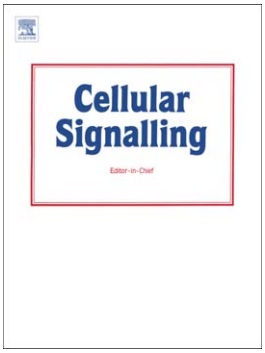

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PRINCIPLES FOR INTEGRATING REACTIVE SPECIES INTO *IN VIVO* BIOLOGICAL PROCESSES: EXAMPLES FROM EXERCISE PHYSIOLOGY

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ABSTRACT

The equivocal role of reactive species and redox signaling in exercise responses and adaptations is an example clearly showing the inadequacy of current redox biology research to shed light on fundamental biological processes *in vivo*. Part of the answer probably relies on the extreme complexity of the *in vivo* redox biology and the limitations of the currently applied methodological and experimental tools. We propose six fundamental principles that should be considered in future studies to mechanistically link reactive species production to exercise responses or adaptations: 1) identify and quantify the reactive species, 2) determine the potential signaling properties of the reactive species, 3) detect the sources of reactive species, 4) locate the domain modified and verify the (ir)reversibility of post-translational modifications, 5) establish causality between redox and physiological measurements, 6) use selective and targeted antioxidants. Fulfilling these principles requires an idealized human experimental setting, which is certainly a utopia. Thus, researchers should choose to satisfy those principles, which, based on scientific evidence, are most critical for their specific research question.

KEY WORDS: free radicals, hydrogen peroxide, oxidative stress, physiology, redox signaling

1. INTRODUCTION

1.1 The problem

There is considerable knowledge available on the detailed chemistry of individual reactive oxygen and nitrogen species and their progeny (collectively termed "reactive species" herein) together with the oxidative and nitrosative reactions that can occur within biological systems (Brewer et al. 2015, Imlay 2013). There is also considerable evidence from chemical biology research that some reactive species serve as signaling molecules (Forman et al. 2014, Holmström & Finkel 2014), reinforcing the concept that reactive species play purposeful roles in biology. Indeed, the translational biology literature is rich in claims that reactive species are involved in virtually every biological process (e.g., aging and stress adaptation; Tomanek et al. 2015, Viña et al. 2013) and most human diseases (e.g., cancer and type II diabetes; Cheng et al. 2011, Giustarini et al. 2009, Manda et al. 2015). Although substantial progress on the understanding of the molecular functions of reactive species has been attained *in vitro*, this has rarely been translated into the *in vivo* human condition. The equivocal role of reactive species in exercise adaptations is an example clearly showing the inadequacy of current redox biology research to shed light on fundamental biological processes *in vivo*. In fact, despite many high-quality and influential research efforts (Gliemann et al. 2013, Gomez-Cabrera et al. 2008, Khawli & Reid 1994, Michailidis et al. 2013, Moopanar & Allen 2006, Reid 2001, Richardson et al. 2006, Ristow et al. 2009, Paulsen et al. 2014, Strobel et al. 2011, Venditti et al. 2014), it is still uncertain whether and how reactive species regulate exercise-induced responses and adaptations. Why did it prove to be so difficult to reveal the role (if any) of reactive species in exercise adaptations?

1.2 The cause

Part of the answer probably relies on the extreme complexity of the *in vivo* biology, the difficulty to replicate the *in vitro* mechanistic set ups into *in vivo* experiments and the nascent nature of exercise redox biology as a discipline. The latter is further compounded by the lack of definitive information from the parent discipline (i.e., redox biology) on some basic redox mechanisms [e.g., whether one-electron oxidants can signal (Winterbourn 2015) or whether redox potential is a driving force of redox reactions (Berndt et al. 2014)]. Most aspects of redox biology are characterized by a delicate specificity. This is best exemplified by the complex nature of the reactive species production and their subsequent metabolism. Exercise increases the production of the parent reactive species (i.e., superoxide anion and nitric oxide) in skeletal muscle from multiple sources (e.g., mitochondria, NADPH oxidases and xanthine oxidase; Gomez-Cabrera et al. 2015, Sakellariou et al. 2014). In addition, these exercise-induced reactive species may or may not exert a biological role (e.g., regulating vital metabolic pathways) depending on several intensely regulated factors such as specificity against target proteins, kinetic rates and site of production (Forman et al. 2014). The same complexity holds also true for the molecules at the other end of the redox continuum, namely, the antioxidants. In particular, despite the wide array of the antioxidant enzymes available, only specific isoforms seem to be selectively expressed, dictated by the type, extent and/or site of the reactive species production (Halliwell & Gutteridge 2015). Interestingly, this spatiotemporal specificity may also indicate that antioxidant enzymes are redox hubs regulating defense and signaling (Forman et al. 2014, Winterbourn 2013).

Contrary to the aforementioned complexity and specificity of the redox network, the most common approach to establish the role of reactive species in exercise responses (i.e., after a single exercise session) and adaptations (i.e., after repeated exercise sessions) is the measurement of oxidative stress biomarkers and antioxidant agents in blood or, less frequently, in muscle biopsies (Theodorou et al. 2014). However, the use of such generic biomarkers provides neither mechanistic information (e.g., type and site of reactive species production), nor in-depth insights about the oxidation taking place (e.g., cysteine oxidation possibly reporting on redox signaling). Another frequent methodological choice is to exogenously administer pleiotropic compounds with purported generic antioxidant properties (principally ascorbate and/or α-tocopherol), aiming to decrease global reactive species levels and, thus, to attenuate exercise-induced redox signaling. However, beyond theory and speculation, there is a paucity of evidence supporting the notion that antioxidant supplementation interferes with exercise-induced redox signaling via a redox-dependent "scavenging" mechanism (Cobley et al. 2015a) (Box 1). Figure 1 illustrates the conventional approach to reveal the role of reactive species in exercise responses and adaptations and the three major accompanying limitations.

1.3 The "solution"

To describe as accurately as possible the potential biological role of reactive species in exercise responses and adaptations, we believe that researchers should take into account the delicate redox specificity and try to overcome the major methodological shortcomings of the currently used experimental designs. Considering that redox biology of exercise is a relatively new field, often research is driven more by intuition and less by sound experimental evidence. Thus, it is desirable to reach a consensus on key influencing factors that investigators should take into account when designing studies in the area of redox biology. To this end, we propose six fundamental principles that should be considered in future studies with a purpose to provide more accurate mechanistic insights into the causality between reactive species production and exercise responses or adaptations. Some of these principles have also been proposed for other biological processes and medical conditions (Buettner 2015, Halliwell & Whiteman 2004, Imlay 2015, Jones & Sies 2015, Murphy et al. 2011, Nikolaidis et al. 2012a, Winterbourn 2008). The recontextualization of these principles in an exercise environment and *in vivo* biology is not an easy task. It is emphasized, particularly considering the inherent complexity of redox biochemistry, that the following principles are based on current knowledge, therefore, they can always be amended or disproved by new evidence and should not be regarded as final.

2. PRINCIPLES FOR INTEGRATING REACTIVE SPECIES INTO *IN VIVO* BIOLOGICAL PROCESSES

2.1 Principle 1: Identify and quantify the reactive species

Since the very first studies that reported increased reactive species production during exercise in skeletal muscle (Dillard et al. 1978, Davies et al. 1982), important advances have been made as regards to their identification (principally) and their quantification. In particular, a series of studies have demonstrated that contracting skeletal muscle transiently overproduces parent (i.e., superoxide anion and nitric oxide) and secondary (i.e., hydrogen peroxide and peroxynitrite) reactive species, both intracellularly and extracellularly (i.e., on the outer side of the plasma membrane) (Bailey et al. 2004, Balon & Nadler 1994, Close et al. 2005, Gomez-Gabrera et al. 2010, Kobzik et al. 1994, Palomero et al. 2008, Reid et al. 1992a, Reid et al. 1992b). These reactive species received great attention after several studies have suggested their important role in exercise responses (e.g., muscle force production, muscle fatigue, muscle glucose uptake and vascular tone; Andrade et al. 1998, Cobley et al. 2011, Gomez-Gabrera et al. 2010, Khawli & Reid 1994, Moopanar & Allen 2006, Reid 2001, Richardson et al. 2007, Roberts et al. 1997, Sandstrom et al. 2006, Toyoda et al. 2004) and adaptations (e.g., mitochondrial biogenesis, muscle hypertrophy and angiogenesis; Cobley et al. 2014, Cobley et al. 2015c, Gomez-Gabrera et al. 2005, Handayaningsih et al. 2011, Irrcher et al. 2009, Ito et al. 2013, Kang et al. 2009, Kim & Byzova 2015, Makanae et al. 2013, Miranda et al. 1999, Scheele et al. 2009, Ushio-Fukai 2006, Zmijewski et al. 2010).

The exact mechanism through which a reactive species may regulate a response or an adaptation after exercise strongly depends on the particular characteristics of each species. Taking into account the large heterogeneity among reactive species (e.g., reactivity and half-life; Halliwell & Gutteridge 2015), it becomes apparent that in future studies the exact reactive species implicated in the pathway of interest should be clearly identified and confirmed. It is equally important to decipher those particular biochemical features that render this specific reactive species competent to be implicated in the investigated responses and adaptations (Murphy et al. 2011). The necessity for this critical identification of the reactive species is, however, in contrast to the common practice encountered in the literature, where all reactive species are crudely consolidated under the abbreviations “RS”, “ROS”, “RNS” and “RONS”. These terms can occasionally be useful as a general description of these species and also in cases where the exact implicated reactive species are either multiple or not strictly specified yet (Forman et al. 2015). However, in most cases, these abbreviations are used misleadingly, by neglecting the range of the species included and, more importantly, by disregarding their diverse biochemical characteristics (Murphy et al. 2011). Evidently, the unequivocal characterization of a reactive species *in vivo* is always a hard task, considering the fleeting nature of reactive species and their multiple interconversions. Nevertheless, the use of collective terms is counterproductive and should be avoided when the species is known or, at least, strongly suspected.

