in untreated

sedentary hearts, but the difference did not reach statistical significance. These data suggest that the exercise-induced cardioprotective effects require eNOS uncoupling during IR injury.

Discussion

Recently eNOS activation has been linked to the cardioprotective effects of voluntary exercise [5]. In the present study, we confirm that eNOS is a major player in the mechanism of exercise-induced cardioprotection and show that, during IR, such cardioprotective effects requires eNOS uncoupling.

In accordance with previous works [5, 21, 53], we confirmed that exercise training increases baseline eNOS-P^{Ser1177} and eNOS dimerization. Both modifications lead to eNOS activation and this might, in part, explain the finding that exercise training increases NO metabolites storage, such as nitrite and SNO [5], which is also obvious in our work. Modulation of eNOS activity is also dependent on L-arginine availability. However, exercise was previously reported to have no effect neither on L-arginine concentration [44] nor on its influx into the cells [32]. Increased eNOS-P^{Ser1177} is an efficient way to protect the myocardium [2] and is an essential trigger of exercise-induced cardioprotection [5]. Accordingly, here we show that eNOS plays a major role in exercise-induced cardioprotection because eNOS inhibition (by L-NAME or more specifically

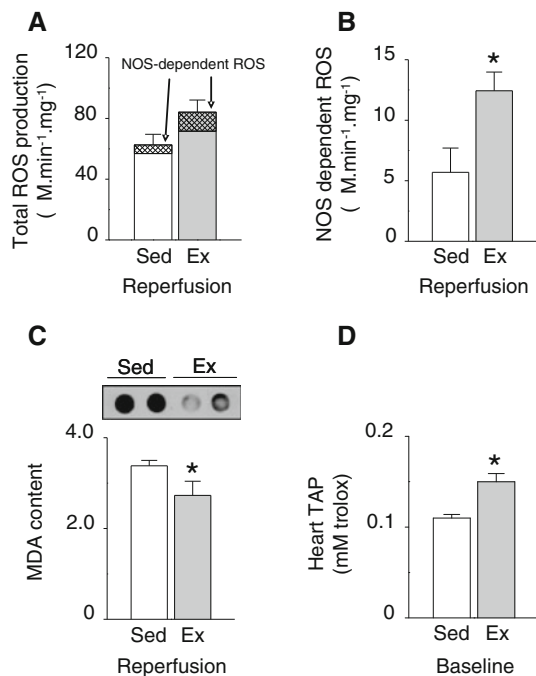


Fig. 5 eNOS uncoupling is associated with increased NOS-dependent ROS production, but reduced ROS-induced cell damages during early reperfusion in exercised rat hearts. **a** Total ROS production measured by electron paramagnetic resonance (EPR) in fresh frozen LV homogenates of sedentary (Sed) and exercised (Ex) hearts after 10 min of post-ischemic reperfusion. **b** NOS-dependent ROS production measured by electron paramagnetic resonance (EPR) in fresh frozen LV homogenates. Values represented correspond to the difference between ROS measurements obtained in the presence or absence of L-NAME. **c** Malondialdehyde (MDA) content, used as an index of lipid peroxidation, measured by dot blot after 10 min of post-ischemic reperfusion in hearts from sedentary and exercised rats. **d** Heart total antioxidant power (TAP) measured in homogenates of sedentary and exercised hearts. TAP is expressed in mM of Trolox equivalent antioxidant capacity. Values are the mean \pm SEM. * $p < 0.05$ vs. sedentary group

by L-NIO) during IR abolished the protective effects of exercise training on heart sensitivity to IR, whereas no effect of eNOS inhibition was observed in sedentary hearts. However, the role of eNOS activation modulation (i.e., phosphorylation and coupling) during IR in such cardio-protective effect was never investigated.

Here, we show that eNOS-P^{Ser1177} and eNOS coupling strongly decrease in exercised heart during ischemia and also during early reperfusion. As increased eNOS coupling is considered to have beneficial consequences in pathological states [31, 34, 48] and that exercise is an efficient strategy to protect the myocardium [13, 39, 40, 50], eNOS uncoupling in exercised myocardium during reperfusion was unexpected. Then, to evaluate whether this phenomenon could play a role in exercise-induced cardioprotection or was only a collateral damage of reperfusion without consequences on heart vulnerability to IR, we perfused hearts with BH₄. An essential point of this work is that

