

Xenotopic expression of alternative oxidase (AOX) to study mechanisms of mitochondrial disease

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ABSTRACT

The mitochondrial respiratory chain or electron transport chain (ETC) facilitates redox reactions which ultimately lead to the reduction of oxygen to water (respiration). Energy released by this process is used to establish a proton electrochemical gradient which drives ATP formation (oxidative phosphorylation, OXPHOS). It also plays an important role in vital processes beyond ATP formation and cellular metabolism, such as heat production, redox and ion homeostasis. Dysfunction of the ETC can thus impair cellular and organismal viability and is thought to be the underlying cause of a heterogeneous group of so-called mitochondrial diseases. Plants, yeasts, and many lower organisms, but not insects and vertebrates, possess an enzymatic mechanism that confers resistance to respiratory stress conditions, *i.e.*, the alternative oxidase (AOX). Even in cells that naturally lack AOX, it is autonomously imported into the mitochondrial compartment upon xenotopic expression, where it refolds and becomes catalytically engaged when the cytochrome segment of the ETC is blocked. AOX was therefore proposed as a tool to study disease etiologies. To this end, AOX has been xenotopically expressed in mammalian cells and disease models of the fruit fly and mouse. Surprisingly, AOX showed remarkable rescue effects in some cases, whilst in others it had no effect or even exacerbated a condition. Here we summarize what has been learnt from the use of AOX in various disease models and discuss issues which still need to be addressed in order to understand the role of the ETC in health and disease.

1. Introduction

About 2 billion years ago, an endosymbiotic event occurred between a hydrogen-supplying archaeal host and a hydrogen-consuming α -proteobacterial partner adapted to life under anoxia eventually giving rise to the so-called “last eukaryotic common ancestor” (LECA) [1–3]. This endosymbiotic bond has remained to this day, resulting in mitochondria. Mitochondria are intracellular organelles surrounded by a double membrane, the outer membrane and inner membrane, which enclose the intermembrane space and mitochondrial matrix (Fig. 1).

The inner membrane harbors the mitochondrial respiratory chain or electron transport chain (ETC), which includes four high-molecular weight multienzyme complexes, namely NADH:ubiquinone oxidoreductase (cI), succinate:ubiquinone oxidoreductase (cII), ubiquinol:cytochrome *c* oxidoreductase or cytochrome *bc*₁ complex (cIII) and

cytochrome *c* oxidase (cIV, or COX) (Fig. 1) [4]. In addition, the ETC includes coenzyme Q (CoQ) and cytochrome *c*, two mobile redox partners which shuttle respiratory equivalents between cI and cIII, and cIII to cIV, respectively. Respiratory complexes may be further organized into supercomplexes [5–7], which constitute platforms for the assembly and maturation of the respiratory chain complexes [8]. The ETC oxidizes reducing equivalents, *i.e.*, NADH and FADH₂, extracted from nutrients through a series of redox reactions ultimately resulting in the reduction of oxygen to water at cIV [9]. This process is essential for redox homeostasis and for the activity of all upstream metabolic pathways including the tricarboxylic acid (TCA) cycle, pyrimidine nucleotides synthesis, fatty acid oxidation and many others [10].

A crucial aspect of the ETC is that electron flow through complexes cI, cIII, and cIV is coupled to the translocation of protons across the inner membrane. The resulting asymmetric distribution of positively charged

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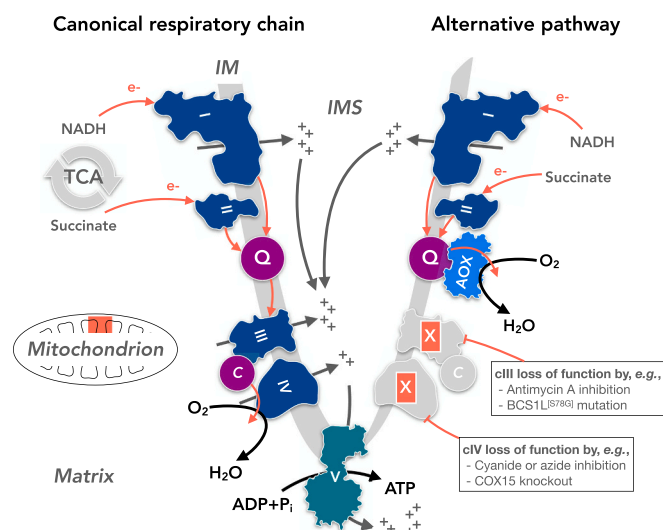


Fig. 1. The canonical respiratory chain and the alternative pathway.

