Review

Muscle redox disturbances and oxidative stress as pathomechanisms and therapeutic targets in early-onset myopathies

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ABSTRACT

Because of their contractile activity and their high oxygen consumption and metabolic rate, skeletal muscles continually produce moderate levels of reactive oxygen and nitrogen species (ROS/RNS), which increase during exercise and are buffered by multiple antioxidant systems to maintain redox homeostasis. Imbalance between ROS/RNS production and elimination results in oxidative stress (OxS), which has been implicated in ageing and in numerous human diseases, including cancer, diabetes or age-related muscle loss (sarcopenia). The study of redox homeostasis in muscle was hindered by its lability, by the many factors influencing technical OxS measures and by ROS/RNS important roles in signaling pathways and adaptive responses to muscle contraction and effort, which make it difficult to define a threshold between physiological signaling and pathologic conditions. In the last years, new tools have been developed that facilitate the study of these key mechanisms, and deregulation of redox homeostasis has emerged as a key pathogenic mechanism and potential therapeutic target in muscle conditions. This is in particular the case for early-onset myopathies, genetic muscle diseases which present from birth or early childhood with muscle weakness interfering with ambulation and often with cardiac or respiratory failure leading to premature death. Inherited defects of the reductase selenoprotein N in SEPN1-related myopathy leads to chronic OxS of monogenic origin as a primary disease pathomechanism. In myopathies associated with mutations of the genes encoding the calcium channel RyR1, the extracellular matrix protein collagen VI or the sarcolemmal protein dystrophin (Duchenne Muscular Dystrophy), OxS has been identified as a relevant secondary pathophysiological mechanism. OxS being drug-targetable, it represents an interesting therapeutic target for these incurable conditions, and following preclinical correction of the cell or animal model phenotype, the first clinical trials with the antioxidants N-acetylcysteine (SEPN1- and RyR1-related myopathies) or epigallocatechin-gallate (DMD) have been launched recently. In this review, we provide an overview of the mechanisms involved in redox regulation in skeletal muscle, the technical tools available to measure redox homeostasis in muscle cells, the bases of OxS as a primary or secondary pathomechanism in early-onset myopathies and the innovative clinical trials with antioxidants which are currently in progress for these so far untreatable infantile muscle diseases. Progress in our knowledge of redox homeostasis defects in these rare muscle conditions may be useful as a model paradigm to understand and treat other conditions in which OxS is involved, including prevalent conditions with major socioeconomic impact such as insulin resistance, cachexia, obesity, sarcopenia or ageing.

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Abbreviations: AP-1, activator protein 1; ARE, antioxidant response element; CMs, congenital myopathies; CMDs, congenital muscular dystrophies; ColVI, collagen VI; CPK, creatine phosphokinase; DMD, duchenne muscular dystrophy; ER, endoplasmic reticulum; GPx, glutathione peroxidase; GSH, glutathione; MEF, myocyte enhancer factor; MRF, myogenic regulatory factor; NAC, N-acetylcysteine; NF-κB, nuclear factor kappa B; NOX, nitric oxide dimutase; NRF2, nuclear factor (erythroid-derived 2)-like2 related factor; OxS, oxidative stress; PTP, permeability transition pore; ROS, reactive oxygen species; RNS, reactive nitrogen species; RyR1, ryanodine receptor type 1; SEPN1, selenoprotein N; SOD, superoxide dismutase; SRF, serum response factor; XG, xanthine oxidase.

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1. Introduction

Early-onset myopathies (EOM) are inherited muscle conditions which present during infancy or early childhood and have so far no specific treatment. Aside from the metabolic, neuromuscular junction or systemic defects that can affect skeletal muscle, the two main groups of structural primary congenital muscle disease are congenital muscular dystrophies (CMDs) and congenital myopathies (CMs) [1,2]. They typically present from birth or infancy with muscle weakness and hypotonia, delayed motor development and difficult or absent ambulation, often associated with orthopaedic complications, respiratory failure or heart disease that can lead to premature death [2]. The muscle biopsy pattern classically differentiates CMDs from CMs. CMDs present with endomyositis fibrosis with or without muscle fiber necrosis and regeneration (dystrophic lesions), whereas CMs muscles are non dystrophic but are defined by characteristic changes in the internal fiber architecture. The CMD and CM present heterogeneous pathophysiological mechanisms, but typically involve defects in proteins which are essential for muscle function, including components of i) the extracellular matrix or sarcolemmal membrane (such as collagen VI (ColVI) or alpha-dystroglycan); ii) the sarcomere (contractile and/or scaffolding proteins such as alpha-actin, myosin, nebuline or titin); iii) the triadic junction, the structural basis of excitation-contraction coupling (such as the ryanodine receptor Ryr1 or StaC3); iv) the redox regulation system of the endoplasmic reticulum (selenoprotein N encoded by SEPN1); v) the inner nuclear envelope ( lamin A/C) [1,3]. In addition to CMDs and CMs, one of the most prevalent muscle diseases in childhood (1/3500 male newborns) is Duchenne muscular dystrophy (DMD), due to mutations in the DMD gene encoding dystrophin and typically presenting with muscle weakness and elevated CPK levels before the age of 3 years.