An additional key issue for elucidating the role of reactive species in biological processes is to quantify their amount in response to exercise. On this notion, during the last 15 years, several concepts have been proposed about how reactive species may affect exercise responses and adaptations (Dröge 2002). Two interpretative frameworks have attracted most researchers' interest. The first framework supports the idea that small to

moderate transient increases in reactive species production are beneficial triggering signaling adaptive events, while the production of large amounts of reactive species do not confer further favorable effects or even cause some detrimental effects (Figure 2A). The second, which is the most widely accepted framework, is hormesis. Despite the lack of a generally agreed definition, most researchers concur that hormesis is a dose–response phenomenon characterized by either a U-shaped or an inverted U-shaped dose response depending on the end-point measured (Costantini 2014, Peake et al. 2015, Radak et al. 2005, Ristow 2014; Figure 2D). In hormesis, dose response is characterized by low dose stimulation and high dose inhibition, leading to the biphasic, hormetic dose–response curve (Costantini 2014, Peake et al. 2015, Radak et al. 2005). In an exercise context, this biphasic curve results in beneficial exercise responses and adaptations when amounts of reactive species are low/moderate, whereas higher levels of reactive species can cause cellular damage, culminating in detrimental exercise outcomes. It is important to emphasize that in order to characterize a dose–response relationship as hormetic, both the stimulus (e.g., an exercise-induce increase in a reactive species) and the response (e.g., exercise response or adaptation) should be measured concurrently and repeatedly over an adequate time interval (Nikolaidis et al. 2012a). Equally important is that the stimulus must be monotonic (i.e., either only increasing or decreasing) whereas the response must be non-monotonic (i.e., increase followed by decrease or the reverse; Nikolaidis et al. 2012a). Although appealing, these two cause-effect relationships are based on *in vitro* and *ex vivo* experiments, thus, lacking *in vivo* confirmation. Namely, in a series of pioneering studies, the effect of reactive species production on muscle function (e.g., force production) was evaluated after the addition of a reactive species and/or an antioxidant agent in intact muscle fibers/cells *ex vivo* or *in vitro*. More specifically, hydrogen peroxide represents the most common oxidant agent utilized and has been applied in several different concentrations (e.g., from 10^2 to 10^4 μM; Andrade et al. 1998, Reid et al. 1993). As regards to antioxidants, both enzymatic (e.g., catalase and superoxide dismutase, Reid et al. 1993) and non-enzymatic agents (e.g., N-acetylcysteine and dithiothreitol; Andrade et al. 1998, Khawli & Reid 1994) have been utilized. In particular, catalase was applied in concentrations from 1 to 10^5 U/ml and superoxide dismutase from 10 to 10^4 U/ml, while N-acetylcysteine and dithiothreitol were applied in concentrations from 10^2 to 6×10^4 μM and from 5×10^2 to 10^3 μM, respectively. Since *in vivo* confirmation is still elusive, the possibility remains that other frameworks may also explain the relationship between reactive species production and exercise responses and adaptations (Figure 2B and 2C, indicating a hyperbolic and a threshold relationship, respectively).

Identification and quantification of reactive species have proven a challenging and technically demanding task. In particular, reactive species are produced in very small quantities (picomolar levels for some species) and are relatively unstable, rapidly reacting and degraded by the endogenous antioxidant buffers (e.g., thiol proteins and enzymes). Several assays and tools have been implemented for the identification of the reactive species in diverse *in vivo*, *ex vivo* and *in vitro* experimental models, including electron spin resonance (ESR), fluorescence assays, cytochrome c reduction models, chemiluminescence, hydroxylation of salicylate and nitration of phenylalanine (Murrant & Reid 2001). Some of these tools are nowadays regarded as outdated and unreliable, while others, along with newer ones (especially ESR, magnetic resonance imaging techniques and fluorescence with targeted probes combined with mass spectrometry) still represent the state of the art (Cocheme et al. 2011, Dickinson & Chang 2008, Kalyanaraman et al. 2012, Meyer & Dick 2010, Pouvreau 2014, Winterbourn 2014). During the last decade, great advances have taken place in the development of probes that are highly selective and sensitive to particular reactive species (e.g., HyPer, Amplex red and aromatic boronates against hydrogen peroxide and MitoSOX against superoxide; Belousov et al. 2006, Miller et al. 2005, Mishin et al. 2010) and of probes targeted to a specific cell organelle (e.g., MitoSOX for mitochondria; Dickinson & Chang 2008, Dikalov & Harrison 2014, Smith et al. 2011, Robinson et al. 2006). Despite these advances, improved techniques, tools and probes are still needed, possessing the following qualities: i) selectivity against redox agents other than reactive species (e.g., thioredoxins or peroxiredoxins), ii) quantifiability of fluorescent probes, iii) insensitiveness to biological parameters such as pH, iv) inertia so as not to disturb cell function and v) ability to detect transient waves of altered reactive species production via reversible fluorescence (Dickinson & Chang 2011, Meyer & Dick 2010, Pouvreau 2014). Certainly, the aforementioned qualities cannot be fully met by current technology.

2.2 Principle 2: Determine the potential signaling properties of the reactive species

Cell signaling represents a fundamental process that coordinates activities enabling cells to respond to various internal and external stimuli (Kholodenko et al. 2010, Smock & Gierasch 2009). According to the prevailing theory,

cell signaling predominately, but not exclusively (see also section 2.4), takes place through channeled biochemical reactions of specific and reversible compartmentalized modifications. Accumulating evidence supports the notion that reactive species regulate cell signaling processes, thus, giving birth to the term "redox signaling" (Brigelius-Flohé & Flohé 2011, Dickinson & Chang 2011, Forman et al. 2010, Forman et al. 2014, Holmström & Finkel 2014, Janssen-Heininger et al. 2008, Thomas et al. 2008, Winterbourn & Hampton 2008, Winterbourn 2008). However, given the different characteristics of each reactive species and the fundamental principles of redox signaling (e.g., high specificity, reaction kinetics and compartmentalization), apparently only few reactive species produced during exercise fulfill the requirements to serve as signaling molecules, at least in this canonical fashion.

A typical example of a species that does not fulfill the requirements to serve as a signaling molecule is hydroxyl radical. Hydroxyl radical upon its production reacts in a random and indiscriminate manner with almost all vicinal biomolecules owing to its diffusion-controlled reactivity (Halliwell & Gutteridge 2015, Halliwell 2012). This lack of specificity is in contrast to the conventional mechanisms regulating cell signaling and, thus, hydroxyl radical seems chemically unable to exert direct signaling effects (Holmström & Finkel 2014). Potential indirect/non-canonical redox signaling effects could derive from the hydroxyl radical–mediated oxidized lipid, DNA, and protein adducts in synergy with proteins implicated in repair mechanisms (Cobley et al. 2015b, Niki 2009, Radak et al. 2013). However, this scenario is still largely counterintuitive to the fundamental aspect of specificity in cell signaling (Kholodenko et al. 2010, Smock & Gierasch 2009) and, additionally, cannot be attributed to hydroxyl radical *per se*.

In contrast to hydroxyl radical, hydrogen peroxide fulfills the criteria to serve as a signaling molecule. In particular, it is a two-electron oxidant (one electron oxidations more likely lead to uncontrolled chain reactions; Jones 2008), is relatively stable and typically selective as regards its reaction with reactive cysteine residues on target proteins (Giorgio et al. 2007, Forman 2007, Winterbourn 2013). This selectivity towards cysteine residues represents the basic mechanism of the hydrogen peroxide–mediated redox signaling, usually influencing the reversible regulation of kinases, phosphatases, transcription factors and co-activators (Brigelius-Flohé & Flohé 2011, Marinho et al. 2014, Winterbourn & Hampton 2008). Considering, however, the tight spatiotemporal regulation of hydrogen peroxide levels by abundant and kinetically rapid intracellular peroxidases (i.e., peroxiredoxins, glutathione peroxidases and catalase), it becomes enigmatic how hydrogen peroxide reacts with the cysteine residues and transmits the signal. There are two most widely accepted mechanisms. The first mechanism, known as the "redox relay", assumes that the redox enzymes receive the initial oxidation by hydrogen peroxide and subsequently transfer the oxidation to the intended target protein (Reczek & Chandel 2015, Sobotta et al. 2015). The second mechanism, known as the "floodgate model", theorizes that hydrogen peroxide inactivates the redox enzymes flooding the area, thus, allowing oxidation of the cysteine residue in the target protein (Reczek & Chandel 2015, Wood et al. 2003). Two less widespread mechanisms suggested are the dissociation of the target protein (and its subsequent activation) from the redox enzyme following oxidation of the redox enzyme by hydrogen peroxide and a modified version of redox relay, whereby the redox enzyme transmits the oxidation to the target protein through an intermediate redox protein such as thioredoxin (Reczek & Chandel 2015), as is the case for apoptosis signal-regulating kinase 1 (Saitoh et al. 1998).

Hydrogen peroxide is not the only reactive species able to exert signaling effects, but is most probably the most widely studied. Other oxygen, nitrogen and chlorine species also exert such effects, with the most salient being nitric oxide, hypochlorous acid and peroxynitrite (Affourtit et al. 2015, Collins et al. 2012, Schieven et al. 2002, Thomas et al. 2008, Thomas 2015). An interesting case is peroxynitrite, an oxidant exhibiting great reactivity against vicinal biomolecules, which is similar to that of hydroxyl radical. However, the great reactivity of peroxynitrite does not preclude it from being a signaling agent, possibly due to spatial clustering (i.e., close proximity between source and target), involvement in redox relays with peroxiredoxins and induction of signaling mechanisms mediated by the radical products of its fast reaction with carbon dioxide, namely carbonate and nitrogen dioxide (Augusto et al. 2002, Carballal et al. 2014, Ferrer-Sueta & Radi 2009). Notably, not all of the reactive species produce the same outcome. In fact, there is a great interaction between species (i.e., synergy or antagonism) that leads to a wider array of signals and outputs and this likely allows fine-tuning of the biological processes. This is illustrated by the context-dependent coordination (e.g., co-activation of signaling pathways)

and antagonism (e.g., inhibition of the activity of nitric oxide synthases) of nitric oxide signaling by another endogenous gas, hydrogen sulfide (Eberhardt et al. 2014, Hancock & Whiteman 2014). Namely, it is becoming evident that hydrogen sulfide plays a key role in mammalian biology by interacting with nitric oxide (e.g., in pro/anti-inflammatory processes and vasodilation/vasoconstriction; Li et al. 2009). This was verified in experiments utilizing either hydrogen sulfide "donors", such as sodium hydrosulfide, which induce an explosive hydrogen sulfide burst for a few seconds (Kubo et al. 2007, Li et al. 2008) or water-soluble hydrogen sulfide–releasing molecules, such as GYY4137, which induce a slow and low-level release of hydrogen sulfide that peaks after 20 minutes (Li et al. 2013, Li et al. 2008). Noteworthy, based on the latest and more accurate analytical methods, hydrogen sulfide concentration in mammalian tissues and fluids is in the nM range (Giustarini et al. 2012, Shen et al. 2012, Stein & Bailey 2013). Thus, the concentration frequently achieved via sodium hydrosulfide or other hydrogen sulfide "donors" in *ex vivo* or *in vitro* set-ups (e.g., 10^3 μM), exerting nitric oxide synthases inhibiting effects, is physiologically irrelevant and the interpretation of the results needs caution.

