Exercise-Induced Cardioprotection via eNOS: A Putative Role of Red Blood Cell Signaling

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Abstract: Moderate exercise training is a key aspect of primary and secondary prevention strategies. Shear-induced upregulation of eNOS activity and function in the vascular endothelium is considered as one of the main molecular mechanisms of exercise-induced protection against myocardial ischemia/reperfusion (I/R) injury. It has been reported that levels of plasma nitrite, which are largely dependent on eNOS activity, were increased in healthy subjects after acute exercise, while this increase was abolished in coronary artery disease (CAD) patients. Our group and others demonstrated that RBCs contain a functional eNOS, which contributes to systemic nitrite homeostasis and to cardioprotection; moreover, expression and activity of red cell eNOS are decreased in CAD patients and significantly correlated with flow-mediated dilation, a diagnostic marker of endothelial function. Therefore, in addition to vascular eNOS, also red cell eNOS (or in more general terms NO metabolic activity of RBCs) may play a role in exercise-dependent changes of NO-bioavailability. In this review, we will focus on what is known and what is unknown about the role of RBCs in exercise-dependent cardioprotection with emphasis on RBC signaling and red cell eNOS. In detail, we will discuss the effects and molecular mechanisms of shear stress and exercise training on RBC signaling and function, review how these changes may influence blood rheology and systemic hemodynamics and highlight the potential role of red cell eNOS-mediated cardiovascular protection induced by physical activity against myocardial injury in animal and human studies and in clinical settings.

Keywords: eNOS, red blood cells, shear stress, exercise, ischemia/reperfusion, myocardial infarction, cardioprotection.

1. INTRODUCTION

Moderate exercise training is a key aspect of primary and secondary prevention strategies [1-5]. Human epidemiological studies demonstrated that regular exercise reduces the risk of death after acute myocardial infarction [5, 6]. Moreover, numerous animal studies confirmed that regular bouts of aerobic exercise protect the heart from ischemia/reperfusion (I/R) injury [7-9].

Acute bout of exercise increases heart rate and blood flow and changes frequency and magnitude of hemodynamic forces on the vascular wall. Mechanical forces, applied to endothelial cells (ECs) and other cell types including red blood cells (RBCs) activate different signaling cascades [4, 5, 10, 11]. One of the main components of mechanotrasduction pathways in ECs is the endothelial nitric oxide synthase (eNOS). Shear stress induced eNOS activation increases nitric oxide (NO) production [12-16], an important determinant of vascular tone and systemic hemodynamics. While vascular eNOS activation is important for arteries with the diameter of 100 µm, smaller arterioles are mainly controlled by metabolic, myogenic and others unknown factors [4].

In this review, we discuss the putative role of RBC signaling and function in exercise-dependent protection against myocardial infarction. RBCs may actively participate in exercise-induced changes of vascular tone, especially in the microcirculation not only via transporting NO-metabolites but possibly also by shear-stress induced release of vasoactive mediators (NO, ATP) which can act as a paracrine regulators of vascu-
lar calibers. Importantly, RBCs themselves express functionally active eNOS [17, 18], which was proposed to contribute to constitutive NO production and nitrite homeostasis [19]. Furthermore, shear-induced elevation of NO release from the endothelium into the vascular lumen and in RBCs themselves might change erythrocytes ability to deform in response to mechanical forces and thus may influence blood viscosity and tissue perfusion. Considering the important role of eNOS activation in cardioprotection by exercise, we here put forward the hypothesis that the increase in systemic shear stress by exercise training induces mechanosensing and mechanotransduction events in RBCs leading to the activation of red cell eNOS, increase of circulating NO-pool, improvement of rheological properties of blood, tissue perfusion and thus cardiovascular protection. Exercise-induced effects and molecular mechanisms triggering changes of RBC functional characteristics and red cell eNOS activity, as well as a specific role of red cell eNOS (versus eNOS in endothelial cell and in cardiomyocytes) in cardioprotection against I/R remain largely unknown.

This review article will (1) provide a brief summary of the molecular mechanisms of exercise-induced protection against myocardial infarction; focus on (2) the role for eNOS in mediating cardioprotective effects of exercise and (3) in setting of exercise capacity and (4) highlight exercise- and NO-dependent changes in rheological properties of blood. Finally, (5) we will introduce the idea that exercise-induced changes of functional characteristics of RBCs, their NO metabolic activity, including activity of red cell eNOS, may play an important role in mediating cardioprotective effects of exercise and discuss potential molecular mechanisms of mechanosensing and mechanotransduction in RBCs.

2. EXERCISE-INDUCED CARDIOPROTECTION

Exercise-dependent cardioprotection is a multifactorial process mediated by 1) elevated myocardial antioxidant capacity, 2) increased expression of cardiac heat shock proteins (HSP), 3) activation of NO-dependent pathways, as well as 4) increase in activity of ATP-dependent potassium channels (K_{ATP}). As highlighted by Powers et al. [20]: “Exercise-induced cardioprotection could be achieved by any physiological adaptation that attenuates one or more of the damaging events that occur during I/R”. In this chapter we will give a brief overview of damaging events of I/R myocardial injury and cardioprotective mechanisms with a focus on the NO–dependent signaling pathways and eNOS.

