

# Redox basis of exercise physiology

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## ABSTRACT

Redox reactions control fundamental processes of human biology. Therefore, it is safe to assume that the responses and adaptations to exercise are, at least in part, mediated by redox reactions. In this review, we are trying to show that redox reactions are the basis of exercise physiology by outlining the redox signaling pathways that regulate four characteristic acute exercise-induced responses (muscle contractile function, glucose uptake, blood flow and bioenergetics) and four chronic exercise-induced adaptations (mitochondrial biogenesis, muscle hypertrophy, angiogenesis and redox homeostasis). Based on our analysis, we argue that redox regulation should be acknowledged as central to exercise physiology.

## 1. Introduction

The multiple physical and mental health-related benefits of regular exercise have long been acknowledged and are nowadays undeniable [1,2]. As a logical consequence, researchers over the years commenced to search for the underlying molecular and biochemical mechanisms that drive these benefits. Since the first studies that implemented pioneering techniques (e.g., muscle biopsies) about 45 years ago [3–5], a great progress has been made in the field. For instance, it is currently well-established that exercise “releases” a set of local and systemic stressors that trigger integrated acute responses, which in the long-term result in phenotypic adaptations in all human body systems [6–8].

Redox processes are increasingly recognized as an integral part of the exercise-associated metabolism. Despite the traditional perception that reactive species are exclusively detrimental molecules, mounting recent evidence suggests that exercise-induced reactive species are essential upstream signals for the activation of redox-sensitive transcription factors and the induction of gene expression associated with exercise [9,10]. Terms such as “ROS”, “free radicals” and “oxidative stress” are more and more often included in leading exercise physiology reviews as regulators of responses and adaptations [11–13]. However, in most cases, the implication of redox processes in exercise physiology processes is either not supported by direct mechanistic evidence or is promoted as a subordinate element. Thus, the main purpose of the present review is to highlight the fundamental redox basis of exercise

physiology, by outlining the contribution of redox network in 4 characteristic acute exercise-induced responses (i.e., muscle contractile function, glucose uptake, blood flow and cell bioenergetics) and 4 well-known training-induced adaptations (i.e., mitochondrial biogenesis, skeletal muscle hypertrophy, angiogenesis and redox homeostasis). We do not aim to comprehensively describe the underlying mechanisms for each of the 8 aforementioned exercise-related processes, but rather to concisely decipher the corresponding redox components probably involved.

Taking into account that the field of exercise redox biology is still in its infancy, the exercise responses and adaptations data presented in this review was selected irrespective of the exercise type (i.e., aerobic or anaerobic, endurance or resistance) or the experimental approach implemented in the original studies. Thus, exercise studies that utilized nutritional antioxidants (e.g., vitamin C) or reactive species source inhibitors (e.g., apocynin), transgenic rodent models over- or under-expressing redox related genes (e.g., G6PD-Tg mice), in-vitro, in-situ or ex-vivo set-ups as well as studies that implemented more invasive techniques, such as synergistic ablation or hindlimb ischemia, have been included. Moreover, the review is inevitably focused on skeletal muscle, due to lack of research in other tissues. Mechanistic information from other scientific fields (e.g., biochemistry, nutrition and clinical medicine [14–16] regarding well-established redox sensitive signaling pathways has been incorporated in particular cases as well. Collectively, our review aspires to tentatively translate molecular and

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**Abbreviations**

Akt	protein kinase B
AMPK	5' AMP-activated protein kinase
ARE	antioxidant response element
c-Src	proto-oncogene tyrosine-protein kinase
CaMK	Ca <sup>2+</sup> + /calmodulin-dependent protein kinase
cGMP	cyclic guanosine monophosphate
ERK	extracellular signal-regulated kinase
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	reduced glyceraldehyde 3-phosphate dehydrogenase
GSH	reduced glutathione
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HIF-1 $\alpha$	hypoxia-inducible factor 1- $\alpha$
IGF-I	insulin-like growth factor 1
IKK	I $\kappa$ B kinase
JNK	c-Jun N-terminal kinases
Keap1	Kelch-like ECH-associated protein 1
LKB1	liver kinase B1
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
NADPH	reduced nicotinamide adenine dinucleotide phosphate

NF $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NO $\bullet$	nitric oxide
Nrf2	nuclear factor erythroid 2-related factor 2
O $\bullet^-$	superoxide radical
OGG1	8-oxoguanine glycosylase
OH $\bullet$	hydroxyl radical
ONOO-	peroxynitrite
p70S6K	ribosomal protein S6 kinase beta-1
PGC-1 $\alpha$	peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$
PI3K	phosphoinositide 3-kinases
PPP	pentose phosphate pathway
Rac1	Ras-related C3 botulinum toxin substrate 1
RONS	reactive oxygen and nitrogen species
RyR	ryanodine receptors
SERCA	sarco/endoplasmic reticulum Ca <sup>2+</sup> + -ATPase
SOD	superoxide dismutase
TnI	troponin I
Trpv1	transient receptor potential cation channel subfamily V member 1
VEGF	vascular endothelial growth factor.

biochemical redox data into physiological real-life exercise situations and, vice versa, to identify likely redox mechanisms underlying common physiological exercise responses and adaptations.

## 2. Redox-regulated exercise responses

### 2.1. Muscle contractile function

Redox regulation of muscle contractile function probably represents the “archetypal” example of how reactive oxygen and nitrogen species are implicated in exercise responses (the umbrella abbreviation RONS will be used throughout the paper, but the precise species will be named when available [17]). During the first half of the 1980's, two studies that used electron paramagnetic resonance spectroscopy demonstrated that the skeletal muscle tissue from rabbits [18] and humans [19] produces detectable amounts of RONS at rest, and this production greatly increases during contraction. These findings were further supported by more recent studies implementing elegant experimental approaches in exercising humans [20,21]. As a result, the key question about the potential biological role of RONS in skeletal muscle function sensibly emerged. Ever since this question was firstly placed, our understanding on the role of RONS on muscle contractile function has greatly progressed under both physiological (mild or transient RONS increase) and pathological (excessive or chronic RONS increase) conditions [22]. Most of the evidence however is inevitably based on studies using isolated muscle preparations and animal models due to limitations in existing analytical tools (i.e., volatile nature of RONS and low bioavailability of redox probes in humans).

An interesting series of studies were conducted in intact mouse fibers (i.e., fibers along with their entire redox environment consisting of RONS sources, antioxidants, and target molecules) [23–25]. In brief, transient exposure (i.e., 4 min) or low concentration (i.e., in the nM and pM range) of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or tert-butylhydroperoxide (t-BOOH) increased submaximal muscle force production in fast-twitch muscle fibers, while the addition of the antioxidant dithiothreitol (DTT) resulted in a progressive force decline. On the other hand, prolonged exposure (i.e., 8 min) or high concentration (i.e., in the mM range) of H<sub>2</sub>O<sub>2</sub> decreased submaximal muscle force production (indication of muscle fatigue development) and this was reversed by DTT addition. Interestingly, transient exposure of unfatigued intact fast-twitch muscle fibers to DTT decreased submaximal force production and this was

reversed after the treatment with the opposite redox stimulus, namely H<sub>2</sub>O<sub>2</sub> [23–25]. Thus, an “optimal” cellular redox state seems to be the key determinant for normal muscle force production, whereas a more reduced (e.g., at rest) or oxidized state (e.g., during fatiguing protocols) negatively regulates this process (as illustrated in the classic biphasic model proposed by Reid [26]).

Similar studies using mechanically-skinned muscle fiber preparations showed that H<sub>2</sub>O<sub>2</sub> per se, in concentrations as high as 10 mM, does not necessarily exert an effect in force production [27] yet in some cases comparable or even higher levels of H<sub>2</sub>O<sub>2</sub> were found to negatively affect contractile function due to oxidation of specific cysteine and methionine residues at the actomyosin interface [28–30]. In a more physiological context, Murphy et al. [31] treated skinned muscle fibers with myoglobin and reduced glutathione along with low H<sub>2</sub>O<sub>2</sub> concentrations (100–300  $\mu$ M). They found that glutathione partially prevented the decline in muscle force production induced by the H<sub>2</sub>O<sub>2</sub>–Fe<sup>2+</sup>/myoglobin reaction in the slow-twitch muscle fibers, while in the fast-twitch muscle fibers, glutathione actually increased Ca<sup>2+</sup> sensitivity (at least in the initial phase) [31]. This was subsequently explained by the interaction of glutathione with the oxidized cysteine residues on the fast isoform of troponin (TnI) that increased myofibrillar Ca<sup>2+</sup> sensitivity [32]. Generally, in most of the aforementioned studies, it was demonstrated that H<sub>2</sub>O<sub>2</sub> regulates muscle force production either positively or negatively mainly by altering myofibrillar Ca<sup>2+</sup> sensitivity as indicated by the marginally affected free cytosolic Ca<sup>2+</sup> concentration [33].

Beyond H<sub>2</sub>O<sub>2</sub>, the superoxide radical has been also demonstrated to affect muscle force production, however this was facilitated through a different mechanism, that is, an altered sarcoplasmic reticulum Ca<sup>2+</sup> release (and not Ca<sup>2+</sup> sensitivity) [34,35]. Regarding nitric oxide (the parent reactive nitrogen species) and its effects on force production, experiments on intact fast-twitch muscle fibers have shown that nitric oxide affects contractile function by altering the myofibrillar Ca<sup>2+</sup> sensitivity [36]. Moreover, a study that used skinned muscle fibers treated with S-nitroso-N-acetylpenicillamine and nitrosoglutathione (two nitric oxide donors) reported decreased Ca<sup>2+</sup> sensitivity in fast-twitch fibers, however maximum force was not affected [37]. In contrast to fast-twitch fibers, the same study showed that the slow-twitch muscle fibers treated with the two nitric oxide donors did not exhibit altered myofibrillar Ca<sup>2+</sup> sensitivity. Other studies have demonstrated that, along with Ca<sup>2+</sup> sensitivity, reactive nitrogen species may indeed

affect muscle force production as well [38] and this may stem from the S-nitrosylation of the myosin heads by peroxynitrite, a highly reactive species produced by the reaction of superoxide radical with nitric oxide [39–41]. Finally, nitrosative modifications, along with other redox reactions, of calcium release-reuptake proteins, such as the ryanodine receptor/ $\text{Ca}^{2+}$  release channel (RyR1), have been also described. These structural alterations also affect muscle contractile function indicating that RONS regulate muscle force production and fatigue development by altering  $\text{Ca}^{2+}$  release [42–44].

In light of the above, several studies sought to investigate the effectiveness of acute antioxidant supplementation as an ergogenic strategy against muscle fatigue induced by RONS. Several nutritional and pharmaceutical compounds with purported antioxidant function (i.e., vitamins C and E, quercetin, resveratrol, coenzyme  $\text{Q}_{10}$ , food-derived polyphenols, spirulina and N-acetylcysteine) have been utilized, typically in several-fold greater doses compared to the recommended dietary allowance guidelines. For most of them, the results are mixed but mostly disappointing [45]. Even among the studies that showed beneficial effects on performance, a certain antioxidant dose, time point of supplementation or exercise type and intensity could not be identified as key factors. N-acetylcysteine, as cysteine donor for the endogenous (re)synthesis of glutathione, seems to be the only antioxidant with promising ergogenic potential after acute supplementation that may offer a performance benefit around competition time [46] (although the antioxidant potential of N-acetylcysteine seems multifaceted from a mechanistic perspective [47,48]). This has been reported in many human exercise studies utilizing whole-body or specific muscle group performance tests, such as repeated bouts of running, electrical stimulation of the tibialis anterior muscle and isometric handgrip maneuvers [49,50,52–54]. Yet, it should be highlighted that in many studies NAC was delivered via infusion and not orally, as was the case for most of the other antioxidants. It is worth mentioning that antioxidant supplementation can also confer beneficial performance effects when reversing a particular antioxidant deficiency (e.g., Vitamin C supplementation only in vitamin C-deficient individuals) [55]. This is also in line with the recent consensus statement of the International Olympic Committee, which states that “supplements aimed at correcting nutrient deficiencies need to be judged on their ability to prevent or treat suboptimal nutrient status, with the benefit accruing from the removal of the associated impairment of health, training capacity or performance” [56].