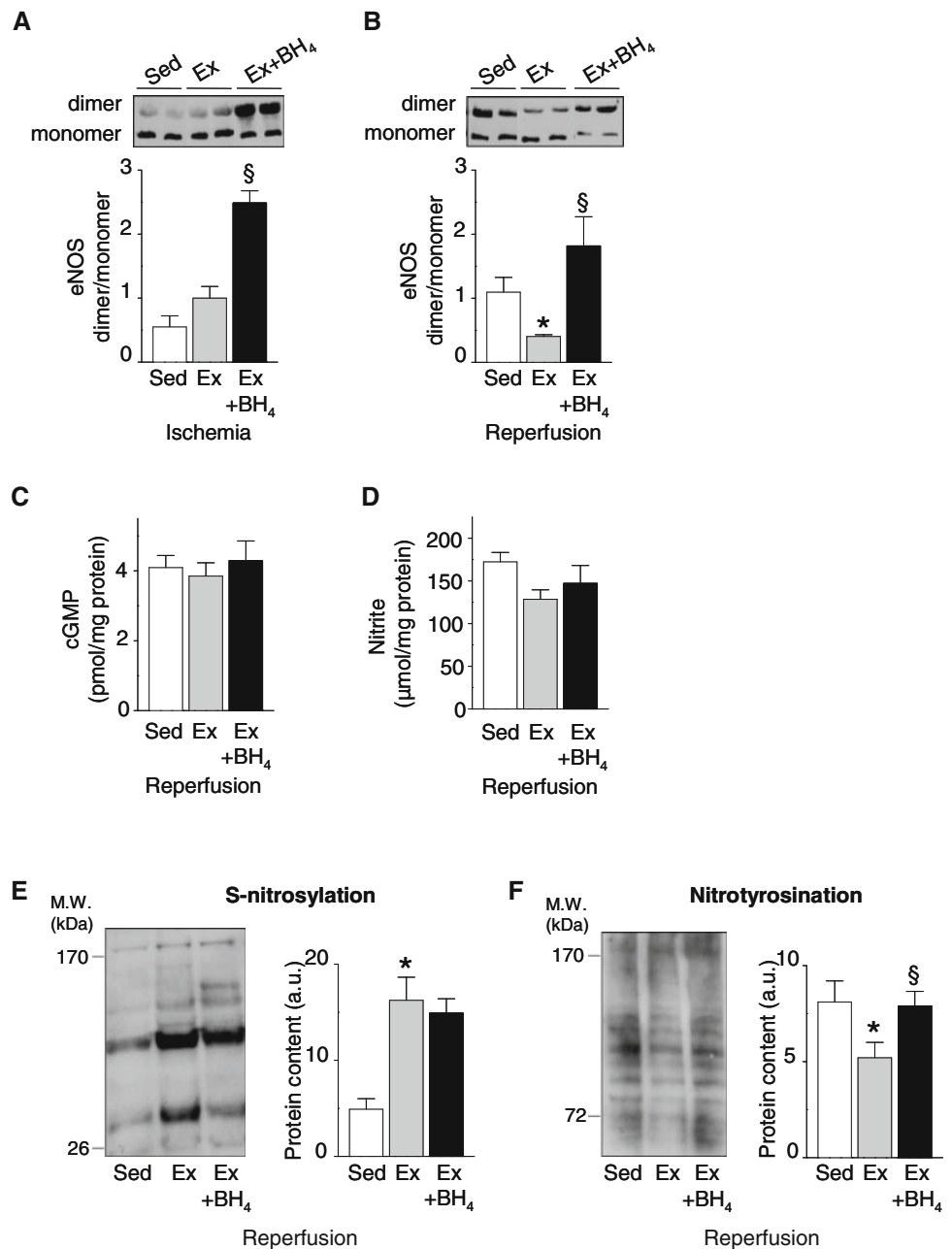
pharmacological maintenance of eNOS coupling in exercised hearts perfused with BH₄ before and after ischemia or during reperfusion only (online Fig. 5) was associated with total loss of exercise-induced cardioprotection, as indicated by the increased infarct size and reduced myocardial function. This result clearly shows that eNOS uncoupling is required for exercise-induced lower heart sensitivity to IR.