2.1. Damaging Events in Myocardial I/R Injury

I/R signaling events involve numerous biochemical and metabolic alterations in cardiomyocytes and other cell types leading to the myocardial damage [5, 20, 21]. Briefly, as reviewed elsewhere [20, 22-24], oxygen supply is blocked during ischemia leaving no energy for oxidative phosphorylation in the mitochondria. To meet myocardial energy demand, cellular ATP is generated via glycolysis leading to the simultaneous changes of intracellular pH (which can reach values as low as 5.5) and to the increase of cytosolic lactate levels. Decreased cellular ATP production impairs the activity of SERCA and Na/K ATPase resulting in the Na⁺ and Ca²⁺ overloads, the impairment of depolarization processes and contractile dysfunction. Ca²⁺ overload in cytosol, in its turn, activates different phosphatases, proteases (e.g. calpain), ATPase and other enzymes involved in reactive oxygen species (ROS) production, and lipid peroxidation leading to the pathological events and cell death. In reperfusion phase, restoration of blood and oxygen supply promotes further burst of ROS and facilitates permeabilization of the mitochondrial outer membrane, opening of mitochondrial permeability transition pore (MPTP), and necrotic cell death [22-24].

2.2. Mechanisms of Exercise-induced Cardioprotection

It is well established that exercise training provides cardioprotection against myocardial infarct injury [4, 5, 21, 25-27]. The main molecular mechanisms underpinning exercise-dependent protection against I/R damage are summarized in several recent reviews [4, 20, 25, 27, 28]. These mechanisms include 1) increased cardiac antioxidant capacity, 2) changes in mitochondrial metabolism, 4) enhanced function of sarcolemmal and/or mitochondrial ATP-sensitive potassium channels (K_{ATP}) and increased expression of K_{ATP} channels and 5) altered NO signaling.

2.2.1. Increased Cardiac Antioxidant Capacity

Mitochondrial ROS generation significantly contributes to the cell death following I/R [20, 29]. A number of studies indicated exercise-induced activation of redox-sensitive signaling pathways in the heart, on a first line superoxide dismutase 1 (SOD1) in cytosol and mitochondrial matrix [30, 31] and SOD2 in mitochondrion [32]. However, data on changes in expression of other antioxidative enzymes in the heart such as catalase, glutathione peroxidase, thioredoxin are inconsistent: some authors reported upregulation of these en-
zymes [33, 34], while others found no changes [35, 36].

2.2.2. Elevated Expression of Cardiac Heat Shock Proteins (HSP)

Several HSPs are associated with cardioprotection against I/R, especially the members of HSP70 family. It is well established that HSP72 protects the heart against I/R injury [37, 38] and that exercise training increases cardiac HSP72 [39]. However, several studies revealed that exercise-induced cardioprotection can be also achieved without the increase in HSP72 levels [40, 41] suggesting that, although being involved in cardioprotective effects of exercise, HSP72 is not a prerequisite for cardioprotection [20].

2.2.3. Changes in Mitochondrial Proteins and Metabolism

It is proposed that mitochondria are central target in exercise-induced cardioprotection [20]. Exercise-induced cardioprotection is mediated, at least in part, through mitochondrial adaptations resulting in a cardiac phenotype resistant to the Ca$^{2+}$-induced opening of MPTP, an important apoptotic and necrotic signal [32]. Among other cardioprotective changes in mitochondria after exercise are reduced generation of ROS in cardiac mitochondria [42] and downregulation of monoamine oxidase (MAO)-A, an important source of increased hydrogen peroxide generation [43].

2.2.4. ATP-sensitive Potassium Channels ($K_{ATP}$)

Exercise training was reported to increase sarcolemmal $K_{ATP}$ expression on cardiomyocytes [44] and exercise-induced short-term and long-term cardioprotection was lost after inhibition of these channels [44, 45]. Sarcolemmal $K_{ATP}$ dependent protection of the heart against I/R injury appears to accelerate cardiomyocyte repolarization process, shortening of the action potential and prevent Ca$^{2+}$ overload by reducing the opening rate of the L-type Ca$^{2+}$-channel [46]. Likewise, a role for mitochondrial $K_{ATP}$-channels in cardioprotective effects of training has been suggested [47].

2.3. Cardioprotection by Exercise: Focus on eNOS

eNOS activity and NO bioavailability play an important role in the direct protection afforded by exercise against myocardial infarction. Several studies showed that exercise-induced cardioprotection against I/R was abolished by inhibition of NOS [48] and in eNOS-deficient mice [9, 25]. Exercise-induced increase of NO levels was detected by elevated circulating and tissue levels of nitrite or/and nitrosothiols, both being considered as biomarkers for NO-bioavailability and endothelial function [9, 49, 50], however the exact mechanisms, eNOS cellular localization and mediators of eNOS-dependent cardioprotective action are still under evaluation [20, 21, 25, 51].

Current tenets hold that cardioprotection by NO/eNOS is mediated by various effects on vessel tone, thrombosis, cell death signaling, inflammation and energy conservation, and a long list of proteins/enzymes targeted by nitrosation, including guanylate cyclase, caspases, NF-$\kappa$B, and mitochondrial proteins, has been investigated [20, 25, 28, 52]. In the heart the vast majority of eNOS is localized in the vascular endothelium. Exercise-induced upregulation of eNOS mRNA was first observed in dog coronary arteries [13] and then confirmed in many other species [16, 48, 53, 54]. Likewise, exercise-induced changes in vascular eNOS activity including complex pattern of intracellular eNOS regulation by phosphorylation, translocation to the caveolae and other mechanisms have been reported in response to exercise in different animal models [9, 10, 55] and humans [16].