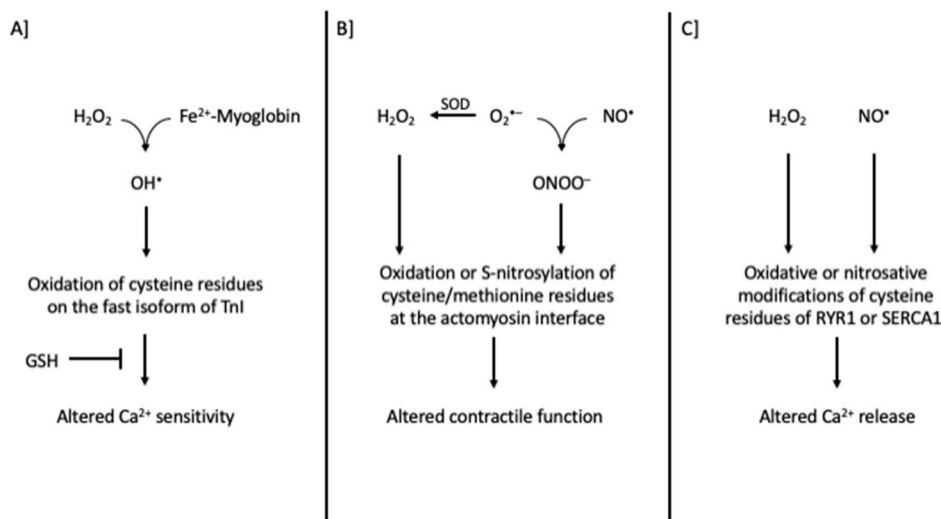
Collectively, RONS-regulation of muscle contractile function has been predominantly verified in fast-twitch muscle fibers rather than in slow-twitch muscle fibers and during submaximal rather than maximal intensities (e.g., during repeated tetanic stimulations). Moreover, RONS-induced alteration in  $\text{Ca}^{2+}$  sensitivity and sarcoplasmic

reticulum  $\text{Ca}^{2+}$  release, rather than the impairment in membrane excitability, seem to be the main cellular processes regulating muscle contractile function; yet, the exact redox mechanisms still remain to be fully elucidated [e.g., oxidation, S-nitrosylation or S-glutathionylation of the skeletal muscle RyR1 and of the calcium binding subunit of troponin [57,58,405] (Fig. 1). For an in-depth analysis of the topic, the reader is referred to reviews about the redox-regulation of muscle contractile function and force production [59,33], the redox-mediated muscle fatigue [60,61] and the effectiveness of antioxidants as ergogenic aids [45,51,62,63].

## 2.2. Glucose uptake

During exercise, skeletal muscle tissue upregulates the utilization of substrates, predominantly carbohydrates and fats depending on the duration and intensity of exercise, to meet the increased energy demands. Diverse signaling transduction pathways have been described that coordinate the complex metabolic process of glucose uptake by skeletal muscle during exercise [64]. Up till now, none of these pathways appears to be the main governor orchestrating the whole process; instead, redundant mechanisms most probably account for the delivery, transport across the muscle membrane and intracellular metabolism of glucose [65]. Among these mechanisms, there is building evidence that reactive oxygen and nitrogen species, either individually or in combination play a key role in skeletal muscle glucose uptake during exercise (the peculiar role of RONS in insulin-mediated glucose uptake has been covered elsewhere [66,409]).

Regarding reactive oxygen species, Sandström and colleagues [68] treated an ex-vivo muscle preparation (i.e., extensor digitorum longus; EDL) with the non-specific antioxidant N-acetylcysteine or Ebselen (a glutathione peroxidase mimetic [67]; and demonstrated that both antioxidant interventions attenuated the increased muscle glucose uptake during contractions (but not resting uptake). Interestingly, this effect was accompanied by a decrease in CM-H2DCF fluorescence (a non-specific indicator of ROS production) pointing towards a redox implication in glucose uptake. In the same study, the EDL muscle from mice that over-expressed superoxide dismutase exhibited greater glucose uptake in response to ex vivo contractions compared to EDL from wild type mice. Given that increased superoxide dismutase activity normally results in increased  $\text{H}_2\text{O}_2$  levels (due to dismutation-reduction of the superoxide radical) and Ebselen mimics glutathione peroxidase activity, it was concluded that  $\text{H}_2\text{O}_2$  is the key oxygen species involved [68]. This idea was also supported by a study which showed that catalase, an enzyme that catalyzes the decomposition of  $\text{H}_2\text{O}_2$  to water and oxygen, but not superoxide dismutase inhibits EDL muscle glucose



**Fig. 1.** Proposed mechanisms on how RONS regulate muscle contractile function. Panel A based on [31,32]; Panel B based on [28,29,39] and [30]; Panel C based on [42–44]. GSH, reduced glutathione;  $\text{H}_2\text{O}_2$ , hydrogen peroxide;  $\text{NO}^\bullet$ , nitric oxide;  $\text{OH}^\bullet$ , hydroxyl radical;  $\text{ONOO}^-$ , peroxynitrite; RyR, ryanodine receptors; SERCA, sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase; SOD, superoxide dismutase; TnI, troponin I.

uptake in response to a hypoxanthine-xanthine oxidase superoxide-generating system in rats [69].

In an effort to acquire a mechanistic perspective, the authors also evaluated AMPK activity and reported hampered activity after N-acetylcysteine treatment, indicating that RONS-regulation of glucose uptake is mediated by AMPK [68]. This scenario seemed to be biologically possible, since  $H_2O_2$  has been shown to selectively activate the  $\alpha 1$  isoform of AMPK via modifications of redox-sensitive residues in particular muscles, such as the epitrochlearis muscle [70,71]. However, Merry and colleagues [72,78–80] conducted a study to specifically examine whether AMPK indeed stimulates glucose uptake in response to exercise-induced reactive oxygen species by using AMPK kinase-dead mice and N-acetylcysteine administration during ex-vivo contractions of EDL and soleus muscles. Their findings did not verify the relationship between reactive oxygen species and AMPK activity in glucose uptake, since N-acetylcysteine hampered glucose uptake similarly in AMPK kinase-dead and wild type mice [72]. This notion was indirectly supported by the findings from Jensen et al. [73], who provided evidence for an essential role of  $\alpha 1$  AMPK in contraction-stimulated EDL glucose uptake in a  $H_2O_2$ -independent manner. However, recent findings reconcile previous discrepancies and fundamentally redefine the role of AMPK activation in exercise, suggesting that AMPK is important for maintaining glucose permeability in skeletal muscle following exercise, but not during exercise in vivo [74–76]. In other mechanistic ex-vivo studies, p38 MAPK, the calmodulin family of kinases (CaMK) and phosphatidylinositol 3-kinase (PI3K) have been also directly or indirectly implicated in the reactive oxygen species regulation of muscle glucose uptake [69,77,410, 411]; yet mechanistic redox studies with a particular focus on exercise energy metabolism and muscle glucose uptake are scarce and, in some cases, data are controversial [78].

In a more physiological context, and in contrast to ex-vivo studies that suggested a role for reactive oxygen species in muscle glucose uptake, some in-vivo and in-situ data did not confirm this viewpoint. In particular, two studies from the same group, who administered N-acetylcysteine in humans during cycle ergometry (at about 60% of  $VO_{2max}$ ) and in rats' hind-limb in-situ during repeated contractions, did not report any effect on muscle glucose uptake [79,80]. These contradictory findings may stem from the fact that in-vivo and in-situ set-ups maintained their circulating and local redox milieu and the contraction and exercise intensities applied were more physiologically relevant compared to the prolonged ex-vivo tetanic stimulations of isolated fibers (eliciting artificially high RONS production) and the supra-physiological doses of exogenously added oxidants (in the mM range) [81]. On the other hand, a very recent in-vivo study by Henríquez-Olguín and colleagues [82], who exploited the advantages of some modern and sophisticated analytical methods with genetically encoded biosensors, demonstrated that NADPH oxidase 2-originated reactive oxygen species play a pivotal role in muscle glucose uptake and GLUT4 translocation during moderate intensity exercise [82]. In addition, intravenous infusion of N-acetylcysteine attenuated thigh net glucose uptake during exercise after, but not prior to, blood flow restricted training in humans, which coincided with an elevated muscle abundance of nNOS and the antioxidant enzymes Cu/Zn-SOD and GPX-1. This finding indicated that the contribution from ROS to muscle glucose regulation in humans may depend on the trained state of skeletal muscle [83]. In sum, the role of reactive oxygen species in muscle glucose uptake remains, at least, elusive.

Regarding reactive nitrogen species, the case of muscle glucose uptake is slightly clearer at least in humans. More specifically, it has been demonstrated that local infusion of the nitric oxide synthase inhibitor NG-monomethyl-L-arginine (L-NMMA) in the femoral artery of human subjects impaired the increase in leg glucose uptake by ~45% during cycling at 60%  $VO_{2max}$  and this adverse effect was reversed by infusion of L-arginine (i.e., a key precursor for nitric oxide production by synthases) [84]. The effect of the L-NMMA administration was exacerbated in diabetic patients (i.e., up to 75% impaired glucose uptake

during exercise [85], which is of utmost importance for this cohort taking into account the different mechanisms between insulin- and exercise-mediated glucose uptake [86]. Moreover, administration of L-arginine in healthy humans significantly increased glucose disposal during exercise as assessed via glucose disappearance rate (stable glucose tracer infusion method was used) and glucose clearance rate [87]. A point worth to mention is that nitric oxide synthase inhibition at this level may affect muscle glucose uptake without altering leg blood flow, blood pressure, or arterial plasma glucose content as indicated by a rat study [88]. In contrast to human studies, rodent studies show contradictory results with NO synthase inhibition yielding positive, negative or neutral effects. Although diverse methodological set-ups had been implemented, such as in-vivo L-NAME ingestion [89,90], in-situ sciatic nerve electrical stimulation followed by ex-vivo exposure to L-NMMA [91] and in-vitro muscle contractions undertaken in the presence of L-NMMA [92,93], the major difference compared to human studies is that, except one [94], all other rodents studies evaluated muscle glucose uptake 20 or more min after the “exercise” stimulus and not during exercise. Thus, methodological practicalities may largely account for the discrepancy between human and rodent studies [95].

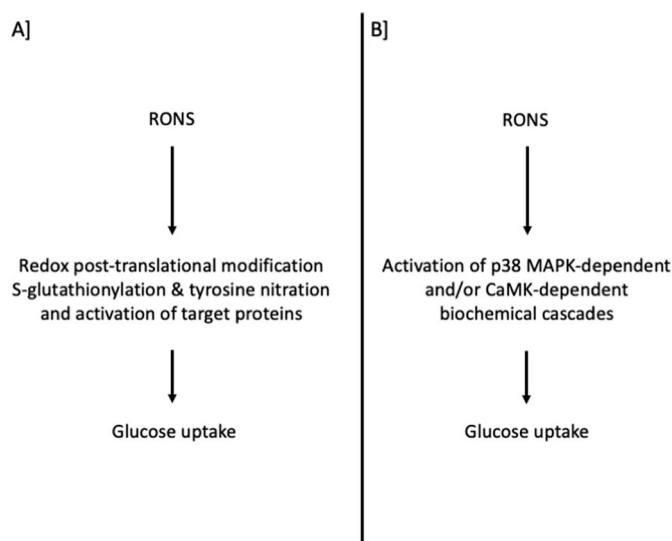
The mechanisms underlying the reactive nitrogen species regulation of muscle glucose uptake during exercise were for a long time based on the combination of two distinct hypotheses. In particular, it has been previously demonstrated that nitric oxide production induced by a nitric oxide donor (i.e., spermine NONOate) triggered glucose uptake in isolated human skeletal muscle and L6 skeletal muscle cells at rest and this was mediated by the typical cyclic guanosine monophosphate (cGMP)-cGMP-dependent protein kinase (PKG) pathway and AMPK [96]. In addition, Lau and colleagues [97] demonstrated that contracting fast-twitch skeletal muscles increase nitric oxide production, which subsequently increases cGMP concentration [97]. By linking the findings from these two conditions (one at resting state and the other during contraction), it was frequently argued that the nitric oxide/cGMP/PKG pathway also regulates muscle glucose uptake during exercise. However, this was not verified in a study by Merry and colleagues [72,78–80] who reported that skeletal muscle glucose uptake during ex vivo contractions takes place via a cGMP/PKG-independent pathway [78]. Other mechanisms that can be proposed for the reactive nitrogen species-mediated insulin signaling and glucose uptake include specific redox post-translational modifications, such as S-nitrosylation, S-glutathionylation and tyrosine nitration of target proteins, such as p21ras and ERK [98–101]. However, and to the best of our knowledge, studies to validate these scenarios in an exercise setting are lacking [78]. Interestingly, Hong and colleagues [103] recently reported that nitric oxide regulates skeletal muscle glucose uptake during ex-vivo contractions of the EDL muscles independently of nNOS $\mu$  (the primary isoform of nitric oxide synthase expressed in skeletal muscle following exercise [102], adding further complexity to the field [103].

