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Effects of reactive oxygen and nitrogen species on actomyosin and their implications for muscle contractility

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Abstract

Experimental evidence accumulated during recent years is pointing out that numerous pathological conditions in skeletal and cardiac muscle are associated with an oxidative stress-induced muscle injury. Additionally, it has been postulated that several oxidants can directly alter contractile function by oxidative modification of the myofibril proteins –

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actin and myosin. Peroxynitrite (ONOO-), a potent biological oxidizing agent formed in the nearly instantaneous reaction of nitric oxide with superoxide anion, is increasingly recognized as playing a major role in the skeletal and cardiac muscle dysfunction. This is supported by detection of 3-nitrotyrosine, a protein modification produced by the reaction of peroxynitrite with tyrosine, on skeletal and cardiac muscle proteins during aging or in diseases associated with myocardial inflammation or ischemia/reperfusion insults. Although some studies point to a correlation of protein nitration with functional and structural modifications, the mechanism by which peroxynitrite may impair muscle contractility remains far from being elucidated. In the present review we address the role of reactive oxygen and nitrogen species on the structural and functional impairment of actomyosin ATPase activity and their implications for muscle contraction with particular emphasis on the oxidative modifications promoted by peroxynitrite on actin and myosin.

1. Introduction

Free radicals produced by pathological events or by natural consequences of cellular metabolism are known to damage proteins, nucleotides and lipids inducing cellular injury. Oxygen (ROS) and nitrogen (RNS) reactive species mediated protein oxidation is considered to be implicated in a broad variety of diseases. The cell type, the chemical characteristics of the oxidant and its site of generation will determine the primary cellular targets of oxidative stress. Studies conducted over the past decade have demonstrated the importance of ROS/RNS in muscle damage associated with aging and several contractile dysfunctions. Of particular biological relevance is peroxynitrite ($ONOO^-$), whose formation is an established event in multiple insults leading to cardiac dysfunction resulting in selective nitration of protein tyrosine residues and disruption of cellular energetic control. Nevertheless, conclusive evidence regarding the susceptibility of muscle proteins which differ in structure, function and intracellular localization to the different ROS/RNS remains far from being elucidated.

This review will outline our current understanding on the susceptibility of actomyosin filaments to oxidative stress and the functional consequences of eventual modifications for muscle contractility. We will focus primarily on peroxynitrite actions, starting by summarizing the systems involved in its formation and describing the multiple muscle disorders in which this potent oxidant has been implicated. These basic aspects of oxidative and nitrosative stress are followed by a discussion of the mechanisms through which peroxynitrite and other ROS can impair actomyosin structure and function.

2. Reactive oxygen species production in muscle

Skeletal muscle requires very rapid and coordinate changes in energy supply and oxygen flux for contractile activity. Many studies have demonstrated that exercise is associated with an increase generation of reactive oxygen and nitrogen species [1,2]. It has also been postulated that during contractile activity the increase generation of ROS is directly related to the elevated oxygen consumption that occurs due to an increased mitochondrial activity. In normal aerobic metabolism, 2-5% of the total electron flux in mitochondria results in the formation of ROS [3]. ROS production by skeletal muscle plays important roles in signalling adaptive responses to contractile activity [4]. However, during aging and in some disease states these redox-regulated processes can be modified rendering muscle particularly vulnerable to damage.

(a) Superoxide and nitric oxide generation in muscle. The primary ROS generated by skeletal and cardiac muscle are nitric oxide (NO) and superoxide anion (O_2^-) [for reviews on this topic see 4-9] (Figure 1). Superoxide anion is produced at a low rate in resting muscle but its production is dramatically enhanced during contractile activity. Besides being a by-product of mitochondrial electron transport, superoxide may be generated through the xanthine oxidase pathway, NADP(H) oxidase activity and NO synthases, although this latter source is poorly characterized. Superoxide anion is relatively innocuous in chemical terms, when compared to the highly reactive hydroxyl radicals. Nevertheless, superoxide radicals can give rise to other more reactive and damaging species, such as peroxynitrite ($ONOO^-$) formed by the reaction of O_2^- with NO. In fact, recent evidence indicates that most of the cytotoxicity attributed to NO is due to peroxynitrite [10]. Nitric oxide is continuously generated in muscle via constitutive NO synthase isoforms, namely endothelial (eNOS) and neuronal (nNOS), both activated by calcium. NOS metabolize L-arginine and oxygen to generate NO with citrulline as a by-product. While nNOS is strongly expressed in fast-twitch muscle fibers and localizes to the muscle sarcolemma, eNOS is associated with muscle mitochondria. This basal NO production is believed to modulate contractility and other intracellular processes such as mitochondrial oxygen metabolism, glucose homeostasis and blood flow. In heart it has been recently suggested that NO also regulates cardiac function through S-nitrosation of effector molecules such as L-type calcium channels and the sarcoplasmic reticulum (SR) ryanodine receptor (RyR) [11]. In contrast, high levels of NO production, via inducible NOS (iNOS) are associated with several contractile dysfunctions including inflammation/infection induced alterations in skeletal muscle function or cardiac dysfunctions. iNOS is not regulated by calcium, being limited only by substrate availability (L-arginine, which is abundant in muscle). As a result, iNOS produces NO at much higher rates than the two constitutive isoforms. This contributes to potentiate NO action on affected