In addition to ECs, other cell types express eNOS, including cardiomyocytes [56] and blood cells [17, 18]. In the myocardial tissue, eNOS dependent S-nitrosation of myocardial proteins (beta-arrestin, cardiac ion channels, GPCR kinase-2 which influences heart contractility, myocyte survival and function) was demonstrated [57-61]. S-nitrosation of target cardiac proteins during myocardial I/R can attenuate apoptosis [62] and inflammation [60]. For instance, caspase-3 activity is known to be inhibited by protein S-nitrosation [63]. Furthermore, mitochondrial ROS production can be decreased during I/R, since S-nitrosothiols are able to modify complex I of the mitochondrial electron transfer change [64] and prevent opening of the mitochondrial permeability transition pore [65]. In recent study by Farah et al. a potential key role of the endothelial layer in exercise-induced cardioprotection has been suggested [51]: the authors reported that eNOS-dependent cardioprotective effects of exercise are attributed to coronary EC activity and conclude that the endothelium is a key trigger of the eNOS/NO-dependent cardioprotection afforded by exercise training. However, this study was performed using ex vivo I/R injury model in isolated heart, where no blood component was present, making it difficult to extrapolate these finding for the in vivo situation.
To summarize, NO-metabolites play a major role in the beneficial effects of exercise and provide an acute and sustained protection against myocardial I/R [9, 28]. Voluntary running for 4 weeks resulted in eNOS-mediated increase in plasma, myocardial and skeletal muscle levels of nitrite and nitrosothiols before I/R in trained mice [9]. Nitrite or NO-metabolites stored during exercise in skeletal muscle [66] and RBCs ([67], Chapter 5.2.) can be converted into NO under hypoxic conditions in working organ and this pathway appears to be essential in promoting the cardioprotective effects of training that persist after exercise has ceased [9, 28]. Although many studies demonstrated the necessity of increased NO bioavailability for the exercise-induced cardioprotective effects [4, 9, 20, 28], the underlying mechanisms, main mediator(s) and especially whether eNOS expressed in the endothelium or other cell types is responsible for NO-dependent cardioprotection in exercise remain largely unclear.

3. EXERCISE CAPACITY DEPENDS ON THE INTEGRITY OF THE ENOS PATHWAY

eNOS is an important enzyme for maintaining of proper exercise capacity and energy metabolism. Human studies revealed that impaired eNOS function, either achieved via administration of pharmacological inhibitors in healthy individuals or due to disease conditions associated with endothelial dysfunction, leads to reduced exercise capacity [49, 50, 68]. NO influences exercise performance by regulating skeletal muscle function, contractility, blood flow, calcium and glucose homeostasis and importantly – mitochondrial respiration and biogenesis [4, 20, 69, 70]. Likewise, experiments using global eNOS KO demonstrated that eNOS is crucially necessary for maintaining adequate level of physical activity. Momken et al. reported that maximal work performed and distance run by eNOS KO were decreased about 50% as compared to the wild type mice [69] and this was confirmed by us using voluntary running exercise model in another eNOS KO strain [71] (Fig. 1).

Voluntary training (Fig. 1A) represents endurance training model with a lower but constant training intensity. This type of physical activity matches the preferred running time and speed for each individual mouse and is a natural habitat of mice. In addition to the reduced mean running distance (Fig. 1B), voluntary exercise had little effect on heart weight/body weight ratio in eNOS KO mice due to the cardiac hypotrophy reported in this eNOS KO strain by us [72] and in another eNOS KO strain by others [69]. Nevertheless, other anatomical parameters of eNOS KO (such as soleus weight, soleus weight/tibia length and soleus weight/body weight ratios) were increased by exercise comparably to the wild type mice [73]. Whether eNOS in ECs, cardiomyocytes or erythrocytes contribute to the reduction of exercise performance in voluntary exercise is unknown and detailed investigation is still awaiting.

Fig. (1). Voluntary exercise training in mice. (A) Details of the cage used to house and to exercise mice. For voluntary training, the mice were housed individually in cages supplied with running wheels (0.25 m in diameter, Tecniplast, Germany) and equipped with counters to record the daily running distance [73]. (B) The mean running distance/24 h of eNOS-deficient mice [71] was significantly lower than running distance of C57BL/6J mice. Published data suggest that reduction in running distance in eNOS KO strain might be due to the impaired mitochondrial biogenesis, which has been reported in this mouse strain [74]. Thus, mitochondrial oxidative capacity is influenced by global eNOS deficiency, in fact, eNOS KO mice had markedly reduced energy ex-
penditure and oxygen consumption as compared to the wild type mice. eNOS deficiency was also associated with decreased mitochondrial content in cardiac and skeletal muscles and defective fatty acid beta-oxidation [74]. These data suggest that, if eNOS is downregulated, moderate exercise training could worsen energy metabolism, specifically in oxidative skeletal muscles [69, 74]. Similar results were reported for cardiac tissue: endurance exercise training boosted eNOS-dependent mitochondrial biogenesis in wild-type mouse heart and this adaptation was lost in eNOS-deficient mice [70]. Mitochondrial size, their number in cardiomyocytes and glucose mobilization were increased in swim-trained wild types, but not in eNOS KO pointing out to an obligatory role of eNOS-derived NO in metabolic adaptation of cardiac muscle to exercise [70]. However, this study, as most of studies performed in exercised global eNOS KO, did not allow discriminating the relative contribution of eNOS from different cell types, e.g. cardiomyocyte, endothelial cells or RBCs.