The vital differences and interactions among reactive species emphasize the need to merge knowledge from chemistry (e.g., kinetic rates) and biochemistry (e.g., selectivity against target residues) in order to gain a clearer outlook on how reactive species may be implicated in exercise responses and adaptations. Based on the available knowledge, the well-described signaling effects of hydrogen peroxide implicate this reactive species in exercise adaptations (via altered gene expression, e.g., mitochondrial biogenesis; Gomez-Cabrera et al. 2015). On the other, the possibility that highly reactive species (e.g., hydroxyl radical) may be responsible for some acute exercise responses (via enzyme inactivation through sulfhydryl oxidation, e.g., muscle fatigue; Lamb & Westerblad 2011) should not be discounted.

2.3 Principle 3: Detect the sources of reactive species

Sources of reactive species during exercise have been substantiated in all tissues and organs such as blood, skeletal muscle, lung, liver, kidney and heart (Kruger et al. 2009, Margaritelis et al. 2014, Margaritelis et al. 2015, Nikolaidis et al. 2012a, Sakellariou et al. 2014, Veskoukis et al. 2008, Wadley et al. 2015). It has been reported that differences exist in resting redox composition among different cell types and tissues (Nikolaidis et al. 2012). For instance, erythrocytes lack mitochondria (a significant source of superoxide and hydrogen peroxide in other cell types; Nikolaidis & Jamurtas 2009), while the diverse cell types of the central nervous system as well as the various organs (e.g., liver, heart and skeletal muscle) and systems (e.g., digestive, lymphatic, reproductive, urinary, respiratory and endocrine system) exhibit highly different protein distribution of the thioredoxin, peroxiredoxin and glutathione/glutaredoxin systems (Aon-Bertolino et al. 2011, Brandes et al. 2014, Dammeyer & Arner 2011, Dworakowski et al. 2006, Godoy et al. 2011, Rumora et al. 2007, Sakellariou et al. 2014). The latter strongly supports the view of a different distribution and compartmentalization of reactive species sources (e.g., NADPH oxidases) among the various organs and tissues (Leto et al. 2009). Moreover, diverse redox stimuli (e.g., exercise or endotoxin administration) elicit different oxidative and nitrosative responses among tissues depending on the endogenous antioxidant capacity (Dyson et al. 2011, Liu et al. 2000). Given this organ-, tissue- and cell-type-specific expression patterns, it is reasonable to assume that the reactive species produced during exercise in these tissues may contribute to the local responses and adaptations to exercise in a tissue-specific fashion (Nikolaidis et al. 2012a). This tissue-specificity of the reactive species sources becomes a critical issue when inferring exercise responses from non-muscle cell lines (e.g., redox regulation of transcription factors in cancer cell lines). Analogously, the location of reactive species sources is equally important (Kaludercic et al. 2014).

Nevertheless, the vast majority of exercise studies have been performed in skeletal muscle and, moreover, in whole skeletal muscle and, thus, most of the current knowledge refers only to skeletal muscle and cannot offer spatial insights. It is well-established that skeletal muscle cells contain numerous sources of the parent reactive species superoxide anion such as mitochondria, endothelial and neuronal nitric oxide synthases, NADPH oxidases, xanthine oxidase and phospholipase A_2-family-dependent enzymes, which are localized at the plasma membrane, sarcoplasmic reticulum, cytosol and T-tubules (Espinosa et al. 2006, Javesghani et al. 2002, Powers et al. 2011, Sakellariou et al. 2014, Xia et al. 2003; Figure 3). It is worth mentioning that the contribution of each source in the overall redox state depends on the condition of the organism. For example, mitochondria are generally considered the most important cellular source of superoxide at rest (although some authors have questioned this belief; Brown & Borutaite 2012), whereas during exercise NADPH oxidases are ascribed as the

major superoxide source (Sakellariou et al. 2014). Yet, mitochondrial superoxide production during exercise has perhaps been underestimated due to efficient superoxide dismutation to hydrogen peroxide, that is reaction with superoxide dismutase outcompeting probe oxidation, which is kinetically likely. Moreover, each exercise type has its own redox "signature". For example, muscle damaging exercise has been shown to be accompanied by differential magnitude and time-course redox biomarker alterations compared to non-muscle damaging exercise, indicating activation of diverse reactive species sources (Nikolaidis et al. 2012c).

Considering the specificity of underlying redox biology processes, it is apparent that the variable rate of reactive species production and the strict compartmentalization are not random, as evidenced by high conservation across diverse phyla. Taking into account the high reactivity of the reactive species, it is rational to assume that their sources and targets should be in close proximity. This is exemplified by the findings of Bleier et al. (2015), who reported that even between different sites in the same mitochondrion (i.e., electron transport chain complexes I and III), differential patterns of protein thiol modifications take place. In an exercise setting, this could be translated into different impacts (e.g., satellite cell differentiation or endurance capacity; Hepple et al. 2003, Lee et al. 2011). In support of this spatial specificity, Block et al. (2009) demonstrated that the pores of the outer mitochondrial membrane are larger than the pores of the inner membrane, thereby affecting differentially the transport of NADPH, an important cellular reductant. This indirectly highlights the dissimilar demands for NADPH content among the mitochondrial compartments and, consequently, the importance of redox localization. In a novel metabolic study, mitochondrial hydrogen peroxide emission was manipulated (treating mitochondria with a targeted antioxidant and genetically overexpressing mitochondrial catalase) and the corresponding changes in insulin sensitivity were monitored (Anderson et al. 2009). The authors interpreted insulin sensitivity in the context of mitochondrial redox state and bioenergetics, suggesting that mitochondrial-originated hydrogen peroxide emission serves not only as a "gauge" of energy production but also as a regulator of glucose metabolism (Anderson et al. 2009).

We suggest that future studies exploring potential implications of reactive species in biological processes should focus on a specific cellular site. Although some reactive species, such as hydrogen peroxide, have the theoretical ability to diffuse and exert their effects at distal locations, their random diffusion inside the cell probably lacks specificity and, thus, cannot be regarded as a canonical implication in a biological process. Consequently, the focal point for those researchers interested, for example, in mitochondrial biogenesis, would be the measurement of the reactive species produced in the mitochondrion rather than in the whole muscle.

2.4 Principle 4: Locate the domain modified and verify the (ir)reversibility of post-translational modifications

In correspondence to the necessity of identifying the exact reactive species that are produced during exercise, it becomes equally important to know which specific domain of a biomolecule undergoes redox modification, the extent of the modification and whether this modification is reversible or not. The reversibility of protein oxidation or S-nitrosylation is a regulatory mechanism, which ensures that the oxidative or nitrosative modification is reversed back to its reduced state. This means that if the redox insult is removed or decreased, the corresponding outcome (e.g., the inherent activity of target protein) will be restored (Beltran et al. 2000, Benhar et al. 2008, D'Autreaux & Toledano 2007, Marozkina & Gaston 2012).

Why is the issue of reversibility so important? According to the prevailing theory, redox signaling mainly occurs via reversible oxidation of specific domains in amino acid residues (predominately mediated by two electron oxidants; Cobley et al. 2015b) and/or by the covalent attachment of a nitric oxide moiety to a cysteine thiol (S-nitrosylation; Stamler et al. 2001). Compelling evidence supporting an involvement of one-electron oxidants (e.g., free radicals) in thiol-based cell signaling also exists (Winterbourn 2015). For example, incidents of free radical-mediated enzyme regulation and/or signaling inhibition through classical free radical scavengers (e.g., Trolox) have been reported (Buettner 2011, Chen et al. 2009, Frey et al. 2008, Holmgren & Sengupta 2010, Jourd'heuil et al. 2003, Quintanilla et al. 2005). Yet, the main reason put forward to ascribe a central role for reversible modifications in redox signaling is that these modifications act as regulatory reversible molecular "on-off" switches resulting in temporary post-translational modifications of the residues (e.g., forming disulphide, S-nitrosothiol, sulfenic acid and methionine sulfoxide adducts; Albrecht et al. 2011, Jones & Go 2010, Gould et al. 2013, Patel & Insel 2009,

Poole 2015, Stadtman et al. 2005, Sun & Murphy 2010, Ushio-Fukai 2009). These modifications are associated with altered protein activity, inactivation of an enzyme, altered binding affinity to another protein, translocation and/or modified activity of a transporter or a channel (Forman et al. 2014, Janssen-Heininger et al. 2008, Murphy et al. 2014, Murphy 2009, Winterbourn & Hampton 2008). The aforementioned structural and functional alterations are illustrated in Figure 4. It becomes therefore evident that the termination of the redox "signal" (inferring reversal of the redox modification) is equally important as its activation.

Many protein amino acids such as methionine, arginine, tyrosine and cysteine, when found in specific microenvironments in which they are ionized to the thiolate form, become prone to oxidation and undergo modifications by reactive species at their active side domains (e.g., sulfur-containing functional groups) (Radak et al. 2011, Vogt 1995, Stadtman et al. 2005, Winterbourn & Hampton 2008). However, the only oxidative modifications that are reversible and, thus, likely to be implicated in signaling events are those involved in the oxidation of methionine and cysteine residues. In particular, methionine sulfoxide (the product of the reaction between methionine and reactive species; Stadtman & Levine 2003, Vogt 1995) is reduced back to methionine by two different types of methionine sulfoxide reductases in a thioredoxin-dependent manner at the expense of NADPH (Brot & Weissbach 1983). As regards to cysteine, its high nucleophilic side chain (i.e., sulfhydryl group) when deprotonated may undergo several steps of oxidation, with the corresponding products demonstrating distinct chemical properties (Brigelius-Flohe & Flohe 2011). More specifically, sulfur readily undergoes oxidation (mainly in reaction with hydrogen peroxide) to yield sulfenic acid (Hall et al. 2009). The subsequent fates of sulfenic acid are: i) Formation of sulfenic-amide (S–N) bonds after reaction with the amide nitrogen atom or, most commonly, the formation of intramolecular or intermolecular disulfides (S–S) after reaction with another cysteine or with glutathione (S-glutathionylation) (Dalle-Donne et al. 2009, Reczek & Chandel 2015). All the aforementioned reactions are reversible, namely, the oxidized protein can be reduced back to its initial state through the thioredoxin or glutaredoxin enzymes, at the expense of NADPH (Reczek & Chandel 2015). ii) Further two- or four-electron oxidations to form sulfinic and sulfonic acid, respectively. Both types of overoxidation are typically irreversible and result in the permanent inactivation of the protein (Yang et al. 2002). However, exceptions exist as regards to the reversibility of the sulfinic acid formation. In particular, in the case of peroxiredoxins, sulfiredoxin catalyzes the reduction of sulfinic acid to its sulfenic form via an Mg^{2+}- and ATP-dependent reaction, using glutathione or thioredoxin as electron donors (Biteau et al. 2003, Chang et al. 2004). Along with the oxidative modifications, cysteine residues can also become S-nitrosylated by nitric oxide-derived nitrosylating agents, such as dinitrogen trioxide and nitrosonium ion as well as by trans-S-nitrosylation from low molecular mass S-nitrosothiol proteins (Hogg 2002, Sun & Murphy 2010). The reversibility in the case of S-nitrosylated cysteines depends on two different processes, namely trans-S-nitrosylation and denitrosylation (Lima et al. 2010). During trans-S-nitrosylation, the nitrosyl moiety is transferred to low-molecular-mass thiols, such as GSH to form S-nitrosoglutatione (GSNO), which is subsequently denitrosylated by the GSNO reductase. On the other, denitrosylation is accomplished by diverse enzymatic mechanisms, including inter alia the thioredoxin system, carbonyl reductase, copper-zinc superoxide dismutase and xanthine oxidoreductase (Benhar et al. 2009, Sun & Murphy 2010). All enzymes participating in denitrosylation processes are commonly summarized under the term "denitrosylases". Based on the aforementioned reversibility considerations, methionine and cysteine seem to be the only amino acids that once oxidatively or nitrosatively modified during exercise may be implicated in redox signaling events.

