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Preconditioning Cardioprotection and Exercise Performance: a radical point of view

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Abstract

It is well known that regular exercise training can reduce the incidence of coronary events and increase survival chances after myocardial infarction. Myocardial beneficial effects are due to the reduction of several cardiovascular disease risk factors, such as high cholesterol, hypertension, metabolic syndrome, obesity, etc. Moreover, exercise can reproduce the so-called “preconditioning”: the capacity of brief periods of ischemia to induce myocardial protection against ischemia/reperfusion injury. Pre- and post-conditioning of the myocardium are two treatment strategies that considerably reduce post-ischemic contractile dysfunction and the amount of necrosis. Paradoxically, reactive oxygen and nitrogen species (ROS and RNS) have been identified as essential cardioprotective signaling molecules, in either pre- or post-conditioning phenomena. Several clues demonstrate that preconditioning may be directly induced by exercise, thus leading to a protective phenotype at cardiac level without the necessity of causing ischemia. Also exercise appears to act as a physiological redox-sensible stress that induces antioxidant beneficial myocardial adaptive responses at cellular level. The purpose of the present work is to review the role played by factors released during exercise in improving exercise performance and in triggering cardioprotection *via* a redox-sensible mechanism.

Keywords: *Exercise performance; Preconditioning; Postconditioning; Endothelial factors; Redox balance.*

Introduction

Cardiovascular diseases (CVDs) will be the major cause of death in the world as a whole by the year 2020, and will determine a human and economic costs that will be unmatched by any other disease [1]. *Physical inactivity* is now recognized as a major risk factor for CVD. For example, the relative risk of coronary artery disease has been estimated to be *circa* 2 fold higher for inactive subjects compared to physically active individuals [2]. Recent estimates suggest that about 12% of the cost of CVD can be attributed to physical inactivity [3], making physical inactivity a multi-billion € problem.

Regular exercise is beneficial for the cardiovascular system

A significant amount of time, effort and resources are devoted to methods of prevention trying to reduce the burden that ischemic heart disease poses to our health care system. The notion that *regular exercise* may prevent and cure CVD is supported by numerous epidemiological studies. Physical inactivity is a risk factor for these pathologies and physical activity is cardioprotective. Actually, exercise is one of the oldest therapeutic interventions recommended for the treatment or prevention of diseases. In fact it was recommended by the ancient Chinese, Indians, Greeks, and Romans in various forms [4]. More recently, we can find prescription of regular exercise for the prevention of CVD already in the 1850s in Scotland, Scandinavia and Germany [4], as well as in 1904 in the USA [5]. Nowadays the concept that regular exercise confers protection against coronary disease can be traced to the seminal work of Morris and co-workers [6] and it has been extensively investigated since in a number of studies which have demonstrated that regular exercise is beneficial for the cardiovascular apparatus. Regular physical activity (for about 2-4 years),

was associated with a 27% reduction in total mortality and a 31% reduction in cardiac mortality [7]. Moreover, exercise has been suggested as a useful tool in rehabilitation for stable coronary insufficiency and after infarction [8-10], and there are evidences supporting the beneficial effect of regular physical activity in patients with coronary disease [11]. It has been reported that exercise reduces the incidence of arrhythmias [12-14], improves coronary vascular reactivity [15-17] and decrease myocardial stunning [18,19] in hearts experimentally exposed to ischemia/reperfusion (I/R). In humans, exercise decreases the incidence of myocardial infarction and increases the chances of survival after coronary events [20-30]. Furthermore, it is well established that *exercise capacity* is a good predictor of reduced risk of death from any cause in both healthy subjects and in those with CVD [31].

However, the mechanisms through which regular exercise protects against chronic and acute CVD have not yet been completely elucidated.

The putative mechanisms of cardiovascular protection by exercise

It has been proposed that physical training operates by:

- 1) *improvement of endothelial function*. Indeed, the vasculature is the largest organ in the body and endothelium is important in regulating some key functions in homeostasis such as platelet aggregation, immune responses, and vascular permeability. Moreover, the endothelial cells produce numerous substances, including nitric oxide (NO), which is important in regulating vasomotor function, thereby determining blood flow distribution to each tissue and in maintaining the health of the vascular wall [32]. It is now established that exercise improving endothelial functions and endothelial-dependent vasodilatation increases gene expression for endothelial NO synthase (eNOS) [16,33-36].

- 2) *reduction of vascular resistance and structural adaptations in the coronary tree (i.e. increased number of capillaries and number and size of arteries and arterioles)*, thereby enhancing the blood transport capacity at this level [15-17];
- 3) *reduction of several risk factors* related to cardiovascular pathologies, including high blood pressure, dyslipidemia, obesity, insulin resistance, and autonomic deregulation [3,8,37,38].
- 4) cardioprotection by exercise includes also *modified gene expression*. In fact, it has been demonstrated that even mild exercise can trigger gene modifications that may become relevant for cardioprotection [39,40]. In particular, exercise induces the increase of cytoprotective molecules, including heat shock proteins (HSPs) and antioxidant defense (see below) [19]. However, there is a need for studies that utilize an integrated “omics” approach (genomic, proteomic and metabolic analysis) with subsequent robust network bioinformatics analysis to identify key regulatory networks, signaling hubs and confounders in response to exercise as well as to other conditioning stimuli.