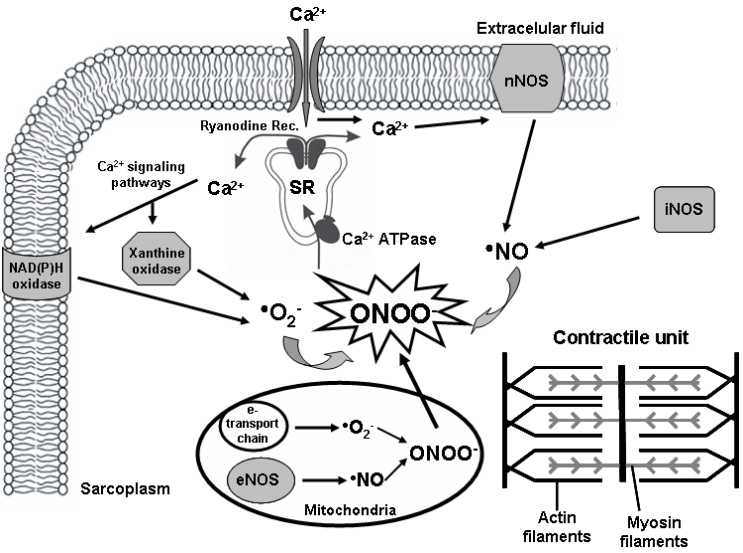

Figure 1. Schematic diagram illustrating the different sources of superoxide anion (O_2^-) and nitric oxide (NO) in muscle cells. NO is produced endogenously via three isoforms of NO synthase (NOS): endothelial (eNOS) and neuronal (nNOS) constitutive isoforms both activated by calcium and inducible isoform (iNOS) expressed in some inflammatory conditions. Mitochondria generates both O_2^- (by-product of the electron transport chain) and NO (via eNOS). O_2^- can be also enzymatically synthesized by xanthine oxidase or NAD(P)H oxidase, two pathways regulated by calcium homeostasis. Synthesis of NO in the vicinity of O_2^- results in the production of peroxynitrite ($ONOO^-$), a highly cytotoxic species more potent than its precursors.

cells and can result in nitrosative stress. NO and superoxide coexist in a delicate balance. Slight variations in the concentration of these radicals will dictate whether oxidative or nitrosative pathways will be followed [12]. Peroxynitrite, being generated from superoxide and nitric oxide may reduce the effective bioavailability of both radicals [10]. This has been suggested to be an adaptative mechanism that potentially influences cellular responses.

(b) Antioxidants in skeletal muscle. Muscle cells are protected from ROS-induced damage by an array of endogenous enzymatic and non-enzymatic antioxidants. Three ROS-scavenging enzymes are expressed constitutively by skeletal or cardiac muscle: (i) superoxide dismutase (SOD) that catalyzes the dismutation of superoxide anion to hydrogen peroxide; (ii) catalase, in turn, catalyzes the conversion of hydrogen peroxide to water and molecular oxygen; (iii) glutathione peroxidase (GPX) also catalyses the reduction of hydrogen

peroxide to water using reduced glutathione (GSH) as the electron donor. Another endogenous defence system involves the up-regulation of stress or heat shock proteins (HSPs) via activation of redox-responsive transcription factors [13,14]. Other enzyme systems (e.g. thioredoxin) may also contribute to antioxidant activity although their actions in muscle have not been well characterized. The most important non-enzymatic antioxidant system in mammalian cells is GSH. In skeletal muscle, GSH ranges from 0.5 to 3 mM [7]. It has direct antioxidant properties (independent of GPX) and in addition functions to recycle other antioxidants, namely α-tocopherol and ascorbate.

(c) ROS and muscle injury. Cellular damage arising from an imbalance between ROS-generating and ROS-scavenging systems has been implicated in a number of muscle disease states. The rise in ROS production that occurs during strenuous exercise has been shown to contribute to the development of acute muscle fatigue [1,2,7,15,16]. Conversely, oxidative stress has been implicated in muscle atrophy during periods of prolonged disuse [9,17]. This paradox can be integrated in a homeostatic model proposed by Reid [7] that predicts an optimal intracellular redox state for force generation in which deviations from this optimum, towards low or high oxidant levels, lead to loss of skeletal muscle force. In cardiac muscle, ROS formation has been proposed as a pivotal mechanism underlying contractile dysfunction occurring in ischemia-reperfusion insults such as heart infarct [18,19]. The ischemia-reperfusion injury results from the reestablishment of blood flow after ischemia which is essential to recover tissues, but leads to a burst of reactive oxygen species causing an extensive damage of the tissue. Inflammatory processes have also been shown to increase the oxidant load to which muscles are exposed in different diseases, such as hyperthyroid myopathy, sepsis or malignant hyperthermia [20-22]. Finally, impairment of motor function is often associated with the specific aging-related changes in skeletal muscle, termed sarcopenia, which include progressive decline in skeletal muscle mass, ability to generate force, an enhanced susceptibility to injury and a poor ability to repair [23-25].