In our work, ischemia induced eNOS uncoupling in both sedentary and exercised hearts. This may be due to the fact that ischemia strongly reduces BH₄ content and this reduction is proportional to the duration of the blood flow restriction [11]. In addition, Chen et al. [6] proposed recently that increased eNOS phosphorylation at Ser¹¹⁷⁷ could favor eNOS uncoupling during stress, which could notably be obvious in the post-ischemic heart. To test this hypothesis in our model, sedentary rats received a single bolus of epinephrine prior IR (online Fig. 6A) to rapidly increase, in a β -adrenergic receptors-dependent manner, eNOS-P^{Ser1177} (online Fig. 6B), mimicking then the effects of exercise on eNOS pathway [5]. Epinephrine increased the level of eNOS-P^{Ser1177}, but had no effect on eNOS coupling during early reperfusion (online Fig. 6D). This suggests that eNOS uncoupling at reperfusion is an exercise-dependent mechanism, in which the increase of eNOS-P^{Ser1177} before IR is not directly involved. Then, how exercise decreased eNOS coupling at early reperfusion remains to be investigated. In addition, β -adrenergic receptors-dependent increase of eNOS-P^{Ser1177} performed on sedentary animals was previously reported to reduce heart sensitivity to IR [2, 24], which is also obvious in our work (online Fig. 6C). Nonetheless, we can note that increased eNOS-P^{Ser1177} induced by epinephrine was maintained at early reperfusion (online Fig. 6E), whereas it was clearly reduced in exercised hearts. Taken together, these results mainly suggest that eNOS uncoupling and decreased eNOS-P^{Ser1177} at early reperfusion constitute specific exercise-dependent mechanisms, and that catecholamine-dependent increase of eNOS-P^{Ser1177} is not sufficient to mimic them.

Classically, eNOS uncoupling results in NO decrease [27]. In the present study, eNOS uncoupling increased after reperfusion without affecting NO bioavailability as indicated by the higher SNO and unchanged cGMP content in exercised hearts. Considering that during myocardial ischemia, NO originates mostly from NO metabolites [30], we propose that the higher NO metabolites content, produced in exercised hearts and measured in the present study, constitutes a storage for NO that will be available during ischemia and reperfusion. A previous report using genetic mouse model reached similar conclusions that increased NO metabolites storage by exercise in baseline plays a major role in the exercise-induced cardioprotection against IR, without exploring NO homeostasis during IR

Fig. 6 S-nitrosylation remains higher in exercised rat hearts during early reperfusion despite eNOS uncoupling. eNOS recoupling with BH₄ is not able to improve NO bioavailability, but significantly increases protein nitrotyrosination.

a Effect of BH₄ on eNOS dimer/monomer ratio in hearts from exercised rats, measured at the end of the ischemic period. BH₄ was perfused during the last 5 min of stabilization. **b** Effects of BH₄ on eNOS dimer/monomer ratio in hearts from exercised, measured after 10 min of post-ischemic reperfusion. BH₄ was perfused during the last 5 min of stabilization and the first 5 min of reperfusion. **c** Effects of exercise on cGMP concentration measured after 10 min of post-ischemic reperfusion in hearts perfused or not with BH₄. **d** Effects of exercise on nitrite concentration measured after 10 min of post-ischemic reperfusion in hearts perfused or not with BH₄. **e** Effects of exercise on protein S-nitrosylation measured by Western blot, using the switch assay TMT method, after 10 min of post-ischemic reperfusion in hearts perfused or not with BH₄. **f** Effects of exercise on protein nitrotyrosination measured by Western blot after 10 min of post-ischemic reperfusion in hearts perfused or not with BH₄. Values are the mean ± SEM. **p* < 0.05 vs. sedentary group; §*p* < 0.05 vs. homolog group treated with BH₄



[5]. In addition, such NO metabolites may contribute, during ischemia and early reperfusion, to S-nitrosylation of specific targets, which constitute essential triggers for cardioprotection [7].

In our model, despite eNOS uncoupling and increased NOS-dependent ROS production, total ROS production slightly increased in exercised hearts without reaching statistical significance. In addition, oxidative stress damages were markedly reduced in exercised hearts as indicated by lower lipid peroxidation after reperfusion compared with sedentary hearts. This could be explained by the previously described beneficial effect of exercise on the antioxidant capacity [17, 23] that was confirmed in the

present study (higher total antioxidant capacity in exercised than in sedentary hearts). In addition, increased eNOS-P^{Ser1177}, which normally enhances eNOS-derived NO production, increases eNOS-derived ROS production when eNOS is uncoupled [6]. Therefore, the decrease in eNOS-P^{Ser1177} observed during IR in exercised rat hearts could be beneficial by limiting the amount of eNOS-dependent ROS production. During IR, the protective or detrimental effect of NO may largely depend on the concentration NO and O₂⁻ and their ratio, promoting a pathological concentrations of ONOO⁻ or not [14]. In our work, eNOS uncoupling, by limiting excessive amount of NO synthesis during early reperfusion could contribute, in pro-oxidant