Respiratory complexes I-IV (cI-cIV) of the canonical electron transport chain (ETC) are embedded in the mitochondrial inner membrane (IM). Complexes cI and cII oxidize NADH and succinate, respectively, and reduce the mitochondrial ubiquinone pool (Q) to ubiquinol. ETC complex cIII oxidizes ubiquinol and transfers electrons *via* cytochrome *c* (c) to cIV where oxygen is reduced to water. Respiratory substrates are replenished by the TCA cycle (tricarboxylic acid or Krebs cycle) located in the mitochondrial matrix. Electron flux is coupled to proton translocation across the IM into the intermembrane space (IMS) at cI, cIII and cIV thereby generating a proton electrochemical gradient (+) which forms the so-called protonmotive force (*pmf*) for ATP production at cV (or F_1F_0 -ATP synthase). Respiratory chain inhibitors such as antimycin A (inhibitor of cIII) or cyanide and azide (inhibitors of cIV) disrupt electron flux through the ETC which increases ubiquinol level and causes ROS production by reverse electron transport (RET). AOX restores electron flux, TCA activity, redox balance, heat production and prevents RET-mediated ROS production [107,108].

protons creates a proton electrochemical gradient ($\Delta\mu_{H^+}$) or protonmotive force (*pmf*, $\Delta p = \Delta\mu_{H^+}/F$), comprised of chemical (ΔpH) and electrical potential ($\Delta\psi$) components, which is the driving force for ATP synthesis at complex V (cV, or F_1F_0 -ATP synthase). This mechanistic coupling of oxygen consumption to ATP production is termed oxidative phosphorylation (OXPHOS) [11].

Mitochondria possess their own mtDNA [12] which in mammals is circular (approx. 16.5 kb) and encodes 13 core subunits of cI, cIII, cIV and cV, 22 tRNAs and 2 rRNAs [4]. The latter two components are required for translation of mtDNA-encoded proteins. In healthy individuals, mtDNA are all genetically identical, a condition referred to as homoplasmy. However, pathogenic mtDNA mutations, which may arise due to higher rate of mutation of mtDNA compared to nuclear DNA, may coexist with wildtype mtDNA (a condition termed heteroplasmy). When heteroplasmy exceeds a certain threshold, mitochondrial dysfunction may eventually arise leading to disease [13]. Notably, the rest of the more than 1000 proteins of the mitochondrial proteome are encoded by the nuclear genome and translated in the cytosol and imported into the organelle by an ATP consuming process mediated by highly sophisticated machineries [14]. In addition to being the site of OXPHOS, mitochondria also play key roles in other pathways such as mtDNA replication, transcription and translation, mitodynamics (*i.e.*, mitochondrial fusion and fission), reactive oxygen species (ROS) production and detoxification, apoptosis, biosynthesis of pyrimidines, to name but a few [4]. Given the biochemical complexity and the peculiarities of mitochondrial genetics it is not surprising that numerous mitochondrial dysfunctions have been identified as the underlying mechanism for a broad spectrum of pathological conditions [15].

Defects related to the mitochondrial OXPHOS system are referred to

as primary mitochondrial diseases (PMDs), which may arise from both pathogenic mutations in nuclear and/or mtDNA. Point mutations in mtDNA may be homoplasmic or heteroplasmic. Examples of homoplasmic mtDNA mutations include Leber's hereditary optic neuropathy (LHON) [16] whereas mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), neurogenic weakness, ataxia, and retinitis pigmentosa (NARP), and Leigh's syndrome (LS) are examples of heteroplasmic point mutations [16]. Rearrangements (single deletions or duplications) of mtDNA may sporadically occur and cause progressive external ophthalmoplegia (PEO), Kearns-Sayre syndrome (KSS), and Pearson's syndrome [16]. In contrast, large-scale rearrangements are invariably heteroplasmic, and are not transmitted through the germ line. Secondary mitochondrial disorders, *i.e.*, conditions that not arise from mutations directly impacting on the respiratory chain, have been associated with numerous common diseases, including cancer [17–19], cardiac contractile failure [20,21], chronic obstructive pulmonary disease (COPD) [22], and obesity and diabetes mellitus [23,24]. Regardless of the underlying cause (primary or secondary), there is currently no cure for OXPHOS defects [25].