3. Peroxynitrite and contractile dysfunction

Studies performed in the last decade have suggested that peroxynitrite may be responsible, at least in part, for the skeletal or cardiac muscle dysfunction observed in several of the pathophysiological conditions mentioned above. This assumption is supported by the observed increase in protein 3-nitrotyrosine (3-NT), a chemically stable protein modification produced by the reaction of peroxynitrite with tyrosine. The accumulation of nitrated proteins in tissues has been widely used to define the phenotype of biological aging and different pathologies. It has been reported a significant

age-dependent accumulation of 3-NT on proteins in skeletal [25] and cardiac muscle [26,27]. The extent of protein-3NT has also been shown to increase in skeletal muscle and heart in numerous disease conditions including sepsis, acute lung injury, myocarditis, atrial fibrillation, doxorubicin cardiomyophathy or ischemia/reperfusion insults [27-29]. In addition, some reports indicated that administration of either superoxide scavengers or NOS inhibitors prevent the development of contractile dysfunction in endotoxin-induced sepsis [30-33]. On the other hand, direct administration of peroxynitrite or peroxynitrite-generating compounds in muscle tissue was shown to induce a form of dysfunction analogous to that observed in the pathophysiological conditions being studied [33,34]. Moreover, peroxynitrite has been shown to be a major trigger of cardiac myocyte apoptosis, a crucial mode of cell death in many cardiac conditions, both *in vitro* in cultured H9C2 cardiomyocytes and *in vivo* in a rat model of myocardial ischemia-reperfusion [35]. Another study of Wang and collaborators [36] demonstrated that the enhancement of survival and prevention of apopotosis in hypoxic/reoxygenated cardiomyocytes by hypoxic postconditioning are associated with the reduction in peroxynitrite formation induced by hypoxia/reoxygenation.

(a) Potential sites of muscle dysfunction induced by peroxynitrite. Nitric oxide as a neutral and small hydrophobic molecule is able to freely diffuse across biological membranes contrarily to superoxide, which is anionic at neutral pH. Therefore, peroxynitrite formation will occur predominantly close to the sites of superoxide generation. Peroxynitrite may be generated at the muscle cell surface trough the reaction of superoxide anion with nitric oxide produced by endothelial cells or through the infiltration of neutrophils into muscle, a phenomenon seen in sepsis or ischemia/reperfusion. Considering that peroxynitrite has a relatively long half-life when compared with other free radical species and is able to diffuse across biological membranes via anion channels ($ONOO^-$) or passive diffusion (ONOOH) [37], intracellular constituents could be potential targets for peroxynitrite action. On the other hand, peroxynitrite is highly reactive and the reaction with cellular components is completed within seconds of exposure. Thus, in this particular case, peroxynitrite would be expected to preferentially react with outer cell structures altering action potential propagation and calcium homeostasis, thereby affecting muscle force generation and relaxation characteristics. Peroxynitrite may also be generated within cells, reaching especially high concentrations in or near mitochondria and plasma membrane as the redox chains of these two systems are able to produce large quantities of both substrates (superoxide anion and nitric oxide) needed for the formation of peroxynitrite. Formed in this way, peroxynitrite would have the opportunity to directly react with a variety of intracellular constituents. Bauer and collaborators [34,38-42] have originally demonstrated that the myofibrillar

compartment is a predominant site of peroxynitrite formation and concomitant protein nitration in both acute and chronic states of cardiac dysfunction. Among the several myofibrillar proteins showing increased tyrosine nitration, the most significant increases occurred in creatine kinase and myosin, two critical energetic controllers of cardiomyocyte contractility mediating production and utilization of ATP, respectively. An age-dependent accumulation of 3-NT was also found in sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) skeletal and cardiac muscle isoforms with significant yields of nitration on individual proteins (~ 4 mol and ~3 mol of 3-NT/mol of skeletal and heart SERCA2a, respectively) [25,43]. Recently, proteomic analysis of protein nitration in skeletal and cardiac tissue of old rats identified 3-NT containing sequences of different cytoskeletal proteins including the myosin heavy chain, tropomyosin, nebulin-related anchoring protein, actinin and actin [26,27]. This selectivity of protein nitration in skeletal muscle may be caused by the combination of different factors, such as (i) protein environment during oxidative stress; (ii) the microenvironment and the number of Tyr residues in the protein; (iii) the relative abundance of the protein; (iv) the proximity to the subcellular sites of peroxynitrite generation; and (iv) the turnover of the nitrated proteins.