Similarity between preconditioning and exercise

Besides exercise, a growing number of strategies have been described as possible treatment to protect the heart from I/R injury. Among them, one the most powerful is the so called “*ischemic preconditioning*” phenomenon, first described by Murry et al. [41], where a series of short periods of ischemia separated by brief episodes of reperfusion before a long *index/infarcting* ischemia greatly reduced infarct size. Since then, several researchers have put forward the idea that cardiovascular protection by exercise and by ischemic preconditioning may share several mechanisms.

The similarity and differences between exercise and preconditioning have been analyzed in some recent reviews [42-48]. Similarly to exercise, conditioning protocols may affect gene expression, as elegantly reviewed by Ferdinandy's group [49]. Here we consider only some aspects of the mechanisms of preconditioning and exercise.

After a general introduction on preconditioning and exercise we will specifically analyze mitochondrial and endothelial function as they are of paramount importance both in exercise and preconditioning. In particular, we will describe *a)* the role of some autocrine and paracrine factors in influencing endothelial function in exercise and preconditioning, *b)* the role of mitochondrial reactive oxygen species (ROS) in these two phenomena, *c)* redox aspect and interaction with gasotransmitters on the endothelial function during exercise and preconditioning.

Conditioning by brief ischemia as protective procedure

Ischemic Preconditioning

As said, one of the most promising approaches in reducing myocardial I/R injury is ischemic preconditioning (I-PreC), a phenomenon induced by repetitive brief episodes of ischemia during early reperfusion before a prolonged ischemic insult [42,50-52] In recent years, it has been shown both in the experimental and clinical settings that the infarct-limiting effect of ischemic preconditioning is due to the autocrine/paracrine effects of ligands released by the preconditioned tissue (see below). It is likely that ligand formation and release is favored by oxygen tension drop in the tissue, which occurs also in exercise [53]. Of note, the hypoxia also promotes an increase of mRNA levels of protective expression of

hypoxia-inducible factor 1 (HIF-1) target genes, such as HSPs (for extensive reviews, see [49,54]). However, it has been suggested that hypoxic preconditioning is due to the reoxygenation rather than to hypoxia *per se* [55].

The Trigger phase: the ligands may couple to G-protein-linked receptors, or tyrosine kinase receptors, or may activate directly intracellular signaling pathways, including kinases, such as protein kinase C (PKC), nitric oxide synthase (NOS), mitochondrial ATP-sensitive potassium channels (mKATP), which may promote ROS production. Nitric oxide and ROS may react to obtain reactive nitrogen species (RNS). Both ROS and RNS have a fundamental signaling role. In fact, antioxidant given in this phase avoids the induction of preconditioning cardioprotection [42,43,50-52,56].

The mediation phase: cardioprotective mechanisms and pathways similar to those observed in the trigger phase are operative in the reperfusion phase. These ROS/RNS signaling, as well as protective signaling pathways, namely cGMP/PKG (cyclic guanosine monophosphate/Protein kinase G), RISK (Reperfusion Injury Salvage Kinase) and SAFE (Survivor Activating Factor Enhancement), which converge on mitochondria. Thus, for the endogenous ligands the final targets of the protective pathways are the mitochondria, where the signaling induces protection by preventing mitochondrial permeability transition pore (mPTP) formation, which is considered the end-effector of the protective pathway. Thus, regardless the fact that the ligands are formed during the pre-conditioning procedure, the real protection occurs against reperfusion injury, which follows the index ischemia [43,56].

In brief, ***three mainstream cardioprotective pathways*** are described, namely the *cGMP/PKG pathway*, which starts from nitric oxide formation and guanylyl cyclase (GC)

activation, the *RISK pathway*, which includes activation of protein kinase B (PKB, also known as Akt) and extracellular signal-regulated kinase (ERK)1/2, and the *SAFE pathway*, which requires the activation of the signal transducer and activator of transcription 3 (STAT3). These protective pathways, here only mentioned, have been described in several excellent reviews, to which the reader is kindly redirected; see for example [43,52,57-61].

Other conditioning protocols

Besides ischemic preconditioning the heart may be “conditioned” by other approaches. *Ischemic postconditioning* can be triggered by short cycles (a few seconds) of I/R at beginning of reperfusion, immediately after ischemia. It is a protective mechanisms observed in early phase of reperfusion after an infarcting ischemia and has also shown to be protective against I/R injury [59,60,62-64]. Similarly to preconditioning, postconditioning reduces: infarct size, apoptosis, post-ischemic arrhythmias and endothelial-dysfunction/activation.