One attractive possibility which recently emerged is the use of the alternative oxidase (AOX) to bypass genetic and non-genetic cIII- and cIV-related defects [26–30]. AOX incorporation would, in principle, facilitate the restoration of electron flux and metabolic flexibility. Indeed, it has been shown to promote CoQ redox-cycling and enhance the activity of dihydroorotate dehydrogenase, a mitochondrial enzyme with a critical role in the *de novo* synthesis of pyrimidine [31,32]. The general viability of such an approach has been tested in mammalian cells [28,33], *Drosophila melanogaster* [34–38], and a number of mouse models [39–44]. In this review we critically assess what has been learnt about the role of the respiratory chain in health and disease through the expression of AOX and discuss which areas require further experimental clarification.

2. The alternative oxidase (AOX), a naturally evolved rescue mechanism

AOX, a ubiquinol-oxidoreductase, belongs to the diiron carboxylate superfamily which includes methane monooxygenase, ribonucleotide reductase R2 subunit, fatty-acyl desaturase and rubrerythrin [45]. AOXs are present in yeasts, plants and lower eukaryotes as alternative components of the respiratory chain which oxidize reduced CoQ (ubiquinol) and reduce molecular oxygen to water thereby bypassing the cytochrome segment of the respiratory chain, *i.e.*, cIII to cIV (Fig. 1) [45]. Unlike canonical respiratory complexes, AOXs are single polypeptides and do not couple electron flux to proton translocation and thus do not *per se* contribute to the *pmf*. Instead AOXs dissipate free energy as heat. AOXs are encoded by the nuclear genome and the lack of prosthetic groups such as heme renders them insensitive to the common OXPHOS inhibitors such as cyanide, hydrogen sulfide, azide, nitric oxide, carbon monoxide and others [42,46–48]. Of particular importance is that AOXs are only catalytically engaged under specific conditions and, generally, do not compete with cIII [49] for reducing equivalents. In most tissues catalytic engagement of the AOX occurs only when the reduction level of the CoQ pool has achieved a threshold value of approximately 35–40%. This was originally demonstrated to be the case in plants [50] and later confirmed in mice following xenotopic AOX expression [41]. The inability to outcompete cIII under non-stressed conditions is due to AOX requiring a much higher K_m for ubiquinol than cIII [49,51].

In addition to these universal features, different AOXs from varying species have been shown to be activated by specific metabolites, such as pyruvate and other organic acids, which may accumulate when the respiratory chain is impaired [52–54]. Structural analyses also indicated that the catalytic engagement of AOXs rely on specific features within the hydrophobic channel which connects the hydrophobic environment of the membrane with the active site thereby dictating the interaction

with individual metabolites [55]. Another important aspect is that AOX normally exist as a dimer, which in plants is activated by breakage of the inter-subunit disulfide bridge operated by thioredoxins suggesting a role in redox stress [56]. Finally, in its original hosts, some AOXs have been shown to be transcriptionally upregulated by several metabolic stresses including drought, heat, cold, salt and pathogen invasion [57,58].

Much information about the biological function of AOX has been gleaned from studies in plants particularly with respect to its role in guarding against excessive ROS production during the daylight. High photosynthetic ATP production tends to limit respiratory electron flux through the ETC during daylight and consequently increases mitochondrial ROS production. Engagement of AOX decreases ROS and hence enables TCA cycle metabolism and redox homeostasis to continue under these conditions [58,59]. Furthermore, it is known that AOXs play important roles in thermogenesis, due to the dissipation of redox energy as heat, in aging, since expression of AOXs restores the NAD^+/NADH balance thereby reactivating primary electron flow to support growth, and in pathogenicity through increasing metabolic flexibility resistance to many forms of metabolic stress [58,60,61]. Preventing ROS damage has been proposed as a major function of AOXs since engagement of the oxidase only occurs at high ubiquinol levels thereby decreasing ROS production. It should be noted that ubiquinol itself is a source of ROS via the passage of single electrons to oxygen both at cIII and at cI mediated by reversed electron transport (RET) [62]. The notion that AOXs are a naturally evolved rescue mechanism of vital significance is reinforced by the finding that these enzymes have been found in all kingdoms of life, including bacteria and in some metazoans, arthropods, and chordates. Conversely, AOXs appear to have been lost during evolution in vertebrates, most insects, and molluscs. Both the reason for this loss and the biological significance of AOXs in the animals in which they have been preserved are poorly understood [30,57]. One reason for gene loss could be that since AOXs decrease ROS production they could interfere with the ROS signaling pathways with potentially harmful consequences [41,44,63]. It is equally possible that AOX fosters tumorigenesis through changes in CoQ redox-cycling and activity of dihydroorotate dehydrogenase [31,32] although data from our models expressing AOX do not support this notion.