Deregulation of redox homeostasis has emerged in the last years as a common pathogenetic mechanism and potential therapeutic target in Collagen VI-related congenital muscular dystrophies, in RYR1-related myopathies (RYR1-RM), in SEPN1-related myopathy (SEPN1-RM) and in DMD, as well as in other more prevalent processes such as age-related muscle loss (sarcopenia). This review will focus on the importance of redox homeostasis for muscle integrity and function, on the methods to detect oxidative damage and on the different mechanisms underlying oxidative stress (including mitochondrial defects, calcium signaling alteration or reduction of antioxidant defenses) that represent hallmarks of these early-onset myopathies and targets for current and future therapeutic interventions.

2. Muscle redox homeostasis

Homeostasis is the property of a system to regulate its internal environment so as to maintain a stable condition (equilibrium). Tight regulation of homeostatic processes at the cell, tissue, organ and organism levels is necessary for life, and disturbance of homeostasis is associated with many diseases. In particular, redox homeostasis is finely regulated, since low or moderate levels of reactive oxygen species (ROS) or reactive nitrogen species (RNS) (reactive molecules and free radicals derived from molecular oxygen and/or nitric oxide) are important physiological signaling molecules. Oxidative stress (OxS), an alteration of redox homeostasis consisting in an imbalance between the ROS and/or RNS production and a biological system’s ability to readily detoxify the reactive intermediates or to repair the resulting damage, is frequently associated with pathological conditions. Mitochondria, the powerhouse of the cell, produce most of the cellular energy and are commonly considered as the main production site of free radical formation and oxidative damage. However, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), lipoxygenases or xanthine oxidase (XO) are other relevant sources of ROS [4].

Skeletal muscle, the largest type of tissue in the human body, has specific characteristics which require a tight regulation of redox homeostasis, such as intense energy requirements, calcium signaling and metabolism. It also has the capacity to regenerate after lesion by recapitulating the myogenesis process, which depends on the activation of muscle stem cells (satellite cells) that can differentiate into myotubes and ultimately muscle fibers [5]. Maintenance of the muscle architecture, which is highly organized to optimize contraction and force generation, requires basal energy. In addition, muscle cells consume a high amount of oxygen that is significantly increased during exercise, and this O2 consumption is associated with continuous ROS/RNS generation, although, contrary to early reports, the rate of ROS production by mitochondria during exercise has been probably overstated [6]. These reactive species are important signaling molecules necessary for muscle function and for flexible adaptive responses to stress and/or effort [4]. Thus, they are involved in regulation of muscle cell growth, proliferation or differentiation [6–8], contractile performance during exercise [9,10], calcium signaling [11], glucose uptake [12] and mitochondrial biogenesis [6]. However, excessive ROS/RNS levels unchecked by antioxidant defenses have negative impact on muscle contractile proteins, mitochondrial phospholipids, or DNA, and have been involved primarily or secondarily in the pathophysiology of muscle ageing (sarcopenia), of muscle fatigue [13] and of various muscu-
lar disorders like DMD, congenital myopathies with core lesions or malignant hyperthermia.

2.1. ROS/RNS and the antioxidant defense system

Most ROS/RNS are labile and their effects depend on multiple factors such as local environment and/or dose, making it difficult to define a threshold between physiological signaling molecules and damaging factors leading to pathological conditions. Superoxide radical O$_2^•$-, the “primary” ROS species produced mainly by mito-
chondria, is converted into “secondary” ROS, directly or through enzyme- or metal catalyzed processes [14] (see Fig. 1). Thus, O$_2^•$- is rapidly converted into hydrogen peroxide H$_2$O$_2$ by spontaneous dismutation or by superoxide dismutases (SOD). H$_2$O$_2$ can also be converted via the Fenton reaction in the presence of iron into the hydroxyl radical OH•. Compared to O$_2^•$- and OH•, the secondary ROS H$_2$O$_2$ has the lowest reactivity, the highest stability with its half-life values around 10$^{-5}$ seconds and the highest intracellular concentration, around 10$^{-6}$M [14–16]. O$_2^•$- can also react with nitric oxide (NO•) to generate peroxynitrite (ONOO$^-$) which is a ROS and a RNS species.