(b) Functional implications of protein nitration. It is well known that tyrosine nitration may affect protein structure and function, nevertheless the magnitude of such effect will depend on the extent and location of the nitrated tyrosine residues within the protein. The molecular basis for enzyme inactivation by tyrosine nitration is still not well understood. The introduction of a NO_2 group onto a tyrosine ring may prevent the accessibility of the substrate to the active site, block protein phosphorylation and by introducing a negative charge (pKa changes from 7.5 to 10 when tyrosine becomes nitrated) interfere with inter/intra molecular interactions of ionic nature or induce conformational changes. The best described example of functional impairment by tyrosine nitration is the mitochondrial manganese superoxide dismutase (Mn-SOD) inhibition by peroxynitrite. Nitration of this enzyme, which catalyses the disproportion of superoxide to oxygen (O_2) and hydrogen peroxide (H_2O_2), was found to disrupt the hydrogen-bonding network at the active site leading to an almost complete inhibition of its activity [44]. In skeletal and cardiac muscle tissue, SERCA has been shown to be partially inactivated by Tyr nitration [43,45] which was suggested be responsible, at least in part, for the loss of Ca^{2+}-homeostasis generally observed in biological aging. Protein nitration has also been suggested to have mechanistic relevance in the impairment of the creatine kinase system in the hearts of doxorubicin-treated mice and the peroxynitrite-treated cardiac trabeculae of rats [38,42]. In vascular smooth muscle it was hypothesized that peroxynitrite-induced F-actin nitration could lead to a depolymerization of the filament with subsequent loss

of myogenic tone in cerebral arteries [46]. Alternatively, tyrosine nitration of α-actinin, a structural protein essential for maintenance of the Z-line and for the integrity of the sarcomeres was suggested to be tightly coupled to the deterioration of the myofibrillar cross-striation pattern and consequently to the reduction of isometric force production in permeabilized human ventricular myocytes exposed to peroxynitrite [29]. Although several studies point to a correlation of protein nitration with functional and structural modifications, the molecular mechanisms by which nitrated proteins impair muscle contractility remains far from being elucidated.

4. ROS-induced muscle injury and actomyosin functional impairment

It is now relatively well established the various pathological conditions in skeletal muscle associated with an oxidative stress-induced muscle injury. Nevertheless, fundamental questions remain to be answered. What are the primary molecular targets that respond to changes in the redox state of skeletal muscle? What are the molecular mechanisms underlying the oxidative-induced muscle dysfunctions? The primary molecular targets can diverge depending on the nature of the oxidant, its site of generation (intra- or extra-cellular) and the proximity of the oxidant to a specific cellular component. Therefore, the contractile changes mediated by ROS/RNS are likely to involve more than one molecular target. Proteins related to calcium regulation, such as the ryanodine-sensitive calcium release channel or the sarcoplasmic reticulum calcium-dependent ATPase (SERCA), are prime candidates. Other potential targets are proteins involved in the control and utilization of energy substrates, such as creatine kinase. ROS/RNS may also act on regulatory proteins indirectly via redox-sensitive second messenger systems such as the cyclic guanosine monophosphate (cGMP). A growing body of evidence is also pointing out to the high sensitivity of the contractile filaments to oxidative stress.

The cellular redox balance appears to have an important influence on force production in skeletal muscle fibres. Among all the cellular elements of the contractile machinery, myofibrillar Ca^{2+} sensitivity and the cross-bridge kinetics were shown to be especially susceptible to changes in the oxidation-reduction balance within myocytes, while the SR functions (Ca^{2+} leak and uptake) are less sensitive [47,48]. In fact, several studies associate the age-related decrease in force generation of muscle fibres and of myofibrils with changes in the interaction between myosin and actin during the actomyosin ATPase cycle [24,49,50]. More recently, it has been demonstrated that the decrease in force production in hyperthyroid rat soleus muscle is associated with failure in myofibrillar protein function due to oxidative modifications of the myosin heavy chain [51]. A high sensitivity of muscle myofibrils to free-

radical-mediated oxidative stress had been noticed earlier by Nagasawa et al [52], who showed that skeletal muscle actin and myosin become oxidized *in vivo* following Fe-nitilotriacetate treatment in rats, an experimental model for iron overloaded. Furthermore, in cardiac muscle, the reversible failure of contraction occurring on post-ischemic reperfusion has been proposed to depend on covalent changes of myofibrillar proteins [53].

