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Review

Blood as a reactive species generator and redox status regulator during exercise

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ABSTRACT

The exact origin of reactive species and oxidative damage detected in blood is largely unknown. Blood interacts with all organs and tissues and, consequently, with many possible sources of reactive species. In addition, a multitude of oxidizable substrates are already in blood. A muscle-centric approach is frequently adopted to explain reactive species generation, which obscures the possibility that sources of reactive species and oxidative damage other than skeletal muscle may be also at work during exercise. Plasma and blood cells can autonomously produce significant amounts of reactive species at rest and during exercise. The major reactive species generators located in blood during exercise may be erythrocytes (mainly due to their quantity) and leukocytes (mainly due to their drastic activation during exercise). Therefore, it is plausible to assume that oxidative stress/damage measured frequently in blood after exercise or any other experimental intervention derives, at least in part, from the blood.

Introduction

Exercise is perhaps one of the most characteristic examples demonstrating that reactive oxygen and nitrogen species (hereafter, reactive species) and oxidative stress are not necessarily "harmful" entities, considering that the well-known benefits of regular exercise on muscle function and health are accompanied by repeated episodes of oxidative stress [1]. Nowadays, the field of exercise and reactive species is being rapidly expanded. Indeed, a recent issue of the official journal of the Society for Free Radical Biology and Medicine was devoted to this topic and hosted a dozen of review articles written by eminent researchers (Free Radic. Biol. Med. 44 (2008) 123–230). However, none of these reviews has dealt with the potential role that blood may play at rest or during exercise on reactive species production and/or with the meaning of these changes in blood redox status. This is the case even though the vast majority of the relevant studies have determined redox status in blood. To the best of our knowledge, the same holds true for all the available reviews devoted to this topic, the number of which is enormous (a search in PubMed for "oxidative stress" and "exercise" on 3rd August, 2009 produced 387 review articles). The excellent reviews by Lamprecht et al. [2] and Jenkins [3] stand as notable exceptions as they tried to highlight the role of blood in reactive species production during exercise. Hence, the main aim of this perspective paper is to present a critical synopsis of basic redox biology knowledge relevant to blood and exercise biology rather than a detailed analysis of every subject related to this topic.

Definition of exercise and some basic exercise effects relevant to redox status

In the present paper, by the term "exercise" we include any type of physical activity that has been mostly performed only once and was of sufficient intensity (normally 50–80% of maximal effort) and duration (normally 30–90 min). Most of the human studies that are presented here have used running, cycling or resistance exercise. The most commonly used type of animal physical activity is a rat running on a motor-driven treadmill. Acute muscle-damaging exercise can induce oxidative stress lasting 1–4 days after exercise [4,5], which is in contrast to the return to the resting values few hours after non-muscle-damaging exercise [6,7]. To this end, the studies presented in this paper have used only non-muscle-damaging exercise protocols (i.e., they did not use physical activities that are biased toward eccentric muscle actions).

Exercise induces a multitude of physiological and biochemical changes in blood that may ultimately affect its redox status. Some of the well-described events that arise during exercise are increases in blood temperature [8], decreases in blood pH [9], decreases in blood oxygen partial pressure [10] and increases in the concentration of blood lactate [10]. All these exercise-associated homeostasis disruptions are able to modify blood redox status. Indeed, hyperthermia increased the levels of reactive species within

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the splanchnic circulation of the rat [11]. The formation of reactive species seems to be dependent on pH. In fact, experiments performed in intact respiring mitochondria, with the pH varying from 6 to 8, revealed that alkalization of the medium strongly increased the rate of reactive species generation [12]. Inside hypoxic tissues (i.e., when oxygen partial pressure is low), xanthine dehydrogenase can be converted into xanthine oxidase [13]. During reoxygenation, superoxide radical ($O_2^{\bullet-}$) can be formed by a reaction catalysed by xanthine oxidase between oxygen, hypoxanthine and xanthine [13]. Finally, lactate has been shown to scavenge hydroxyl radical ($HO^{\bullet}$) and $O_2^{\bullet-}$ [14]. It is worth mentioning that the above exercise-modifiable factors can also act by affecting one another. For example, one of the early responses during hypoxia includes increased levels of glycogen degradation and glycolysis leading to increased lactate production [15].