Cardioprotection by *pharmacological postconditioning* has also been described. It is obtained with the infusion of some of the agents able to induce preconditioning, but these agents must be applied very soon in reperfusion, because the first minutes of reperfusion are critically important [59,60,65,66].

More recently, *remote ischemic pre-, per- and post-conditioning* have also proved to be protective against cardiac I/R damage [67-73]. These approaches consist in subjecting a limb or organ, remote from the heart, to brief cycles (a few minutes) of ischemia/reperfusion, *prior to or during or after* cardiac ischemia, respectively (Fig. 1).

Cardiac ischemic and remote preconditioning have been shown to induce both early (the above described phenomenon) and delayed cardioprotection [73-76]. This delayed protection, is also named “*second window of protection*” (SWOP).

While the early protective phenomenon is active immediately after conditioning ischemia and lasts for about 2-3 hours, SWOP begins 12-24 hours after the initial conditioning ischemia and lasts 72-90 hours conferring a delayed cardioprotection [77,78]. This late phase of protection is particularly interesting in a clinical perspective because of its sustained duration. Besides duration, other differences between early preconditioning and SWOP exist. For instance, SWOP is more effective than early preconditioning in attenuating myocardial stunning, whereas the early phase is characterized by a pronounced infarct-sparing effect [42,79,80]. Notably, a cardioprotective role of HIF-1 and related target genes have been demonstrated in both delayed and early phase of preconditioning as well as in remote limb ischemic preconditioning [43,81].

The redox signaling in cardioprotection (preconditioning and postconditioning)

Here we report some details about the role of ROS/RNS signaling in pre- and postconditioning of the heart. Transient pre-ischemic ROS/RNS formation is of paramount importance in triggering preconditioning cardioprotection. Whether redox signals arise during the reperfusion phase which follows the brief preconditioning ischemia [82] or during preconditioning hypoxia/ischemia itself [83], is a matter of controversy. Although preconditioning modulates ROS/RNS production during the infarcting ischemia [82-85], it also limits, but not avoids, ROS/RNS production in reperfusion [52,58,61,83,85,86].

Actually, the acidosis and redox signaling at the beginning of reperfusion contribute to trigger cardioprotective pathways, comprising several processes of activation/inhibition of key enzymes. Moderate acidosis plays a critical role in the prevention of mPTP opening. In fact, similarly to ischemic postconditioning a slight acidosis treatment in the initial phase of reperfusion is protective. Acidosis cardioprotective role has also been clearly demonstrated in postconditioning experiments and has been attributed to the direct action on mitochondria, where mPTP formation is prevented [87]. Consistently, transient preconditioning with acidosis is cardioprotective [88]. Preconditioning not only decreases tissue acidosis and anaerobic glycolysis during the subsequent sustained ischemic period [84], but also avoids quick pH recovery in the early reperfusion [58]. Therefore, in the initial phase of reperfusion a slight acidosis plays a critical role in the cardioprotection against reperfusion injury both in pre- and post-conditioning. Thus preventing mPTP opening, slight acidosis limits ROS production and avoids the ROS-induced ROS release (RIRR) phenomenon, which is irreversible and will lead inevitably to cell death. Therefore, ROS/RNS signaling is also cardioprotective at the beginning of reperfusion. In fact, ROS/RNS are double-edged swords whose harmful role is well known in I/R context. Nevertheless their cardioprotective role has been unequivocally demonstrated in postconditioning experiments. This redox sensible protection has been attributed to the direct and indirect modulation of pro-survival kinases activity, including PKC [52,61,82,89] (Fig 2). In summary, both in pre- and post-conditioning the persistence of a slight acidosis and a slight production of ROS/RNS in the early phase of reperfusion play crucial roles in the cardioprotection against I/R injury.

The redox signaling in exercise

It is clear that acute exercise modifies redox homeostasis in every fluid, blood cell, tissue and organ. In fact, several studies have found alterations in redox homeostasis after acute exercise in several tissues [90-100] including the heart [101].

There are several types of exercise and ROS/RNS production may be typical for each type of exercise protocol and this makes this issue particularly complex. From the point of view of redox signaling, it may be important/useful to divide the acute exercise in non-muscle- and muscle-damaging exercise. It seems clear that *non-muscle-damaging exercise* induces changes in redox homeostasis that lasts in tissue and blood a few hours after the exercise bout [102-106]. This form of exercise seems more similar to the ischemic conditioning, where brief non-damaging ischemia triggers protection. Exercise may be a paradigmatic example of the beneficial effects induced by transient oxidative signaling [107-109], which also comprises an up-regulation of eNOS [110,111] and other cytoprotective molecules, including HSPs. HSPs up-regulation occurs as a consequence of both acute exercise and, especially, of chronic cardioprotective exercise, and persists over training cessation [112,113].