To maintain ROS/RNS at non deleterious level, skeletal muscle and myogenic cells are equipped with strong enzymatic and non-
zymatic antioxidant defenses, making them responsive to redox environment changes. Among enzymatic antioxidants, superox-
ide dismutase (SOD) catalyzes transformation of O$_2^•$- to H$_2$O$_2$. Three isoforms exist in mammals, SOD1 in the cytosol, SOD2 in the mitochondria and SOD3 which is predominantly extracellu-
lar. SOD1/3 (Cu/ZnSOD) contains copper and zinc and SOD2 (MnSOD) contains manganese in the active site [17]. In rats, exer-
cise training increases superoxide dismutase activities specifically in the soleus muscle, an oxidative muscle characterized by a high mitochondrial content [18]. Absence of SOD1 imposes elevated oxidative stress and accelerated age-dependent atrophy in skele-
tal muscle [19]. Catalytic decomposition of H$_2$O$_2$ into H$_2$O and oxygen can be mediated by several enzymes such as the heme-
dependent enzyme catalase (CAT), peroxiredoxins or glutathione peroxidases (GPx). The latter belong to the family of selenopro-
teins, characterized by the presence of at least one seleniumcysteine, an aminoacid that represents the biological form of selenium. In mammals, there are at least five selenocysteine-containing GPx (GPx1–4 and GPx–6) [20]. GPx reduce lipid hydroperoxides to their corresponding alcohols and reduce free H$_2$O$_2$ to H$_2$O, coupled to oxidation of reduced/monomeric glutathione (GSH, present in millimolar concentrations in muscle cells) to glutathione disul-
fluide (GSSG). Reduced glutathione is then recycled from GSSG by glutathione reductase, the GSH/GSSG ratio being a good indicator of the cellular redox status [20]. Like for SOD, GPx activities are increased in muscle after exercise, while catalase tends to remain unchanged [18]. Almost all the other members of the selenoprotein family whose function is characterized have antioxidant capaci-
ties. This includes selenoprotein N (SEPN1, encoded by the SEPN1 gene), whose mutations cause SEPN1-related myopathy (see below and [20]). Defects in the transcriptional machinery required for selenocystein cotranslational incorporation and leading to defi-
cency of several selenoproteins have recently been associated in 3 families with systemic diseases which include early-onset muscle weakness and, at least in one case, markers of systemic oxidative stress [21–23]. The antioxidant network also includes soluble non-
zymatic antioxidants like vitamin A, C and E, creatine, bilirubin and its derivative bilirubin and glutathione [4,17].

Cellular response to oxidative stress also involves at least four key factors involved in transcription regulation: nuclear fac-
tor kappa B (NF-κB), activator protein 1 (AP-1), nuclear factor (erythroid-derived 2)-like 2 related factor (known as NFE2L2 or NRF2) and peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) [4,6,17]. The promoter of each of the three main antioxidant enzymes SOD2, CAT and GPx contains, among other specific sites, a NF-κB and AP-1 consensus sequence [24]. Oxidative stress upregulates NF-κB activity, and AP-1 dimer formation depends on the redox environment [8]. Similarly, phys-
iological expression of the PPARα1 gene encoding Pgc-1α requires an optimal concentration of ROS in skeletal muscle [7,25], and NRF2 activity is determined by the redox status [8]. Under normal conditions, NRF2 is rapidly degraded by the ubiquitin-proteasome pathway through its interaction with Keap1 (a substrate adaptor protein of Cul3-based E3 ubiquitin ligase). Under oxidative condi-
tions, reactive cysteine residues in Keap1 are oxidized leading to NRF2 dissociation from Keap1 and activation. The free NRF2 then translocates to the nucleus and forms heterodimer with Maf pro-
teins. This complex activates the transcription of cytoprotective genes (HO-1, NQO1, PRDX1, GST, etc.) through binding to the antiox-
idant response element (ARE) present in their promoters [8,17]. One stress indicator identified in the OKD48 mouse is based on this Keap1-Nrf2 pathway (see below).

2.2. Redox homeostasis and myogenesis

A central characteristic of the early-onset myopathies is the presence of muscle weakness before birth or in the first years of life, suggesting a potential alteration of myogenesis as part of the disease mechanism. The establishment of muscle through myo-
genesis is necessary during embryogenesis as well as for muscle regeneration. Satellite cells are multipotent cells able to prolifer-
ate into new progenitors or to differentiate into skeletal muscle cells upon activation. Non-proliferative quiescent satellite cells, which are identified by their location between the sarcolemma and the basal lamina and by expression of the transcription fac-
tor Pax7, once activated will proliferate as myoblasts and then fuse to become myotubes [26]. A decreased content of satellite cells has been reported in several EOM, in particular in CM, with minicore lesions due to mutations of the satellite cell gene MEGF10 or of SEPN1 [27,28].

Although a clear relation between oxidative stress and loss of satellite cells has not been defined in early onset myopathies, trans-
forming growth factor beta-activated kinase 1 (TAK1) has been involved in stem cell homeostasis and skeletal muscle repair [29]. Ogura and collaborators found that TAK1-deficient satellite cells have high level of oxidative stress and undergo spontaneous cell death. More specifically, TAK1-mediated activation of JNK is essen-
tial to inhibit oxidative stress and to enhance differentiation of satellite cells.