Collectively, mounting evidence suggests that RONS production represents one of the multiple and redundant mechanisms that mediate muscle glucose uptake during exercise [64,104] (Fig. 2). However, further work is needed, especially for the reactive oxygen species, to reveal the potential underlying mechanisms involved. We specifically focused on insulin-independent glucose uptake during exercise and not on the enhanced insulin-stimulated glucose uptake following exercise (i.e., insulin sensitivity [105,106]). For an in-depth analysis of the topic, the reader is referred to reviews about the oxidative [81,107] or nitrosative [95,108] regulation of muscle glucose uptake during exercise.

### 2.3. Blood flow

One of the most well-known physiological changes that immediately occur during exercise is the hemodynamic reorganization of blood flow to organs. For instance, blood flow in skeletal muscles may increase up to 100-fold in order to match the oxygen and energy substrate demands





**Fig. 2.** Proposed mechanisms on how RONS regulate tissue glucose uptake. Panel A based on [98,101,108]; Panel B based on [69,77,410]. CaMK,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase; MAPK, mitogen-activated protein kinase; RONS, reactive oxygen and nitrogen species.

of exercise and to remove metabolic by-products [109,110]. Diverse central (e.g., increased cardiac output) and peripheral (i.e., vascular smooth muscle relaxation) mechanisms have been implicated in the regulation of blood flow during exercise, and although none of them seems to be clearly dominating, nitric oxide appears as the central node molecule of most pathways [111]. Optimal exercise performance dictates a physiological-based “hierarchical” redistribution of blood flow in almost all human organs and tissues, such as brain [112], skin [113] and liver [114], however in the present section we will focus on the role of RONS in vascular function and in blood flow to active skeletal muscles.

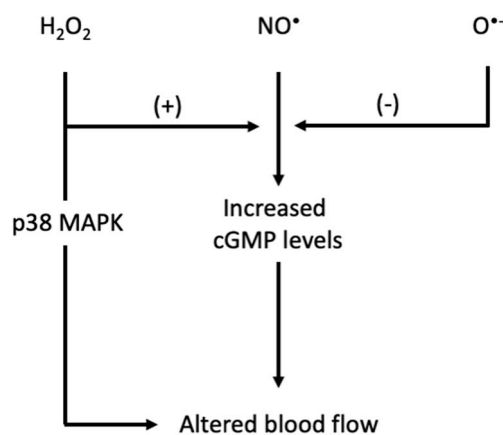
Both the vasculature and the endothelium are tissues that encompass many typical RONS sources, such as NADPH oxidases, xanthine oxidases and nitric oxide synthases [115,116]. The potential role of RONS in vascular function during exercise, along with their interaction (e.g., superoxide reduces the bioavailability of nitric oxide [117], as well as their synergistic or antagonistic effects with other vasoactive molecules (e.g., acetylcholine) remain to a large extent unknown, at least from a mechanistic perspective [118]. As already stated, nitric oxide is regarded a potent inherently vasoactive RONS under diverse physiological conditions including exercise [119,120]. A series of exercise studies performed in humans during the ‘90s demonstrated that nitric oxide positively regulates vascular tone, whilst interesting findings were reported about the time course of this effect, with some studies arguing that nitric oxide is predominantly implicated at rest and during recovery, and not at the initiation or maintenance of active hyperemia [121,122]. Partially contradictory findings were observed regarding the role of nitric oxide in exercise-induced skeletal muscle hyperemia, possibly stemming from the different limbs involved in the original studies (i.e., lower limbs compared to upper limbs) or from the complex redundancy in this process (e.g., simultaneous activity of prostanoids and endothelial derived hyperpolarizing factors) [84,123–127]. In general, these classic exercise physiology studies indicated that nitric oxide is implicated in vascular function during exercise and especially in the peri-exercise period, while its role in muscle blood flow is probably compensatory to other factors.

In addition to nitric oxide, two more recent redox-oriented studies employed a generic antioxidant treatment, consisting of Vitamin C, vitamin E and alpha lipoic acid, and demonstrated that in healthy young males the antioxidant scheme impaired handgrip exercise-induced brachial artery vasodilation (assessed via Doppler ultrasound)

and this was accompanied by a remarkable reduction in RONS production (assessed via electron paramagnetic resonance spectroscopy; EPR) [128,129]. Given that the use of generic antioxidants and the probes of EPR do not always provide insights about the precise reactive species involved in the process, it is reasonable to argue that RONS, other than nitric oxide, may also be implicated. For instance,  $\text{H}_2\text{O}_2$  [130], peroxynitrite [131] and peroxy-radicals [132] have been reported to exert vasodilatory effects under particular, yet non-exercising, conditions. Interestingly, an opposing outcome was observed when the same exercise and antioxidant treatment was applied to aged individuals. In fact, antioxidants restored the impaired vasodilation in this population during an acute exercise bout; however, after a training period the old subjects “mimicked” their young counterparts exhibiting normal resting redox status and impaired exercise-induced vasodilation after antioxidant supplementation [128,133]. In another elegant study, Sindler and colleagues [134] treated soleus muscle resistance arterioles from rats habituated to treadmill running with Tempol (an “antioxidant” redox cycling nitroxide) and/or catalase and reported that both treatments eliminated flow-induced vasodilation indicating a key role of RONS in this process [134]. In light of the above, it seems that under physiological conditions an exercise-induced pro-oxidant environment is necessary for vasodilation, which should not be disturbed with exogenous antioxidants. On the other hand, under pro-oxidative conditions (e.g., ageing or disease) exogenous antioxidants “normalize” resting redox balance facilitating optimal vasodilation both at rest [135] and in response to exercise [133].

Regarding muscle-specific blood flow during exercise, it seems that the regulation of the microvascular tone, coordinated by resistance arteries, arterioles and capillaries, is probably the key biological component and that the tissue blood flow is not a matter of the macrovascular tone (i.e., larger conduit vessels) [136]. This is exemplified by the vasoactive role of RONS (assessed using the spin trap  $\alpha$ -phenyl-tert-butyl nitron and EPR) in a conduit vessel (i.e., brachial artery) during handgrip exercise in humans without a concomitant increase in forearm muscle blood flow [129]. Similarly, hyperemia in the lower limbs of young males during passive knee extension was impaired after L-NMMA infusion (a nitric oxide synthase inhibitor) without a concomitant decrease in femoral artery diameter [137]. Interestingly, the findings by Ref. [128,129] that the antioxidant cocktail, consisting of generic antioxidants, did not exert any significant effect on muscle blood flow questioned the role of reactive oxygen species in this process. On the other hand, some studies have favored the role of  $\text{H}_2\text{O}_2$  in muscle blood flow during exercise. For instance, Copp and colleagues (2009) reported reduced blood flow in young rat spinotrapezius muscle both at rest (–61%) and during contractions (–40%) after acute antioxidant infusion with Tempol [412]. Similar findings were also reported by Hirai and colleagues [138], who reported increased mean spinotrapezius muscle blood flow at rest and during contractions after  $\text{H}_2\text{O}_2$  treatment compared to placebo [138]. Thus, in young individuals, the role of oxygen species in muscle blood flow remains equivocal. Similar to vascular function, studies conducted under pro-oxidative conditions, such as in aged [139] and diseased populations [140] or after exposure to hyperoxia [141], described a beneficial effect of antioxidant treatments (either generic or mitochondria-targeted [142]; in skeletal muscle blood flow. This emphasizes the importance of the resting redox status when evaluating the likely “biphasic” regulatory potential of RONS on skeletal muscle microvascular tone and tissue blood flow [143].

Collectively, most of the available evidence supports a clear role of RONS in vascular function during exercise, while their contribution in skeletal muscle blood flow remains partially obscure due to complex interaction with other local vasoactive molecules (Fig. 3). In addition, it seems that the background redox status (i.e., physiological or pathological) largely affects the magnitude and the biological impact of the exercise-induced RONS production in blood flow. For an in-depth analysis of the topic, the reader is referred to reviews about the



**Fig. 3.** Proposed mechanisms on how RONS regulate blood flow. Based on [117,138,412] and [111]. cGMP, cyclic guanosine monophosphate;  $\text{H}_2\text{O}_2$ , hydrogen peroxide; MAPK, mitogen-activated protein kinase;  $\text{NO}^+$ , nitric oxide;  $\text{O}^-$ , superoxide radical.

regulation of vascular tone and blood flow to skeletal muscle during exercise [114,119] and the role of free radicals in this process [111,144].

#### 2.4. Cell bioenergetics

Redox-regulation of cell bioenergetics (also termed “redox bioenergetics”) represents an intriguing concept and a recently developed area of redox biology [145]. The interplay between cellular metabolic processes and certain delicate redox pathways is increasingly recognized as a fundamental feature of the cell, transducing signals that fine-tune a variety of responses, such as cell proliferation, differentiation and apoptotic procedures [146,147]. The fact that the mitochondrion is a key source, regulator and at the same time target of many RONS, which control both mitochondrial and distinct nucleic and cytosolic signaling pathways as second messengers (e.g., the “retrograde” signaling pathway [148], is a first clear indication regarding the involvement of redox processes in cell energetic metabolism [149]. A second indication is that diverse redox reactions modulate the function of many phosphatases, kinases and transcription factors strongly associated with exercise metabolism and energy production [e.g., glyceraldehyde 3-phosphate dehydrogenase (GAPDH), creatine kinase (CK), pyruvate dehydrogenase kinase-4, phosphatase and tensin homolog (PTEN) and nuclear factor erythroid 2-related factor 2 (Nrf2)] [66,150–153]. Given that the studies in redox bioenergetics specifically focused on physical activity are scarce [154], selected mechanistic information is derived from other scientific fields.

It is nowadays well-established that mitochondria, along with their fundamental duty to generate ATP, are also critically involved in redox-sensitive cellular pathways either by directly producing RONS or by regulating  $\text{NAD}^+/\text{NADH}$  and  $\text{NADP}^+/\text{NADPH}$  homeostasis [155,156]. Although our knowledge about the mitochondrial topology and contribution of the various sites of superoxide and hydrogen peroxide production have rapidly expanded [157], other cellular RONS sources are also considered major contributors (i.e., NADPH oxidases, nitric oxide synthases, xanthine oxidases and cytochrome P450). Especially regarding exercise, a study by Sakellariou and colleagues [158], [196] examined the mitochondrial and cytosolic sites that contribute to superoxide production in mature single muscle fibers at rest and during contractile activity and reported that NADPH oxidase is the major contributor under both conditions [158]. In another elegant study, Frasier and colleagues [159] demonstrated that NADPH oxidase-originated RONS mediate the cardioprotective effects of exercise (i.e., isolated hearts from trained rats were exposed to an ischemia/reperfusion challenge). In fact, this beneficial effect was abolished in the presence

of apocynin or Vas2870 (i.e., NADPH oxidase inhibitors), whereas mitoTEMPO or Bendavia (i.e., mitochondria-targeted antioxidants) did not exert any significant impact [159]. A potential mechanism that has been previously proposed about the cardioprotective effects of NADPH oxidase-originated RONS during exercise includes the S-glutathionylation of the ryanodine receptor-2 (RyR2), which leads to increased calcium release rates in isolated sarcoplasmic reticulum vesicles [160]. In addition, Pearson and colleagues [161] have shown that cytosolic superoxide levels increased in response to muscle contractions, but this was not accompanied by a similar change in mitochondrial superoxide availability and that inhibition of nitric oxide synthases influenced mitochondrial superoxide availability but not cytosolic [161].

Two more recent *in vivo* studies provided additional evidence to support this notion. In particular, one study demonstrated that apocynin injected intraperitoneally for 3 days in mice impaired early muscle gene expression (i.e., p38 MAP kinase, ERK1/2, and NF $\kappa$ B signaling pathways) induced by a single swimming exercise bout [162]. The second study reported that three weeks of MitoQ supplementation in humans (i.e., the antioxidant coenzyme Q10 is conjugated to the lipophilic cation triphenyl phosphonium, which accumulates selectively in mitochondria driven by the plasma membrane potential [163]; did not affect blood and skeletal muscle adaptations induced by endurance exercise training in healthy men and did not affect performance as assessed via a  $\text{VO}_2\text{max}$  test [164]. Nevertheless, allopurinol administration (a xanthine oxidase inhibitor), akin to NADPH oxidase inhibitors, adversely affected cell signaling pathways in gastrocnemius muscle from rats submitted to exhaustive exercise resulting in suppressed MnSOD mRNA increases [165]. The latter finding stresses the need to reveal the potential mechanisms underlying the crosstalk between subcellular compartments and organelles generating RONS [155].