Here a first question arises: *is exercise-induced cardioprotection due to an enhanced ability to produce ROS/RNS or to an ameliorated capacity to scavenge ROS/RNS?*

It must also be considered that amount of products deriving from the anaerobic metabolism (such as ADP, adenosine, lactate *etc.*) is released into the blood during exercise, even without the need of flow reduction used to cause remote preconditioning [114-116]. Among these substances in the blood are also found factors modifying the redox homeostasis [102-106].

Thus a second question arises: *are these substances and factors produced during exercise by remote organ to trigger cardiac preconditioning?*

To the best of our knowledge there is not a definitive answer to these two questions. Nevertheless, for what concern the second question, it has been recently demonstrated that dialysate plasma from humans undergoing high-intensity exercise reduced infarct size in isolated rabbit hearts after ischemia-reperfusion injury. This phenomenon was also present with plasma from humans exposed to remote ischemic preconditioning [117]. These data suggest that exercise-induced cardioprotection is, at least in part, similar to remote conditioning, *i.e.* it is mediated by systemic release of one or more humoral factors reaching the heart. Similar results were also obtained in the mice heart perfused with dialysate plasma from highly trained humans (swimmers) undergoing a protocol of ischemia-reperfusion to trigger the remote preconditioning phenomenon. In this study, along with the infarct reduction effect in the mice heart, the remote ischemic preconditioning maneuvers were also able to enhance the athletic performance during swimming [118].

All together the above data support an important role for the substances produced by “remote organs” in protecting the hearts also during exercise. However the metabolic demand of the heart increases almost in parallel with exercise effort, thus a contribution from the autocrine/paracrine effects of factors released by the heart itself is more than likely.

For what concern the first question (*is exercise-induced cardioprotection due to an enhanced ability to produce ROS/RNS or to an ameliorated capacity to scavenge ROS/RNS?*). Either phenomena may be true. The predominance of one or the other mechanism may depend from several factors.

As candidates for triggering cardioprotection after acute exercise, the activation of adenosine, bradykinin and/or opioid receptors, and/or surges in inflammatory adipocytokines, as well as transient ROS/RNS production have been put forward (Fig. 2).

Several studies reported that the improvements in cardiac function and infarct-sparing effects triggered by exercise, are lost when antioxidants are assumed by the individuals during the exercise bouts [119-121]. Moreover, exercise increases the activity of myocardial NADPH oxidase (a ROS producers), and its inhibition abrogated the cardioprotective effects of acute exercise [122]. A small ROS burst may increase antioxidant buffering capacity by promoting gene expression and protein synthesis, similar to what observed in skeletal muscle [123].

In the heart there are several antioxidant enzymes, which can upregulate their activity after exercise. However, it seems that this is not a feature of acute exercise, which may be, however, able to induce preconditioning-like cardioprotection. In fact in animals voluntary free wheel running [124] and low-intensity treadmill running [125-127] do not increase myocardial *manganese-dependent superoxide dismutase* (MnSOD) levels. Moreover, neither MnSOD mRNA [128] nor protein levels [129] are augmented after acute exercise. A clear upregulation of MnSOD, in both activity and enzyme expression, can be seen only in studies examining exercise protocols of longer duration [125,130-133] a feature that appears to be preserved in the aged heart [132,133]. While, heightened enzymatic scavenging of superoxide anion (O_2^-) seems a feature of adaptation to chronic exercise, an enhanced enzymatic set of hydrogen peroxide (H_2O_2) scavengers does not seem to be necessary for exercise-induced cardioprotection. In fact, only a few studies have reported and enhanced *catalase* activity in the heart [130], with most studies reporting no difference

in cardiac catalase and *glutathione peroxidase* activities after exercise training [14,124,134-136]. Moreover, myocardial *thioredoxin* appears to be unaffected by exercise [134]. Finally, whether *glutathione reductase* is involved in exercise-induced cardioprotection remains to be clarified. Some studies observed enzyme increase with exercise [132,137] whereas others reported no change [124,131,134].

Moreover, the results with exogenous antioxidants are controversial. In fact, while several studies suggest that antioxidant supplementation induces a positive effect [138,139], others studies report either a negative [140,141] or a neutral effect [142-144] on exercise performance. Similarly, several studies have reported that antioxidant supplementation limits oxidative stress [145,146], others report a pro-oxidant effect [147] and others describe a neutral effect on redox homeostasis [144].

In summary, it seems that a modest increase in reactive species may trigger cardioprotection, whereas this positive effect may be reversed at higher ROS/RNS concentrations in a dose-dependent manner. This is in line with the idea that antioxidant supplementation on redox homeostasis are dependent on the antioxidant concentration and effect: too much generation of ROS/RNS may be harmful whereas modest generation may be beneficial [148].