4. EXERCISE-INDUCED SHEAR STRESS, RHEOLOGICAL PROPERTIES OF BLOOD AND NO

Rheological properties of blood are highly affected by exercise training. A “triphasic” action of exercise on blood rheology has been proposed [75, 76]. It is reported that short-term effect of exercise is an increase in blood viscosity, mostly due to a rise in hematocrit and plasma viscosity induced either by fluid shift, water loss, and release of sequestered RBCs from spleen or water trapping in muscle [76-78]. Likewise, alterations in functional characteristics of erythrocytes have been shown [77, 79, 80]. The most classical acute changes are a decrease in RBC deformability, increase in RBC aggregation and increase or no change in RBCs rigidity [81]. These effects are generally not found after exercise when RBC deformability is investigated after resuspension of the cells in a buffer, suggesting that changes of RBC deformability are due to plasma factors (such as blood lactate levels) rather than to intrinsic properties of erythrocytes [75, 82]. Reversal of the hyperviscosity starting at 24 h after exercise is described as “autohemodilution” followed by several profiles of “hemorheological fitness” with a low hematocrit values, improvement of RBC deformability and decrease in erythrocytes aggregation [80, 83]. Interestingly, the popular belief that “the more RBCs you have, the fitter you are” seems to disagree with the physiological truth. The important paradox concerning hematocrit in exercise physiology is that “the fitter the athlete – the more fluid is his blood” (hematocrit paradox) [76, 84].

Effects of exercise on hemorheological pattern critically depend on the number of factors, such as: 1) duration of exercise, 2) mode and intensity of exercise training, 3) basal level of physical activity and the training status of the individual, 4) training discipline (please refer to [76, 77, 85, 86] for extensive review). For instance, body builders have been reported to have no improvement in blood rheology after training [80, 84] while an increase in viscosity was demonstrated in rugbymen [79].

Acute rise in blood viscosity during exercise was associated with increased aerobic performance, it decreases vascular resistance and increases tissue perfusion [77, 87]. A marked increase in blood viscosity is necessary for eNOS-dependent NO production in ECs and, possibly, in RBCs, leading to the adequate vasodilation and beneficial peripheral blood distribution in exercise. This could also explain the hematocrit paradox in athletes - decreased blood viscosity is related to improved aerobic fitness [76, 84]. Having decreased resting blood viscosity could be a way to obtain the larger rise in blood viscosity [77] in response to exercise leading to eNOS activation and NO production [76, 80, 87]. This is in line with the finding by Martini et al., who showed that increasing hematocrit has a biphasic effect on systemic hemodynamics and blood pressure [88]. Blood pressure was initially lowered due to stimulation of NO release by increased shear stress; afterwards this effect was counterbalanced by an increase in peripheral vascular resistance due to the increase in hematocrit leading to an exponential increase of blood viscosity [88].

It is important to mention that effects of NO on RBC deformability are controversial [89] and need further thorough investigation. Administration of NOS inhibitors decreased RBC deformability as assessed by ektacytometry [90] while low concentrations of NO donors increase RBC deformability [90], membrane fluidity [91], and RBC filterability [92]. In the microcirculation of the chorioallantoic membrane of the chicken egg, NOS inhibition and NO donors affected RBC deformation and velocity independently from changes of the vascular diameter [93].

Increase of RBCs deformability, generally reported after regular exercise training, enables erythrocytes an easier transit through the capillaries resulting in the improvement of oxygen supply to skeletal muscle and cardiomyocytes. The improvement of RBC functional characteristics by shear forces may have significant
effects on blood flow dynamics, especially in tissues supplied by blood vessels with impaired vasomotor reserve, and may therefore serve as a compensating mechanism for the maintenance of adequate microcirculatory perfusion in cardiovascular disease state. The question remains how these effects are regulated at molecular and cellular levels.

5. EFFECTS OF SHEAR STRESS AND EXERCISE TRAINING ON RBC SIGNALING AND FUNCTION

Endothelial cells sense changes in local hemodynamic patterns (= mechanosensing) by the presence of mechanoreceptors localized on the luminal side of the membrane, in junctional complexes connecting two adjacent cells, in focal adhesions in the subluminal side of the cell membrane or inside the cell (i.e. the cytoskeleton), and respond to the mechanical signal by activation of signaling pathways (= mechanotransduction) leading to physiological changes [10, 11]. Similar to endothelial cells, RBCs are also subjected to significant mechanical forces while recirculating. There is an emerging evidence that they are equipped with mechanosensing molecules, which may transduce these mechanical stimuli in autocrine, paracrine and endocrine fashion. Capacity of RBCs to change their shape under different flow conditions (= deformability) is a pivotal functional characteristic of erythrocytes. The mean diameter of human RBCs is about 8 µm, yet they are able to pass through vessels with a diameter of only 3 µm. Such elasticity and flexibility of RBCs are required for the efficient oxygen and nutrients delivery to the tissues, and allow RBCs to participate to the flow. Furthermore, as discussed above, changes of RBC characteristics (RBC deformability and aggregability) contribute to define rheological properties of blood and tissue perfusion. In this chapter we will focus on the important putative mechanosensors in erythrocytes and signaling cascades of mechanosensing and mechanotransduction in RBCs and discuss shear stress- and NO-dependent changes of RBCs functional characteristics.