3. AOXs to bypass to challenge disease paradigms and alternatives to cure

3.1. AOX use in cells with OXPHOS defects

The general features of AOXs outlined above prompted the proposal to use xenotopic expression of AOX in species which have lost the enzyme during evolution [29]. The first use of AOX to by-pass a block of the respiratory chain induced by cyanide was investigated in 2006 [26]. In this study, AOX from the tunicate *Ciona intestinalis* (CiAOX) was expressed in human cells thereby conferring a markedly increased cyanide resistance. One of the reasons why CiAOX was originally favored over others was that it is the closest known relative to vertebrates that still expresses a functional enzyme [64]. Notably, and in keeping with data on protein biochemistry, CiAOX itself had no obvious effect on cellular growth. Another important finding was that AOX activation upon antimycin A inhibition of cIII dramatically decreased ROS production through re-oxidation of ubiquinol.

Furthermore, it was demonstrated that CiAOX was activated by pyruvate, comparable to that described for plant AOXs, thus making them amenable to metabolic control [26]. Later, it was shown that AOX can rescue a detrimental metabolic phenotype in COX deficient human fibroblasts [28]. Following the same rationale, AOX from another source was used, i.e., the filamentous fungus *Emericella nidulans* [65]. In this study, the expression of AOX in $\rho 0$ cells, i.e., cells lacking mtDNA, was able to restore the capacity to oxidize ubiquinol thus restoring uridine and pyruvate auxotrophy. These authors demonstrated that AOX could reactivate several pathways, including pyrimidine synthesis, the TCA

cycle, glycerol phosphate shuttle and β -oxidation, all of which are stalled when ubiquinol levels are high as a result of a faulty respiratory chain.

3.2. AOX use in flies with OXPHOS defects

In addition to the proof-of-concept studies in mammalian cells, a series of studies were devised in which CiAOX was expressed in a variety of model organisms and disease models including *Drosophila melanogaster* [34–37,66–68] and mice [39–44,69–71]. It was initially confirmed that CiAOX protein was correctly imported into mitochondria using its intrinsic N-terminal mitochondrial targeting peptide and furthermore its expression in isolated mitochondria and cells conferred resistance to OXPHOS inhibitors such as antimycin A, azide, and cyanide. Most importantly, however, AOX expression in both in *Drosophila* and mice did not induce a detrimental phenotype at least under non-stressed conditions [34,40]. It should be noted, however, that a more recent study indicated that AOX expression does reduce male reproductive fitness in the fly [72].

Apart from this result, a particular important observation was the finding that AOX protected against physiological stresses related to mitochondrial dysfunction comparable to those observed in human pathologies. Although AOX was unable to rescue a null mutation in COXVIC subunit of cIV in flies [34], it did rescue, at least in part, cIV defects due to partial knockdown of COX4, COX5A, COX5B, COXVIC, COX7A and SURF1 [36]. In addition, AOX alleviated the effects of a heteroplasmic mtDNA mutation affecting cIV in the fly [73] and improved the phenotype of fly models of both Parkinson's and Alzheimer's diseases [35,37]. Understanding the underlying mechanisms of effects in the different neurodegenerative conditions does, however, warrant further investigation. Conversely, AOX was unable to correct the phenotype of the so-called technical knockout (tko) fly, a *Drosophila* model of mitochondrial deafness [67,74]. This model of mitochondrial translational defects, characterized by developmental delay and mechanically induced seizures, is associated with a decreased activity of all four OXPHOS complexes which are dependent on mitochondrial translation [67]. AOX also failed to rescue mutants in the mtDNA helicase (Twinkle) and DNA polymerase γ (Poly) [75] however it did rescue the phenotype of a fly model carrying a point mutation in *stress-sensitive B1* (*sesB*¹), the fly orthologue of the adenine nuclear translocase (ANT) [68].