The beneficial effects of exercise on properties of endothelial cells and vasculature: a redox perspective.

Endothelium plays a role of paramount importance in exercise regulation [149,150]. Endothelial cells (ECs) form a multifunctional *signal-transducing surface*, which regulates several fundamental processes, including blood flow and pressure response to exercise. ECs

present numerous ion channels and enzymes and produce many factors affecting the response to exercise. Some of these factors are produced “on demand” (NO, endothelins, PGI₂, and apelin), whereas others compounds (tissue plasminogen activator, von Willebrand factor, tissue factor pathway inhibitor, etc.) are stored in granules and released by exocytose, tonically and in response to various stimuli. Among stimuli there are both chemicals/neurotransmitters (such as apelin, histamine, acetylcholine, angiotensin, bradykinin, ATP, ADP, thrombin, growth factors) and mechanical stimuli (such as increased pulsatility and shear stress) [149-152]

The increase of shear stress and pulsatile pressure during acute exercise represent the mechanical stimulation of the arteries and arterioles. These two important mechanical stimuli “augment” the endothelial regulation of vascular tone to ameliorate blood flow where needed. Shear stress and cyclic tension may thus operate to maintain the viability and phenotype of normal endothelium [153,154]. During acute exercise, in particular, the mechanical forces acting on ECs induce the release of NO and other vasodilator factors, that in some vascular district (see infra) dilate preferentially arteries and more proximal arterioles and may act as a local vasodilator amplifier of the metabolites released by the muscle, which act mainly on smaller arterioles [155,156]. Therefore metabolites (such as adenosine or K⁺ acting on K⁺ ATP sensible channels) and endothelial factors (e.g. NO acting also *via* cGMP) present a synergistic action in determining in specific vascular regions their vasodilator effects.

Even when the function of the endothelium is compromised, as it can occur in aging and/or exposure to risk factors for cardiovascular disease, exercise represents an important and beneficial effect and this effect is correlated with improved production of NO [157]. The

beneficial effects of exercise, such as increased vasodilators and reduced vasoconstrictors, and lowered blood pressure, may contribute to reverse the endothelial dysfunction possibly through an increased availability of NO due to an increased production and/or inhibition of NO degradation and scavenging by lowered lipoprotein levels. Importantly, the improvement of endothelial function during exercise is correlated with an increased extracellular superoxidismutase (ecSOD) activity leading to a reduction of ROS and increasing NO half-life [110,158,159]. In fact ROS have a major role in NO degradation. It is likely that exercise inducing the formation of NO and scavengers of O_2^- , limits the formation of the deleterious peroxynitrite ($ONOO^-$) and favors positive processes of S-nitrosylation [52,56]. However, as said above, it is not clear whether and how different exercise types and training alters antioxidant defense system and NO availability in humans. Intriguingly, animal studies suggest that at certain point of training an increased NO availability is no longer detectable [160,161]. It has been suggested that short-term exercise training enhances NO production and bioactivity to induce vasodilation and to buffer increased shear stress. After extended training increased NO production, and possibly other factors, induces structural changes to the vessel resulting in an increase in lumen diameter. Hence, shear stress is constantly normalized and endothelial NO production returns towards initial levels [161]. However, a regional differences in effects must be taken in account as endothelial and/or vascular adaptations are specific to certain regions, regardless if the skeletal muscles of the considered region are active or not during training bouts [157,162,163].

Autocrine/paracrine protective effects of factors released during I-PreC or Exercise

Several substances released by exercising tissue may be responsible of the observed protective effects. Among these are included adenosine, apelin, bradykinin, opioids and gasotransmitters (CO, H₂S and NO); several of these have been widely described in several recent reviews [42-48,52,57-61,164], here we consider only apelin and H₂S giving particular emphasis to their redox signaling role.

Apelin

Adipokines or adipocytokines are active polypeptides produced by the adipocytes. They exert a plethora of functions in physiological and pathological conditions and several of them are considered as predictor markers for cardiovascular diseases [165].

Production and concentration of various adipocytokines, such as apelin, visfatin, resistin, leptin, adiponectin, IL-6, MCP1 and TNF- α are influenced by physical exercise. Because of the role of exercise in protecting myocardium against I/R injury, it may be argued that changes in the production of one or more adipocytokines could affect the exercise-induced protection. Actually, the exercise-induced changes in leptin, TNF-a, adiponectin, IL-6 and MCP1 release have already been extensively discussed in recent reviews [166,167].

Here we focus our attention on apelin, an adipokine widely studied in cardiovascular system for its cardiac protective effect. The most active isoforms on cardiovascular system are apelin 13 and, to a lesser extent apelin 36, while the predominant in the heart and plasma is the pyroglutamyl apelin 13 ((pyr)-apelin 13) [168-170]. Adipose tissue is not the only source of apelin. It is also expressed in various organs and tissue, as, e.g., heart (right

atrium), lungs, kidney, liver, gastrointestinal tract, brain, adrenal gland and endothelium of large conduit vessels and endocardium [171,172].