5.1. Mechanosensing and mechanotransduction in RBCs

A number of studies suggest that RBCs respond to variations in hydrodynamic shear stress by releasing ATP [94-97]. Sprague et al. proposed that increase in shear stress causes the RBC membrane to deform [98] and that the deformation triggers ATP release from RBCs which involved cystic fibrosis transmembrane conductance regulator CFTR [99] or pannexin-1 ion channels [100]. ATP released from RBCs might be an important mediator in local metabolic sensing and signal transduction between the RBCs, endothelial and smooth muscle cells.

The exact mechanosensing mechanism(s) inside RBCs remains unknown, however, few possible mechanosensors were suggested which include 1) pannexin-1, 2) a conserved family of mechanosensitive non-selective cation channels Piezo1; and 3) cytoskeletal protein spectrin.

5.1.1. Pannexin-1

Immunohistochemical and electrophysiological data suggest that RBC express the mechanosensitive gap junction protein pannexin 1[101]. It was also shown that erythrocytes can release ATP after cell swelling, which is not thought to result from cell lysis and has been proposed to occur via pannexin-1 [101]. Although it is not clear whether swelling-induced ATP release and ATP release induced by mechanical stimuli are essentially the same, mechanosensitive and mechanotransduction pathways of ATP releasing channel pannexin-1 [101] have been proposed [99], but not investigated in details [99, 101].

5.1.2. Piezo1

Piezo1 protein encoded by the Piezo1/FAM38A gene has recently been identified as mechanically activated cation channel in many mammalian cells including erythrocytes [102]. Piezo1 was shown to form homomultimeric complexes sufficient to mediate stretch-induced cation currents in RBCs; this was demonstrated by applying force to the cell surface, while monitoring transmembrane currents using patch-clamp technique [103, 104]. Furthermore, Piezo1 dependent calcium entry in RBCs in response to mechanical stretch has been reported [105]. Intracellular Ca\(^{2+}\) increases may hit multiple downstream targets in RBCs, including eNOS-signaling pathway and ATP release through pannexin-1 or CFTR channels (Fig. 2). Noteworthy, the absence of Ca\(^{2+}\) in incubation medium did not fully block ATP release from RBCs, indicating that also Ca\(^{2+}\)- independent mechanotransduction pathway(s) are present in RBCs [106].

Piezo1 is also known to regulate cell volume homeostasis in erythrocytes. Gain-of-function PIEZO1 mutations are linked to dehydrated hereditary stomatocytosis, a pathophysiological condition of decreased intracellular erythrocyte volume and mild hemolysis [107]. RBCs from blood-cell specific Piezo1 conditional knock-out mice are overhydrated and exhibit increased fragility in vitro and in vivo [105]. Interest-
ingly, in a recent genome-wide association study of RBC indices, mean corpuscular hemoglobin concentration was linked to a single nucleotide polymorphism in the Piezo1 gene locus [108]. The ability of RBCs to reduce their volume in response to mechanical forces could improve their ability to traverse through small-diameter capillaries and could aid in oxygen/CO₂ exchange in the periphery by concentrating Hb within RBCs, which may promote release of oxygen from Hb as it has been shown by optical tweezers [109].

Piezo1 is important for the endothelial shear stress sensing in the vasculature [110]. Recently, a role of mechanosensing by Piezo1 in blood pressure control has been reported [111]. Using *in vitro* and *in vivo* studies on inducible endothelium-specific PIEZO1 deficient mouse models, the authors demonstrated that endothelial Piezo1 mediates flow-induced ATP release and subsequent P2Y2/Gq/G11-dependent activation of downstream signaling *via* phosphorylation and activation of AKT and eNOS, leading to NO formation, reduction of vascular tone and arterial blood pressure [111]. Whether these mechanosensing and mechano-transductive pathways discovered in ECs are relevant for the regulation of red cell eNOS remains to be elucidated. However, RBCs possess Piezo1 channel and express functional eNOS. By analogy to ECs, exercise-training and increase of shear stress on RBCs may activate Piezo1 which, in its turn, is linked to red cell eNOS activity and thus may contribute to the improvement of local and systemic NO-bioavailability by exercise training.

### 5.1.3 Cytoskeletal Spectrin

The RBC membrane is highly dynamic structure, which enables erythrocytes to undergo considerable deformations. The complex structural organization of the RBC membrane components is responsible for its unique features of extensive deformability and mechanical stability [112]. In RBCs, the major cytoskeletal proteins spectrin, actin, band 3, and ankyrin form a pseudohexagonal structure, which creates a dynamic network and supports integral membrane proteins [113]. The flexibility of the RBC membrane depends 1) on the interactions of the cytoskeletal proteins spectrin and actin [114], 2) on the intrinsic plasticity of the
Spectrin is a highly flexible molecule, but it is not clear how its flexibility is achieved. It is a long protein composed of 2 parallel chains α- and β- spectrin oriented in opposite directions and both are structured in triple helical repeats [118]. Recent study showed that spectrin is sensitive to shear and both spectrin dissociation from the network [117] and spectrin unfolding [119] appears to contribute to the RBC response to mechanical stress (Fig. 3). Subjecting human RBC membranes to fluid shear stress at level similar to in vivo blood flow revealed a stress-enhanced cysteines labeling in spectrin - this suggests that mechanical force unfolds cysteine residues, which normally are buried in protein folds and protein-protein interactions but possess a highly reactive thiol in cysteine [119]. The structure of spectrin affects not only its mechanical properties and RBC membrane stability, but also specific interactions with other proteins that are bound to it.