From the two aforementioned amino acids, cysteine has attracted the greatest attention, and hence, the discussion herein will be limited to this amino acid. To date, approximately 214,000 cysteine residues have been identified in the human proteome, with approximately 5–12% of these cysteines being residually oxidized in cells (Go & Jones 2013). This implies that, at rest, the majority of cysteines are not oxidized (Go et al. 2015, Jones 2008), yet, the broad distribution of cysteines among structural and regulatory proteins is essential for maintaining normal protein function (Miseta & Csutora 2000, Jones 2010). This percentage, however, can be increased up to >40% in response to an oxidizing stimulus (Go & Jones 2013, Jones 2008). With regard to exercise, it is still unknown which specific proteins are oxidatively modified and whether these modifications are reversible or not. Identification of the proteins that are oxidized during exercise can be achieved with redox proteomics (Gould et al. 2015; McDonagh et al. 2014). We are unaware of any cysteine-based exercise proteomics study. It is true that proteomics approaches in skeletal muscle are difficult mostly due to the fact that signaling proteins are partially

masked by the abundance of contractile and metabolic proteins (Malik et al. 2013). However, redox proteomics could provide the first evidence for the occurrence of distinct protein networks that undergo redox signaling through specific cysteine modifications in response to exercise (Gould et al. 2015). In the same vein, a recent study performed a global phosphoproteomic analysis of human skeletal muscle revealing an impressive number of 8,511 unique phosphorylation sites, of which approxiamately 12% were regulated with exercise (Hoffman et al. 2015).

Contrary to the reversibility of cysteine modifications, irreversible oxidations of biomolecules during excessive or persistent production of reactive species are mostly mediated by one-electron oxidants (i.e., free radicals; Jones 2008). One-electron reactions generally lack sufficient selectivity to trigger a specific biochemical cascade and, therefore, it was argued that irreversible oxidations do not fulfill the requirements to exert any conventional signaling transduction effect (Jones 2008, Radak et al. 2011). However, this is not always the case, since some studies have enriched the redox signaling repertoire with cases showing that signaling may also be mediated by irreversible modifications. For instance, the cytosolic pathway of nuclear factor erythroid 2-related factor 2 (NRF2) and Kelch-like ECH-associated protein 1 (KEAP1) (NRF2–KEAP1 pathway) is regulated by the irreversible modification (S-alkylation) of a KEAP1 thiol group by electrophiles and/or oxidants (Higdon et al. 2014, Kobayashi & Yamamoto 2006). In addition, it has been suggested that redox signaling might also occur as a stress-response to excessive reactive species production, a fact that implicates one-electron oxidants signaling initiators (Winterbourn 2015). A typical example supporting this idea is the free radical-mediated lipid peroxidation product, 4-hydroxy-2-nonenal, which has been implicated in redox signaling processes acting either as oxidant or as an electrophile (Brigelius-Flohe & Flohe 2011, Forman et al. 2014, Winterbourn 2015). Figure 5 demonstrates how 4-hydroxy-2-nonenal may link the two intertwining pathways of exercise-induced redox signaling (adaptations) and oxidative stress (responses).

A crucial point in redox signaling is that typically requires only one or few redox modifications of a thiol group in an amino acid residue and not extensive structural alterations of a protein, such as the carbonylation of the acto-myosin complex that most likely impacts exercise responses (e.g., muscle fatigue; Castro et al. 2012, Moen et al. 2014). Yet, even in this case, the existence of repair mechanisms for the oxidized proteome (Arnold & Grune 2002, Catalgol et al. 2010, Chondrogianni et al. 2014) represents a potential mechanism offering the required reversibility for signaling. Regarding thiol modifications, it is of upmost importance to quantify the fraction of the protein that needs to be oxidized or S-nitrosylated in order to initiate a signaling cascade or to elicit a functional/physiological effect during exercise. Several mass spectrometry-based analytical approaches have been implemented towards this aim combined with the biotinylated iodoacetamide method (BIAM), the Isotope Coded Affinity Tag (ICAT) and its modified version oxICAT, the stable isotope labeling by amino acids in cell culture (SILAC), tandem mass tags (TMT) and isobaric tags for relative and absolute quantification (iTRAQ) (Baez et al. 2015, Leonard & Carroll 2011). In general, the combination of mass spectrometry with the aforementioned approaches increases the sensitivity and specificity regarding the identification of thiol modifications and affords quantitative insights. Studies that utilized such model systems demonstrated that the average percentage of the oxidation or S-nitrosylation of a given protein at rest and under physiological conditions varies from 1% to 20% (Go et al. 2011, Kohr et al. 2012), although S-nitrosylation is frequently underestimated (Doulias et al. 2013). Yet, this value can increase up to 50% or 60% upon a redox stressor depending on the cysteine residue (Kohr et al. 2012). Of interest, during labeling and quantification of cysteine redox modifications several analytical and biochemical issues should be taken into account, including artifact-prone sample handling, potential overlap or crosstalk between oxidations and S-nitrosylations, complementing poly-oxidations of a given protein in diverse sites and cysteines as well as cases of rapid reversibility (e.g., denitrosylation) (Baez et al. 2015, Gould et al. 2013, Leonard et al. 2009, Tsikas 2012, Tsikas 2008).

Therefore, in order to acquire more informative mechanistic details about the role of exercise-induced reactive species in responses and adaptations, it is crucial to identify i) the protein oxidized, ii) the domain of the modification, iii) the extent of the modification iv) whether the modification is reversible and v) the function, regulation and physiological consequence of the modification. Generally, reversible oxidations of specific sulfur-containing amino acid residues (i.e., methionine and cysteine) are associated with signaling events and long-term

adaptations, whereas irreversible oxidative modifications seem more reasonable to be associated with oxidative or nitrosative stress and short-term responses.

2.5 Principle 5: Establish causality between redox and physiological measurements

An increasing number of biochemical pathways is identified and associated with diverse exercise responses and adaptations (Egan & Zierath 2013, Hawley et al. 2014). Reactive species have been implicated in these pathways and are proposed to act either directly (e.g., by oxidizing a target molecular domain) or indirectly (e.g., by disturbing mitochondrial function causing an aberrant ATP/ADP ratio) (Cardaci et al. 2012, McConell et al. 2012, Merry & McConell 2009). However, a commonly encountered ambiguous method is to establish a cause-effect relationship between reactive species and an exercise response or adaptation by correlating the production rate of a reactive species with the magnitude of alterations in exercise responses or adaptations via calculation of a correlation coefficient. The situation becomes even less specific when the correlation analysis is performed between a generic oxidative stress biomarker and an exercise response or adaptation. For instance, the measurement of F_2-isoprostanes does not provide identity or spatial information about the potential underlying redox mechanisms of an exercise response or adaptation. This is due to the fact that several reactive species, such as the hydroxyl, alkoxyl and peroxyl radicals, can abstract a bis-allylic hydrogen atom from the arachidonic acid carboxyl chain (Nikolaidis et al. 2011). In addition, arachidonic acid can be found in various subcellular compartments in muscle cells, including plasma membrane, mitochondria and endoplasmic reticulum (Fiehn et al. 1971). On this basis, the frequently reported correlation between the level of oxidative stress biomarkers and exercise responses and adaptations cannot convincingly establish a causal redox-mediated relationship.

In the light of the above, it is imperative to know whether the reactive species produced during exercise are chemically competent to be implicated in the pathway of the response or adaptation under study (e.g., by oxidizing a key molecule of that specific pathway). Mitochondrial function and glucose metabolism are two exemplar processes that are regulated by 5' adenosine monophosphate-activated protein kinase (AMPK) during exercise (Karagounis & Hawley 2009). Despite that AMPK is mainly activated in response to energy disturbances (increased AMP/ATP ratio) acting as an energy "sensor", recent evidence suggests that reactive species can activate AMPK without a concomitant increase in cellular AMP levels (Cardaci et al. 2012, Emerling et al. 2009, Toyoda et al. 2004, Zhang et al. 2008). More specifically, hydrogen peroxide can oxidatively modify cysteine residues (by S-glutathionylation) of the α- and β-subunits of AMPK, which subsequently cause an allosteric transition of the AMPK-αβγ heterotrimer towards its active state (Cardaci et al. 2012, Zmijewski et al. 2010). These findings indicate the existence of a redox-related and energy-independent mechanism of AMPK activation linked to mitochondrial function and glucose metabolism. Indeed, in a comprehensive study Morales-Alamo et al. (2013) have shown that reactive species play a critical role in AMPK-mediated signaling after sprint exercise in human skeletal muscle. However, this study used a cocktail of non-specific antioxidants (i.e., α-lipoic acid, ascorbate and α-tocopherol) and it is likely that some of the effects observed may not be redox-related. If future research directly demonstrates that AMPK oxidation is causally linked with some short- or long-term biological outcomes (e.g., contractile force, fatigue, mitochondrial biogenesis or insulin sensitivity; Costill & Hargreaves 1992, Gomez-Gabrera et al. 2005, Hawley et al. 1992, Kuwahara et al. 2010, Lee et al. 2014, Piantadosi 2012, Sahlin et al. 2006, Xie et al. 2006, Zhang & Casadei 2012), then a clear example for the implication of reactive species in the exercise responses or adaptations could be established.