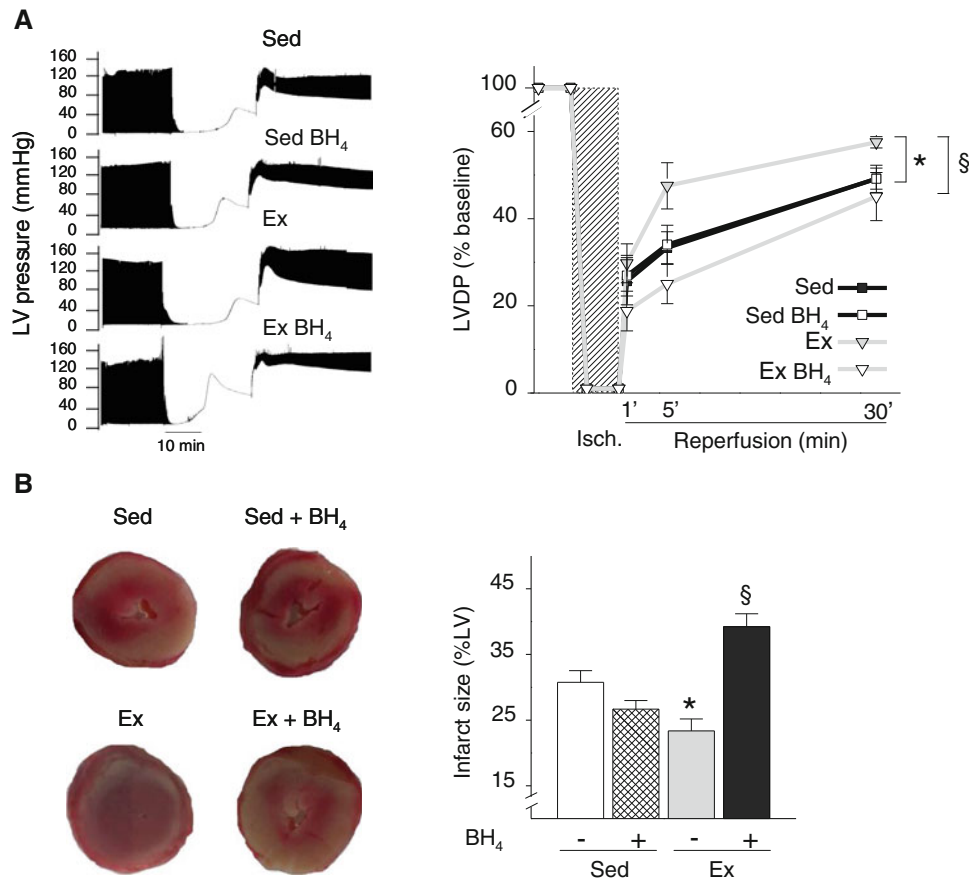


Fig. 7 Exercise training-induced cardioprotection requires eNOS uncoupling at early reperfusion. **a** *Left panel* left ventricular (LV) pressure traces in hearts mounted on a Langendorff apparatus, perfused or not with BH₄ during the last 5 min of stabilization and the first 5 min of reperfusion after 30 min of total global ischemia. *Right panel* left ventricular developed pressure (LVDP) in hearts from sedentary and exercised rats, perfused or not with BH₄. LVDP is expressed as percentage of the baseline value. **b** *Left panel* heart

sections stained with triphenyltetrazolium chloride (TTC) to show the infarct size after 30 min of total global ischemia and 120 min of reperfusion. *Right panel* infarct size in hearts from exercised and sedentary rats, treated or not with BH₄ as described in **a**, following ischemia–reperfusion. Infarct size is expressed as percentage of the left ventricular section area. Values are the mean \pm SEM. * $p < 0.05$ vs. sedentary group; § $p < 0.05$ vs. homolog group treated with BH₄

conditions such as post-ischemic reperfusion, to prevent the toxic reaction between NO and O₂⁻ to form the potent cytotoxic ONOO⁻. Indeed, such cardiac phenotype was associated with lower protein nitrotyrosination, suggesting that ONOO⁻ formation was reduced in exercised hearts during IR. Interestingly, the recoupling of eNOS with BH₄ had no beneficial or detrimental effects on NO bioavailability, since both SNO and cGMP were not altered in exercised hearts by this pharmacological strategy. However, a main point of this work is that in the presence of BH₄, protein nitrotyrosination was markedly increased in exercised hearts and reached the level observed in sedentary rats. This could also contribute to maintain a high level of SNO in exercised hearts perfused with BH₄, since ONOO⁻ also reacts with thiols to form SNO [14]. Altogether, the results strongly suggest that, during early reperfusion, excessive level of NO produced by eNOS rapidly reacts with O₂⁻ to form ONOO⁻ and then

aggravates nitro-oxidative stress. In summary, in exercise-induced cardioprotection, eNOS uncoupling and eNOS dephosphorylation at Ser¹¹⁷⁷ during early reperfusion in association with increased antioxidant capacity to buffer ROS may contribute to prevent the reaction between NO and O₂⁻ to form ONOO⁻.