Information on the blood constituents relevant to redox status

Plasma

Reactive species production

The vast majority of the relevant human studies have measured the redox status of plasma or serum (for the sake of simplicity, we use the term "plasma" even for studies in which serum was analysed). This is probably done because it is assumed that plasma better reflects tissue redox status and due to the ease of plasma collection. Blood plasma is the liquid component of blood, in which the blood cells are suspended. It makes up about 55% of the total blood volume. It is composed mostly of water (90% by volume) and contains dissolved proteins, glucose, lipids and mineral ions. Reactive species can be produced in plasma mainly through reactions with metals. For example, hydrogen peroxide (H_2O_2) reacting with ferrous ion (Fe^{2+}) in plasma can produce strong oxidants. In fact, it has been demonstrated that coincubation of human plasma with Fe^{2+} and H_2O_2 resulted in almost three-fold increase in lipid peroxides [16]. Plasma contains $\approx$0.25 μM H_2O_2 [17], since it is continually produced in virtually all tissues and mixes readily with plasma [18]. Although almost all of the tissue iron is bound to transferrin and ferritin (in ferric state; Fe^{3+}) under normal conditions, Fe^{2+} release (the bound Fe^{3+} is reduced to Fe^{2+} upon release; [19]) can occur from injured cells in conditions such as inflammation [20]. Therefore, Fe^{2+} and H_2O_2 can generate Fe^{3+}, hydroxide ion (OH^-) and the highly reactive $HO^{\bullet}$ by means of the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$ [15]. However, it appears that no study has measured Fe^{2+} concentration in plasma after exercise.

Antioxidants

Admittedly, to define the term "antioxidant" is a difficult task. In this paper, antioxidant is defined as any substance that delays, prevents, or removes oxidative damage to a target molecule [15]. Antioxidants protect the hydrophilic (e.g., urate and albumin) and lipophilic constituents (e.g., polyunsaturated fatty acids (PUFA[1]) and cholesterol) of plasma against damage by reactive species [21]. What is the most important antioxidant in human plasma? This is a difficult question to answer, largely because the answer depends on the reactive species and the target molecule in question [22]. These are probably the main reasons why different assays for measuring plasma antioxidant activity produce divergent results. Generally, the most important antioxidants in plasma are considered to be urate, abundant proteins (mainly albumin; [23]), ascorbate and α-tocopherol [24].

Oxidizable substrates and oxidative damage

The term "oxidizable substrate" includes almost every molecule found in vivo [15]. Blood plasma carries a multitude of substances that can be oxidized. In fact, the proteome, lipidome and glycome (i.e., the entire complement of proteins, lipids and carbohydrates expressed at a given time under defined conditions) encountered in plasma is probably the richest and most complex of all tissues [25]. Oxidative damage has been defined as the biomolecular damage caused by reactive species attack upon the constituents of living organisms [15]. Despite the fact that theoretically all molecular targets can be oxidatively damaged, we focus our discussion on proteins and lipids, which are the most investigated targets and constitute the major components of most body fluids and tissues including plasma and blood cells [26].

What factors determine which protein or lipid is going to be oxidized? It appears that the most important factors are the rate constants for reaction of reactive species with proteins and lipids as well as the abundance of these macromolecules [26]. For example, the rate constants for reaction of a common reactive species, such as $HO^{\bullet}$, with plasma albumin and linoleic acid, the former being the most abundant protein, and the latter the most abundant PUFA in plasma, are 8×10^{10} [25,26] and 9×10^9 [26,27], respectively. These data show that oxidation of proteins and lipids varies to a relatively small extent, and therefore, the overall rate of reaction (which is the product of the rate constant and the concentration of the target), will be mainly determined by the concentration of the target [26]. Undoubtedly, other factors such as the location of reactive species and the target, the occurrence of chain reactions and the effects of repair processes also affect the reactivity of oxidants [26].

More than 300 proteins have been detected in human plasma differing in abundance by a factor of 10^{10} (from the highly abundant albumin present at 35–50 mg/ml to the less abundant interleukin 6 present at 0–5 pg/ml; [25]). Albumin alone makes up approximately 55% of the total plasma protein content, whereas together with the other ten most abundant proteins found in plasma (including, immunoglobulins, transferrin, fibrinogen and apolipoproteins) account for more than 90% of all plasma proteins [25]. Several studies have consistently reported increased protein oxidation (most frequently measured as protein carbonyls) in plasma after exercise (e.g., [7,28]). The origination of protein carbonyls in plasma is unknown and it is not clear whether they are derived from the muscle and/or other tissues. Considering that oxidized proteins are being degraded inside tissues (i.e., in our case mainly skeletal muscle) and remain there until cell death [29], it is unlikely that they can be exported to the plasma. In the case of oxidized amino acids, though, the situation might be different as amino acids can readily appear in plasma [30]. However, the concentration of the free amino acid pool in skeletal muscle accounts only for $\approx$1/20 of the total amino acids [31]. Thus, the increased protein oxidation commonly measured after exercise should be derived mainly from oxidation of albumin and the other major plasma proteins. Supporting the antioxidant role of albumin, comprehensive studies have found that plasma albumin is reversibly shifted to an oxidized state after exercise [32,33].