Three main families of transcription factors are involved in the different steps of skeletal muscle progenitors proliferation and dif-
ferrentiation. Firstly, the myogenic regulatory factors (MRFs) MyoD, Myf5, Myf6 and Myogenin; secondly, the myocyte enhancer fac-
tors (MEFs), and lastly the serum response factor (SRF) [4]. There are controversial data about how the redox cell environment affects myogenesis, although oxidative stress appears to mostly reduce the efficiency of myogenic differentiation. ROS induces an important decrease of the intracellular GSH pool, which favors NF-κB activation thus contributing to reduce MyoD expression [30]. However, it is not well understood if this is a specific and direct effect of ROS or a secondary consequence of cell suffering [4]. Exposure of differen-
tiated primary human skeletal muscle cells and isolated rat skeletal muscle cells to H$_2$O$_2$ led to a profound increase in MEF2 DNA bind-
ing via pathways that are dependent of p38 mitogen-activated protein kinase (MAPK), Protein Kinase C, PI 3-kinase and AMPK [31]. The importance of these pathways for muscle function has been confirmed in Drosophila, in which over-expression of p38MAPK extends lifespan in a MnSOD-dependent manner through the transcrip-
tion factor MEF2, while inhibition of p38MAPK causes early
3. How can oxidative stress be measured in muscle?

Measuring redox status in a reliable and meaningful way in skeletal muscle is difficult, requiring specific expertise and tight control of many interfering factors. This has been one of the main limitations in identifying the role of ROS/RNS in muscle function and disease. ROS signaling is doubtless complex: it can be multiphasic (having an immediate and a delayed phase) and it depends on the reactive species involved, on their global and local intracellular concentration, the tissue and cell environment and the stress exposure timing (chronic vs acute). Also, it is highly sensitive to technical artifacts depending on oxidation of the samples upon exposure to ambient O₂. The “perfect” ROS indicator does not exist yet due to the long list of specific criteria required, which would include detection of one or several types of ROS species, high signal to background contrast, high dynamic range, non-toxicity, lack of sensitivity to environment parameters (like pH) or specific subcellular compartmentalization [39,40]. However, important progress in ROS quantification has been made the last decade, particularly due to major advances in synthetic and genetically encoded fluorescent based ROS detectors and in vivo imaging technology [16,39–42]. Table 1 summarizes the redox sensors commonly used and stresses their indications and limitations. As this is not primarily a methodological review, we will focus on summarising the main tools which are useful to measure reliably oxidative stress in muscle cells, and discuss their potential applications [16,39,41,43–53].

3.1. Analysis of carbonylated proteins

Oxidation of proteins can induce both structural and functional alterations which can be beneficial or harmful. Formation of carbonyl groups is the most frequent irreversible oxidative transformation, but no direct method exists to detect carbonylation as this is not associated with distinguishable fluorescence/spectrophotometric absorbance properties [41,46]. Global analysis is mainly based on derivation of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) followed by spectrophotometric analysis or immunodetection of DNPH:DNP (2,4-dinitrophenylhydrazone) [41,46]. More detailed analyses have been possible with proteomics and mass spectrometry approaches [54–56].

3.2. Small-molecule fluorescent probes

Small molecule fluorescent probes are also called chemical reporters. Hydroethidine (HEt) and its mitochondria-targeted variant Mito-HEt (known as MitoSOX-red) cross phospholipid bilayers and detect superoxide radicals [16,44]. However, detection is difficult due to short half-life of the latter and conversion by SOD [14–16]. Moreover, Mito-HEt accumulation in mitochondria depends on its concentration and on the mitochondrial membrane potential.

CM-H₂DCFDA, composed of a chloromethyl group, non-fluorescent 2',7'-dichlorodihydrofluorescein (H₂DCF) and diacetyl ester, optimized for cell permeability and reduced leakage, is oxidized by intracellular oxidants into fluorescent 2',7'-dichlorofluorescein (DCF) [16]. Thus, it is commonly used to measure global oxidant activity in living cells. CM-H₂DCFDA lacks specificity on the nature of the oxidants measured, as the probe has low reactivity toward superoxide radicals or hydrogen peroxide [45]. The formation of DCF should rather be considered as a general marker of cellular oxidant level [16,45].

C11-BODIPY³/⁸/⁵⁹¹ is used to measure lipid peroxidation. Upon oxidation, the red fluorescent reduced form of the probe is con-
verted into a green emitting oxidized form [57]. Because of its lack of lipid specificity which reflects peroxidation of all membranes, a compound derived from C11-BODIPY581/591 has been developed to target only mitochondria lipids [47]. This, termed MitoPerOx, contains the boron dipyrromethane difluoride (BODIPY) fluorophore conjugated to a triphenylphosphonium lipophilic cation to be selectively taken into mitochondria [47] and allows the measurement of mitochondrial lipid peroxidation.