The mitochondrial RONS production at rest and during exercise has been greatly revised throughout the years. In particular, it was for a long time reported that at rest 1–3% of electron flow during mitochondrial respiration produces reactive species. However, these values most probably overestimated the free radical leak in mitochondria, with studies that implemented more advanced techniques and physiologically relevant substrate concentrations reporting significantly lower values as much as 0.15% of the electron flow [166,167]. With respect to exercise, the increased mitochondrial respiration during physical activity was also incorrectly translated into increased electron leakage and reactive species production. In fact, Gonçalves and colleagues [154] investigated the superoxide and hydrogen peroxide production by isolated muscle mitochondria *ex vivo* under conditions mimicking rest and exercise (i.e., incubated in media containing the cytoplasmic substrate and effector mix of skeletal muscle during rest and exercise) and demonstrated a steep decline in total mitochondrial superoxide/ $\text{H}_2\text{O}_2$  production during “exercise” conditions [154]. Interestingly, the more increased the mitochondrial respiration rate between “rest”, “mild exercise” and “intense exercise” conditions the lower the superoxide/ $\text{H}_2\text{O}_2$  production, which imitates the rate of state 3 respiration. This was further supported recently by an *in vivo* study that reported lower mitochondrial  $\text{H}_2\text{O}_2$  production during exercise [82]. In another study by Quinlan and colleagues [168] it was demonstrated that the contribution of each mitochondrial site to the total RONS production in rat skeletal muscle is largely dependent on the available oxidizable substrates (i.e., succinate, glycerol 3-phosphate, palmitoyl-carnitine plus carnitine and glutamate plus malate) [168]. This is of utmost importance taking into account that the exercise- and redox-sensitive Nrf2 impacts cellular bioenergetics by controlling the substrate availability for mitochondrial respiration [151] and provides a redox perspective on the health-promoting effects of regular exercise and calorie restriction (nicely summarized by Ref. [169]. However, whether the improved cellular redox status after these situations is exclusively due to a decreased mitochondrial superoxide/ $\text{H}_2\text{O}_2$  production or also mediated by the more reduced glutathione pool remains a matter of debate [170]. For instance, it was shown that both pharmacological (i.e., treatment of

permeabilized myofibers with the glutathione-depleting agent 1-chloro-2,4-dinitrobenzene) and physiological oxidation of the cellular glutathione pool (i.e., high fat feeding in rodents) elevates pyruvate dehydrogenase-induced  $\text{H}_2\text{O}_2$  emission [171].

The  $\text{NAD}^+/\text{NADH}$  and  $\text{NADP}^+/\text{NADPH}$  couples orchestrate many catabolic and anabolic processes, respectively, to conserve cell survival at the interface between energy metabolism and redox signaling. Of note, these molecules synchronize complex cellular pathways, such as the energy-consuming redox circuit between the pyruvate dehydrogenase complex and nicotinamide nucleotide transhydrogenase, to defend homeostasis during periods of positive and negative energy balance [172]. From a redox standpoint, NADPH serves as substrate for NADPH oxidases generating RONS, but at the same time it represents the most important reductive substrate for major antioxidant enzymes, such as peroxiredoxins and glutathione peroxidases [173,174]. The dominant role of NADPH in maintaining redox homeostasis is best exemplified by studies that reported a dynamic rerouting of the carbohydrate flux and acute activation (within seconds [175]) of the pentose phosphate pathway (PPP) as a first-line (i.e., transcription-independent) metabolic response to oxidative insults [176–179]. Several mechanisms have been proposed regarding how this immediate PPP activation is achieved and include the highly conserved  $\text{H}_2\text{O}_2$ -induced reversible inactivation of GAPDH (via mechanisms distinct from those relating to the reaction between its active site cysteine and the glycolytic substrate [180]; and the enhancement of G6PDH activity [181,182]. Based on the above, exogenous supplementation with NADPH precursors, such as nicotinamide riboside which is currently regarded the most efficient molecule [183], has been utilized in conditions with increased underlying oxidative stress (e.g., disease and ageing) and have yielded promising results [184–186]. However, when similar treatments were used in young male humans or rats either neutral [185] or negative outcomes [187] were observed, respectively, in exercise performance.

Collectively, a strong interplay exists between cellular energetic processes and redox reactions, since energy metabolism is both a source and target of RONS (Fig. 4). In fact, reactions of catabolic pathways generate RONS, which subsequently regulate (structurally or functionally) selective target-molecules of metabolic cascades, while the antioxidant mechanisms coordinate this process. Considering that redox reactions have been linked, at least in part, to some central metabolic molecules strongly associated with exercise metabolism, such as AMPK [188], TCA enzymes [189], succinate dehydrogenase [190], ATP synthase [191], creatine kinase [192], cytochrome c oxidase [193], SIRT3 [194], G6PD [195] and GAPDH [180], it becomes evident that the field of redox bioenergetics is one of the most attractive, yet unexplored, areas of exercise physiology. For an in-depth analysis of the topic, the reader is referred to reviews about the spatiotemporal production of RONS at rest and in response to exercise [157,196], the role of RONS as cellular messengers regulating kinases and phosphatases [150] and the emerging field of redox bioenergetics [145].

### 3. Redox-regulated exercise adaptations

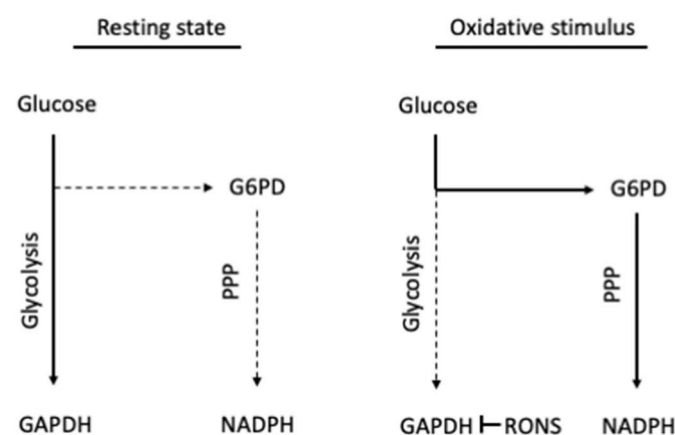
#### 3.1. Mitochondrial biogenesis

If we accept muscle contractile function as the archetypal example of redox-regulated exercise responses, mitochondrial biogenesis represents the respective archetypal example in the category of redox-regulated exercise adaptations [197]. Endurance exercise training increases skeletal muscle mitochondrial number and volume density leading to improved oxidative capacity [198]. Since cells do not produce mitochondria *de novo*, mitochondrial content is determined by the dynamic balance between the synthesis and degradation (also known as mitophagy) of these organelles. In particular, already existing highly functional mitochondria become segregated from the defective ones, which are disposed, and are subsequently set for proliferation [199].

This highly complex process is accomplished by a coordinated bigenomic network. In fact, proteomic analyses revealed that almost 1500 proteins are present in mitochondria and the vast majority of them are encoded inside nucleus and not within the mitochondrion (i.e., only 13 polypeptides are encoded by mtDNA and synthesized there) [200–202].

The key factors that orchestrate the communication between the mitochondrial and the nuclear genome are the mitochondrial transcription factor A (mtTFA) and the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2). However, almost all upstream signals that stimulate muscle contraction-induced mitochondrial biogenesis (e.g., AMPK, CaMK, mitogen-activated protein kinases p38 and p53) converge to the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), which is therefore regarded the major governor of the specialized regulatory proteins involved in mitochondrial biogenesis [203,403]. Several studies in rodent (mainly) and human skeletal muscles have underlined the key role of PGC-1 $\alpha$  in exercise-induced mitochondrial biogenesis. It has been demonstrated that an acute exercise bout can impact PGC-1 $\alpha$  at both transcriptional and post-translational level regulating its expression and activity, respectively [204–209]. In addition, overexpression of PGC-1 $\alpha$  was found to significantly enhance the oxidative phenotype of muscle fibers, as indicated by increases in the activity and content of many enzymes involved in oxidative metabolism, such as cytochrome oxidases and citrate synthase [210]. Our knowledge about how redox processes are implicated in exercise-induced mitochondrial biogenesis comes from studies that utilized redox treatments along with an exercise protocol and measured markers of mitochondrial biogenesis (e.g., PGC-1 $\alpha$  mRNA and COX4 content) as well as from mechanistic studies that investigated the role of RONS in the regulation of the upstream molecules, such as AMPK.

Gomez-Cabrera and colleagues [211] were the first to report that daily vitamin C administration in rats blunted the exercise-induced expression of key transcription factors involved in mitochondrial biogenesis (i.e., PGC-1 $\alpha$ , mtTFA and NRF-1), while it also prevented the exercise-induced expression of cytochrome c [211]. One year later, another study reported that an antioxidant scheme consisting of vitamin C and E impaired mRNA responses in several markers of mitochondrial biogenesis, including PGC-1 $\alpha$ , in healthy young men subjected to a training intervention [212]. The adverse effect of antioxidant supplementation on cellular exercise-induced adaptations



**Fig. 4.** A representative example of the interplay between cellular energy metabolism and redox homeostasis. At rest, glucose is directed mainly towards glycolysis, whereas under increased oxidative stress conditions, ROS inactivate GAPDH, as a first-line metabolic response, and carbohydrate flux is dynamically rerouted to the pentose phosphate pathway to produce NADPH (the major reductant of key antioxidant enzymes). Based on [177–179]. G6PD, glucose-6-phosphate dehydrogenase; GAPDH, reduced glyceraldehyde 3-phosphate dehydrogenase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PPP, pentose phosphate pathway; RONS, reactive oxygen and nitrogen species.

was further supported by the findings of Strobel and colleagues [213] who reported that an antioxidant scheme consisting of vitamin E and  $\alpha$ -lipoic acid negatively affected several markers of skeletal muscle mitochondrial biogenesis in rats after treadmill training (i.e., PGC-1 $\alpha$  mRNA and protein concentration, COX4 protein content and citrate synthase activity) [213]. In the same line are generally the findings from 5 other studies performed in rats [214,215], mice [216] and humans [217,218], which used generic antioxidants in combination or separately, and reported blunted increases in the mRNA levels and protein content of some (but not all) factors related to mitochondrial biogenesis (i.e., COX4, PGC-1 $\alpha$ , mtTFA, NRF-1 and NRF-2). Finally, two studies on the topic followed alternative approaches. The first was conducted by Strobel and colleagues (2014), who treated rats with diethyl maleate in order to deplete skeletal muscle glutathione, and showed that exercise induced greater oxidative stress and augmented muscle PGC-1 $\alpha$  gene expression [219]. In the second study, the authors used allopurinol along with exercise to selectively inhibit xanthine oxidase and found lower PGC-1 $\alpha$ , NRF-1, and mtTFA levels compared to the exercise only group [220].