(a) Myosin sensitivity to oxidative stress. Force production in muscle is generated by the myosin cross-bridges which interact with actin filaments and allows for the sliding of the thin actin filaments over the myosin thick filaments. This is achieved by structural alterations of the myosin head inducing the transition of the actomyosin complex from states of weak to strong interactions (Figure 2). Briefly, in the actomyosin ATPase cycle, ATP binding starts to reduce the affinity of myosin for actin, and the subsequent hydrolysis of ATP results in a ternary complex between myosin, ADP and Pi, which can be blocked by γ-phosphate analogues such as vanadate (VO_4^-). The M-ADP-Pi is in rapid equilibrium with the AM-ADP-Pi complex but this is a low affinity interaction. This step is followed by a slow transition to a high-affinity state of the actomyosin complex. Release of the hydrolysis products is then catalyzed by the rebinding of myosin to actin giving rise to about 10-nm displacement of the actin filament relative to the myosin filament [54]. On the basis of this model, it has been suggested that the age-related decrease in Vmax could be accompanied by alterations in the structural states of myosin affecting the transition from weak to strong interactions. Specifically, it was proposed that the changes in force production during aging result in an accumulation of the weak binding complexes slowing down the actomyosin ATPase cycle [24,55]. On the other hand, in muscle fibres, free radicals were shown to induce a process that enhanced the dissociation of ADP and vanadate from the myosin heads, and forced the transition of cross-bridges from weakly binding state into rigor [56]. These results are consistent with *in vitro* studies performed in our lab with purified rabbit skeletal myosin showing that peroxynitrite impairs the ability of vanadate to induce the intermediate catalytic transition state M-ADP-Pi [57]. The decrease of conformational flexibility of the myosin catalytic domain was than suggested to cause inhibition of the F-actin-activated myosin ATPase activity observed upon exposure of myosin to SIN-1 producing peroxynitrite fluxes close to 1 μM/min.

(b) Actin sensitivity to oxidative stress. Most investigations elucidating the mechanisms of the oxidative-induced degeneration of muscle have normally focused on myosin, but there are evidences that oxidative modifications of actin may also affect actomyosin ATPase activity and contractility. An example of that is the carbonyl groups and oxidation of cysteine residues found in actin from isolated rat hearts subjected to ischemia/reperfusion [58,59]. This

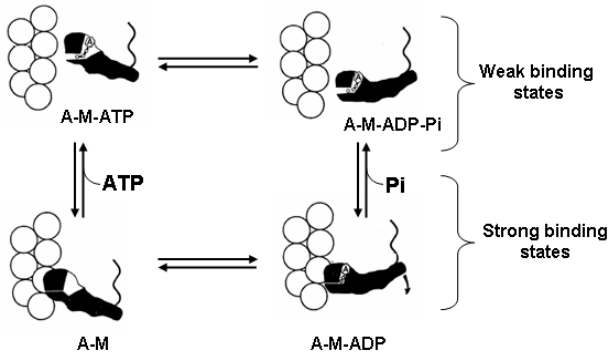

Figure 2. Reactive oxygen species are suggested to induce alterations of the structural states of myosin which lead to an impairment of the actomyosin ATP hydrolysis and decrease of force production. The relevant steps of the actomyosin ATP hydrolysis cycle are schematically represented indicating the weak and strong binding states of actin (A) and myosin (M). Adapted from Rayment et al [54].

has been confirmed by a study demonstrating post-ischemic oxidation of actin in the myocardium [60]. The novel physiological relevance of actin polymerization regulated by S-glutathionylation (reversible formation of disulphides of cysteinyl residues on proteins with gluthathione) under conditions of oxidative stress is also an evidence for the high sensitivity of actin to the cellular redox balance [61]. Moreover, S-nitrosylation of actin was demonstrated to inhibit annealing of short actin filaments after mechanical fragmentation [62]. Annealing is thought to constitute an important repair mechanism *in vivo* and myosin can exert enough force to break actin filaments during muscle contraction [63].

Monomeric actin, G-actin, contains very specific functional sites on its surface such as myosin-binding and ATPase-activating sites, as well as numerous other protein-binding sites. By itself G-actin does not stimulate the myosin ATPase activity. In the presence of suitable concentrations of bivalent cations, G-actin starts to form relatively unstable nuclei followed by rapid elongation and formation of filamentous actin (F-actin). In muscle cells, the polymerization process is very important to maintain the thin filaments required for contraction. Oxidative conditions are known to alter both the kinetics of actin polymerization and interaction with myosin. Slow oxidation of sulfhydryls has been suggested to account for increases in the shear elasticity and fragility of purified actin observed after aging [64]. Several studies have also demonstrated that modification of the highly reactive sulfhydryl, Cys-374 affects functional interaction with actin (both in G and F

forms)-binding proteins, including myosin, as well as the supramolecular organization of actin [65,66]. Actin modifications can also alter muscle function through effects on actin's interaction with proteins other than myosin. For instance, oxidative modifications of actin were shown to inhibit the interaction with α-actinin [67,68] and it is known that the mutation-induced changes in α-actinin binding sites of actin is implicated in the mechanism of myocyte dysfunction and heart failures in dilated cardiomyopathy [69].