3.3. Genetically encoded ROS detectors

Until recently, much of the data on ROS production have been obtained using isolated mitochondria, much less in intact cells and rarely at the organism level. In the last decade, promising in vivo systems for monitoring oxidative stress have been developed. Protein-based ROS reporters, first developed in single cell experiments, can target specific cell compartments since they contain targeting sequences that allow selective expression in nucleus, ER, mitochondrial matrix, mitochondria inner/outer membrane or plasma membrane [16,39,40]. Another advantage is that they can now be used for adenovirus-associated virus (AAV) mediated transduction or to generate transgenic animals. Indeed, genetically-encoded reporters of oxidative stress appear to be a highly convenient technology for noninvasive detection of oxidative stress in mice. Moreover, these tools will help to characterize bioactive molecules such as ROS for which it is important to determine where (subcellular origins), when (spatio-temporal distribution) and how (redox couple involved) they are produced [58]. One of the limitations of this approach is that each transgenic mouse can only bear a unique redox indicator.

Five main families of genetically encoded redox indicators have been developed (cf. Fig. 2):

1) Modified fluorescent proteins like the redox sensor roGFP, made by the introduction of two cysteine residues on the surface of GFP [59], have been applied in vivo using AAV technology [60] and also for generation of transgenic mice [61]. Formation of a reversible disulfide bond between the two cysteines upon oxidation induces an important decrease in the intrinsic fluorescence [62]. This green redox-sensitive (roGFP) as well as the yellow redox-sensitive YFP (rxYFP) based on the same principle [62] allows to monitor the thiol/disulfide ratio [40].

2) These redox-sensitive proteins have also been combined to the redox-active enzyme glutaredoxin-1 to create the chimeric sensors rxYFP-Grx1p developed by Bjornberg et al. [63] and Grx1-roGFP developed by Gutscher et al. [64]. In these, glutaredoxin is oxidized by the disulfur bond-forming oxidized cysteine residues in rxYFP or roGFP and reduced non-enzymatically by GSH. These chimeric sensors allow improved specificity and faster kinetics to measure the redox process, and their reactivity toward other oxidants remains low, giving almost absolute glutathione specificity and allowing to measure the GSH/GSSG ratio.

3) A superoxide biosensor has also been developed thanks to a circularly permuted yellow fluorescent protein targeted to the mitochondria (mt-cpYFP) [65]. In the transgenic mouse expressing the mt-cpYFP ROS biosensors, brief bursts of superoxide production also called superoxide flashes have been detected in skeletal muscle in basal conditions and are increased after glucose or insulin challenges [65]. These transient increases in fluorescence intensity (flashes) have some similarities with calcium sparks, that represent elemental Ca²⁺ release signals mediated by the ryanodine receptors in the sarcoplasmic reticulum. Further analyses are required to understand the crosstalk between mitochondria and sarcoplasmic Ca²⁺ release, which probably represents a critical step in the activation of stress response.

4) A genetically encoded sensor for H₂O₂ called HyPer has also been developed by the insertion of cpYFP into the H₂O₂-sensing regulatory domain of the E. coli transcription regulator OxyR
Despite similarities between HyPer and roGFp (fluorescence change upon formation of a disulfide bond between two cystein residues), the mechanisms of these reactions are different. In HyPer the cystein 199 is inaccessible to superoxide and GSSG because of its localization in the hydrophobic pocket of the regulatory domain of OxyR, but can be oxidized by the amphiphilic molecule of hydrogen peroxide [66]. So this probe allows targeted measuring of H₂O₂. Improved versions of HyPer (HyPer-2 and HyPer-3) have been developed and were used successfully in vivo in zebrafish [67].

In addition, there is at least one biosensor not based on a protein but on a stress-inducible promoter, and is dually regulated by induction at the transcriptional level, and by protein stabilisation at the post-translational level in the Keap1-Nrf2 pathway. The OKD48 transgenic mouse (Keap1-dependent Oxidative stress Detector, No-48) expresses ARE stress-inducible promoters controlling a Nrf2 fragment fused to luciferase [68]. The OKD48 oxidative stress detector responds in vivo specifically to oxidative stressors such as sodium arsenite (ASN) and dimethylmaleate, and is barely responsive to ER stressors, reducing agents and cell death induced by DNA damage [68]. In vivo, the OKD48 transgenic mouse shows high level of bioluminescence after ASN intraperitoneal injection or UV-A irradiation [68].

These tools have been used to monitor oxidative stress in vivo in skin [69], in dopaminergic neurons [70], in red cells [61] or in whole body [68] with different transgenic mouse models. Muscles have not been extensively studied so far, although these genetically encoded ROS biosensors can bring important progress in our understanding of oxidative stress-related diseases such as the early onset myopathies discussed below. Development of more sensitive oxidative stress detectors are required to allowed more precise ROS mapping. Indeed, most of the experiments above have been performed in anesthetized animals. Since exercise and muscle activity have a major impact in muscle redox homeostasis, monitoring oxidative stress in freely moving animals would give additional valuable information, although it would necessitate precise tracking of the mouse and short time exposure for the ROS detection.