5.1.4. Other Mechanisms

There is some indication for mechanosensitivity of a ligand-gated Ca\(^{2+}\) channel in RBCs, e.g. erythroid-N-methyl D-aspartate receptor (NMDAR), which mediates Ca\(^{2+}\) uptake upon stimulation with agonists glutamate and glycine [82]. NMDARs activated by acute bout of exercise and induced an elevation in intracellular Ca\(^{2+}\). However, these effects appear to be mediated rather by exercise-induced increase in plasma glutamate levels then by direct mechanosening of the NMDARs [82].

Taken together, studies using RBCs subjected to the mechanical stimuli have shown a Pannexin1 or Piezo1-mediated mechanotransductive release of ATP, or changes in cytoskeletal protein spectrin configuration, but the role of RBC eNOS and NO in these processes needs to be fully explored.

**Fig. (3). Proposed mechanism of RBC mechanosening by cytoskeletal protein spectrin.** In response to mechanical stress spectrin may dissociate from cytoskeletal protein ankyrin and unfold its shear-stress sensitive cysteine residues (modified from [119]). S-nitrosation of the shear-sensitive cysteines in spectrin molecule by exercise-induced increase of NO-bioavailability/levels may change RBC membrane stability and deformability and thus represent a possible underlying mechanism of shear stress- and NO-dependent changes of RBC functional characteristics.
5.2. Role for RBCs in Nitrite Homeostasis and Exercise Induced Cardioprotection

Under traditional view RBCs by themselves were originally considered as the compartment responsible mostly for NO breakdown. The discovery that RBCs represent an important reservoir for NO metabolites and mediate hypoxic vasodilation implies that RBCs are able to transport endothelium-derived NO as bioactive metabolites and to release NO “on demand” under hypoxic conditions [120-122], when eNOS activity is impaired. Thus, RBCs by themselves represent a source of NO/NO-metabolites and play an active role in maintaining of NO bioavailability [123, 124].

Several candidates were suggested for the role of bioactive NO metabolite and mediator of hypoxic vasodilation. The first was S-nitrosohemoglobin (SNOHb), which was proposed to be produced within the RBC by the reaction of endothelium-derived-NO with the highly conserved β-chain Cys-93 residue of Hb. During oxygenation, a still not identified intermediate is released from SNOHb that is exported from RBCs as a low-molecular-weight S-nitrosothiol, leading to hypoxic vasodilation [125]. Later on, nitrite was shown to be the substrate of non-enzymatic NO synthesis in RBCs, which is converted into NO by a reaction shown to be the substrate of non-enzymatic NO synthesis [125].

First evidence that red cell eNOS is important for the modulation of systemic hemodynamics and the degree of myocardial damage in I/R, has been obtained using chimera mouse models generated by the transplantation of bone marrows from eNOS KO into irradiated wild types and vice versa [19, 134]. Mice lacking eNOS in blood cells had lower nitrite levels in blood plasma, impaired erythrocyte deformability, larger infarct size and myocardial contractile dysfunction suggesting that red cell eNOS contributes to constitutive NO production and nitrite homeostasis and seems to be cardioprotective in myocardial I/R [134].

In addition to intracellular NO metabolic activities, RBCs were also proposed to participate in the regulation of endothelial eNOS activity by releasing ATP [97] into the intravascular lumen. Once released from RBCs, ATP can activate purinergic receptors, expressed on the vascular endothelium leading to the synthesis and release of NO. The resultant increase in vascular diameter, in its turn, would decrease the stimulus for deformation-induced ATP release and increase oxygen delivery to the tissue, resulting in improved matching of oxygen supply with metabolic demand [135]. Likewise, recent paper of Xu et al. [136] highlighted the important role of RBC ATP release in the regulation of vascular tone in response to shear stress. They found that the levels of shear stress-induced release of ATP from RBCs at physiological hematocrits were about 3 orders of magnitude higher than those from ECs. Moreover, shear-stress-induced EC released ATP alone was insufficient to increase [Ca^{2+}]i in ECs. While shear stress-induced endothelial NO production occurred in both cell free fluid and blood perfused vessels, shear stress-induced increases in EC cell [Ca^{2+}]i required the presence of RBCs, attributing to shear-induced pannexin-1 channel dependent release of ATP from RBCs [136]. The authors concluded that changes in blood flow alter vascular endothelial function through both wall shear stress and shear stress exerted on RBCs, and RBC released ATP plays an essential role in changes of EC signaling and vascular function.
All together, these findings indicate a complex role of RBCs in the regulation of systemic NO metabolism, however, more research is needed to understand how RBCs sense mechanical forces and whether red cell eNOS is involved in mechanosensing and mechanotransduction processes in RBCs.

5.3. Red Cell eNOS and Exercise Training of Healthy Humans

There is not much known about the effects of exercise training on red cell eNOS activity in healthy individuals and cardiovascular disease patients. Very few studies, which investigated the effects of exercise training on erythrocyte eNOS signaling and function and their main results are resumed in Table 1.