On the cardiovascular system, apelin acting on its own receptor (*i.e.* APJ, a G protein coupled receptor) induces vasodilatation and cardiac inotropy, attenuates arrhythmias and I/R injuries, and promotes angiogenesis. The inotropic effect of apelin has been considered a component of the response to increases in pre- and afterload [172]. Nevertheless, apelin inotropic efficiency is a matter of controversy because apelin was seen to produce either a powerful [168,173,174] or a slight (18%) and brief (1-3 min) increase in left ventricular developed pressure [170,175-177].

As above-mentioned and similarly to other endothelial factors, apelin may be released by endocardial endothelium and vascular endothelium after an increase of shear stress [172]. In fact, it has been reported that an exercise-induced increase in shear stress triggers apelin-mediated vasodilatation and improve cardiac contractility [172]. Actually, the apelin vasodilator effect is endothelium- and NO-dependent [168,177,178]. In fact, in the presence of endothelial dysfunction or NOS inhibition, apelin acts directly on smooth muscle causing vasoconstriction *via* a PKC activation and an increase in intracellular calcium concentration [177,179].

Intriguingly, it has been observed that aerobic exercise induces an increase of NOS mRNA expression and NO production, together to an increase in plasma apelin concentration [180]. This is in line with the observation that the *i.v.* administration of apelin induces NO production and promotes eNOS mRNA expression in vascular endothelial cells [181], *via* PI3K/Akt pathway [182].

Apelin plasma level doubled after training with repeated aerobic exercise in both middle-age and old healthy subject [180]. Moreover, in spontaneous hypertensive rats, which were characterized by a down-regulation of apelin, swimming training induced an increase of apelin and APJ levels in plasma, myocardium and aorta, together with a reduction in blood pressure. These results suggest that the effect of training on hypertension may be mediated by an up-regulation of apelin/APJ system [183].

An increase of apelin concentration was observed in plasma of type 2 diabetes patients, after training with aerobic, but not with resistance exercise. In these patients, aerobic training induced also an increase in insulin sensitivity and attenuated carotid intima-media thickness progression [184]. In this study there was no difference in apelin level between healthy and diabetic subjects before training. On the contrary Krist *et al.*, [185] report a higher apelin mRNA expression in adipose tissue and concentration in plasma of diabetic patients. Since in the studied diabetic cohort the fat mass percentage was significantly higher than in the control, it can be argued that the increased apelin concentration was related to the overweight.

Consistent with what described right above, apelin plasma level is augmented in obese subjects, but decreased in women when aerobic exercise training causes a reduction of body fat mass [186]. However, this does not occur in males in spite of a reduction in fat mass; interestingly, apelin mRNA expression does not change in adipose tissue but increased in skeletal muscle [187].

In the case of exercise training also a protection against I/R injury takes place. The protection consisted in the limitation of the infarct size and the improvement of post-ischemic mechanical recovery [188]. Due to the timing of exercise training, this kind of

protection must be taken as a sort of preconditioning. However, in isolated rat hearts, protection was obtained only if apelin was infused at beginning of reperfusion (that is postconditioning), but not if given before the infarcting I/R [177]. Since exogenous apelin acts as postconditioning agent only, it may be argued that the relevant endogenous apelin in trained subjects is acting in reperfusion.

Apelin exerts its protective effect against I/R by delaying the opening of mPTP via PI3K/Akt and NOS signaling pathway [189]. Apelin displays also antioxidant properties. In fact, ROS production was reduced in cardiomyocytes when apelin was added to the medium before hypoxia-reoxygenation [190]. Attenuation in O_2^- and $ONOO^-$ levels was seen also in isolated hearts when apelin was given before I/R [191]. Moreover, apelin affects O_2^- and H_2O_2 production in mitochondria [192]. These antioxidant effects of apelin seem to be due to the recovery of the activity of antioxidant enzymes, such as Cu- and Zn-SOD, catalase, and glutathione peroxidase, in myocardium after I/R [191,193]. In particular, the involvement of catalase in mediating apelin-induced removal of H_2O_2 has been observed also in pressure overload mice hearts, where this effect was also seen to prevent myocardial hypertrophy [194]. As reported above, this is in part at variance with the antioxidant effects of chronic exercise, in which SOD expression increases, but peroxide scavenging activities are not usually up-regulated. Notably, apelin effects on redox conditions are also mediated by pro-survival signaling pathways, comprising PI3K, PKC, and/or mKATP channels [191] (Fig 2). Nevertheless, it has been reported that after 2 weeks of apelin injection in male rats, an increase in mitochondrial enzyme activity and respiratory chain protein content can be observed in triceps, but not in soleus muscle and in myocardium [195]. The absence of this

increase was attributed to the already high levels of mitochondria in high oxidative tissue, such as the myocardium.