3.4. Not to forget

Analysing oxidative stress, despite all the available and developing tools, remains delicate and not straightforward. Precautions and expertise are required to avoid over- or underestimation of oxidative stress, artifacts and non-reproducible data [46]. Each technique has its own limitations but, independently of the methods used, careful ROS/RNS titration requires extensive controls to exclude background and interfering signals. A number of variables has to be taken into account to measure oxidative stress flux, such as the ROS/RNS species type, concentration, cell differentiation status (i.e. myoblasts versus myotubes) or intracellular compartmentation [4,46], aside from the global environment (animal facility conditions, exercice, diet).

4. Oxidative stress is a primary pathophysiological defect and therapeutic target in SEPN1-related myopathy

SEPN1-related myopathy is so far and to our knowledge the unique inherited muscle disease due to a primary defect in redox homeostasis. SEPN1, a member of the selenocysteine-containing protein selenoprotein family, is a transmembrane protein localized to the endoplasmic reticulum (ER) membrane, involved in redox-modulated calcium homeostasis and in protection against oxidative stress [20]. The precise function of this protein is still unknown.
Sequence analysis revealed a potential EF-hand domain (calcium interacting site), a putative reductase catalytic site and several predicted glycosylated sites [71]. Different mutations in the SEPN1 gene have been found in CMS or CMDs including rigid spine muscular dystrophy [72,73], multimyoclonic disease [74], desmin-related myopathy with Mallory body-like inclusions [75] and congenital fiber-type disproportion [76]. All these are now considered to be part of the histopathological spectrum of presentations of the same, unique condition termed SEPN1-related myopathy. All patients with SEPN1 mutations have a severe weakness of neck and trunk muscles, leading to scoliosis, a variable degree of spinal rigidity and life-threatening respiratory insufficiency. In contrast, ambulation and limb strength are relatively preserved. The pathophysiological presentation of SEPN1-RM is quite large and heterogeneous, suggesting a polymorphic mechanism.

Surprisingly, in postnatal tissues SEPN1 is weakly expressed [77] but its expression is more important in all proliferating cells as well as in muscle and spinal cord during embryonic development [78]. SEPN1 is completely dispensable during mouse development as the Sepn1--/ mice is viable, and its growth and lifespan is normal [80]. Consistently, antisense morpholinos against sepn1 decreased zebrafish mobility without damaging muscle formation [78]. However, SEPN1 has been shown to be involved in muscle regeneration and satellite cell maintenance [28]. In fact, the level of Pax7+ satellite cells is reduced in uninjured adult muscle in Sepn1--/ mice and, after 2 cardiotoxin injections, muscle regeneration (efficient restoration of the muscle fibers) is impaired in this model [28]. The mechanism underlying the loss of satellite cells is still unknown and thus it is difficult to know if this is a cause or a consequence of muscle dystrophy. The SEPN1 gene promoter contains predicted sequences for NF-kB, an ER stress response element and an AP-1 consensus sequences [79], underlying a potential gene expression regulation by cell stress [20]. Along these lines, Sepn1-deficient mice displayed limited motility and body rigidity after physical exercise and stress conditions (forced swimming test) [80]. In addition, SEPN1-deficient myotubes from patients displayed increased basal oxidative activity and protein oxidation associated with reduced survival upon H2O2 treatment, all of which could be abrogated by pre-treating cells with the antioxidant N-acetylcysteine (NAC) [81]. These results are at the origin of the first clinical trial in this condition, using oral NAC (Pharmacological treatment of a rare genetic disease: N-acetylcysteine in selenoprotein N-related myopathy (SELNAC), ClinicalTrials.gov Identifier: NCT02550587). Moreover, SEPN1-deficient patient myotubes displayed an increased resting cytosolic calcium concentration and reduced sarcomplasmic reticulum Ca2+ load compatible with redox-mediated abnormalities in calcium homeostasis [81]. In line with these abnormalities, SEPN1 has been shown to interact with the redox-sensitive calcium release channel RyR1 (ryanodine receptor 1) [82] and the ER calcium import pump SERCA2 (sarcoplasmic reticulum calcium transport ATPase 2) [83]. Furthermore, SEPN1 levels matched those of an endoplasmic reticulum oxidoreductin 1 (ERO1) and have been proposed to counteract peroxide formation and SERCA oxidation by this ER protein thiol oxidase [83]. Remarkably, the ryanodine receptor 1 has been involved in the same cellular differentiation events as SEPN1 and is required for calcium fluxes in the zebrafish embryo [82]. Deficiency of either SEPN1 or RyR1 leads to muscular disease with abnormal sensitivity to redox conditions [82]. Similar histological (namely core lesions) and clinical signs can be observed in myopathies associated with SEPN1 or with RyR1 mutations. These data suggest that ER redox and calcium homeostasis are interlinked pathways essential in muscle function, and their deregulation by abnormalities of either SEPN1 or RyR1 is a major cause of congenital muscle disease.