3.3. AOX use in mice with genetic OXPHOS defects

Despite the relative heterogeneity of outcomes, data from fruit fly studies have provided the groundwork for further studies with AOX in the mammalian system to eventually test its potential application in human disease. In several landmark-studies, mitochondrial disease mouse models were crossed with an AOX transgenic mouse, obtained by knock-in of CiAOX into the *Rosa26* locus (*AOX*^{Rosa26}) (Fig. 2) [40]. The *AOX*^{Rosa26} strain was first crossed with a muscle-specific knockout for *Cox15* (COX15-KO), an enzyme involved in the biosynthesis of the prosthetic group of cIV heme A [41]. COX15-KO mice are characterized by a severe mitochondrial myopathy with profound cIV deficiency and a markedly decreased lifespan [76]. Surprisingly, the double mutants showed a worsening of the myopathic phenotype (Fig. 3). Initially, this result appeared highly counterintuitive since AOX restored mitochondrial deficiencies in COX15-mutant cells in previous studies [27,28]. The unexpected failure to rescue the COX deficiency *in vivo*, we suggest, is related to the disruption of ROS signaling which is necessary for compensatory mitochondrial biogenesis and recruitment of satellite cells (Fig. 3) [41].

A suitable, albeit simplistic, explanation on how activation of mitochondrial biogenesis can rescue cell function even if mitochondria remain genetically impaired is that the increase in mitochondrial content may result in increased ATP production. In line with this idea, in

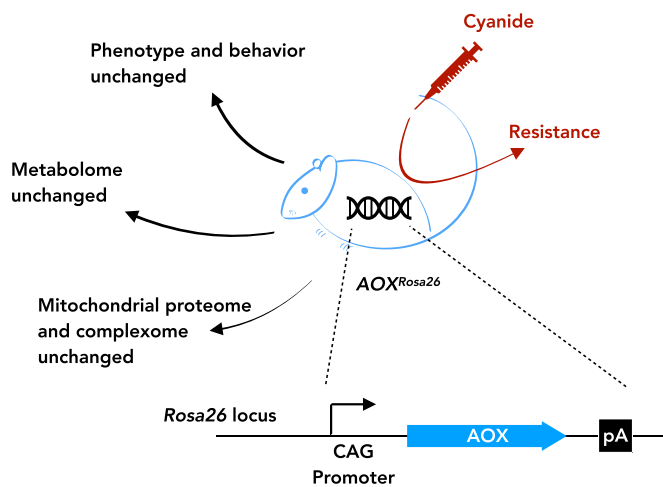


Fig. 2. Generation and characterization of the AOX^{Rosa26} strain. The coding sequence of AOX from *Ciona intestinalis* was integrated into the *Rosa26* locus. A CAG promoter controls ubiquitous AOX expression. AOX^{Rosa26} mice and wildtype littermates are remarkably similar with respect to metabolic, behavioral, morphological, immunological, cardiac, and neurological parameters. Yet, AOX^{Rosa26} mice are cyanide resistant [40].

patients with mitochondrial disease, muscle fibers often observed to possess ragged red fibers [77,78], a sign of tentative mitochondrial biogenesis. Alternatively, it is plausible that nuclei obtained from satellite cell fusion can transitionally restore mitochondrial functions until Cre-mediated ablation of *Cox15* occurs. In support of this notion, we observed altered stress responses, expression of mitokines, and a decreased median lifespan of 60 days in AOX-expressing COX15-KO mice compared with 150 days for COX15-KO alone. This work has thus highlighted the fact that mitochondrial ROS signaling may act as a

hormetic signal [79,80] and questioned the benefits of using antioxidants in the treatment of mitochondrial diseases. Indeed, in this COX15-KO model, mitochondrial ROS generation was likely mediated by RET, although succinate levels, which should be increased to promote RET, were not directly assessed. Our results suggest that ROS may trigger compensatory processes which ultimately determine adaptive organ remodeling, such as increased recruitment of satellite cells [41,44,81]. It is still unclear, however, if ROS is the sole mediator of the observed organ remodeling or whether succinate and/or other metabolic intermediates or changes in the redox balance are required to facilitate adaptation. The extent to which AOX affects such mediators is currently unknown.