Gasotransmitters, physical exercise and cardioprotection

The role of gaseous signaling molecules in physiology and pathophysiology has been extensively described in recent literature [e.g. 33-35,162-164,196]. Concomitantly, gasotransmitters (CO, NO and H₂S) effect on physical exercise has received scientist attention with particular focus on NO. By definition, gasotransmitters are toxic at high concentrations whilst indispensable at low doses that are constitutively produced [164,196]. Impaired basal production of these molecules leads to altered cardiovascular functions and all gasotransmitters have been involved in cardioprotection [164]. In addition, as said, there are a plethora of evidences for cardioprotection conferred by physical exercise *via* NO metabolites [164,197] suggesting an important role of gasotransmitters in exercise-induced cardiac protection. Although NO is a paramagnetic species and as such can act as gasotransmitter in short distance (in neighboring cells) during organ's conditioning procedures, its halftime in the circulation is brief and would not allow long traveling distance as remote conditioning would need. Nevertheless, nitrite may represent a major bioavailable pool of NO, on which hemoglobin may act as a nitrite reductase, thus potentially contributing to NO-mediated hypoxic and exercise vasodilatation, even in remote organs [198]. Despite of the large amount of research about CO and its cardiovascular effects [164], very few studies considered a possible involvement in the cardioprotective effect of physical exercise. The advent of CO-releasing molecules (CO-RMs), as compounds capable of carrying and liberating controlled quantities of CO, may help to overcome the dangerous limitations of CO as gasotransmitter [199]. In particular,

Vanergriff *et al* provided the first evidence for CO-MP4, a CO-carrier agent, as deliverer of CO to the circulation is able to reduce I/R injury in rats [200]. Intriguingly, an increase in the mRNA levels of heme-oxygenase-1 (HSP32), the endogenous CO generator with antioxidant properties, has been observed in skeletal muscle after repetitive contractions [201]. This may underlie an inducible antioxidant pathway in muscle responsive to metabolic stresses associated with repeated muscle contractions. However, to the best of our knowledge, an upregulation of heme oxygenase-1 (HSP32) after exercise in cardiac muscle has not been described so far.

NO and Physical exercise. As seen above, NO is the member of the gasotransmitters family which has received the major attention. Its beneficial effects in terms of endothelial function, response to ischemia and blood pressure regulation are also involved in the adaptation to physical exercise. A pivotal mechanism against myocardial I/R injury, which deserves few words of discussion, relies on the β -adrenergic stimulation of NOS. In fact, circulating catecholamines are elevated upon voluntary physical exercise and eNOS activity increases *via* stimulation of β_3 -adrenergic receptors and after injection of epinephrine [202]. However, while β_1 and β_2 stimulation increases inotropism and lusitropism, β_3 receptors are shown to enhance production of NO from eNOS leading to a negative inotropic effect [203]. However, another putative mechanism is based on the sympathetic drive that, during physical exercise, stimulates β_2 receptors both on endothelial and cardiac cells upregulating eNOS activity. In fact, recent evidences show how β_2 stimulation can activate eNOS *via* a pro-survival Src kinase-PI3K/Akt-dependent pathway although independently from cAMP/PKA, MAPK, and AMPK [204]. As said above NO may exert both positive and

negative inotropic regulations [164], thus an interesting relation exists with sympathetic signals, which deserves further attention.

H₂S and Physical exercise. Hydrogen sulfide has been shown to influence exercise capacity during sub-maximal and maximal exercise [205] and, concomitantly, several evidences confirm a potent cardioprotective effect against ischemia\reperfusion injury and a mediatory role in both pre- and postconditioning [164,206,207]. A possible link between the two effects probably relies in the ability of H₂S to influence mitochondrial activity. At sub toxic doses, H₂S can serve to the respiratory chain through Sulphide Quinone Reductase [208]. Considering these experimental evidences it seems reasonable to assume a potential role for H₂S in physical exercise-mediated cardioprotection. However, probably due to the recently emerged attention to the latest member of the gasotransmitters family, there is no animal study showing a link between the subjects. The only report that somehow approaches the problem is Tiagy's review on the effect of H₂S on myocytes metabolism [209]. Unfortunately, aside from analyzing power output capacity upon inhalation of different concentration of H₂S, there is no evidence linking H₂S mediated cardioprotection and physical exercise.

Along with the protective action on the myocardium, emerging evidence supports the notion that preconditioning may be beneficial also for the skeletal muscle, thereby improving exercise performance.