5. Redox abnormalities associated with Ca2+ handling alterations or mitochondrial dysfunction as a secondary pathophysiological defect in RyR1- and COLVI-associated congenital muscle diseases

Redox imbalance, associated with dysfunctions of mitochondria or calcium handling, have been observed in muscle disorders due to primary defects in the COL6 and RyR1 genes, and is thus emerging as an interesting drug-targetable therapeutic target for these untreatable diseases. This is particularly relevant since mutations in RyR1 represent the most prevalent form of congenital myopathy (see review by Jungluth et al. in this issue), and COL6 mutations are also relatively common.

RyR1, also known as skeletal muscle calcium release channel, is an essential component of the excitation-contraction coupling apparatus. RyR1, which assembles as a homotetramer, is a key regulator of calcium homeostasis. Mutations in the RyR1 gene are responsible for core myopathies typically characterized by slowly or non-progressive proximal weakness involving hip and axial muscles, but also for the allelic pharmacogenetic condition malignant hyperthermia (MH) [84]. Abnormal excitation-contraction coupling secondary to impaired calcium release or to uncoupling is a key primary defect in RyR1-related myopathies [85] and review by Treves et al. in this issue). This is closely related with redox homeostasis, since oxidative and/or nitrosative stress can be regulated by calcium and conversely calcium homeostasis (via channels and transporters) can be a target of these stresses [86]. RyR1 is a paradigm of redox-sensor ion channel, as this receptor contains the highest number of reactive cysteines which are redox-sensitive and strongly involved in calcium handling [87]. One RyR1 monomer comprises around 5000 amino acids, including more than 300 cysteins half of which are, under basal conditions, in the reduced form [88,89]. Modifications of multiple key cysteine residues by S-glutathionylation, S-nitrosylation, S-nitrosothiolation and unspecified S-oxidation induce functional modulation of the RyR1 channel properties [89–92]. Redox remodeling of the RyR1 complex causes “leaky” channels in RyR1-MH and in muscle fatigue after exercise [93,94]. Consistently, the Y522S RyR1 K1 (a mouse model of MH) shows lipid peroxidation and reduced maximal developed force which was prevented by NAC treatment [93]. Interestingly, using the relatively relaxed zebrafish (ryr, a spontaneous mutant having defective expression of one of the ryr1 fish isoforms) and cultured myotubes from patients with RyR1-RM, excessive production of oxidants by mitochondria, a major Ca2+ dependent source of ROS, and diminished survival under oxidant conditions have been observed [95]. Moreover, improvement of muscle function and histology and restoration of the myotube phenotype were obtained with the use of the antioxidant NAC. These results are at the origin of the first clinical trial in RyR1-related myopathy using oral NAC (Antioxidant therapy in RyR1-related congenital myopathy, ClinicalTrials.gov identifier: NCT02362425).

On the other hand, mutations in the genes encoding the extracellular matrix protein Collagen VI (COL6A1, COL6A2 and COL6A3) cause a continuous spectrum of muscle diseases including four recognized clinical forms of collagen VI related myopathy: Ulrich Congenital Muscular Dystrophy (UCMD), Bethlem Myopathy (BM), congenital myosclerosis and limb-girdle muscular dystrophy [96]. While BM can have a later onset and displays a relatively mild and slowly progressive phenotype, UCMD is usually more severe with significant weakness of skeletal muscles in the first year of life, more rapid progression of symptoms and life-threatening respiratory failure [96,97]. In fibers from skeletal muscles of ColVI null mice (Col6a1--/-) as well as in myoblasts from UCMD patients, mitochondrial dysfunction and apoptosis have been observed [98]. These alterations could be prevented by cyclosporine A (CsA), a potent
inhibitor of the mitochondrial permeability transition pore (PTP) and a by a nonimmunosuppressive CsA, Debio25 [99]. However, in fibroblasts, the main producer of collagen VI [100], abnormal PTP opening was not found either using cells derived from UCMD patients or from other forms of muscular dystrophy [101]. More work is needed to confirm the previous findings and to decipher the relationship between PTP dysregulation and UCMD pathogenesis. Nevertheless, accumulation of dysfunctional mitochondria due to defective autophagic degradation has been observed in Col6a1-/– mice [102]. Consistently, a strong decrease in Beclin1 and Bnip3, two key players in the autophagic process, was also found in muscle biopsies from UCMD and BM patients. Remarkably, forced activation of autophagy by dietary, genetic and pharmacological agents restored myofiber homeostasis and improved the dystrophic phenotype of Col6a1-/– mice [103]. Recently, monoamine oxidase (MAO) which catalyzes the oxidative deamination of neurotransmitters generating H2O2, has been suggested as responsible for mitochondrial dysfunction in myoblasts from patients affected by Col6a1 myopathies [103]. Inhibition of MAO by pargyline, which prevents ROS formation, led to recovery from the dystrophic phenotype.

Interchange between calcium microdomains and mitochondria is essential for subcellular physiology. In RYR1- and Col6a1-related myopathies, oxidative stress due to defects in calcium homeostasis or to altered mitochondrial function appears to be a common signature of the muscular disease and therefore a potential therapeutic target.