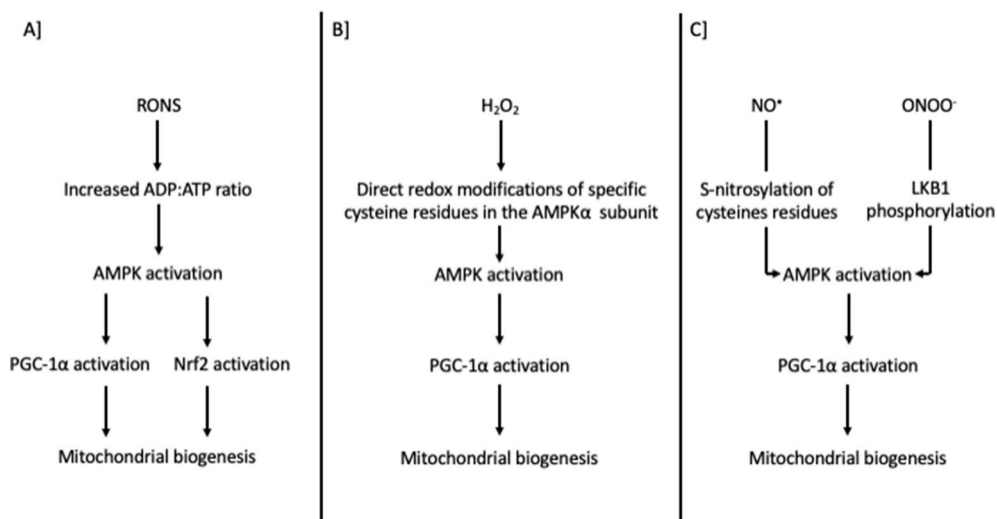
Certainly, opposing findings also exist. Two studies that used antioxidant schemes consisting of vitamin C and E did not report adverse effects on mitochondrial protein content in rat triceps muscle [221] or on the activity of citrate synthase in human vastus lateralis [222]. Likewise, Petersen and colleagues [223] reported that N-acetylcysteine infusion in humans impaired many early adaptive responses to exercise, however, PGC-1 $\alpha$  was not included in the hindered factors [223]. Interestingly, two other studies, which utilized generic [216] or targeted antioxidants [224], demonstrated that although some acute exercise-induced changes in mitochondrial markers were disturbed (e.g., mtTFA mRNA levels), training-induced adaptations were not affected in terms of mitochondrial biogenesis (e.g., normal mitochondrial mass). Hence, it should be cautioned that mitochondrial biogenesis signaling (i.e., gene expression following an acute bout of exercise) is not always linked to mitochondrial biogenesis per se (i.e., changes in protein/enzyme content after exercise training). Even more important is the fact that, in some cases, the molecular and biochemical impairments observed after antioxidant supplementation did not translate into worse performance. For instance Ref. [211], reported impaired increases in endurance capacity in rats after the supplementation period, but this was not statistically verified in humans despite the large difference in VO<sub>2</sub>max improvement between groups after the training protocol (i.e., 22 vs. 10.8%) [211]. Similarly, the hampered cellular adaptations observed after vitamin C and E supplementation by Paulsen and colleagues [218] were not projected in VO<sub>2</sub>max and running performance. This issue emphasizes the need to combine molecular and biochemical

data with physiological and/or performance outcomes in order to support the translational potential of cellular findings [10,225].

Regarding reactive nitrogen species, it is nowadays beyond doubt that nitric oxide promotes mitochondrial biogenesis at rest via canonical (i.e., cGMP) and non-canonical (i.e., S-glutathionylation) pathways [226,227]. However, the data about the role of nitric oxide in exercise-induced mitochondrial biogenesis are scarce and, thus, this scenario remains far less clear [228]. In fact, few studies that used either the nitric oxide synthase inhibitor L-NAME [229,230] or recruited mice deficient in endothelial or neuronal nitric oxide synthase (eNOS<sup>-/-</sup> and nNOS<sup>-/-</sup>) have yielded both positive and negative results in mitochondrial biogenesis markers after exercise, with some of them (i.e., mtTFA) being affected only in particular muscles. As a result, the contribution of nitric oxide in mitochondrial biogenesis in response to exercise remains, at least, equivocal. Another interesting case regarding mitochondrial biogenesis is polyphenols and especially resveratrol supplementation [231]. Many studies that used resveratrol along with exercise (nicely summarized in Ref. [58]) have yielded promising results; however, whether the effects are mediated by redox mechanisms cannot be safely argued due to the multiple and pleiotropic biological properties of resveratrol (e.g., AMPK activator and Sirt1 agonist) and its poor bioavailability and, consequently, low antioxidant potential of this polyphenol in humans [232,233].

From a mechanistic point of view, PGC-1 $\alpha$  regulation at rest and during exercise depends on several (most probably redundant) factors. There is nowadays considerable evidence that AMPK, Nrf2 and p38 serve as the principal intermediate molecules connecting RONS with PGC-1 $\alpha$  activation and/or mitochondrial biogenesis markers [165,220,224,234–237]. Regarding AMPK, Choi and colleagues [238] demonstrated that AMPK becomes activated under oxidative stress conditions induced by H<sub>2</sub>O<sub>2</sub> and this is mediated by energy disturbance (AMP:ATP ratio) [238]. In the same vein, another study that used AMP-insensitive cell lines demonstrated that AMPK signaling was abolished under oxidative stress conditions, pointing towards an energy-mediated redox regulation of AMPK [239]. Likewise, a study that also used AMP-insensitive mutant cultured cells showed that changes in adenine nucleotides content, rather than a direct oxidative modification, is the major driver of AMPK activation during oxidative stress [240].

On the other hand, some studies have shown that reactive oxygen species and especially H<sub>2</sub>O<sub>2</sub> may also promote AMPK activation via direct redox modifications (i.e., oxidation and S-glutathionylation) of specific cysteine residues (i.e., 299 and 304) independent of energy imbalances [241–243]. Interestingly, Shao and colleagues [244] have shown that oxidation of other cysteine residues (i.e., 130 and 174) in the AMPK $\alpha$  subunit leads to disulphide bonds formation and



**Fig. 5.** Proposed mechanisms on how RONS regulate mitochondrial biogenesis. Panel A based on [188,238–240]; Panel B based on [241–243]; Panel C based on [245,247]. AMPK, 5' AMP-activated protein kinase; cGMP, cyclic guanosine monophosphate; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; LKB1, liver kinase B1; NO<sup>•</sup>, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; ONOO<sup>-</sup>, peroxynitrite; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$ ; RONS, reactive oxygen and nitrogen species.



aggregation of AMPK [244]. Thus, oxygen species may both directly activate or deactivate AMPK by modifying redox-sensitive residues at the  $\alpha$  subunit. Overall, the predominant idea suggests that energy imbalance derived from pro-oxidant conditions is the central means of redox-mediated AMPK activation compared to the possible direct oxidative modifications of target cysteine residues at AMPK subunits [70,245]. Reactive nitrogen species have been also implicated in the regulation of AMPK. Similar to oxygen species, the most common pathway that has been described is the modulation of the ATP production in mitochondria via inhibition of the cytochrome c oxidase [406]. In addition, nitric oxide is known to activate soluble guanylyl cyclase, which leads to intracellular  $\text{Ca}^{2+}$  release and CaMKKb-mediated activation of AMPK [246]. Direct regulatory nitrosative modifications of AMPK have been also described, such as the S-nitrosylation of target cysteines (i.e., 299 and 304) and nitration of tyrosine residues, as well as the peroxynitrite-mediated activation of AMPK via LKB1 phosphorylation [245,247].

p38 MAPK and particularly the p38 $\gamma$  isoform mediates PGC-1 $\alpha$  phosphorylation and translocation to nucleus to trigger mitochondrial biogenesis [220,248,249]. It is well-established that diverse redox processes, especially in response to stress stimuli, activate p38 which subsequently facilitates cell signaling events [250,251]. Redox activation of p38 takes place indirectly through activation of upstream redox-sensitive kinases (e.g., ASK1 [252]); or inactivation of redox-sensitive inhibitory phosphatases (JNK-inactivating phosphatases; [253]). However, data from Galli and colleagues [254] provided supporting evidence for dose-dependent direct activation of p38 by the oxidation of the Cys162 within the docking domain of p38, affecting thereby its interaction with other upstream MAPKs [254]. Finally, regarding Nrf2 it has been suggested that both  $\text{H}_2\text{O}_2$  and NO-induced increases in mitochondrial biogenesis markers are dependent on Nrf2 and NRF-1, yet only NO-mediated mitochondrial biogenesis gene expression requires PGC1 $\alpha$  [236]. However, since most of the available mechanistic data presented above come from studies outside the exercise field, it is still an active debate whether these mechanisms also orchestrate exercise-induced AMPK-, Nrf2 and p38-mediated activation of PGC-1 $\alpha$  and mitochondrial biogenesis.

Collectively, mounting evidence supports the key role of RONS in mitochondrial biogenesis at rest and in response to exercise (Fig. 5). More specifically, the majority of exercise studies that used antioxidants or RONS inhibitors substantiate the role of reactive species in mitochondrial biogenesis, however, the mechanistic basis (especially for reactive nitrogen species) warrants further investigation. For an in-depth analysis of the topic, the reader is referred to reviews about the redox-regulation of exercise-induced mitochondrial biogenesis [197] and the role of RONS in the activity of upstream molecules, such as PGC-1 $\alpha$ , AMPK and Nrf2 [70,245,255].

### 3.2. Skeletal muscle hypertrophy

Skeletal muscle hypertrophy, as the phenotypic result of net increase in protein synthesis relative to protein degradation, is the most common adaptation observed in response to resistance exercise training. Diverse mechanical (e.g., loading) and chemical (e.g.,  $\text{Ca}^{2+}$  influx) processes have been identified as signals that trigger anabolic cellular transduction pathways leading to muscle hypertrophy, including the insulin/IGF-1-PI3K pathway, mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase (p70<sup>S6k</sup>), eukaryotic translation initiation factor 4E (eIF4E) and ERK1/2 [256,257]. Although the vast majority of redox exercise studies have focused on endurance training adaptations (e.g., mitochondrial biogenesis; [197]), increasing evidence now suggests that redox processes may also regulate complex resistance training-mediated adaptations and we will present the existing literature regarding the role of reactive oxygen and nitrogen species in muscle hypertrophy.

The findings from 5 studies that used generic antioxidants (i.e.,

vitamin C only or in combination with vitamin E) along with a muscle hypertrophic stimulus yielded mixed results. In particular, Makanae and colleagues [258] demonstrated that the antioxidant treatment impaired overload-induced (i.e., gastrocnemius and soleus ablation) skeletal muscle hypertrophy in the plantaris muscle of rats [258]. This effect was partially supported by the data regarding the phosphorylated form of ERK1/2 and p70<sup>S6k</sup> as well as by the expression of atrogin-1. That is, although antioxidant group was not different compared to placebo, it was also not different compared to the sham group, indicating a tendency for blunted anabolic hypertrophy signaling and potent catabolic signaling. Another study showed that in healthy young men and women, subjected to a heavy-load resistance exercise protocol, antioxidant supplementation did not impair lean body mass gains (via DXA), cross-sectional areas of several muscle groups (via ultrasound imaging) or the acute changes in protein synthesis in the vastus lateralis muscle. However, the increased phosphorylation of ERK1/2 and p70<sup>S6k</sup> induced by training was blunted by the antioxidant scheme [259]. A recent human study by Dutra and colleagues [260] demonstrated that antioxidant supplementation did not affect quadriceps muscle thickness assessed via ultrasound after a strength training program. However, performance measurements (i.e., peak torque and total work assessed via isokinetic dynamometer) were adversely affected by supplementation [260]. The rest two studies were conducted in aged populations and reported contradictory findings as well. In particular, Bjørnsen and colleagues [261] reported greater total lean mass gains and rectus femoris thickness after a strength training program in the placebo group compared to the antioxidant group (although other muscles groups exhibited similar increases) [261]. On the other hand, Bobeuf and colleagues [262] reported no difference in fat free mass after a resistance training protocol between placebo and antioxidant groups of older individuals [262].

Handayaningsih and colleagues [263] investigated the role of RONS in IGF-1 signaling in C2C12 myocytes and used both generic (i.e., N-acetylcysteine) and targeted antioxidants (i.e., Tempol and Tiron as superoxide dismutase mimetics). The authors reported that both endogenous and exogenous  $\text{H}_2\text{O}_2$  increased phosphorylation of the IGF-I receptor and subsequently up-regulated the downstream signaling pathway (i.e., Akt-mTOR-p70<sup>S6k</sup>). However, this effect was abolished when cells were treated with all antioxidants. These signaling warnings were further supported by the impaired increase in myotube diameter after N-acetylcysteine treatment [263]. Two rat studies aimed to investigate the role of nitric oxide in muscle hypertrophy and used targeted antioxidant treatments [i.e., L-NAME and 1-(2-trifluoromethyl-phenyl)-imidazole (TRIM)] followed by synergistic ablation (i.e., removal of gastrocnemius and soleus) to induce hypertrophy in the plantaris muscle unilaterally. The first study demonstrated that L-NAME treatment partially impaired the hypertrophic adaptation induced by synergistic ablation; however, the effect was present only in specific muscle fiber types (i.e., Type IIA) [264]. The second study reported up-regulation of skeletal  $\alpha$ -actin and type I Myosin Heavy Chain mRNA after ablation; however, this effect was abolished after L-NAME and TRIM treatment. On the other, antioxidant treatments did not affect induction of the growth factors VEGF and IGF-1 as well as the expression of total p70<sup>S6k</sup> (instead the ratio of phosphorylated to total p70<sup>S6k</sup> was significantly higher in the antioxidant groups) [265].