Actin structural and functional impairment has been extensively investigated by treating purified G-actin or F-actin with different oxidizing agents. These studies indicated that oxidative injuries on actin are specific and related to the chemical characteristics of the oxidants. Some oxidants such as hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (t-BOOH), hypochlorous acid (HOCl) or chloramine-T (CT) were shown to affect actin dynamics by decreasing the maximum rate and extent of polymerization, by forcing the disassembly of actin filaments and by affecting the interaction with DNAse-I [67,70,71]. In contrast, the specific thiol oxidant diamide was found to decrease the extent of polymerization without influencing the polymerization rate, actin filaments disassembly or DNAse-I interaction [72].

5. Peroxynitrite oxidative modifications of actomyosin

Peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH; pKa = 6.8) can be toxic by reacting with several biological molecules including fatty acids, low molecular mass antioxidant molecules (glutathione, ascorbate and α-tocopherol) and proteins. In addition to 3-NT formation, this oxidant can alter the structure and function of proteins by modifying critical amino acid residues. It can directly produce oxidation of protein cysteines to thiyl radicals ($RS^{\cdot}$), disulfides (RSSR) and eventually to sulfenic (RSOH), sulfinic (RSO_2) and sulfonic (RSO_3) acids [73,74], although at physiological pH the two electron oxidation leads predominantly to disulfides [75]. To a lesser extent peroxynitrite can oxidize other amino acids including methionines and tryptophans [76] or amino acid side chains which can lead to the formation of protein carbonyls [77]. Additionally, peroxynitrite can modify proteins by acting through radicals generated during its spontaneous decomposition in biochemical buffers and biological fluids, particularly hydroxyl ($HO^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$) radicals [78,79]. As $ONOO^-$ is a very short-lived chemical molecule at physiological pH, less than 1 s in buffer solutions at pH 7 [75] and less than 0.1 s in the cytosol [80,81], the efficiency of the $ONOO^-$ treatment in a single "bolus" is much lower than that expected to be attained under conditions where it is slowly released, mimicking the "*in vivo*" situation. The exposure of cells, or isolated subcellular components, to slowly released $ONOO^-$ can be mimicked by 3-morpholinosydnonimine (SIN-1), a

peroxynitrite-producing agent. SIN-1 slowly decomposes in neutral and weakly alkaline water solutions releasing nitric oxide ($NO^{\cdot}$) and superoxide anion ($O_2^{\cdot-}$) [82], which react each other to produce peroxynitrite with a second order rate constant of (4-7) $\cdot 10^9$ $M^{-1} \cdot s^{-1}$ close to the diffusion limit for chemical reactions. This reaction is three times more efficient than SOD in scavenging superoxide and therefore is preferred where ever both species are present [83]. It is important to recall that peroxynitrite "dosage" should be calculated as a concentration x time product. In some pathophysiological conditions such as during ischemia/reperfusion syndrome and inflammation, generations rates of 0.1-1μM/min peroxynitrite have been reported to be reached in tissues [75,84], where the duration of the tissue insult can last from 30 min to several hours.

(a) Modified residues on myosin. Thiol oxidation by peroxynitrite has been previously attributed to loss of function in a number of proteins. On the other hand, the decrease in the free cysteine content of myosin has been previously associated with age-related inhibition of the actomyosin ATPase activity. Skeletal muscle myosin contains 40 cysteines including two highly reactive cysteines, Cys-707 (SH1) and Cys-697 (SH2). These critical residues are located close to the nucleotide binding site and its modification has shown to have functional consequences to the actomyosin complex and to force development of muscle contraction. Oxidation of SH1 is known to inactivate the K^+-EDTA ATPase while simultaneously activates the Ca^{2+}-dependent ATPase activity. In contrast, oxidation of both reactive cysteine residues inhibits all the myosin ATPase activities including the physiological actin-activated Mg^{2+}-ATP hydrolysis activity [85]. The observed decrease of both physiological and non-physiological ATPase activities upon exposure of purified myosin to the peroxynitrite releasing agent SIN-1 indicates that both Cys707 and Cys697 are oxidized by this reagent [57] (Figure 3). Moreover, the obtained IC_{50} values were very close to that found for the inhibition of the actin-activated Mg^{2+}-ATPase activity demonstrating a tight correlation between the oxidation of the highly reactive sulfhydryls and the loss of actomyosin ATPase activity induced by SIN-1. These observations are in agreement with previous studies showing that the NO donor sodium nitroprusside (SNP) exerts direct effects on actin-myosin cross-bridge cycling by modulating critical thiols on the myosin head in permeabilized fibers [86].