The relationship between an oxidation and a physiological outcome should not be advocated *a priori* by researchers without establishing a plausible biochemical mechanism of action. However, the link between molecular cascades and physiological manifestations cannot be easily established using isolated and biologically distant measurements (e.g., trying to statistically connect the concentration of hydrogen peroxide with a maximal oxygen consumption value). The communication between the distinct layers of biological organization (from molecular and cellular to organism and whole-body level) has been a subject of debate for a long time (Dupré 1993, Pigliucci 2003). Several researchers have also expressed their concerns with regard to whether changes in some aspects of redox homeostasis can accurately predict exercise phenotypes (Close & Jackson 2014, Cobley et al. 2015a; Paulsen et al. 2014). There are several cogent reasons for this. First, redox signaling is characterized by high level of redundancy and in-built reserve capacity. This conveys that the suppression or the complete inhibition of an upstream signal does not always translate to blunted downstream responses (Cobley et

al. 2015a, Kholodenko et al. 2010, Smock & Gierasch 2009). Second, a physiological response is the reflection of highly regulated processes across several layers of biological organization. Thus, it becomes questionable how the blunted activation of only one or two regulatory proteins will impact this highly integrated response (Cobley et al. 2015a). Third, the molecular outcomes usually encountered in the literature are frequently only part of the stress-response, which is seldom essential for physiological adaptations.

Despite the limitations of the reductionist approach, the complexity of *in vivo* redox biology and the difficulties in linking a molecular mechanism to a physiological manifestation frequently obliges researchers to perform *ex vivo* studies in order to decipher the role of reactive species in exercise responses and adaptations (Davison et al. 2008, Etgen et al. 1997, Higaki et al. 2001, Palomero et al. 2012, Pearson et al. 2014, Reid et al. 1992a, Sandstrom et al. 2006). Two major approaches have been implemented so far. The first approach aims to mimic the increased reactive species production during exercise by adding a reactive species exogenously or by overexpressing its specific source. The second approach encompasses the decrease of reactive species concentration by administering an antioxidant (see principle 6) or by abrogating the production of the reactive species, usually by using transgenic approaches. Although the aforementioned approaches seem at a first glance efficient to establish a cause-effect relationship between the manipulated reactive species and the outcomes measured, the conclusions drawn may be fragmentary and partially misleading (Nikolaidis et al. 2015). In fact, the experimental and subsequently the interpretative isolation of a specific reactive species in such methodologies, inevitably leads to underestimation of the biochemical networks complexity. For instance, the components of a molecular network dynamically respond and adapt to the environment and, thus, any experimental manipulation of this environment (e.g., alteration of the vicinal interactome) can result in completely different outcomes compared to what usually occurs *in vivo* (Forman 2007, Halliwell 2013, Papin et al. 2004). In accordance to this notion, many reactive species and amino acid residues become unstable outside their cellular milieu exhibiting differential reactivity, while antioxidant mechanisms appear highly redundant *in vivo* in response to a reactive species overproduction (e.g., against hydrogen peroxide; Winterbourn 2008).

It is reasonable that researchers are wishing to translate the chemical details of their redox experiments into *in vivo* human biology. Based on the aforementioned considerations, the implementation of complementary and pluralistic approaches would provide a more comprehensive and realistic representation of how reactive species might be implicated in the exercise responses and adaptations. Researchers should try to connect an oxidation with an exercise outcome by establishing a plausible biochemical means of action. *In vitro* and *ex vivo* studies can certainly enrich the *in vivo* findings providing fundamental mechanistic information that cannot be provided otherwise. For instance, Figure 6 displays the levels of superoxide and hydrogen peroxide production by muscle mitochondria in an experimental setting at the interface between *ex vivo* and *in vivo* redox biology, where researchers mimicked resting and exercise conditions (mild and intense exercise intensity) *ex vivo* (Goncalves et al. 2015). Another recent example, successfully linking *in vivo* with *ex vivo* approaches, showed that high intensity exercise induces reactive species mediated ryanodine receptor fragmentation, which might be essential for triggering muscle adaptations (Place et al. 2015). Therefore, despite the difficulty of the *in vitro* set ups to sufficiently mimic the *in vivo* conditions (Halliwell 2014, Kojo 2012, Osterburg et al. 2013, Rice 2012), it is crucial to try validating the translational *in vivo* findings with mechanistic *ex vivo* data.

2.6 Principle 6: Use selective and targeted antioxidants

The vast majority of studies investigating the potential implication of reactive species in exercise responses and adaptations utilize antioxidants to scavenge or inhibit the activity of reactive species produced during exercise and examine the subsequent biochemical and physiological outcomes (reviewed in Braakhuis & Hopkins 2015; Mankowski et al. 2015, Neubauer & Yfanti 2015, Reid 2015). However, in most (if not all) exercise studies, the antioxidants utilized are pleiotropic and non-selective against a certain reactive species and, at the same time, are unevenly distributed in the various tissues, cells and organelles of the human body. As a result, no precise redox-related mechanistic conclusions can be drawn, especially when this approach is allied to the futile practice of inferring redox signaling from oxidative stress biomarkers. For instance, it is possible that the altered (impaired or improved) performance observed after the “antioxidant” supplementation may have actually resulted from redox-unrelated effects of the substance administered (Cobley et al. 2015a). Antioxidants such as vitamin C, vitamin E, NAC and dietary polyphenols are such non-selective antioxidants frequently utilized in the relevant literature

(Nikolaidis et al. 2012b, Nikolaidis et al. 2015, Paschalis et al. 2014). Besides selectivity, an additional drawback of the aforementioned antioxidants is the existence of other kinetically favored competitors against reactive species, mainly, antioxidant enzymes (Cobley et al. 2015a). As an example, vitamin C and E negligibly react with primary (i.e., superoxide, nitric oxide) and secondary (i.e., hydrogen peroxide, peroxynitrite) reactive species and when they react, the rate constants are much lower than those observed between reactive species and antioxidant enzymes (Cobley et al. 2015a).

Based on the above, when using an exogenously administered antioxidant agent to shed more light on the potential underlying redox mechanisms of exercise responses and adaptations, it becomes reasonable that the antioxidant utilized should obey the "rules" of specificity and selectivity and also be able to out-compete the respective redox enzymes. There are two options to accomplish this goal: either to use molecules that selectively block specific sources of reactive species (e.g., febuxostat as inhibitor of xanthine oxidase or VAS2870 as inhibitor of some NADPH oxidase isoforms; Altenhöfer et al. 2012, Halliwell 1987, ten Freyhaus et al. 2006, Takano et al. 2005) or to create novel selective "antioxidant agents". These agents should desirably exhibit the following properties: i) high bioavailability in the human body, ii) safe administration (especially at high doses without toxicity side-effects) and iii) selective reactive species targeting in specific cell compartments. Compartmentalization is of outmost importance for better mechanistic understanding of the role of reactive species in exercise, since each organelle possesses unique redox regulatory mechanisms. Towards this aim, promising advances have been made in biomedical sciences, with mitochondria representing a salient example for targeted antioxidant manipulations (Murphy & Smith 2000). In fact, mitochondria, due to their central role in cell metabolism, energy supply, Ca^{2+} homeostasis and reactive species production, have attracted great attention in the development of (antioxidant) therapies that aim to ameliorate the mitochondrial damage during several diseases (Smith et al. 2011, Szewczyk & Wojtczak 2002, Weissig 2003).

Multiple methods have been developed for the targeted delivery of antioxidants to mitochondria (Sheu et al. 2006). Two approaches that exploit biophysical properties of mitochondria (e.g., high negative internal potential) have received most attention: i) conjugation of the antioxidant to lipophilic cations, typified by MitoC, MitoE, MitoPeroxidase and the most widely studied MitoQ, a coenzyme-Q analog, which encompasses a lipophilic triphenylphosphonium (TPP) cation covalently bounded to ubiquinol through an alkyl carbon chain (Finichiu et al. 2015, Murphy & Smith 2007, Sheu et al. 2006, Smith & Murphy 2010) and ii) incorporation of the antioxidant dimethyltyrosine into synthetic mitochondria-targeted tetrapeptides consisting of an alternating aromatic-cationic residue motif, termed Szeto-Schiller (SS)-peptides (Szeto 2014, Szeto & Schiller 2011, Zhao et al., 2004). Noteworthy, these tetrapeptides are taken up into cells and mitochondria in an energy-independent manner (Szeto 2014). An important prerequisite that both approaches have to fulfill is that they should not adjust normal mitochondrial function, for example, through inhibition of mitochondrial bioenergetics or dissipation of the inner mitochondria membrane potential (Kelso et al. 2001, Reily et al. 2013). Otherwise, the final outcome cannot be safely attributed to the antioxidant delivery and activity. In contrast, if this prerequisite is met, one can more unambiguously ascribe a redox regulatory mechanism between a redox molecular modification (e.g., altered cytochrome c content) and a physiological outcome (e.g., altered respiration) after administration of a mito-targeted antioxidant.

Exercise studies could extend the central idea of these approaches in an exercise setting. For instance, administration of a selective and targeted antioxidant (e.g., MitoQ, which most likely diminishes mitochondrial superoxide production) in combination with redox proteomics approaches (i.e., targeted to mitochondria) could provide sound mechanistic insights into how the exercise-induced reactive species (e.g., superoxide) may regulate the biochemical and/or physiological responses and adaptations to exercise and to establish a "cause-effect" relationship between them (Figure 7).

3. SYNTHESIS AND EPILOGUE

Despite theoretical idealizations, the effort of linking the chemistry of reactive species with physiological phenomena proved to be a Kafkaesque process. Like the characters in a Kafka's novel, doing research in translational redox biology frequently evokes feelings of senselessness and disorientation. This is largely because real-life redox biology is incomprehensibly complex and sometimes seems illogical. This partly explains why

research in redox biology sometimes looks like a literary competition, where researchers compete on the ability of who is telling the most fascinating story. Redox biology science is quite novel, the background knowledge is limited and, as a result, researchers often feel obliged to improvise to breathe life into their data.