Zhang and colleagues [266] recruited a skeletal muscle-specific Sod1-knockout mouse model to scrutinize the role of RONS in muscle mass and contractile force loss during aging. The knockout mice did not exhibit evidence of muscle fiber loss, instead they partially showed increased muscle mass compared to wild-type mice (however gastrocnemius muscle isometric force was compromised). In addition, Akt-mTOR signaling was not disturbed in knockout mice, which suggested enhanced regenerative pathways after CuZnSOD loss [266]. The most comprehensive original study about the role of reactive nitrogen species on muscle hypertrophy was performed by Ito and colleagues [267] who conducted a series of experiments including synergistic ablation in nNOS

null (nNOS<sup>-/-</sup>) mice, nitric oxide synthase inhibitors and peroxynitrite selective scavengers. Ablation increased plantaris muscle weight, fiber size and total protein contents, whereas no such substantial increases were observed in the nNOS-null mice. Administration of L-NAME prevented the increase in muscle weight and fiber size, whereas its inactive enantiomer did not affect hypertrophy indices, indicating a role of nitric oxide in this process especially during the first day after ablation. Interestingly, the canonical nitric oxide-activated soluble guanylate cyclase and cyclic GMP pathway was found not to be involved in this process. Additional measurements using the specific peroxynitrite catalyst and scavenger FeTPPS, the nitric oxide and peroxynitrite donors molsidomine or SIN-1 and the NOX4-specific inhibitor GKT136901 were performed. Collectively, the authors suggested that peroxynitrite was the reactive nitrogen species that promoted normal hypertrophic responses in response to ablation in the nNOS null mice. These findings were also supported by signaling data; that is, activated mTOR signaling and increased phosphorylation of p70<sup>S6k</sup> were found after synergistic ablation, however this effect was absent in nNOS null mice and those treated with peroxynitrite scavengers. Finally, the authors proposed that peroxynitrite regulated muscle hypertrophy through the transient receptor potential cation channel V1-mediated mTOR activation and not through the insulin/IGF-1-P13K pathway [267].

Collectively, despite the fact that the available literature is limited and to some extent controversial [258–260], RONS are increasingly recognized as factors regulating muscle hypertrophic adaptation in response to resistance training (Fig. 6). Studies that implemented targeted redox interventions [265] or multifaceted methodological approaches [267] have shed light on the potential underlying redox mechanisms. Certainly, many aspects still remain unanswered. For instance, although it is clear that skeletal muscle regeneration is controlled by redox processes at several different steps of the process [268], two studies that evaluated overload-induced activation of satellite cells did not report differences between wild-type and nNOS-null mice [267] or after L-NAME treatment [265]. Thus, further work is needed for this type of exercise training and the corresponding adaptations from a redox perspective. For an in-depth analysis of the topic, the reader is referred to a very recent systematic review and meta-analysis about the effect of antioxidants on muscle hypertrophy after strength training [269].

### 3.3. Angiogenesis

Angiogenesis is the physiological process of the formation of new blood vessels from the pre-existing vasculature. The two types of angiogenesis that have been identified during postnatal development are sprouting and intussusceptive (or splitting) angiogenesis. Under conditions of increased demands, such as during exercise, any blood, nutrient or metabolite demand-to-supply mismatch triggers a highly dynamic adaptive response that results in an extended vascular network in the tissue under stress (e.g., skeletal and cardiac muscle). Although several kinds of vascular growth or remodeling have been described, and the term ‘neovascularization’ is frequently used as a collective term, angiogenesis in the context of exercise is most commonly used to describe the formation of new capillaries [270]. The advantages stemming from the increased muscle capillarity are multiple and can be summarized as follows: i) the surface area for diffusion is augmented, ii) the average diffusion path length within the muscle shortens and iii) the length of time for diffusive exchange between blood and tissue increases [408].

Exercise as a potent physiological stimulus integrates a multiset of key angiogenic mechanisms that initiate the enrichment of working skeletal muscle capillarity, such as mechanical forces (e.g., vascular wall tension, shear stress and strain), tissue hypoxia and cycling hypoxia-reoxygenation phenomena as well as humoral biochemical factors and accumulation of metabolic by-products [271–273]. The central factor that largely orchestrates the process of angiogenesis is the vascular endothelial growth factor (VEGF), while a key role for

angiopoietins and platelet-derived growth factor-B has been also reported [274]. Several human and animal studies have demonstrated that exercise elicits both acute (i.e., immediately after and up to several hours post-exercise) and chronic increases in the mRNA levels and activity of VEGF and its receptor tyrosine kinases (VEGFR1 and VEGFR2) [275,276], [277], [408]. The downstream molecules of VEGF/VEGFR pathway that control the process of angiogenesis include a wide variety of redox-sensitive kinases, phosphatases, cytokines and transcription factors, such as c-Src, ERK1/2, JNK, NFkB, PTP1B, PTEN, p38 MAP kinase, protein kinase C and PI3K-Akt [278–281]. In fact, the direct (e.g., oxidative activation of transcription factors) or indirect (e.g., modulation of intermediary regulatory molecules) implication of redox processes in angiogenesis is delicately co-regulated by RONS production and scavenging enzymes or non-enzymatic antioxidants [282–284]. In the next paragraphs we will try to synopsise the evidence suggesting a role for redox molecules in the angiogenic process and to recontextualize current knowledge in an exercise setting, focusing on VEGF. Aberrant angiogenesis, namely excessive or insufficient, which is linked to many pathologies (e.g., cancer and hypertension), has been also associated with RONS production [285,286]. However, such conditions are typically characterized by chronic and uncontrollable overproduction of RONS, denoting a different pattern compared to exercise which is of transient nature.

Although angiogenesis is a complex process necessitating the activity of multiple cell types, including endothelial, smooth muscle and blood cells, it predominantly depends on the recruitment, proliferation and differentiation of endothelial cells. The two principal endogenous RONS sources in endothelial cells are the mitochondrial electron transport chain and the NADPH oxidases, with some particular NOX isoforms (i.e., 1, 2 and 4) being strongly associated with the angiogenic process [287]. Redox processes regulate VEGF fate and metabolism at both transcriptional and signaling level acting as second messengers. In particular, RONS and especially superoxide, H<sub>2</sub>O<sub>2</sub> and nitric oxide produced endogenously or added exogenously in low amounts in cell cultures, have been found to directly up-regulate VEGF expression in diverse cell types [288–290], [291–293], [410, 411]. Thus, RONS with signaling properties and adequate diffusion distances, such as H<sub>2</sub>O<sub>2</sub>, produced during exercise even from extracellular sources (e.g., blood cells) could theoretically induce VEGF expression in endothelial cells and trigger angiogenesis [280]. Although RONS seem to serve mainly as upstream VEGF inducers, intracellular VEGF-related angiogenesis

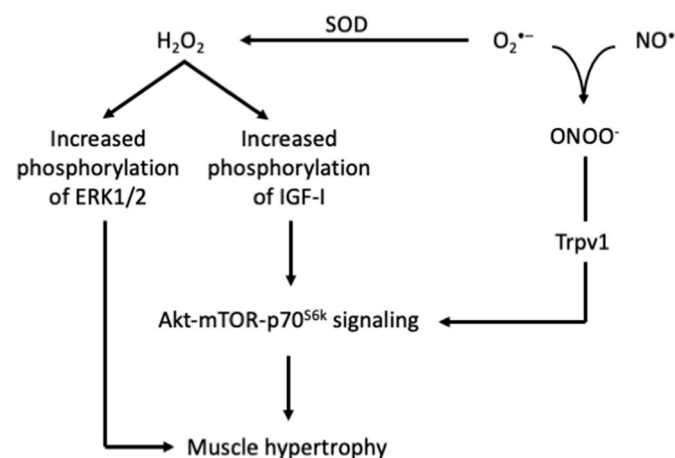


Fig. 6. Proposed mechanisms on how RONS regulate muscle hypertrophy. Based on [258,263,267] and [259]. Akt, protein kinase B; ERK, extracellular signal-regulated kinase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; IGF-I, insulin-like growth factor 1; mTOR, mammalian target of rapamycin; NO<sup>•</sup>, nitric oxide; O<sup>•-</sup>, superoxide radical; ONOO<sup>-</sup>, peroxynitrite; p70<sup>S6k</sup>, ribosomal protein S6 kinase beta-1; SOD, superoxide dismutase; Trpv1, transient receptor potential cation channel subfamily V member 1.

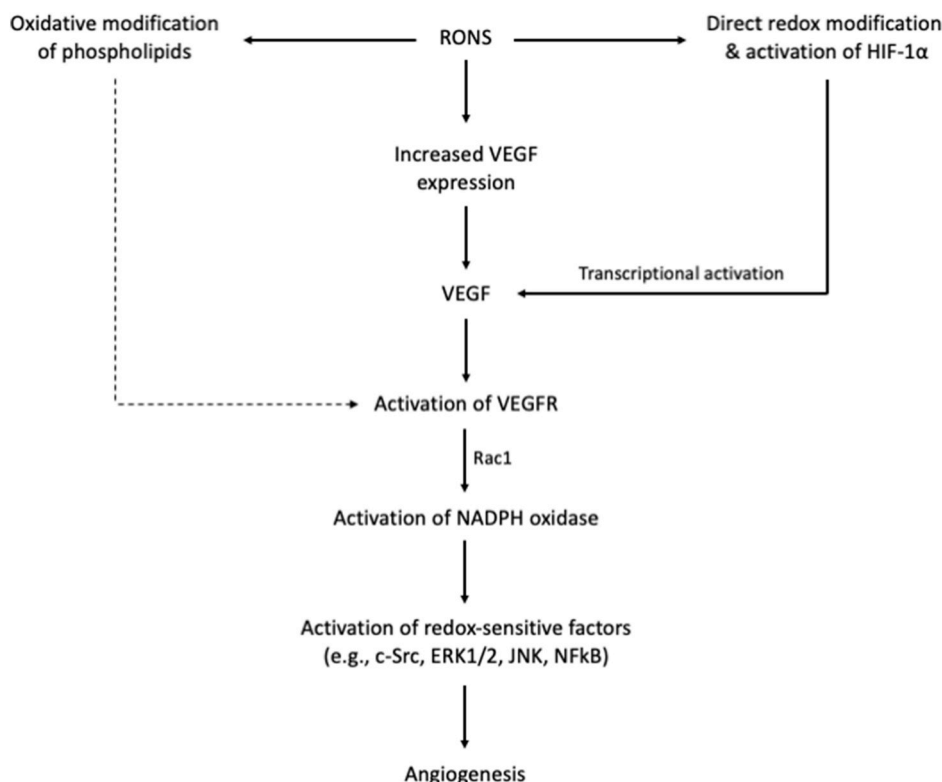
pathways also involve RONS production and redox signaling [294–296]. In particular, VEGF binding to VEGFR2 activates and translocates the small GTPase Rac1 to the plasma membrane, which stimulates RONS production by NADPH oxidase in endothelial cells. RONS subsequently oxidatively inactivate protein tyrosine phosphatases enhancing thereby VEGFR2 autophosphorylation and redox signaling linked to endothelial cell proliferation and migration [295]. Taking into account that exercise increases VEGF content, it seems reasonable to speculate that RONS originated from the VEGF-stimulated NADPH oxidases are involved in exercise-induced angiogenesis. In light of the above, RONS seem to act both as upstream and downstream mediators of VEGF/VEGFR2 signaling and angiogenesis.

Hypoxia is a key physiological characteristic that may also explain the contribution of RONS in exercise-induced angiogenesis. Low oxygen levels in working skeletal muscle is a common characteristic as a consequence of increased oxygen consumption and leads to activation of the hypoxia-sensitive transcription factor hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ) [297]. In fact, a series of studies have shown that acute exercise increases HIF-1 $\alpha$  mRNA expression, while chronic exercise moderates this increase as an adaptation [298–300]. Although the exact molecular mechanisms by which cells sense hypoxia and activate HIF-1 $\alpha$  have not been fully unraveled, redox chemistry appears as a competent candidate [301]. In particular, HIF-1 $\alpha$  activity can be upregulated through reactive oxygen and nitrogen species-induced redox modifications of specific cysteine residues [302–304]. Moreover, several genes have been identified which are transcriptionally activated by HIF-1 $\alpha$  and encode proteins that increase oxygen delivery in hypoxic cells. These genes include, among others, VEGF and nitric oxide synthase [296,305]. The dependence of VEGF expression on HIF-1 $\alpha$  was strongly supported by a study using HIF-1 $\alpha$ <sup>-/-</sup> cells, which demonstrated that VEGF mRNA levels did not increase in response to hypoxia in these cells [306]. Based on the above, it could be hypothesized that RONS produced during exercise may also activate VEGF and promote angiogenesis in a HIF-1 $\alpha$ -dependent manner.