6. Oxidative stress: a pivotal role in dystrophin deficiency?

Duchenne muscular dystrophy is a severe degenerative skeletal muscle disease due to mutations in the large DMD gene, encoding dystrophin and localized in the X chromosome. Its clinical presentation includes skeletal muscle weakness, and elevated CK levels typically detected around 3 years of age. The disease course is progressive, leading to gait loss and causing a premature death due to respiratory and cardiac failure [104,105]. Absence of dystrophin in skeletal myofibers induces sarcolemna damage after muscle contraction, leading to myofiber necrosis. Repeated cycles of damage/inflammation/regeneration lead to important muscle weakness with irreversible damage over time [106]. Various dystrophin-deficient animals have been developed but the main model remains the Mdx mouse, first described in 1984 [107] and whose muscle presents necrosis, inflammation and fibrosis, although to a much lesser degree than that found in DMD patients [108]. Increase of oxidative stress markers has suggested ROS as a central mediator of these lesions [106,108–110]. Various sources of ROS have been suspected, including the inflammatory cells (myeloperoxidase), NOX, XO, mitochondria or decoupling of NOS [106,108,110]. Nonetheless, NOX is suggested to be one of the most important sources of ROS in the development of the dystrophy pathophysiology. Indeed, activation of NOX2 by stretching in dystrophin deficient muscles induced ROS/RNS production that enhances Ca2+ influx and activates Src kinase, which in turn activates further NOX2 by p47phox phosphorylation [111].

Several antioxidants have proven effective in ameliorating the Mdx phenotype, reducing muscle damage and loss of muscle strength, inflammation, fibrosis and muscle oxidative stress. These include antioxidants which act as ROS scavengers, such as NAC [112], epigallocatechin-3-gallate (a polyphenol extracted from green tea extracts) [113] or Idebenone (benzoquinone related to coenzyme Q) [114], and also those which prevent ROS formation like the MAO inhibitor pargyline [115]. Sirt1 muscle-overexpression results in increased levels of utrophin, a functional analogue of dystrophin, and reverses the phenotype of the Mdx mice [116]. Similarly, resveratrol, which activates Sirt1, improves muscle function in the Mdx model by reducing inflammation [117]. Moreover, the iron chelator deferoxamine, which decreases NF-κB levels [118], or low-intensity training which restores SOD1 levels [119] were able to counteract oxidative stress in Mdx mice. Although to date DMD remains without a successful single treatment, these findings are at the origin of a clinical trial using epigallocatechin-3-gallate in DMD (Sunphenon Epigallocatechin-Gallate (EGCG) in Duchenne Muscular Dystrophy (SUNIMUD), ClinicalTrials.gov Identifier NCT01183767).

Very recently, gene editing technology CRISPR/cas9 (clustered regularly interspaced short palindromic repeats)/Cas9 was able to generate a pool of endogenously corrected myogenic precursors in Mdx mouse muscle [120–122]. Correction was not a precise genomic modification by HDR (homology-directed repair) because HDR does not occur in post-mitotic adult tissues such as heart and skeletal muscle, but by “myoediting” non-homologous end-joining (NHEJ). To introduce CRISPR/cas9 able to skip the mutant dystrophin exon in postnatal muscle in vivo, the authors used AAV-9, which displays high tropism for muscle [120–122]. Mdx-CRISPR corrected mice partially recovered dystrophin expression in skeletal myofibers and cardiac muscle with significant enhancement of muscle force. It will be of great interest to see if these corrections are able to impact oxidative stress in DMD models and patients and if combination with antioxidant therapy will further improve dystrophin deficiency.

7. Conclusion: pharmacological correction of oxidative stress as an emerging therapy

Significant advances have been made in the complexity of ROS signaling in a muscle context. Multiple lines of evidence are showing that primary or secondary oxidative stress is a significant pathogenic abnormality in early-onset myopathies. Because redox homeostasis is drug-targetable using drugs that are already in the market and approved for clinical use in patients, these findings and subsequent pre-clinical studies paved the way for the implementation of three pharmacological trials using antioxidants in SEPN1- and RYR1-related myopathies and in DMD. The result of these clinical trials might potentially lead to discovering the first pharmacological treatment for these often devastating inherited disorders which are currently untreatable, and provide important proof-of-concept regarding the efficiency of pathophysiology-based drug therapies in genetic conditions. Furthermore, understanding and treating oxidative stress in these rare muscle conditions may be useful as a model paradigm to understand and treat other conditions in which OxS is involved, including prevalent conditions with major socioeconomic impact such as insulin resistance, cachexia, obesity, sarcopenia or ageing.

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References

Wild-type ARMS knock-in mice had a lower age-related loss of motor function than control mice. These findings suggest that ARMS may play a role in presymptomatic motor neuron disease, possibly by reducing oxidative stress and preserving mitochondrial function. Future studies will be needed to determine the mechanism by which ARMS functions in motor neuron disease and to evaluate the potential of ARMS as a therapeutic target for this disease.
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