Plasma lipids consist of phospholipids, triacylglycerols, cholesterol, cholesterol esters and non-esterified fatty acids [34]. Lipid peroxidation mostly affects PUFA, because they contain two or more carbon–carbon double bonds [15]. Indeed, PUFA contribute largely to the pool of oxidizable biological compounds in plasma [23]. Only a small portion of plasma fatty acids are present in free or non-esterified form, whereas the majority are in fact acyl groups (derived from fatty acids by removal of an oxygen anion; O^-) with-

[1] *Abbreviations used:* PUFA, polyunsaturated fatty acids; NADPH, nicotinamide adenine dinucleotide phosphate; HOCl, hypochlorous acid; ESR, electron paramagnetic resonance.

in phospholipids, triacylglycerols and cholesterol esters [34]. In detail, the concentration of PUFA in human plasma is approximately 2.38 mM in cholesterol esters, 1.02 mM in phospholipids, 0.34 mM in triacylglycerols, while the concentration of non-esterified PUFA is as low as 0.08 mM [35]. Based on this hierarchy, the most likely lipids to be oxidized in plasma are the PUFA esterified to cholesterol esters (incorporated into the core of lipoproteins; [36]).

Erythrocytes

Reactive species production

Plasma interacts with all organs and tissues and, consequently, with many possible sources of reactive species. In contrast, the blood cells are less permeable and their environment may be more controlled than that of plasma. Erythrocytes have a very simple structure, being essentially composed of a membrane surrounding a solution of hemoglobin [37]. Nowadays, there is an increasing recognition of the role of erythrocytes beyond its oxygen-carrying function. Indeed, there is now compelling evidence that erythrocytes contribute to the maintenance of circulatory antioxidant levels [38]. Despite the absence of mitochondria in erythrocytes, reactive species are ceaselessly produced in erythrocytes mainly due to the high O_2 tension in arterial blood and their abundant heme iron content [39]. The major source of reactive species in erythrocytes seems to be the oxygen carrier protein hemoglobin that undergoes autoxidation and produce $O_2^{\bullet-}$. More specifically, when O_2 attaches with the heme iron in deoxyhemoglobin (i.e., hemoglobin in its oxygen-unloaded form) a molecule of oxyhemoglobin (i.e., hemoglobin in its oxygen-loaded form) occasionally releases $O_2^{\bullet-}$ [39]. Since the concentration of oxygenated hemoglobin in erythrocytes is 5 mM, even a small rate of autoxidation can produce substantial levels of $O_2^{\bullet-}$ [39]. Despite the fact that $O_2^{\bullet-}$ is a relatively unreactive oxidant [40], it is substrate (along with H_2O_2) in the iron-catalyzed Haber–Weiss reaction that produces the most reactive $HO^{\bullet}$: $O_2^{\bullet-} + H_2O_2 \rightarrow HO^{\bullet} + OH^- + O_2$.

Antioxidants

As already mentioned, however, erythrocyte is not only a source of reactive species. The mobility of the erythrocyte makes it an ideal antioxidant not only for its own membrane and local environment, but also an oxidant scavenger throughout the circulation [39]. Erythrocyte's efficient intracellular reducing machinery, coupled with its high cell density, renders it an effective "sink" of reactive species [41]. Indeed, erythrocytes can act as scavengers for plasma H_2O_2 and $O_2^{\bullet-}$ [15]. Hydrogen peroxide crosses membranes easily and the erythrocyte has an anion channel through which $O_2^{\bullet-}$ can move [15]. Moreover, erythrocyte is the major scavenger of nitric oxide radical ($^{\bullet}NO$) in circulation, because erythrocytes contain high concentration of hemoglobin [39]. Oxyhemoglobin converts $^{\bullet}NO$ to nitrate (NO_3^-), whereas deoxyhemoglobin binds to $^{\bullet}NO$ to form iron-nitrosylated hemoglobin [42]. Based on the above rationale, the scavenging ability of erythrocytes could benefit not only the blood per se, but more importantly, the entire organism including skeletal muscle.