Suhr et al. reported that moderate exercise training increases red cell eNOS activity, NO production and deformability through the Akt kinase pathway (measured by the intensity analysis of immunostaining against eNOS and its phosphorylated forms) [138], while the high intensity exercise training in athletes induced a downregulation of human red cell eNOS expression and activation [137]. In healthy individuals NO produced by RBC was proposed to modulate RBC deformability through S-nitrosation of the cytoskeletal protein spectrin [142], however, molecular mechanisms triggering changes of red cell eNOS activity and RBC deformability in response to mechanical forces and functional significance of exercise-dependent eNOS activation in RBCs are not understood.

Recently, Medeiros Lima et al. reported that acute bout of treadmill maximal cardiopulmonary exercise test resulted in increase of red cell eNOS activity measured by the conversion of \(^{3}H\)-L-Arg to citrulline and increase of intraerythrocyte cGMP levels immediately after termination of the exercise test [140] giving another proof for shear-stress induced increase of eNOS activity in RBCs and implying the activation of a downstream-activated sGC-cGMP pathway. The authors reported that acute strenuous exercise test induced also erythrocyte fragility which is caused, at least in part, by an increased lipid oxidative damages due to excessive ROS formation [140].

Whether NO formed by RBCs and NO-dependent signaling in RBCs are involved in beneficial exercise-induced changes of rheological properties of the blood, improvement of erythrocyte functional characteristics and in complex relationship between modulation of RBC deformability by NO under shear conditions and blood viscosity is still an open question which awaits future investigations.

5.4. Clinical Aspects of Exercise Training: RBC Function in Cardiovascular Disease

Epidemiological studies have reported a strong association between increases in hematological factors and increased cardiovascular risk [143, 144]. Hematological risk factors predicted cardiovascular disease at least as strongly as traditional risk factors such as blood lipid concentrations [144]. Likewise, analysis of post-training changes in healthy individuals showed a positive impact of exercise on RBC functions – reduced erythrocyte aggregation, hematocrit and red cell rigidity [83, 145]. Studies of hematological, rheological, and biochemical parameters in blood samples of women over 60 years of age (measured at baseline and five months after the exercise program) revealed a significant improvement of erythrocyte count, hematocrit and erythrocyte deformability by lower shear stress levels while no significant changes were noted by the higher shear stress values [146].

In contrast, reports on post-training erythrocyte deformability in cardiovascular disease patients are inconclusive. Recent study comparing the blood count and rheological properties of blood before and after outpatient cardiac rehabilitation program in CAD patients showed no changes in erythrocyte deformability, but a significant reduction of erythrocyte aggregation index [147]. In contrast, enhanced deformability of erythrocytes was found after aerobic interval training in patients with heart failure [148], while no changes in blood rheology were reported after exercise training in patients with chronic heart failure [149]. The diversity of post-exercise rheological changes in the blood depends on the exercise protocols and is analyzed in details in [150]. Likewise, heterogeneity of the study groups and different methods of measuring rheological properties of blood [77] add to the discrepancy in results, therefore, the influence of physical activity on the rheological properties of blood in people suffering from cardiovascular diseases should be determined more precisely.

It is well established that red blood cell distribution width (RDW), a quantitative measure of the variability in the size of the circulating erythrocytes, represents a strong independent predictor of morbidity and mortality after acute myocardial infarction in patients with CAD and heart failure [151, 152]. Higher RDW levels likely suggest immature RBCs production in bone marrow and increased RBCs with incomplete oxygen binding with hemoglobin or rheological abnormalities of RBCs. Importantly, Van Craenenbroeck et al. demonstrated that in patients with chronic heart failure higher
RDW is related to impaired exercise capacity, independently of hemoglobin, iron status, and creatinine clearance [153]. Moreover, RDW values were significantly decreased in patients who participated in a structured, supervised exercise training program [153]. Recently, similar beneficial effects of exercise training on exercise tolerance and RDW have been reported in patients with CAD [154], however it is still an open question whether changes in RDW are just a marker of impaired exercise tolerance or play a pathophysiological role in impaired oxygen transport by erythrocytes [153].

Data from Kelm, Rassaf et al. demonstrated that exercise-induced physiological stimulation of eNOS activity is mirrored by increased levels of circulating NO metabolites [49, 50, 68]. Patients with cardiovascular risk factors and CAD and 25 elderly healthy control subjects without cardiovascular risk factors underwent an ergometric exercise test with the stepwise increase in force [68]. Ergometric exercise stress measured as rate pressure product was similar in both groups, but in healthy subject plasma nitrite was increased by 22 ± 8%, while there was a decrease in plasma nitrite seen in the patients with CAD (-7.0 ± 4 %) [68]. These data indicate an impairment of shear-stress induced activation mechanisms of eNOS in CAD patients, but they do not allow to distinguish endothelial cell eNOS activity and (dys)function from that of red cell eNOS. Whether the lack of exercise-induced increase in nitrite in CAD patients is attributed to the altered RBC signaling and functions and impaired shear stress induced red cell eNOS activation and whether NO-dependent RBC signaling and function in CAD patients can be improved by regular moderate exercise training is still to be investigated.