In a further study, we crossed AOX^{Rosa26} mice with a strain carrying a *Bcs1*^{p.S78G} mutation [82] reproducing a recurrent mutation found in patients with the so-called GRACILE syndrome [83]. This condition is one of several syndromes associated with mutations in this gene characterized by multiple visceral symptoms and premature death [84]. *BCS1L* encodes for a AAA+ protease involved in the insertion of the Rieske protein and of the supernumerary subunit UQCRI1 into cIII [85]. GRACILE mutant mice expressing AOX were viable, showed an extended lifespan (from 210 to 590 days) potentially due to the prevention of a lethal cardiomyopathy. AOX also prevented renal tubular atrophy and cerebral astrogliosis, but interestingly not liver disease, growth restriction, or lipodystrophy. The inability of AOX to rescue the liver phenotype was particularly surprising since the mouse model was originally established and described as a model of mitochondrial hepatopathy [83]. The discrepancy with previous results may be attributed to the fact that the original model was based on a mixture of mutations in the nuclear genome and in mtDNA, thereby incorrectly attributing the results to the *Bcs1*^{p.S78G} mutation alone [86]. It is also conceivable that the cIII defect, specifically in the liver of true *Bcs1*^{p.S78G} mice, may not be strong enough to catalytically engage AOX resulting in cardiomyopathy rather than hepatopathy as the primary cause of disease. It should also

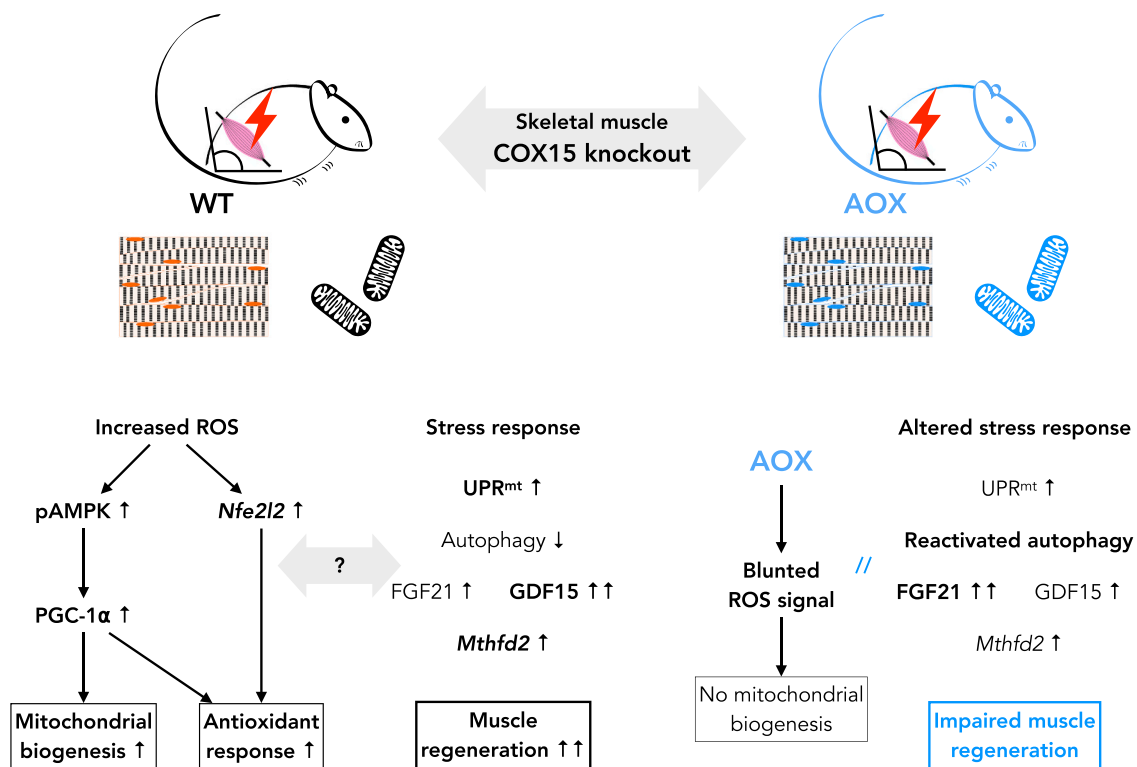


Fig. 3. AOX expression in a mouse myopathy model induced by skeletal muscle-specific ablation of COX15. AOX expression exacerbates a mitochondrial myopathy. Arrows correspond to the degree of change in expression of markers, with one arrow indicating a moderate increase and two arrows indicating a strong increase [41].

be noted that, in contrast to the results observed with the COX15-KO/AOX mice, ROS produced *via* RET did not appear to play an essential role in the etiology as they were similarly increased in all groups, including *Bcs1*^{p.S78G} and *Bcs1*^{p.S78G}/AOX. Furthermore, succinate levels did not increase in either heart muscle or liver [82]. Such data reinforce the importance of thoroughly characterizing disease models, including the use of *in vivo* tools such as AOX, to highlight different etiologies within the heterogeneous group of mitochondrial diseases.