Most of the evidence supporting the regulatory effect of reactive species on exercise responses and adaptations is circumstantial and come from mechanistic (non-physiological) models. In other words, we can reasonably assert that reactive species regulate exercise responses and adaptations, but we were not able to prove it convincingly at the present time. Towards this end, we have proposed six biochemical principles to assist in the development of a methodology that will hopefully lead to more realistic data. Presumably, it is impossible to apply all these principles in a single study. Moreover, many of these principles cannot be satisfied due to technical or ethical limitations of human *in vivo* studies. For example, the direct *in vivo* measurement of a specific reactive species with spatiotemporal resolution is not feasible *in vivo* in humans. Even using synthetic antioxidants (i.e., MitoQ) designed to accumulate in mitochondria it is very likely that the measurement of individual reactive species cannot be achieved. This can be accomplished only by using the redox probe MitoSOX but this probe can be used only in cell cultures, and even in that case, may uncouple mitochondrial respiration if applied at micromolar levels (Roelofs et al. 2015). Thus, fulfilling all the principles proposed in the present article requires an idealized human experimental setting, which is certainly a utopia. Yet, alternative experimental set-ups (e.g., *ex vivo* and *in vitro* studies and animal models) should be designed to serve towards the creation of an "idealistic" model to complement *in vivo* human studies (i.e., principle 5).

We acknowledge the fact that proposing methodological principles for future research is a highly reductionist approach. However, it is stressed that the molecular mechanisms often do not satisfactorily explain how the phenomena at the higher organizational level (i.e., organism) are possible and often cannot accurately predict what the higher-level phenomena will actually be at any given instance (Joyner 2015). With this in mind, the reader will notice that we avoided giving a definitive answer on how to meet any principle that we proposed. This was mainly for three reasons: first, because redox biology is a relatively new research field not having established maturity. As a result, it still lacks a solid theoretical framework and valid experimental tools. Second, because we are skeptical about the feasibility of defining firm principles for *in vivo* human studies with a huge variety in animals, tissues/fluids, analytical tools and exercise protocols. Third, because we believe that establishing "gold standards" in any field of science is implausible. Experimental standardization implies definite answers and particularly current redox biology is much far from that. We think that, instead of a dogmatic approach (e.g., trying to satisfy all six principles), researchers should choose to satisfy some of the proposed principles based on scientific evidence and decide which experimental variables are most critical when dealing with their specific research question. On the other, we also believe that it is fair enough to make readers aware of the possible study limitations, when some the "ideal" principles are not met.

Many redox biology researchers have come to the disappointing conclusion that their redox data are no longer providing much insightful information or to conclude that alterations in redox homeostasis are in many cases just an epiphenomenon not needed to be studied. Although it is straightforward to define redox biology chemically, characterizing the effects of an "oxidant" or an "anti-oxidant" in a biological environment has proven to be a very difficult task. For instance, the targeted "antioxidant" MitoQ can auto-oxidize if deprotonated in the aqueous phase to become a pro-oxidant (James et al. 2005), however, whether this scenario is indeed feasible *in vivo* has been questioned (Rodriguez-Cuenca et al. 2010). We hope that the proposed principles will be a useful tool to assess more realistically redox homeostasis alterations in response to exercise and reach more pragmatic explanations of redox biology phenomena. As exercise is just an example of a stimulus able to alter the redox biology of organisms, we believe that the thoughts expressed in the present article would be also useful in medicine and life sciences.

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FIGURE LEGENDS

Figure 1. The conventional experimental approach to reveal the role of reactive species in exercise responses and adaptations and the three major limitations of this approach.

Figure 2. Theoretical dose-response relationships between reactive species production and exercise responses and adaptations. (A) sigmoidal, (B) hyperbolic, (C) threshold and (D) hormesis curves.

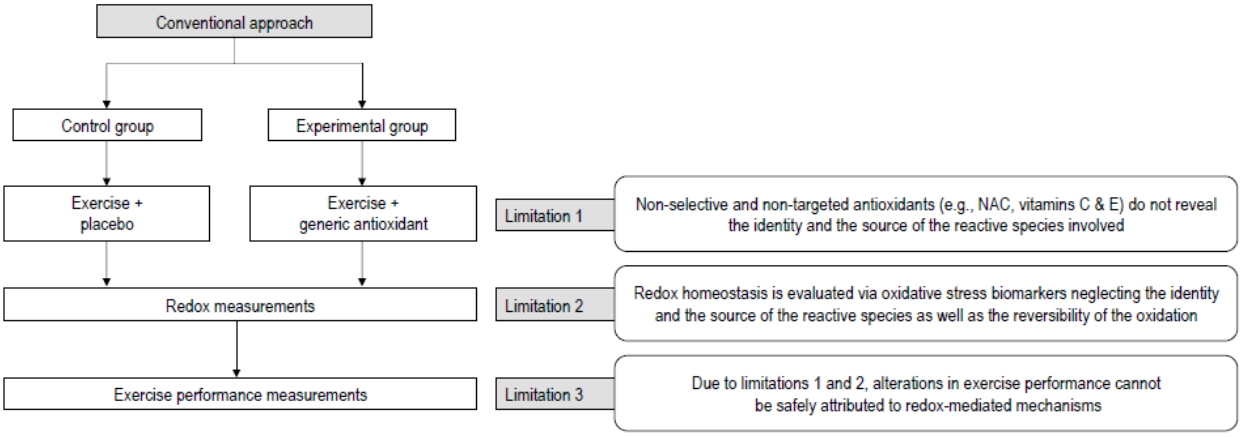
Figure 3. The several sources of superoxide anion in skeletal muscle.
CuZnSOD, CuZn-superoxide dismutase; ecSOD, extracellular superoxide dismutase; eNOS, endothelial nitric oxide synthase; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; MnSOD, Mn-superoxide dismutase; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; $O_2^{\bullet-}$, superoxide anion; $OH^{\bullet}$, hydroxyl radical; PLA_2, phospholipase A_2; XO, xanthine oxidase.

Figure 4. Reversible modifications of amino acid residues by reactive species may result in altered protein activity, inactivation of a enzyme, altered binding affinity to another protein, translocation and/or modified activity of a transporter or a channel.

Figure 5. A conceptual scheme showing two intertwining pathways regulating exercise responses and adaptations via redox signaling and oxidative stress.
4-HNE, 4-Hydroxynonenal; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PARP-1, poly [ADP-ribose] polymerase 1.

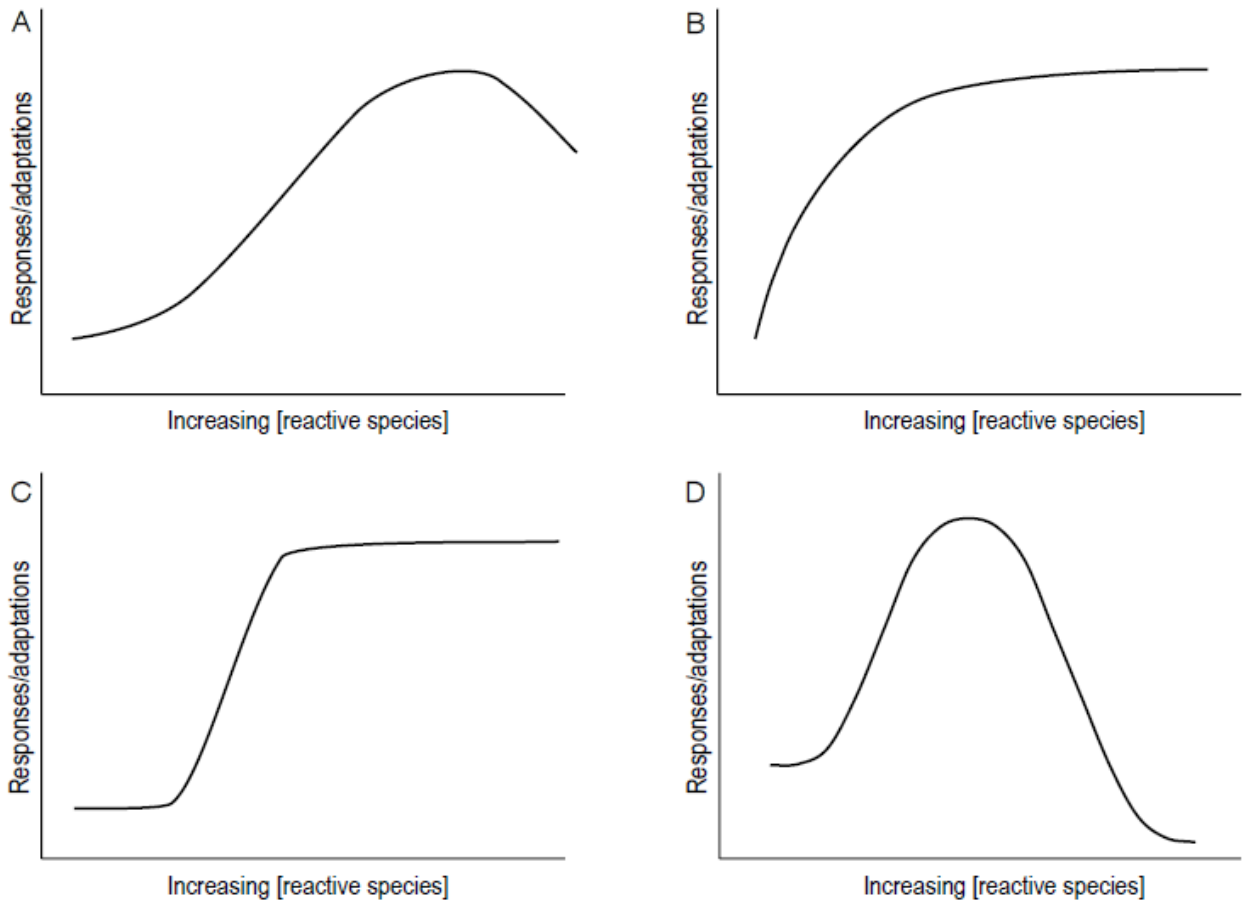
Figure 6. Mitochondrial superoxide/hydrogen peroxide production at rest and during mild and intense endurance "exercise". (A) topology of the diverse sites of superoxide production and (B) relative contribution in hydrogen peroxide production from each site during rest, mild and intense endurance "exercise". This study, at the interface between theoretical and experimental biology, shows that *ex vivo* experiments can provide realistic estimates of esoteric molecular processes not accessible *in vivo* (The figure is based on data from Goncalves et al. 2015).
I_F, flavin in the NADH-oxidizing site of respiratory complex I; I_Q, ubiquinone-reducing site of respiratory complex I; II_F, flavin site of respiratory complex II; III_{QO}, outer quinol-oxidizing site of respiratory complex III; ETC, electron transport chain; ETF:QOR, electron-transferring flavoprotein:ubiquinone oxidoreductase; G_Q, quinone reducing site in mitochondrial glycerol 3-phosphate dehydrogenase; H_2O_2, hydroxyl radical; $O_2^{\bullet-}$, superoxide anion; O_F, flavin in the 2-oxoglutarate dehydrogenase complex.