Two independent groups reported decreased red cell eNOS expression and/or activity in patients with CAD [18, 155]. Likewise, reduced red cell eNOS expression was found in RBCs from end-stage renal disease patients [156] and in patients with microvascular angina [157]. The consequences of all these disease states are not limited to impaired vascular function, but may also affect function of RBCs. Similarly to endothelial eNOS dysfunction, red cell eNOS dysfunction may depend on both decreased protein levels, and changes in eNOS catalytic activity resulting in the impaired systemic NO bioavailability, a condition strongly related to cardiovascular disease state. Importantly, red cell eNOS expression and activity were found to correlate significantly with flow-mediated dilation, a diagnostic marker of endothelial function and eNOS activity [18] suggesting that the characterization of NO metabolic activity of RBCs holds a promise as a circulating biomarker of putative cardiovascular risk. Furthermore, posttranslational modifications of RBC proteins (including eNOS) by S-glutathionylation and S-nitrosation might change

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Intensity</th>
<th>Duration</th>
<th>Main post-exercise changes in eNOS-pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suhr et al. 2009 [137]</td>
<td>12 international-class field hockey players</td>
<td>high-intensity</td>
<td>two days, five training sessions</td>
<td>↓ RBC eNOS expression and activity</td>
</tr>
<tr>
<td>Suhr et al. 2012 [138]</td>
<td>15 healthy males</td>
<td>moderate</td>
<td>acute moderate intensity training test (running on a treadmill) for 1 h</td>
<td>↑ RBC eNOS activation via PI3/Akt pathway</td>
</tr>
<tr>
<td></td>
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<td>↑ NO levels in RBCs</td>
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<td></td>
<td>↑ RBC deformability</td>
</tr>
<tr>
<td>Ladage et al. 2012 [139]</td>
<td>12 non-insulin-dependent type 2 diabetic men</td>
<td>intermittent hypoxia</td>
<td>hypoxia exercise training 4 times a week over a 6 week period (every training period of 90 min)</td>
<td>↓ eryNOS phosphorylation at Ser 1177 after exercise, which was restored after 6 weeks of intermittent hypoxia training.</td>
</tr>
<tr>
<td>Medeiros Lima et al. 2016 [140]</td>
<td>12 young healthy male volunteers</td>
<td>acute maximal intensity</td>
<td>cardiopulmonary exercise test on a treadmill (8-12 min)</td>
<td>↑ RBC eNOS activity, NO production, cGMP levels</td>
</tr>
<tr>
<td>Koli amitra et al. 2017 [141]</td>
<td>38 healthy male subjects</td>
<td>enduring moderate and high intensity training</td>
<td>regular over a 6 week period</td>
<td>↑ of RBC NOS activation, RBC nitrite levels and RBC deformability in high intensity training group</td>
</tr>
</tbody>
</table>
functional activity of RBCs. Measurements of such posttranslational modifications in erythrocytes were suggested as potential biomarkers of oxidative stress and cardiovascular disease [158]. However, current methods of evaluation of NO-metabolic activity of RBC by multilevel analytical approach comprising HPLC, LC-MS/MS, flow cytometry, laser scanning microscopy, and enzymatic assays [18, 155] are relative expensive and impractical for scaling up for high-throughput screening and analysis. Further validation and assay development as well as large-scale clinical studies will be required to establish the role of NO metabolic activity in RBCs as biomarker in diagnosis, prognosis and treatment of cardiovascular disease before it can be adopted as biomarker ready for the clinic.

CONCLUSION

Exercise is an exciting, inexpensive and safe approach to reduce the severity of myocardial infarction following ischemia. Published data suggest that eNOS and eNOS-derived NO metabolites play an essential role in mediating acute and sustained cardioprotective effects of exercise [9, 20, 28]. Here, we propose that exercise-induced changes of functional characteristics of RBCs may influence the cardioprotective effects of exercise training against myocardial injury. RBCs possess several mechanosensing molecules (Pannexin1, Piezo1, spectrin) and are able to transduce the mechanical signals in autocrine, paracrine and endocrine/erythrocyte manner. Furthermore, NO-metabolic activity of RBCs and red cell eNOS may be involved in exercise-dependent cardioprotection.

Although RBC signaling and function have gained considerable attention within the last few years [11, 18, 124], many questions remain unanswered. How RBCs sense mechanical forces and how red cell eNOS is involved in mechanosensing and mechanotransduction process in RBC? What are the trigger(s), mediator(s) and molecular mechanisms of exercise-induced changes in erythrocyte function? How exercise-induced changes of erythrocyte function influence systemic hemodynamics and blood rheology? What is a specific role of eNOS in RBCs and eNOS expressed in other cell types, e.g. in endothelium and in cardiomyocytes in these exercise-mediated changes? Future experiments using tissue specific transgenic mice with the “loss of function” and “gain of function” for eNOS and other key proteins in NO-pathway will help to decipher biological mechanisms by which exercise training protects the heart against I/R.

Better understanding of the signaling cascade induced by exercise in RBCs will provide a basis for the development of therapeutic strategies design to mimic cardiovascular protection by exercise and is essential to further optimize diagnostic tools and training interventions. Future clinical studies are required to clarify whether impaired RBC functionality and red cell eNOS activity and function can be improved by moderate low intensity exercise training (e.g. in a rehabilitation program) and will provide an important outlook for a better secondary prevention in patients with stable CAD.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The work was supported by the Research Commission of the Heinrich-Heine-University’s Medical Faculty, 9772646 to T.S., and by the German Research Council (DFG, CO 1305/2-1 to M.M.C.-K). Artwork for the figures was adapted from Serviers Medical Art (http://www.servier.com/Powerpoint-image-bank).

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Erythrocytes and Exercise-Dependent Cardioprotection