Additional indirect evidence about the implication of RONS in VEGF metabolism and/or angiogenesis comes from studies that used

experimental models that under- or over-expressed either RONS sources (e.g., NADPH oxidases and nitric oxide synthases [307–309]; or antioxidant enzymes (e.g., mnSOD and catalase [310], [410, 411]), as well as from studies that utilized generic (e.g., vitamins C and E and N-acetylcysteine [311,312]); or targeted antioxidants (gp91ds-tat, 1-NAME and Tempol [288], [407]) and nutritional compounds with purported antioxidant properties (e.g., resveratrol and catechins [313,314]). Most of these studies report increased VEGF expression and angiogenesis after RONS production and blunted responses after RONS source inhibition or antioxidant administration. Nevertheless, since the aforementioned studies are outside the exercise field, it is important to clarify that increased levels of angiogenesis is not always a beneficial effect of RONS (e.g., in tumor growth); thus, antioxidants act in essence as homeostatic regulators of the pro-angiogenic activity of RONS, guaranteeing optimal levels of angiogenesis.

Collectively, our knowledge on the role of RONS in exercise-induced angiogenesis is by some means fragmented. In fact, we now have clear evidence that RONS: i) regulate the expression of the central angiogen VEGF, ii) fine-tune the VEGF/VEGFR2 signaling cascade, iii) activate HIF-1 $\alpha$ , which in turn participates in VEGF regulation and iv) cooperate with endogenous antioxidant enzymes to secure optimal levels of angiogenesis. Based on the above, and taking into account that exercise (especially in acute form) upregulates VEGF and HIF-1 $\alpha$  mRNA expression, it seems realistic to argue that RONS produced during exercise partially contribute to the VEGF expression and angiogenesis observed after exercise training either directly or via HIF-1 $\alpha$  (Fig. 7). Other redox mechanisms regarding angiogenesis have been also described, yet available evidence is much lesser especially in a physiological context. These mechanisms involve mainly oxidized phospholipids, which can stimulate angiogenesis via both VEGF/VEGFR2-dependent and independent mechanisms (i.e., via Src and Toll-like receptor activation, respectively) [315,316]. For an in-depth analysis of the topic, the reader is referred to reviews about the redox-regulation of angiogenesis in response to exercise training [280], the central role of endothelial NADPH oxidases [287,317] and the key action of antioxidant enzymes [282,283] in this process.



**Fig. 7.** Proposed mechanisms on how RONS regulate angiogenesis. Based on [280,282,296, 315,317] and [287]. c-Src, proto-oncogene tyrosine-protein kinase; ERK, extracellular signal-regulated kinase; HIF-1 $\alpha$ , hypoxia-inducible factor 1-alpha; JNK, c-Jun N-terminal kinases; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; Rac1, Ras-related C3 botulinum toxin substrate 1; RONS, reactive oxygen and nitrogen species; VEGF, vascular endothelial growth factor.

### 3.4. Redox homeostasis

Probably the most idiosyncratic exercise adaptation mediated by RONS is the “upgraded” redox homeostasis. This is generally represented by the following indices: i) increased gene expression, protein content and activity of antioxidant enzymes, ii) higher resting levels of low molecular weight antioxidants, iii) lower resting levels of oxidative stress biomarkers, iv) increased resistance to oxidative insults and v) improved repair mechanisms for oxidatively modified proteins and DNA. Although for a long time this adaptation was regarded an unpolished stress-response paradigm to RONS production, it is nowadays clear that this revised redox profile is orchestrated by well-organized redox signaling processes [9,255,318]. Moreover, this adaptation is frequently referred to as “improved antioxidant defense”; however, taking into account the prevailing concept that antioxidants fine-tune redox signaling [319,320], we preferred the broader idea of “upgraded redox homeostasis”.

Quintanilha & Packer first reported that endurance training increases the levels of antioxidant enzymes in skeletal muscle of rats [321]. Afterwards, an abundant literature has verified that exercise training increases the activity of superoxide dismutase (especially the MnSOD isoform) [322],[323], glutathione peroxidase (GPx) [324,325] and catalase [322,326]. Along with activity, gene expression and protein content of these enzymes have been also shown to become upregulated with regular exercise [327–330]. Of note, several early studies have demonstrated that these adaptations were fiber type specific [331–334]. The first indication that these adaptive responses were triggered by the exercise-induced redox alterations derived from studies that used antioxidants, RONS source inhibitors or mouse models along with an exercise training protocol. In particular, most studies reported detrimental effects of supplementation in the acute antioxidant responses after an exercise bout (i.e., mRNA levels of enzymes), which subsequently led to blunted long-term improvements in enzyme activity and protein content [211,212,216,335,336]. In line with these findings, a recent study that used Ncf1-deficient mice (i.e., NOX2 deficiency) reported impaired catalase and SOD2 expression after high intensity interval exercise [337]. It should however be mentioned that other studies reported either no long-term effect of supplementation in antioxidant enzyme activity and protein levels (despite a blunted acute signal the hours after exercise) or even no impact in any parameter related to antioxidant enzymes [221,222,338]. In sum, antioxidant treatments seem to blunt the acute redox signals that initiate the antioxidant adaptations after exercise, however the long-term effects on activity and protein concentration are not fully supported.

The underlying mechanisms that largely account for the upgraded redox homeostasis after exercise training include several redox-sensitive factors, such as NFκB and Nrf2 which will be briefly described next. NFκB belongs to the “rapid-acting” primary transcription factors and is normally sequestered in an inactive state in the cytoplasm by the inhibitory IκB proteins. RONS (e.g., H<sub>2</sub>O<sub>2</sub>), among other stimuli, activate IκB kinase (IKK) which drives IκB ubiquitination and its proteolytic degradation by the 26S proteasome. Subsequently, the P50/P65 subunits of NFκB translocate into the nucleus and bind the DNA sequence of target genes related to antioxidant enzymes (e.g., MnSOD and GPx), while the whole process is controlled by glutathione and thioredoxin [339,340]. Some studies reported increased NFκB binding after an acute exercise bout accompanied by increased IKK activity, IκBα phosphorylation followed by degradation and P50 accumulation in the nucleus [341,342]. The study by Gomez-Cabrera and colleagues [165] demonstrated that exercise increased reactive species production and NFκB binding in muscle nuclear extracts and this was accompanied by increased mRNA levels of MnSOD and iNOS. However, allopurinol administration blunted both NFκB binding and mRNA levels of antioxidants strongly supporting the idea that RONS production triggers the expression of antioxidant enzymes [165].

The transcription factor Nrf2 is a member of the basic leucine zipper

family of transcription factors and is found inactive in the cytosol through binding to the homodimeric protein Kelch-like ECH-associated protein 1 (Keap1). In this condition, Keap1 facilitates the degradation of Nrf2 by promoting its ubiquitination and subsequent degradation by the 26S proteasome. RONS do oxidize specific cysteine residues on Keap1 promoting either the stabilization of the Keap1-Nrf2 complex (slowing thereby the degradation process at the proteasome) or the dissociation of Nrf2 from the complex. In the latter case, Nrf2 is translocated into the nucleus where it has the capacity to heterodimerize with small MAF proteins and activate the antioxidant response element (ARE) [343]. Done & Traustadóttir have comprehensively reviewed exercise studies that implemented either acute or chronic protocols and evaluated the effects on Nrf2 signaling and its downstream consequences (i.e., expression of SOD, GPx and catalase), as well as the impact of antioxidant supplementation on these procedures [344]. In brief, both acute and chronic exercise has been consistently shown to activate Nrf2 signaling across multiple tissues and species, whereas this effect was suppressed by generic antioxidants [344]. However, some phytonutrients, that are widely portrayed to feature antioxidant properties, serve in essence as Nrf2 and ARE activators providing synergistic effects with exercise [345].

The redox-mediated upregulation of antioxidant enzymes after exercise training is accompanied by a higher content of non-enzymatic antioxidants. The most representative example is the increased glutathione content after training [346–348]. This is particularly important since glutathione serves both as a direct nucleophile against electrophiles and as reducing agent for key antioxidant enzymes (i.e., glutathione peroxidases, glutaredoxins) and low molecular weight antioxidants (e.g., vitamin C) [349,350]. The improved antioxidant network is represented by the lower resting oxidative stress levels and the increased resistance against a subsequent oxidative stress-inducing stimulus. For instance, it has been recently demonstrated that human individuals who experience larger increases in RONS production after an acute exercise bout exhibit greater redox adaptations (i.e., in F<sub>2</sub>-isoprostanes and protein carbonyls) in the long-term compared to their counterparts who experienced low or minimal increases in acute RONS production [351]. In the same context, repeated bouts of aerobic exercise were found to lead to moderate free radical generation in skeletal muscle, while exercise has been reported to act as a redox preconditioning measure against subsequent hydrogen peroxide-induced oxidative damage in proteins of rat myocardium [352,353]. Two hypothetical yet realistic scenarios about this “paradoxical” redox effect of exercise have been recently described by Ref. [354,355]. The first scenario is called “adaptive homeostasis” and refers to the transient expansion or contraction of the homeostatic range in response to exposure to sub-toxic, non-damaging, signaling molecules or events (in our case exercise-induced RONS production) and the removal or cessation of them [354]. The second scenario refers to the ability of an organism to extend the peak and/or the optimal physiological zones after exercise training in order to better tolerate an upcoming “greater dose” of a redox stimulus [355].

Along with the increased resistance to subsequent oxidative stimuli, the repair mechanisms of redox modifications are also upregulated with training. This is of utmost importance, since oxidized macromolecules are not chemically and biologically inert [356–358]. These molecules rather modulate normal cell signaling either through direct reaction with other functional macromolecules (e.g., reactive lipid species) or by affecting intracellular RONS fate by feedback of their repair mechanisms (e.g., 8-oxoguanine-DNA glycosylase; OGG1). Exercise training has been repeatedly shown to improve these repair mechanisms, especially regarding protein and DNA oxidation adducts [255]. Regarding proteins, it has been shown that the activity of the proteasome complex is increased in response to aerobic training leading to higher protein turnover and lower levels of reactive carbonyl derivatives [359,360]. Of note, some studies have demonstrated that the activity of the proteasome complex and the degradation of oxidatively modified proteins



can be induced by RONS, such as  $H_2O_2$  [353,361], and this effect seems to be ubiquitination-independent [362,363]. Likewise, regular exercise also upregulates the expression of OGG1, reducing thereby oxidative damage to nuclear and mitochondrial DNA [364,365]. Acute RONS production has been reported to activate the DNA repair pathway of OGG1 in order to maintain genomic stability, highlighting the active crosstalk between redox processes and DNA damage in response to diverse cellular stress stimuli [366–369]. In light of the above, the redox-mediated improvement in the repair mechanisms of oxidation products represents a major component of the improved redox metabolism after exercise training.

Collectively, it is clear that the upgraded redox homeostasis after exercise training is mediated by delicate redox processes (Fig. 8). In particular, RONS activate a series of transcription factors that rapidly turn on gene coding for the expression of antioxidant enzymes. The improved repair mechanisms that facilitate more rapid and efficient turnover of oxidatively modified proteins and DNA, as well as the increased resistance to redox modifications in response to a following stimulus, corroborate also towards a more solid redox homeostasis. For an in-depth analysis of the topic, the reader is referred to reviews about the upregulation of antioxidant genes by exercise training [9,318], the oxidative stress biomarkers and the oxidation repair mechanisms [255,370,371] and the regulation of the two central redox-sensitive transcription factors, NFkB and Nrf2 [340,344,372].