Preconditioning and its potential effects on exercise performance

An increasing number of studies have been published and have demonstrated that I-PreC can effectively enhance performance in several kinds of exercise [118,210-212]. However,

these beneficial effects have not been unanimously proved, and some studies reported uncertain [213-216] or even negative effects [217]. These different outcomes could be the consequence of differences in the maneuvers to induce preconditioning, in the different exercise duration and modes, and in timing between preconditioning and exertion. We cannot rule out that the differences are due to the redox status of the tissue.

De Groot and co-workers [210] were among the first to test the I-PreC effect on exercise performance. They found that skeletal muscle I-PreC increased maximal oxygen uptake and power output during maximal cycling performance in healthy and physically fit subjects. The increment in subjects' performance after I-PreC was slight but significant. Similarly, in another investigation it has been demonstrated that skeletal muscle I-PreC slightly improved maximal performance in highly trained swimmers [119]. Of note, these authors were able to demonstrate that the dialysate plasma from the preconditioned athletes could reduce the infarct area of perfused mice hearts. The possibility to enhance exercise performance by I-PreC was substantially confirmed by Crisafulli and co-workers [211] during maximal cycling. However, these investigators failed to detect any benefit of I-PreC during all-out testing, a kind of exercise mostly related to athletes' anaerobic capacity. A very recent paper has confirmed the fact that I-PreC can not ameliorate athletic performance during anaerobic testing [218]. Another very recent investigation confirmed the positive effect of I-PreC on exercise performance and fatigue perception [219]. Collectively, reported data seem to suggest that I-PreC can only produce little improvements in exercise performance (in the order of 2-3%) and that this effect is more pronounced in aerobic exercise (running, swimming, cycling etc.) than in power and sprint performance. It should however be borne in mind that athletic competitions are often decide by a small margin of

differences. Therefore, from an athletic performance point of view improvements of 2-3% may be substantial. That is, increments of this order is highly relevant in elite athletes' competitions.

As far as the mechanisms by which I-PreC operates to increase exercise performance, to date none has provided any definite explanation. It has been suggested that I-PreC produces a more efficient muscle contraction, thereby promoting an ATP-sparing effect which, in turn, led to a larger work load per oxygen consumed. This effect has in fact been demonstrated in a pig model of I-PreC [220]. Authors speculated that the ATP-sparing effect afforded by I-PreC may occur because of a tightening of excitation-contraction coupling and a reduction in futile ion pumping. It is also possible that I-PreC reduces the feeling of fatigue, thus allowing the subjects to exercise longer [211]. This fact could be related to the desensitization of groups III and IV nerve endings in the muscle, which act as mechanic and metabolic sensors. The reduction of signals by these nerve endings may decrease the central sensation of fatigue and could at least in part explain the increased performance. Alternatively, I-PreC-related enhancement in exercise performance may be the consequence of improvements in peripheral vascular functions. Indeed, I-PreC can increase NO production, which induces vasodilation, increments in muscle blood flow and oxygen delivery, thereby improving muscle aerobic capacity [48,74]. This latter mechanism may be particularly relevant in the adaptive effects of physical training, where an enhanced exercise-induced NO production is usually observed in some vascular districts [15,33,35].

Finally, similar to exercise, I-PreC can induce an increase in ROS/RNS production [91,92,96.], followed by an antioxidant and survival proteins up-regulation in both training

and late preconditioning [78,79,133,136], thus reinforcing the concept that I-PreC and exercise can act in the same biochemical pathways. However, we should keep in mind that ROS/RNS have multi-phasic effects on the contractile function of skeletal muscle. The low ROS levels present under basal conditions are essential for normal force production. Selective depletion of ROS from unfatigued muscle by use of SOD or catalase causes force to fall. Conversely, modest ROS supplementation causes force to increase. This positive effect is reversed at higher ROS concentrations; force production falls in a time- and dose-dependent manner [221]

In conclusion, preconditioning has similarity with exercise and both induce a great benefit against I/R injury *via* several mechanisms, which comprises a redox modulation. However, I-PreC induces a small benefit on exercise performance, mainly during aerobic efforts. Further investigation is warranted to better clarify effects and mechanisms of both phenomena: exercise and preconditioning.

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FIGURE LEGENDS

Figure 1.

Ischemic conditioning consists of a series of brief periods of ischemia and reperfusion performed before (preconditioning), during (perconditioning) or after (postconditioning) the infarcting/index ischemia. The conditioning procedure can be performed in a remote organ with respect to the target organ (e.g. the conditioning protocol can be applied to the limbs and the index ischemia to the heart).

Figure 2

The release of several endogenous cardioprotective agents. Cardioprotective agents can be released by several cell types and may act in a paracrine/autocrine fashion to activate membrane receptors and to trigger redox-sensitive intracellular pro-survival pathways. Cardioprotective pathways converge on mitochondria where they prevent mitochondrial permeability transition pore (mPTP) formation.

Adenosine receptors, AR; Bradykinin receptors, BkR; Opioid receptors, OpR, Apelin receptor APJ. For other acronyms see text.