Finally, because eNOS inhibition by L-NAME or by L-NIO abolished the exercise cardioprotective effects, we cannot ignore the potential contribution of eNOS-dependent ROS production in the activation of classical ROS-dependent anti-apoptotic pathways [38]; however, further studies will be needed to investigate this aspect.

In conclusion, we show here that heart preconditioning by exercise is not the result of a simple increase of eNOS-P^{Ser1177} by a catecholamine-dependent pathway, but of a complex interplay of NO metabolites storage, heart antioxidant capacity and eNOS uncoupling at the early stage of reperfusion (Fig. 8). Indeed, despite that eNOS recoupling

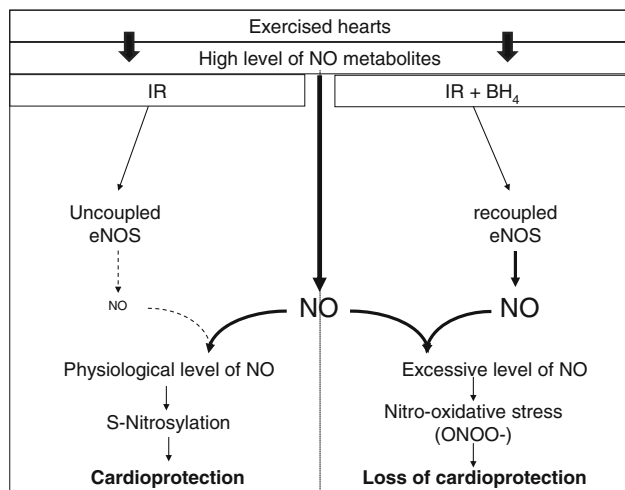


Fig. 8 Proposed mechanism for the role of eNOS uncoupling in exercise-induced cardioprotection. Exercise increased NO metabolites (SNO and nitrites) storage in hearts. During early post-ischemic reperfusion, eNOS of exercised hearts is uncoupled, but NO availability is maintained at high level (increased SNO and no alteration of cGMP concentration), suggesting that NO synthesis is mainly dependent of NO metabolites and is independent of NOS activity. This, associated with the higher capacity of exercised hearts to buffer ROS, results in lower nitro-oxidative stress, higher protein S-nitrosylation, and then cardioprotection. When a recoupling agent (BH₄) is perfused in exercised hearts during ischemia and reperfusion, eNOS is recoupled, which could result in excessive production of NO. However, in pro-oxidant conditions such as ischemia–reperfusion, this excessive NO level does not contribute to increase NO availability (since SNO as well as cGMP are not increased in the presence of BH₄), but increase nitro-oxidative stress, suggesting that excessive NO rapidly reacts with O₂⁻ to form ONOO⁻ and thus the cardioprotection is lost

by BH₄ supplementation during ischemia has been proposed as a potential therapeutic approach to improve post-ischemic functional recovery, in the present study we show that BH₄ perfusion has no beneficial effects on LV functional recovery in sedentary animals and only a very modest, non-significant effect on the infarct size. Moreover, we demonstrate that BH₄ supplementation could dramatically increase nitro-oxidative stress and then heart vulnerability to IR in exercised hearts as keeping eNOS uncoupling during ischemia–reperfusion could be beneficial in some populations. Although more research is needed to better understand how eNOS uncoupling occurs during ischemia–reperfusion, this finding opens new perspectives on the role of the eNOS–NO pathway and puts into perspective the potential therapeutic effects of BH₄ on cardiac health. Our work might offer a new explanation on how exercise protects the myocardium against IR.

Study limitations

Endothelial NOS contributes to coronary blood flow regulation and is highly expressed in coronary endothelial

cells. However, whether this single layer of cells contributes to exercise-induced cardioprotection is not clear. In the present work, no effect of exercise on the recovery of coronary blood flow during IR was observed (online Table 1), suggesting that coronary endothelial function during IR is not altered by exercise. However, the method used by collecting of coronary effluents may not be sensitive enough to detect minor or moderate coronary blood flow changes. In addition, in this work, we did not take into account the potential antioxidant properties of BH₄ [26]. However, the lack of cardioprotective effect of BH₄ perfusion in sedentary rat hearts mainly suggests that, at concentration used in the present work, these effects are insignificant.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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