Oxidizable substrates and oxidative damage

Reactive species produced in erythrocytes and reactive species circulating in plasma may have an impact on the erythrocyte constituents. Indeed, studies have found increased protein and lipid oxidation levels after acute exercise in erythrocytes [43,44]. The erythrocyte membrane (including the erythrocyte skeleton) is composed of ≈50% protein, the ≈75% of which is spectrin [45]. More than 95% of cytoplasmic protein is hemoglobin [39]. Therefore, it appears that the main contributors to protein oxidation are spectrin for the erythrocyte membrane and hemoglobin for the cytosolic proteins. One important technical note: the usual way of erythrocyte fractionation (i.e., lysed by suspension in a hypotonic medium) leaves an empty membrane "sack" after centrifugation (sometimes called a "ghost"), which virtually does not contain any hemoglobin [46]. On the contrary, the supernatant almost completely consists of hemoglobin [46]. Therefore, depending on the specimen collected (either erythrocyte ghosts or suspension), the major protein responsible for the detected level of erythrocyte oxidation varies greatly.

All lipids in the mature erythrocyte are found in the membrane bilayer. They mainly consist of phospholipids and cholesterol and, as erythrocytes lack of organelles, glucose is the only fuel utilized by mature erythrocytes [39]. Individual fatty acids differ greatly in their chemical propensity for oxidative damage [47]. Even though it has been suggested that the rate of PUFA oxidation is unrelated to their degree of unsaturation [48], most authors agree that PUFA are by far more prone to oxidation than monounsaturated and saturated fatty acids [47,48]. Erythrocyte membranes contain significant amount of PUFA, ranging from 28% to 37% of the total fatty acids by weight [49,50], and hence they are prone to peroxidation. We are not aware of any studies that have measured lipid peroxidation of erythrocyte membranes after exercise. The main interest in examining the redox status in erythrocyte membranes is the potential influence that redox status exerts on erythrocyte deformability, thus affecting their ability to transfer oxygen and $^{\bullet}NO$ to tissues [51,52]. This is particularly interesting considering that several studies have reported increased erythrocyte membrane deformability after chronic exercise [34].

Leukocytes

Reactive species production

The leukocytes – through their phagocyte killing mechanism – are probably the first thing that comes to mind when one is thinking of a main source of reactive species production in blood during exercise. In the blood, neutrophils constitute 50–70% of the total number of leukocytes, and their number increases during and after exercise [53]. The main function of neutrophils is to phagocytose and digest cellular debris at sites of damage and inflammation [53]. Neutrophils respond to appropriate stimuli by a marked increase in O_2 uptake, called the "oxidative burst" [54]. The uptake of oxygen arises from the activation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex [54]. The activated complex oxidizes NADPH into $NADP^+$, releasing two electrons in the process that reduce $2O_2$ to $2O_2^{\bullet-}$: $NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet-}$ [54]. Superoxide is poorly reactive, but it can be converted to the highly reactive $HO^{\bullet}$ [54]. This can be done in two reactions. First, $O_2^{\bullet-}$ dismutates (i.e., reacts with itself) to H_2O_2: $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$. Second, H_2O_2 can be converted into $HO^{\bullet}$ by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$ [54]. It is worth mentioning that H_2O_2 can readily exit neutrophils, therefore $HO^{\bullet}$ could be produced in the plasma if adequate amount of "free" or "labile" iron (i.e., iron not bound to ferritin or transferrin) is present. An increase in vitro production of $O_2^{\bullet-}$ by neutrophils has been reported either immediately and few hours after non-muscle-damaging exercise [55], or 1–5 days after muscle-damaging exercise [56]. As a final point, hypochlorous acid (HOCl) is formed by the action of myeloperoxidase utilizing H_2O_2 [40]. Hypochlorous acid is predominantly formed by neutrophils and is a strong oxidizing agent capable of crossing membranes [40] and, if any survives, to exit and oxidize plasma constituents.

Antioxidants

The antioxidant vitamins ascorbate and α-tocopherol appear to play an important role in the function of leukocytes. Neutrophils contain a high level of ascorbate (1–20 mM vs. 50–150 μM in plas-

ma; [57–59]), which it possibly serves to preserve the cell's integrity and protect host tissues by acting as a reducing agent [60]. Neutrophils and lymphocytes also contain a high level of α-tocopherol (≈100 μM in neutrophils and ≈500 μM in lymphocytes vs. 20 μM in plasma; [61,62]). More importantly, it is possible that some neutrophilic ascorbate is secreted into the extracellular space (i.e., plasma) and reduces reactive species near the neutrophil surface [60]. Moreover, immunocytochemical analysis showed that catalase can be also excreted extracellularly from neutrophils after exercise [63]. In support of this finding, four out of the five studies that determined catalase activity in neutrophils after exercise reported decreases in activity [61,62,64,65], whereas one reported no change [66]. However, it is not safe to draw valid conclusions based merely upon changes in antioxidant levels in one cell type alone, as there is a possibility for antioxidant redistribution in various cells/tissues after exercise [67–70]. For example, several studies have clearly shown that the changes in glutathione concentration after exercise are different among tissues, probably because of interorgan glutathione transport [69,70].