#### 4. Some underappreciated issues in (exercise) redox biology research

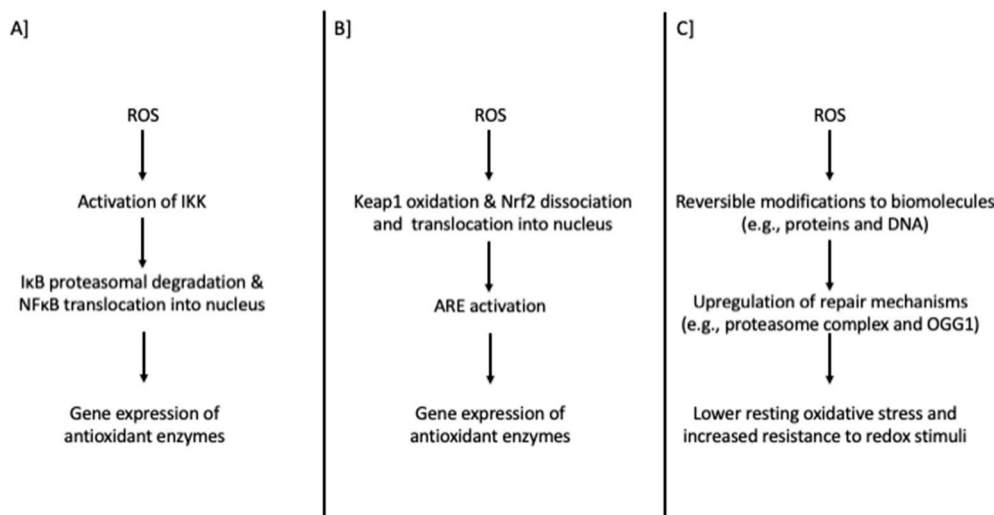
In this section we will try to highlight some frequently neglected issues in the field of exercise redox biology that relate predominantly with the interpretational and translational potential of the current literature, and not with analytical issues which have been covered comprehensively elsewhere [373–378]. Our intention is by no means to set certain “methodological-guidelines-for-redox-research”, but rather to present some features that may have led to contradictions (as is the case frequently in redox biology), especially when there is inevitable absence of clear evidence to assess the truth about the role of RONS in a particular phenotype. Taking into account the extreme nature of the reactive species and the immature state of the exercise redox biology field (e.g., many fundamental questions remain still unanswered), we argue that researchers should realize that the redox stories of the literature may have most probably loose ends [379].

#### 4.1. Exercise-induced redox metabolism: more than a stress-response

In the conclusion of their pioneering study, Davies and colleagues [18] proposed that “*exercise-induced free radicals may cause limited damage to mitochondrial membranes which, in a chronic training situation, may be the initiating stimulus to mitochondrial biogenesis*” [18]. This inspirational statement was actually one of the first tentative efforts to negate the very bad reputation of free radicals those days in both academic and mass culture settings. Despite the fact that important advances in our understanding about the biology of RONS have been achieved since then, exercise-induced RONS production is still widely regarded, especially by exercise physiologists, merely as a stress- or damage-response signal, analogous to the idea of mithridatism. However, this is not the entire truth, or may even describe only a small part of the adaptive response (e.g., via the repair mechanisms or oxidation products themselves [370]). In fact, we now know that RONS directly (and not necessarily as a response) regulate molecular and biochemical transduction pathways (e.g., via oxidation, S-nitrosylation or S-glutathionylation) both at rest and in response to several stimuli including exercise. This is accomplished through firmly controlled redox signaling cascades coordinated by RONS and antioxidants. Thus, the “damaging” part of the process may not be an essential component or, in many cases, could well refer to transient and reversible redox modifications. For instance,  $H_2O_2$  produced during exercise by NADPH oxidases can directly activate AMPK via selective oxidation of cysteine residues in the  $\alpha$  subunit independent of energy imbalance and this may lead to PGC-1 $\alpha$  activation and mitochondrial biogenesis [70].

#### 4.2. Studies with generic antioxidants: interpretational difficulties

A large part of the literature that aimed to investigate the role of RONS in a particular exercise response or adaptation is based on studies with generic antioxidants. Irrespective of the outcomes, the interpretation of such studies needs caution. If antioxidants indeed affect the response/adaptation of interest (either negatively or positively), we cannot be entirely sure that this effect is mediated by redox processes. This is because most (if not all) generic antioxidants are pleiotropic molecules exerting also multiple redox-independent biological effects (e.g., vitamin E as signaling molecule [380]). In addition, these antioxidants are non-selective against a certain reactive species and, at the same time, are unevenly distributed in various cells, tissues and organelles of the human body. Thus, the final read-outs of these studies lack redox mechanistic evidence and additional insights should be gained from in-vitro, in-situ or ex-vivo experiments. On the other, the failure of generic antioxidants as modulators of a specific exercise response/



**Fig. 8.** Proposed mechanisms on how RONS regulate redox homeostasis. Panel A based on [9,318]; Panel B based on [344,372]; Panel C based on [255,370]. ARE, antioxidant response element; DNA, deoxyribonucleic acid; IKK, I $\kappa$ B kinase; Keap1, Kelch-like ECH-associated protein 1; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid 2-related factor 2; OGG1, 8-oxoguanine glycosylase; ROS, reactive oxygen species.

adaptation (e.g., to reduce muscle fatigue) does not necessarily imply that RONS are not implicated in this process. In fact, even the most well-known dietary antioxidants (vitamin C and E) cannot, based on available kinetic data, outcompete antioxidant enzymes in order to appreciably react with redox signaling molecules [381]. Thus, it is probably our inability to exogenously regulate the fate of key RONS in this process and not the real absence of a redox implication. Obviously, we do not disregard this kind of studies, which are the most practical and realistic approach in an in-vivo setting and actually represent the largest part of the present review, especially after taking into account that even the selective and targeted antioxidants may have non-selective or off-target effects [382].

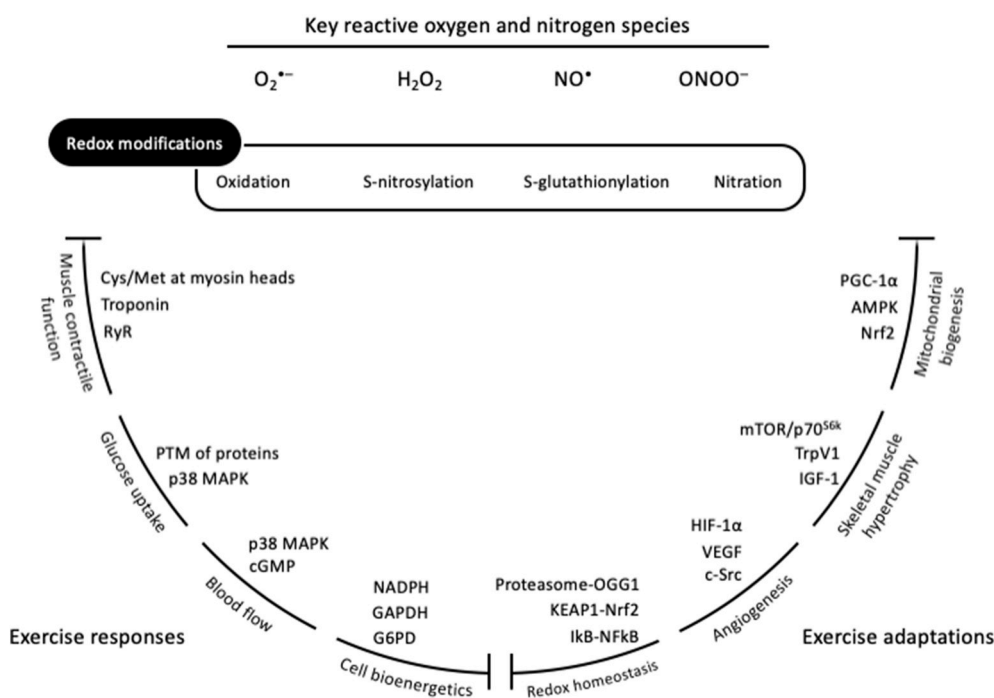
#### 4.3. Antioxidant supplementation: should we identify and target particular deficiencies?

There is an active and polarized debate in the literature about the effectiveness of antioxidants to enhance performance and improve health. Of course, several factors related to the methodological choices of each study can partially explain some discrepancies in the literature (e.g., type, dose and duration of antioxidant supplementation, type of exercise etc.). Halliwell in his viewpoint in Lancet stressed a different concern in antioxidant research by arguing that we should test the effects of antioxidants on the most “rancid” people, who may be those at greatest risk [383]. Put differently, akin to the case of drugs that are administered only in patients with specific needs, antioxidants should be administered only in individuals with specific redox needs. By exploiting this idea, we followed a “targeted” supplementation strategy (instead of administering antioxidants indiscriminately) and found that vitamin C and N-acetylcysteine exert the ergogenic potential only in individuals with vitamin C and glutathione insufficiency, respectively [384,385]. As an additional verification of our concept, we recently examined the effect of both targeted and non-targeted supplementation in deficient individuals and found that only the antioxidant in deficiency yielded ergogenic effects after supplementation (unpublished data). In light of the above, we recently proposed the idea of personalized treatments according to the antioxidant profile of each

individual, a concept we called “redox phenotyping” [55]. The redox background could also rationally explain the differential effect of antioxidants between young and old or diseased populations. For instance, we and others have shown that a detrimental impact of a chronic antioxidant protocol in young individuals (e.g., nicotinamide riboside, vitamin C and E) can be of great importance for older individuals or under a disease state (e.g., chronic obstructive pulmonary disease, diabetes mellitus) [85,185,187,386–388]. Thus, it seems that the basic redox background largely affects the impact of both exercise training and antioxidant supplementation [389], and based on this notion, we tried to refine the original idea of [383] by stating that it is more than necessary to identify the precise antioxidant deficiency that contributed to the increased levels of oxidative stress [55].

#### 4.4. Linking molecular and biochemical data with physiological read-outs

When a specific molecular or biochemical pathway has been found to regulate an exercise response/adaptation, it is very common to examine the role of RONS on molecules involved in this pathway in order to extend the conclusions also in the physiological phenotype of interest. However, if the molecular and biochemical data are not linked with measurements of the investigated physiological phenotype, no safe conclusions can be drawn. For instance, two studies by Paulsen and colleagues reported blunted cellular adaptations (i.e., COX4 and PGC-1 $\alpha$ ) and protein signaling (i.e., ERK1/2 and p70<sup>S6k</sup>) after antioxidant supplementation with vitamin C and E, however the improvements in VO<sub>2</sub>max and running performance and in muscle mass, respectively, were not different between groups [218,259]. If the performance tests were not performed, a completely different outcome would have been drawn in both studies. The indefinite communication between the distinct layers of biological organization (from molecular and cellular to organism and whole-body level) remains still a subject of intense debate in redox biology [10,390], exercise physiology [225,391] and biology in general [392,393].



**Fig. 9.** A summary figure illustrating the central RONS, types of redox modifications and molecules involved in the 4 acute exercise-induced responses (muscle contractile function, glucose uptake, blood flow and cell bioenergetics) and the 4 training-induced adaptations described in the present review. AMPK, 5' AMP-activated protein kinase; c-Src, proto-oncogene tyrosine-protein kinase; cGMP, cyclic guanosine monophosphate; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, reduced glyceraldehyde 3-phosphate dehydrogenase;  $H_2O_2$ , hydrogen peroxide; HIF-1 $\alpha$ , hypoxia-inducible factor 1-alpha; IGF-I, insulin-like growth factor 1; IKK, I $\kappa$ B kinase; Keap1, Kelch-like ECH-associated protein 1; LKB1, liver kinase B1; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NF $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NO $^{\bullet}$ , nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; O $^{\bullet-}$ , superoxide radical; OGG1, 8-oxoguanine glycosylase; ONOO $^-$ , peroxynitrite; p70<sup>S6k</sup>, ribosomal protein S6 kinase beta-1; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PTM, post-translational modifications; RYR, ryanodine receptors; TrpV1, transient receptor potential cation channel subfamily V member 1; VEGF, vascular endothelial growth factor.

## 5. Conclusion

Since the first studies more than 35 years ago that reported increased free radical production during exercise, our understanding on the biology of RONS has greatly advanced in terms of identification (e.g., what kind of RONS are produced during exercise and from which source), quantification (e.g., orders of magnitude change in RONS production after a redox treatment) and mechanistic impact (e.g., structural and functional changes of redox-sensitive transcription factors) [10]. Nowadays, redox reactions are increasingly recognized as a fundamental element of the cell signaling machinery along with other well-established types of biochemical reactions which fine-tune human metabolism (e.g., phosphorylation and ubiquitination). This is best exemplified by the strong interaction between RONS and classic metabolic molecules, such as protein kinases and phosphatases [66,394]. In the present review we provided an overview of how RONS and antioxidants are integrated into molecular and biochemical pathways that regulate 4 exercise responses and 4 exercise adaptations (summarized in Fig. 9). Apparently, there are many other examples of responses and adaptations linked to exercise metabolism that are controlled, at least in part, by redox reactions. Such examples are neuroprotection and cognitive function [395], mechanotransduction [396], muscle regeneration [268], autophagy [397], insulin sensitivity and glycemic control [398,399], heat shock proteins metabolism [400] and nerve-muscle interactions [401]. Taking into account that redox biology research is currently in a key turning point, characterized by more targeted methodological approaches and sophisticated analytical tools, we believe that the “unconventional” relationship between exercise and redox homeostasis (i.e., redox cascades triggered by exercise result in oxidative eustress and well-being [402]) represents a fascinating translational research area, with many challenging questions still unanswered.

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## Declaration of competing interest

None to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101499>.

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