In addition to Cys oxidation, peroxynitrite can also produce 3-nitrotyrosine formation on myosin. This modification was detected in the 25 kDa tryptic fragment of myosin subfragment-1 upon exposure of the purified protein to peroxynitrite [57]. This location is consistent with the four nitrated tyrosines recently identified in the myosin heavy chain extracted from cardiac muscle of aged rats [27] (indicated in Figure 3). However, the extent of this chemical modification was found to be very low in the purified treated myosin, i.e. less than 0.05 moles 3-NT/mole of myosin when 50% of the activity was

already lost suggesting a secondary role of nitration in the inhibition of the actomyosin ATPase activity [57]. The failure of bicarbonate to enhance the inhibition of the F-actin stimulated myosin Mg^{2+}-ATPase activity by peroxynitrite was also fully consistent with this conclusion, because bicarbonate potentiates 3-nitrotyrosines formation by peroxynitrite via a radical pathway involving the tyrosyl radical formation after the attack of $CO_3^{\cdot-}$ [75,87,88].

Three of the major antioxidants present in muscle cells (GSH, ascorbate and NADH) were found to efficiently protect against myosin inhibition by the peroxynitrite-releasing agent SIN-1, with an IC_{50} /SIN-1 ratio close to 1 for all of them [57]. These results pointed out that in normal physiological conditions the sarcoplasm concentrations of these antioxidants are high enough to ensure proper protection against an acute and short lasting peroxynitrite insult. However, the increase in the 3-nitrotyrosine content of myocardial myosin after ischemia-reperfusion [39,42] indicates that these antioxidant defences are largely depleted near myofibrils during acute oxidative insults, suggesting that myofibrils are very close to the subcellular sites of peroxynitrite generation. Indeed, myosin along with creatine kinase are the proteins showing the most significant increase in nitrotyrosine content, in myofibrils, after ischemia/reperfusion episodes [39,42]. This is consistent with the high concentration of myosin in the sarcoplasm of muscle cells and with the fact that the IC_{50} for inhibition of F-actin stimulated S1 Mg^{2+}-ATPase activity by SIN-1 and/or synthetic peroxynitrite (molar ratio SIN-1/myosin of approximately 5.5) [57] is close to that reported for inhibition of creatine kinase [41] and nearly 4-fold lower than the one reported for sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase [43,45,89]. Moreover, the age-induced nitration of the myosin molecule either in skeletal muscle [25] or in cardiac tissue [27] can be related with its long half-life and reduced rate of turnover [90].

(b) Modified residues on actin. Peroxynitrite has been shown to nitrate purified actin *in vitro* and to alter its nucleation and polymerization properties. This is not surprising if we consider that the actin molecule contains 16 Tyr residues (the richest in Tyr of all the major myofibrillar proteins) some of them located in key functional regions. Aslan et al [91] have identified four nitrated residues (Tyr53, Tyr198, Tyr240 and Tyr362) in rabbit actin treated with 0.3 mM peroxynitrite (indicated in Figure 3). Nitration was shown to increase the nucleation and elongation rate as well as to slow down the rate of subunit dissociation. A possible explanation was given by the authors who proposed stabilization of the interactions between the pointed end of G-actin and the barbed end of a growing actin filament. Clements et al. [92] have also found nitration on purified rabbit skeletal actin treated either with a bolus of peroxynitrite or with SIN-1. In these studies, the authors concluded that the effects of peroxynitrite on actin function, i.e., F-actin depolymerization and

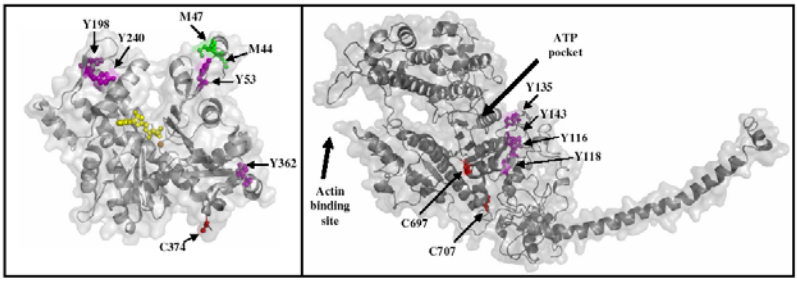

Figure 3. Molecular model of G-actin (PDB Id: 1J6Z) on the left, and myosin subfragment-1 (PDB Id: 2MYS) on the right, depicting the putative residues modified by peroxynitrite. ATP and Ca^{2+} represented as yellow and orange spheres, respectively, are shown just to indicate the active site on the actin structure. Cyst, Met and Tyr residues are represented as stick-and-spheres in red, green and purple, respectively.