Oxidizable substrates and oxidative damage

Reactive species produced by activated leukocytes can be released extracellularly and oxidize several plasma proteins [71]. In addition, the same reactive species can attack the leukocytes generating them [72]. The concentration of most amino acids is higher in neutrophils than in the surrounding plasma [73]. The major proteins of neutrophils include those of the structural matrix, proteins required for its locomotion, chemotactic properties and adhesiveness, as well as the many granule proteins with bactericidal, hydrolytic and inflammatory functions [73]. Despite the fact that leukocytes are rich in proteins, the effect of exercise on leukocyte protein oxidation is unclear, as some studies reported increased protein carbonyls in neutrophils [61] and lymphocytes [62,74], while others found no change in protein carbonyls after exercise in neutrophils [62] and lymphocytes [61].

As it is the case with other cells, plasma membrane and membranes of the intracellular organelles of leukocytes are rich in lipids [73]. Five percent of the neutrophils wet weight is lipid, which is distributed among various classes [73]. Neutrophils and lymphocytes contain approximately 32% and 28% PUFA of the total fatty acids by weight, respectively [75,76]. A number of studies reported increases of malondialdehyde (an index of lipid peroxidation) after exercise in neutrophils [61] and lymphocytes [77], whereas others reported no change [61,62]. Interestingly, it has been reported that exercise increased the percentage contribution of PUFA in neutrophils after exercise, supporting the idea that exercise may modulate neutrophil function through alterations in its fatty acid composition [78]. On the other hand, it has been also suggested that changes in neutrophil fatty acid composition does not always lead to changes in neutrophil redox function, such as $O_2^{\bullet-}$ generation [79].

The reactive species biology of platelets is not well studied and the physiological importance of reactive species produced by these blood cells (primarily $O_2^{\bullet-}$ and $^{\bullet}NO$) is uncertain [15]. Therefore, we will not deal with them in this paper. Nevertheless, given the fact that platelets are found in the blood in significant amounts (more than 30-fold higher than leukocytes; [27]) and contain a lot of PUFA in their membranes (≈30% of total lipids; [80]), it is a promising research target for future studies.

The origin of blood oxidative stress/damage after exercise

From the discussion so far, it is clear that plasma and blood cells are able to produce significant amounts of reactive species and contain considerable quantities of oxidizable substances. Therefore, it is plausible to assume that the oxidative stress/damage measured frequently in blood after exercise or any other experimental intervention derives, at least in part, from the blood. Supporting this hypothesis, several studies have reported increased production of various reactive species after exercise from erythrocytes [81], neutrophils [63], lymphocytes [82] and platelets [83]. Further supportive evidence to this hypothesis is the fact that many reactive species (such as $^{\bullet}NO$, $O_2^{\bullet-}$, H_2O_2 and HOCl) produced during exercise are membrane permeable [40], and, as a result, are potentially able to diffuse out of the plasma membrane into plasma. Therefore, reactive species produced inside blood cells could potentially oxidize substances found in plasma. This appears particularly relevant for $^{\bullet}NO$, H_2O_2 and HOCl, which have relatively long half-life in aqueous solutions (from seconds for $^{\bullet}NO$ to minutes for H_2O_2 and HOCl; [84,85]). Moreover, peroxyl radicals, the main chain propagating intermediate of lipid peroxidation, and probably the most abundant radicals in biological systems [86], have also a half-life of some seconds with a considerable diffusion path length in biological systems [87].

From the above analysis, it is apparent that blood is able to produce reactive species during exercise. Yet, it is equally evident that skeletal muscle is able to produce reactive species during increased contractile activity as well [88–90]. The primary reactive species generated by resting and contracting skeletal muscle are the relatively poorly reactive $^{\bullet}NO$ and $O_2^{\bullet-}$, the latter dismutating rapidly to form H_2O_2 [91]. These substances provide the precursors for generation of the considerably more reactive peroxynitrite ($ONOO^-$; which is also membrane diffusible; [92]) and $HO^{\bullet}$ in the presence of catalytic transition metals [91]. Most of the current data indicate that the production of intracellular reactive species is increased by two- to four-fold during skeletal muscle contractions [91]. Does part of this increased production of reactive species inside skeletal muscle transport into blood? Certainly, the answer to this question strongly depends on the half-life and the membrane permeability of the reactive species in question. In fact, two studies by Bailey et al. [93,94] provided supporting evidence that there is an incremental generation in reactive species outflow across a functionally isolated and energetically active skeletal muscle. Electron paramagnetic resonance (ESR) spectroscopic examination of spin adducts detected ex vivo identified lipid-derived alkoxyl radicals as the probable dominant species [94]. Considering the short half-life of alkoxyl radicals (≈10^{-6} of a second; [95]), it was hypothesized that alkoxyl radicals detected may have evolved during the Fe^{2+}-catalyzed decomposition of extracellular lipid hydroperoxides formed subsequent to primary radical-mediated damage to fatty acids [94]. Nevertheless, given that between skeletal muscle and blood intervenes the vascular smooth muscle cells and endothelial cells, their contribution to the production of these reactive species cannot be ruled out.