Figure 7. The proposed experimental approach and an "idealized" study to more definitely implicate the role of reactive species in exercise responses and adaptations.



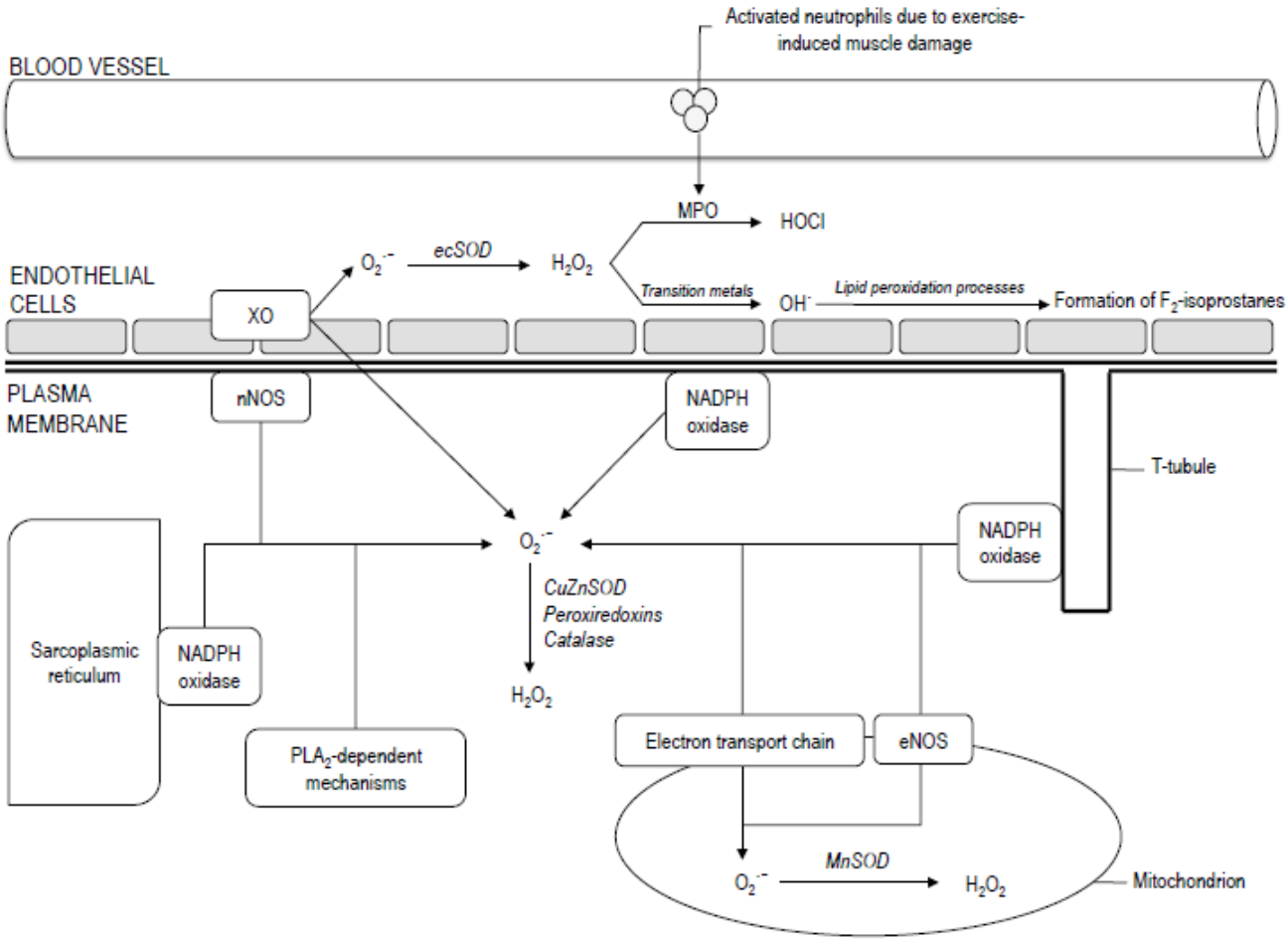
- Figure 1 -

Figure 1



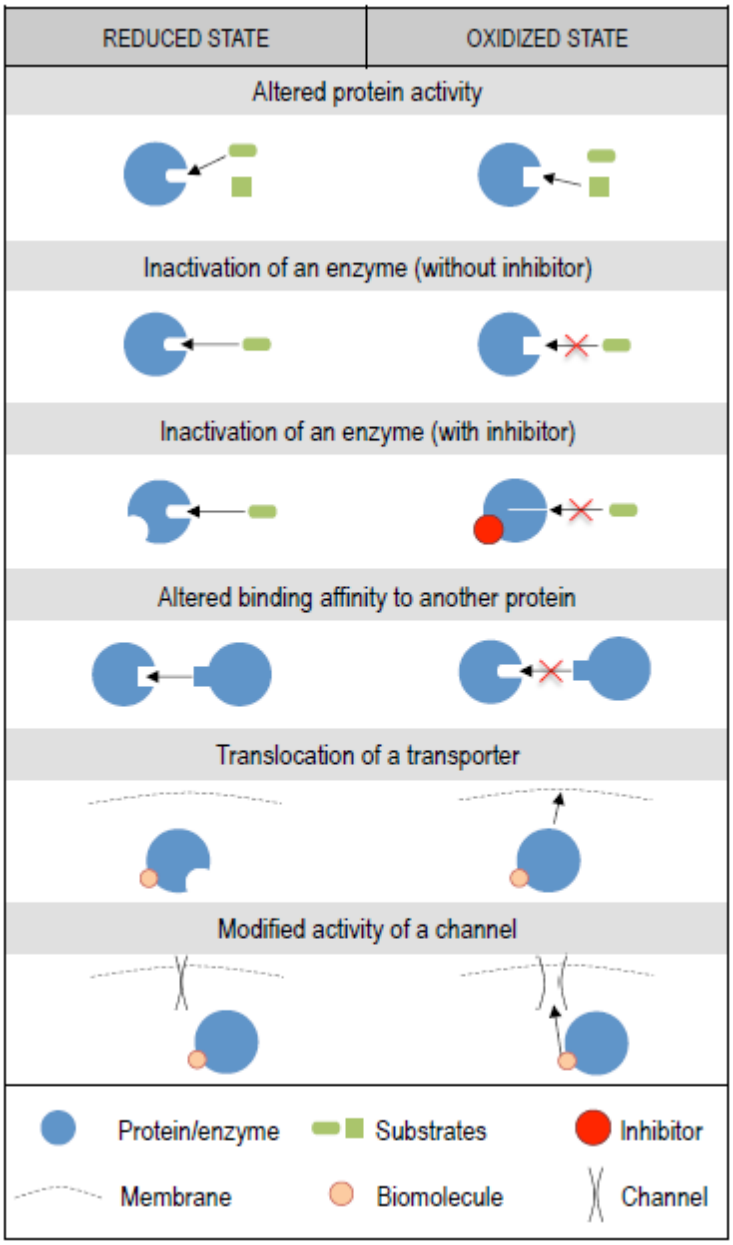
- Figure 2 -

Figure 2



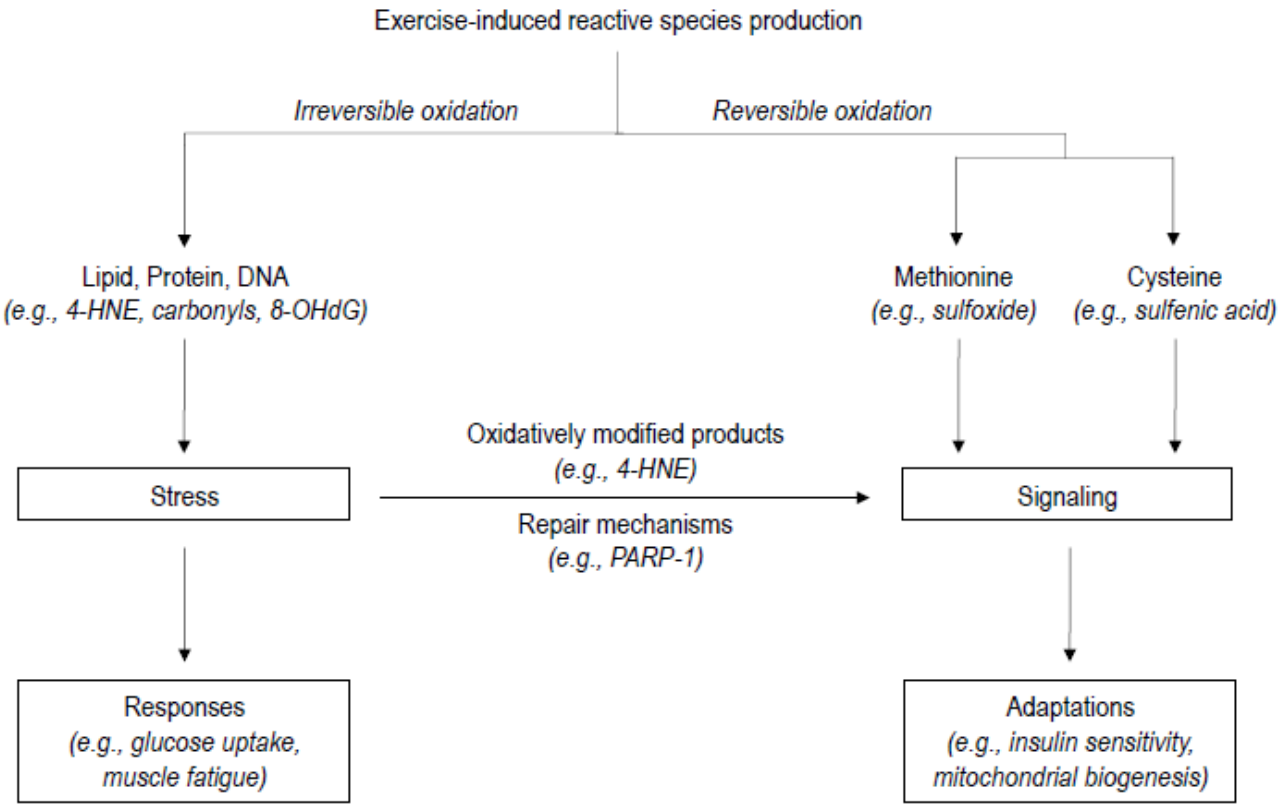
- Figure 3 -

Figure 3



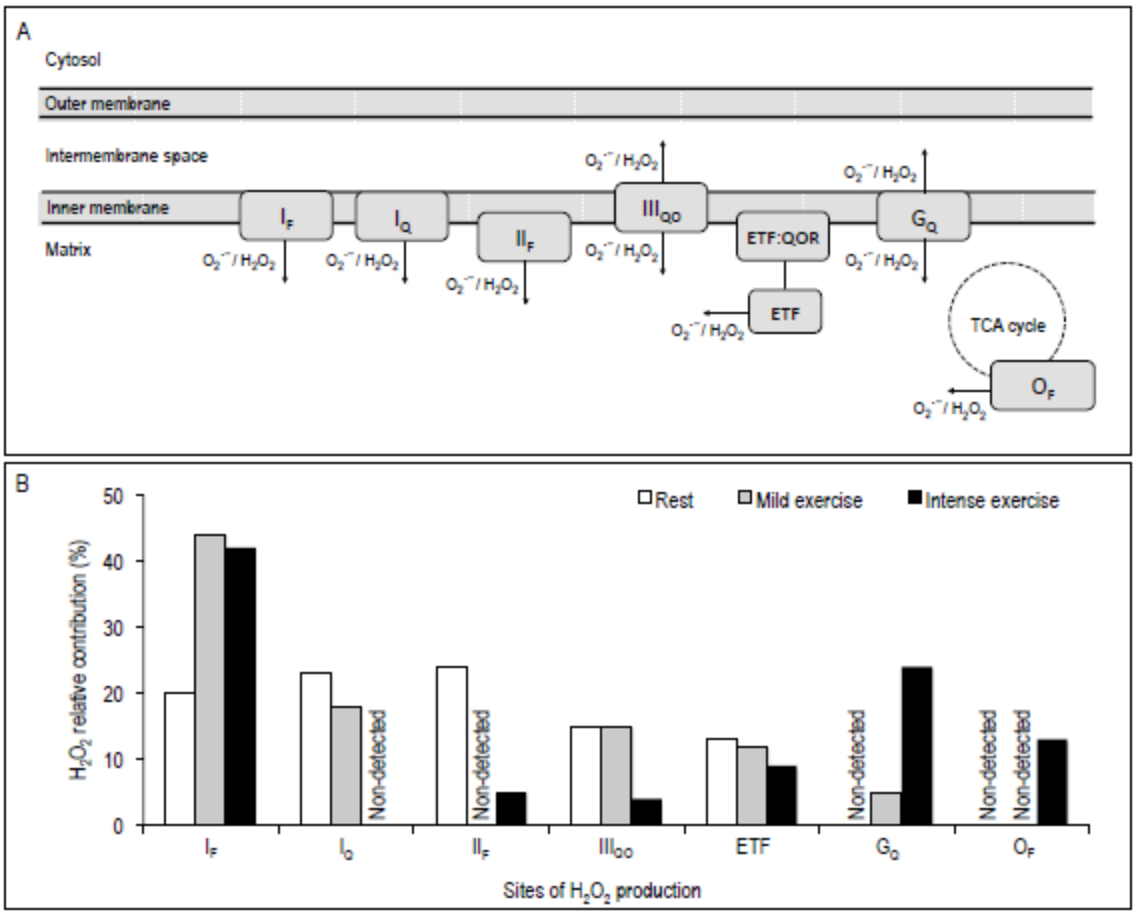
- Figure 4 -

Figure 4



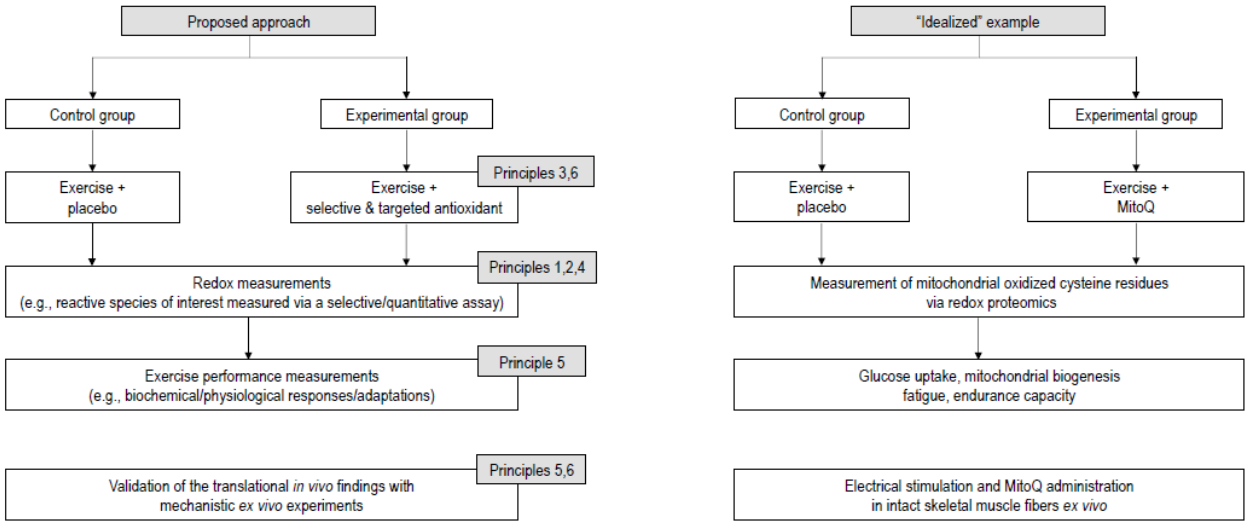
- Figure 5 -

Figure 5



- Figure 6 -

Figure 6



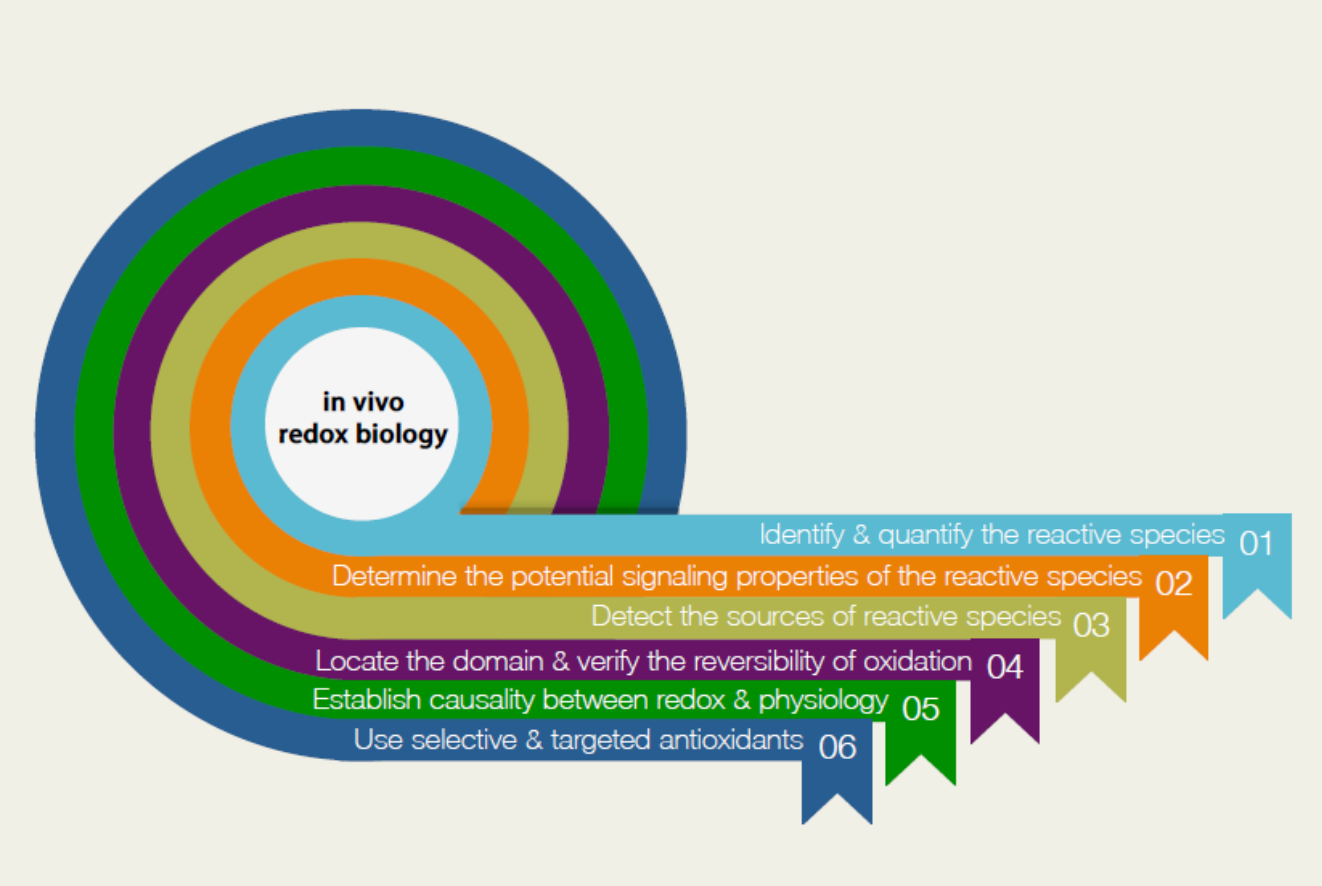
- Figure 7 -

Figure 7

Box 1. Fundamental and experimental limitations for mechanistically implicating reactive species in exerciseresponses and adaptations

■ **The extreme inherent complexity of the *in vivo* (redox) biology** *Example:* Selective expression and redundancy of the antioxidant enzyme isoforms dictated by the type, magnitude and/or site of the reactive species produced
■ **Insufficient and controversial information on major redox biological issues** *Example:* Debate on whether free radicals can signal
■ **The nascent nature of exercise redox biology** *Example:* Absence of any cysteine-based exercise proteomics study
■ **Equivocal identification/quantification of the reactive species and their sources with the available assays** *Example:* Inability to measure NADPH oxidase-mediated superoxide production in plasma membrane
■ **Use of generic oxidative stress biomarkers** *Example:* Measurement of oxidatively modified products such as F_2-isoprostanes
■ **Use of generic antioxidant agents** *Example:* Administration of pleiotropic compounds with purported *in vivo* antioxidant properties such as ascorbate
■ **Difficulty to replicate *in vitro* mechanistic set ups with *in vivo* experiments** *Example:* Unsuitability of all redox probes for human use

Graphical abstract



Highlights

- Inadequacy of current redox biology to shed light on biological processes in vivo
- Six principles to integrate RONS production into exercise responses/adaptations
- The principles is a tool to reach more pragmatic explanations of redox phenomena