inhibition of G-actin polymerization, were primarily a result of nitration. On the other hand, studies performed in our lab indicate only a marginal role of tyrosine nitration for the effects promoted by peroxynitrite. Experiments on purified protein have shown that SIN-1 affects actin dynamics by decreasing the rate of G-actin polymerization and by producing an extensive depolymerization of the actin polymers leading to a complete block of the myosin ATPase activity stimulation [93]. The results highlighted a major role for the highly reactive cysteine and for some critical methionines to the observed functional alterations. Actin, containing five cysteines (all existing in the reduced form), including the highly reactive Cys-374, is a potential target molecule for protein thiol oxidation. Peroxynitrite was found to oxidize nearly 2.5 of the 5 cysteines of G-actin with an IC_{50} of 10 μM for the fast reacting thiol, i.e, a molar ratio of approximately 1 mol SIN-1 per mol of actin. After cysteines, methionine residues are potential sites of oxidative modification and it has been suggested as a major cause of the functional impairment of oxidized actin [71]. Furthermore, along with cysteines and tryptophan, methionines belongs to the amino acids that are able to react directly with peroxynitrite with a second-order rate constant of 10^2 $M^{-1}s^{-1}$ [76]. The actin molecule contains 16 Met residues dispersed along the molecule. A decreased susceptibility to limited proteolysis by α-chymotrypsin and subtilisin of SIN-1 treated G-actin revealed that both Met-44 and Met-47 are oxidized to the sulfoxide derivative. While the reactive sulfhydryl Cys374 is located at the C-terminal region which is the binding site for several actin-binding proteins (including myosin), Met-44 and Met-47 are located in a loop implicated in monomer/monomer contact (Figure 3). The modification of these critical

residues by peroxynitrite could, therefore, affect the functional interaction with myosin as well as the G-actin/F-actin equilibrium.

(c) Structural modifications of actomyosin. In addition to the reduction of biological activity, oxidative damage of proteins can produce a distortion of the folded native structure resulting in an enhanced susceptibility to proteolysis. For the peroxynitrite-induced oxidation, inhibition of the actomyosin ATPase activity is paralleled by a reduce stability of both proteins. This has been shown by the decrease of the unfolding enthalpy (ΔH) and critical temperature (Tc) of SIN-1 treated myosin and G-actin and further confirmed by the increased susceptibility to tryptic digestion. It is well known that unfolded oxidized proteins can be rapidly degraded by the intracellular proteasome [94,95] and peroxynitrite has been previously shown to increase the degradation of several cellular proteins by the proteasome [96]. Taking into account the slow turnover rate of the myofibrillar proteins [90] and the high concentrations of myosin and actin in muscle cells, the peroxynitrite-induced oxidative actomyosin unfolding could account for the rapid loss of muscle mass that can be observed after inflammation or ischemic insults.

6. Concluding remarks

Reactive oxygen and nitrogen species are continuously generated in skeletal muscle cells during normal muscle contraction and relaxation cycles, and it is now relatively well established that derivatives of these species may act as physiological modulators of skeletal muscle function. However, there are conditions in which the delicate balance between oxidant production and antioxidant defence are compromised leading to the development of muscle diseases. The paradox in the role of oxidants as essential molecules in the regulation of muscle function and as toxic by-products of metabolism is particularly relevant for nitric oxide (NO). When produced in low concentrations by the constitutive isoforms of nitric oxide synthase (NOS), NO functions as a signalling molecule mediating regulation of contractility and other intracellular functions. However, when produced in high concentrations by inducible NOS, NO supplies highly toxic oxidants such as peroxynitrite ($ONOO^-$) resulting from the combination of NO with superoxide anion (O_2^-). One of the hallmarks of peroxynitrite-induced oxidation is nitration of protein tyrosine residues. The increase of this post-translational modification in skeletal muscle has been shown to correlate with the development of various pathologies and also with the more complex process of biological aging.

In recent years, there has been a significant shift away from the measurements of protein oxidation products to simply monitor the presence of oxidative stress, to a focus on identifying specific oxidized proteins in diseases and establish the functional consequences of protein dysfunction induced by

oxidative stress. In the present review we have summarized that oxidative modifications of actin and myosin affect actomyosin ATPase activity and actin polymerization state, and that these functional and structural impairments may lead by themselves to skeletal and cardiac muscle dysfunctions observed in pathological conditions associated with an enhanced cellular oxidative stress. Nitration of actin and myosin has been reported in aging and some cardiac disease states, but a correlation of protein nitration with functional and structural modifications is not yet well understood. This is probably related with the fact that the *in vitro* exposure of an isolated protein to peroxynitrite may not always be a good model to mimic protein nitration *in vivo*. In addition, the reaction of peroxynitrite with other amino acids such as cysteine or methionine residues is very likely to precede tyrosine nitration, therefore masking the structural and functional consequences of protein nitration.

The current rapid progress in proteomic analysis allowed us to rationalize the phenotype of biological aging and different pathologies by identifying specific post-translational modifications in skeletal or cardiac muscle proteins. It seems to us that the great challenge now is to identify the most relevant links between protein oxidation, protein dysfunction and muscle diseases, as a step forward for the design of new protective therapies based on more rational molecular grounds.

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