The vascular endothelium is a unique structure building up the inner layer of the vasculature, thus forming an interface between circulating blood and the various organs and tissues [96]. The endothelial cell surface in a 70-kg human is composed of approximately 1–6 × 10^{13} cells, weighs approximately 1 kg and covers a surface area of approximately 1–7 m^2 [97]. Its direct contact with blood (i.e., diffusion distance small) as well as its comparatively large mass and ubiquity are what renders vascular endothelium a potentially important regulator of blood redox status. Vascular endothelial cells produce several reactive species, the most important may be $HO^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, $^{\bullet}NO$ and $ONOO^-$ [98]. Exercise strongly increases the physical forces acting on blood vessels, such as shear stress, transmural pressure and cyclic stretch [99]. In turn, these physical forces have been demonstrated to increase the vascular generation of reactive species and activate NADPH oxidase during exercise [99]. Additionally, it has been shown that endothelial cells can release $O_2^{\bullet-}$ and H_2O_2 extracellularly [100]. While $O_2^{\bullet-}$ barely traverses cell

membranes and is rapidly dismutated by intracellular superoxide dismutases, the resulting product H_2O_2 can diffuse through the endothelial membrane and is much more stable [99].

All blood vessels, with the exception of capillaries, are composed of a subendothelial layer of smooth muscle cells [101]. The smooth muscle cell is mainly responsible for maintaining tension via contraction–relaxation and vessel integrity [101]. The estimated smooth muscle mass in a 70-kg human is approximately 2.4 kg [102]. Smooth muscle cells contain numerous sources of reactive species, including the NADPH oxidases, xanthine oxidase, the mitochondrial respiratory chain, lipoxygenases and nitric oxide synthases [103]. The most important reactive species produced by the above mechanisms may be $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ [104]. Vascular smooth muscle cells are less exposed to shear stress than vascular endothelial cells, but are subjected to transmural pressure and cyclic stretch to a similar degree [99]. How would reactive species produced in vascular smooth muscle get through the endothelium into the blood? In contrast to the endothelium, where the reactive species generated have to cross only a single membrane (that of endothelial cells), the reactive species generated in smooth muscle have to cross a series of smooth muscle membranes along with that of the endothelial cells. It is possible that only the products of lipid peroxidation (e.g., peroxyl radicals) and H_2O_2 produced inside smooth muscle cells, that are long-lived and diffusible [104], are capable of oxidizing macromolecules inside blood.

In addition, it should be taken into consideration that many tissues exhibit increased levels of oxidative damage during exercise (e.g., liver, heart, spleen and lungs; [44,105] and, as a result, may contribute to the oxidative stress detected in blood. Finally, there is now an adequate number of studies that have assessed the effects of various types of exercise on the levels of reactive species in plasma employing ESR spectroscopy. The majority of these studies have generally reported two- to three-fold increases compared to resting levels in humans [6,106–111], whereas some have reported no changes [112], or even decreases [113]. The fact that exercise increases the concentration of reactive species in the blood (i.e., increasing its oxidizing potential) indicates that it is not necessary for any detected oxidized molecules in the blood to have been oxidized inside skeletal muscle or inside any other tissue. Despite the fact that this appears a sound idea, it is sometimes overlooked when data regarding exercise-induced oxidative stress/damage are interpreted in original research articles.

Another conclusion that can arise from the present analysis is that since reactive species are generated by both blood and muscle it is reasonable to assume that there is a bidirectional movement of reactive species from the muscle to the blood, and vice versa, until equilibrium is reached (if the movement is diffusion-controlled). The same may hold true for exchanges among blood constituents, namely, plasma, erythrocytes, leukocytes and platelets, considering that certain basic assumptions are met: reactive species with adequate half-life, ability of the reactive species to cross membranes, and generation of the reactive species at the vicinity of the compartments being considered (e.g., between the skeletal muscle plasma membrane and the membranes of the blood cells inside the capillary veins). An attempt to illustrate the concepts presented above is made in Fig. 1.

As a final point, it is worth mentioning that the effects of exercise on blood redox status are not always straightforward and differences may occur when circumstances change. For instance, the effect of exercise on blood lipid peroxidation seems to be dependent on the partial pressure of oxygen in the inspired air during exercise [114]. In fact, the venous concentration of lipid hydroperoxides increased more during acute exercise under hypoxia compared with normoxia (47% vs. 31%, respectively; [114]). Based on this study, it is clear that acute hypoxia, when combined with the stress of exercise, exacerbated the oxidant load [114].

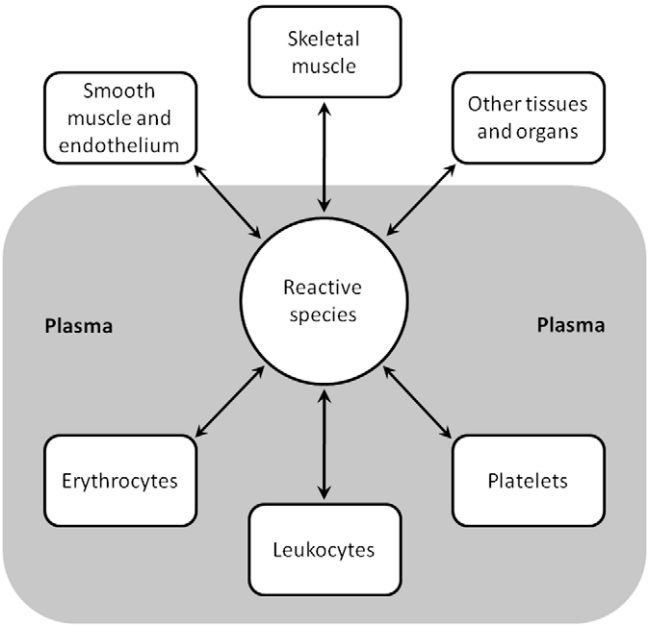

Fig. 1. A simplified diagram showing the main possible sources of reactive species detected in blood plasma. The plasma is seen as a fluid receiving and modifying the reactive species produced in as diverse cells as smooth muscle and erythrocytes. The activity/concentration of the antioxidants in blood (omitted for clarity) greatly affects the extent of reactive species accumulation. The possible movement of reactive species from the tissues to the plasma and the reverse is indicated by the bidirectional arrows. Not all interactions and directions between cells and plasma presented in the diagram have been experimentally verified.

Interpreting changes in blood redox status after exercise: the case of antioxidant enzymes

The primary antioxidant enzymes in erythrocytes are copper- and zinc-containing superoxide dismutase (no manganese SOD is present, as there are no mitochondria), glutathione peroxidase and catalase [39]. Low levels or traces of these enzymes are also found in plasma [15]. The activity of these antioxidant enzymes have been repeatedly measured in both blood compartments (i.e., erythrocytes and plasma) after acute and chronic exercise [115–118]. The assessment of these antioxidant enzymes in both blood compartments was probably performed with the assumption that measurements in plasma reflect systemic changes and measurements in erythrocytes reflect intracellular changes. However, considering that the activity of antioxidant enzymes is much higher in erythrocytes than in plasma (e.g., catalase activity in plasma is about a 1000-fold lower compared to that of erythrocytes; [44,119]), minimal hemolysis could produce inconsistent results. Slight hemolysis during plasma separation is almost unavoidable due to the contact of blood to foreign surfaces and due to centrifugation [120]. Moreover, leakage of antioxidant enzymes from injured muscle cells – which also contain much higher activity of antioxidant enzymes than plasma [121] – may be also a confounding factor, particularly when exercise induces marked muscle damage (i.e., after eccentric exercise). To partially control the influence of cellular lysis on the activity of antioxidant enzymes measured in plasma, hemolysis indices (such as plasma hemoglobin or haptoglobin), and/or muscle damage markers (e.g., plasma creatine kinase), could be also determined.

Based on the above discussion, it becomes apparent that determining antioxidant enzymes in plasma can offer only limited information about the effects of exercise on the enzymes in question. The determination of these enzymes in erythrocytes may be a more useful approach. Yet, it also poses several limitations. As already noted, many studies have measured the activity of antioxidant enzymes in erythrocytes after acute and chronic exercise [116,117]. The evidence regarding the effects of acute exercise on antioxidant enzyme activity in erythrocytes is controversial, with studies reporting increases [122], decreases [116], or no changes [74] after acute exercise. The effects of acute exercise on the antioxidant enzyme activity are further complicated by the lack of a solid explanation on the purported changes in the activity of the erythrocyte enzymes. For example, the same mechanism of enzyme activation (i.e., increased production of reactive species) has been used to explain opposite effects (i.e., both enhancing and inhibitory effects on enzyme activity). In fact, the increased antioxidant enzyme activity after acute exercise has been attributed to increased production of the respective substrate (e.g., increased levels of $O_2^{\bullet -}$ for superoxide dismutase; [121]). This is based on studies showing that partial occupancy of enzymes with their substrates activates them [121]. On the other hand, it has been suggested that increased production of reactive species may limit enzyme activity probably due to mild denaturation of the enzyme [123]. The proposed mechanisms on the effects of chronic exercise on antioxidant enzyme activity are not without ambiguity as well. For instance, increased antioxidant enzyme activity after chronic exercise may obviously indicate that oxidative stress has occurred in the erythrocyte and the erythrocyte has responded to counteract it. However, as erythrocytes cannot synthesize new proteins, this probably indicates that older erythrocytes have been hemolysed, and new erythrocytes have been replaced them, since new erythrocytes exhibit higher activities of antioxidant enzymes compared to older ones [124]. Moreover, older erythrocytes have been found to be more prone to hemolysis [125], and that exercise preferentially lyse them [126]. Consequently, the frequently observed increased activity of antioxidant enzymes after chronic exercise [127] may not fully indicate increased oxidative stress, but it may be partly attributed to the younger erythrocyte population. The factors regulating the activity of erythrocyte antioxidant enzymes in vivo are poorly understood. Elucidation of these factors would enable us to interpret more clearly the effects of exercise on the activities of antioxidant enzymes.

Concluding remarks

We believe that there is a "recontextualization" of knowledge going on from basic redox biology to exercise physiology. Recontextualization is a process that "extracts meaning" from its original context (in our case, redox biology) and introduces it into another context (in our case, exercise physiology) [128]. Thus, "meaning" is borrowed from a different context and is integrated in a new context – thus recontextualized. Something acquires meaning depending upon the context it is used in; therefore, recontextualization implies a change of meaning. Hence, exercise physiology of reactive species inevitably transforms the meanings borrowed and recontextualized from basic redox biology (the present paper does not exclude itself from this process), leading unavoidably to some level of simplification of the complexity of reactive species biology.

The role of blood in reactive species biology has perhaps not received the attention it deserves. The exact origin of reactive species and oxidative damage detected in blood is largely unknown. Blood interacts with all organs and tissues and, consequently, with many possible sources of reactive species. In addition, a multitude of oxidizable substrates are already in the blood. A muscle-centric point of view is frequently adopted (probably again as a result of recontextualization) to explain reactive species generation, thus obscuring the possibility that sources of reactive species and oxidative damage other than skeletal muscle may be also at work during exercise. Plasma and blood cells can autonomously produce significant amounts of reactive species at rest and during exercise. Smooth muscle cells and endothelial cells may also significantly contribute to the reactive species detected in blood. The major reactive species generators located in blood during exercise may be erythrocytes (mainly due to their quantity) and leukocytes (mainly due to their drastic activation during exercise). Considering that reactive species are generated by both blood and muscle, it is reasonable to assume that there is a bidirectional movement of reactive species from the muscle to the blood, and vice versa, until equilibrium is reached. We believe that blood is a tissue of paramount importance in regulating redox status changes appearing during exercise. Nevertheless, much remains to be discovered before we clearly understand the underlying mechanisms governing redox status homeostasis in blood.

Future directions

Researchers planning to investigate the effect of exercise on the blood redox status could:

(i) Compare the effects of an intervention on redox status of different blood cells and plasma in the same experiment, aiming at obtaining the most detailed information possible (in analogy to the recommendation for measuring a battery of redox status indices instead of a single index; [15]).
(ii) Examine cells and tissues (such as platelets and smooth muscle cells) on which data are limited but there is a theoretical background indicating that are important contributors to reactive species appearing in blood.
(iii) Explore mechanisms determining redox homeostasis in blood by exploiting the use of modern technology, such as ESR spectroscopy and microdialysis [89].
(iv) Decide whether systemic redox status or tissue/organ redox status is of interest. If the first is of interest, then blood plasma may be the specimen of choice.
(v) Establish and control the degree of muscle damage and hemolysis.
(vi) Take into account the biological peculiarities of blood cells. For example, erythrocytes are characterized by the presence of hemoglobin at high concentration (≈9 mM), a value considerably larger than that of GSH (≈0.5 mM). Therefore, the potentially important role of sulfhydryl groups of hemoglobin in the detoxification of oxidants by competing with GSH (the molecule traditionally regarded as the most important antioxidant of erythrocyte; [129]) should be considered.
(vii) Analyse through mathematical modeling the complexity of reactive species interactions in blood in order to provide quantitative information. Moreover, mathematical models can be utilized for the validation and screening of the hypotheses offered in the present review [130,131].

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