3.4. AOX use in mouse models of cardiac dysfunction

The requirement of a threshold value of mitochondrial ROS as an essential regulator of adaptive remodeling is further supported by two other studies. In one study, the ability of AOX expression to decrease cardiac ischemia-reperfusion injury was tested since lowering mitochondrial ROS has previously been proposed to be a crucial factor in the exertion of cardioprotective effects [44]. This assumption was mainly based on studies using different antioxidants, including a mitochondrially-targeted CoQ₁₀ (MitoQ) [87–89], mitochondrially-targeted peptides developed by Szeto and Schiller [90], or small molecules which have previously been identified to suppress ROS production at cI (S1QELs) [91]. Unexpectedly, AOX^{Rosa26} mice showed no cardioprotective effects with respect to infarct size or contractile (dys) function after *in situ* ligation and reperfusion of the left anterior descending artery (LAD), or anoxia and reoxygenation in the isolated and perfused (Langendorff) heart (Fig. 4) [44]. The lack of cardioprotection was particularly surprising, considering that AOX does prevent mitochondrial ROS production in post-anoxic isolated heart mitochondria [44]. Even more confusing, however, was our observation that 9 weeks after ischemia and reperfusion cardiac contractility was significantly decreased in AOX-expressing hearts but not wildtype [44]. Analysis of gene and protein expression patterns revealed an increase in gene expression of the hypertrophy marker atrial natriuretic peptide (*Anp*) in wildtype tissue only while its expression was blunted in the presence of AOX. Furthermore, we found a massive increase in proteins involved in extracellular matrix remodeling such as periostin specifically in the post-ischemic tissue of AOX^{Rosa26} mice (Fig. 4) [44].

AOX^{Rosa26} mice have also been used in a mouse model of endotoxemia where the mice and isolated macrophages were treated with lipopolysaccharides (LPS) [69]. LPS treatment induced a pro-inflammatory phenotype shift of macrophages characterized by glycolytic metabolism, high mitochondrial membrane potential, succinate oxidation, RET-mediated ROS production, hypoxia-inducible factor 1 α stabilization and production of proinflammatory cytokines such as interleukin 1 β . AOX treatment reversed the pro-inflammatory phenotype shift of macrophages and restored normal respiratory chain activity, through reduction of RET-mediated ROS production thereby markedly improving survival of mice treated with LPS. Such a result suggests a key role of the ETC in the control of the immune response during endotoxemia.

Since AOX can prevent a pro-inflammatory phenotype shift in macrophages and the development of a cIII-based cardiomyopathy, we proposed that it may also prevent the development of inflammatory cardiomyopathy. Such inflammatory cardiomyopathy was induced by cardiomyocyte-specific expression of the cytokine monocyte chemoattractant protein (MCP-1) (*aMHC-Mcp1*^{tg}) [92]. The *aMHC-Mcp1*^{tg} mouse model is characterized by infiltration of the heart by macrophages, massive remodeling of the extracellular matrix and a gradual but steady development of contractile failure. When AOX^{Rosa26} mice were bred with *aMHC-Mcp1*^{tg} mice the ubiquitous presence of AOX surprisingly did not extend lifespan despite an observed a minor delay of cardiac functional deterioration and cardiac and metabolic remodeling [43].

We hypothesized that different cells in the heart might have different, possibly opposing, effects on disease progression, which would ultimately explain the unexpected outcome. To test this notion, we bred *aMHC-Mcp1*^{tg} mice [92–94] with a conditional AOX mouse strain (*SNAPf-AOX*^{Rosa26}) [43] and activated AOX expression in cardiomyocytes only. Surprisingly, cardiomyocyte-specific AOX expression exacerbated the phenotype and lead to a dramatically shortened lifespan [43]. The finding that ubiquitously expressed AOX was unable to restore contractile function, as in the GRACILE model, whereas it exacerbated the condition when expressed only in cardiomyocytes may indicate that MCP1-induced inflammatory cardiomyopathy is not primarily caused by metabolic disturbances in cardiomyocytes. Yet, the worsening of the

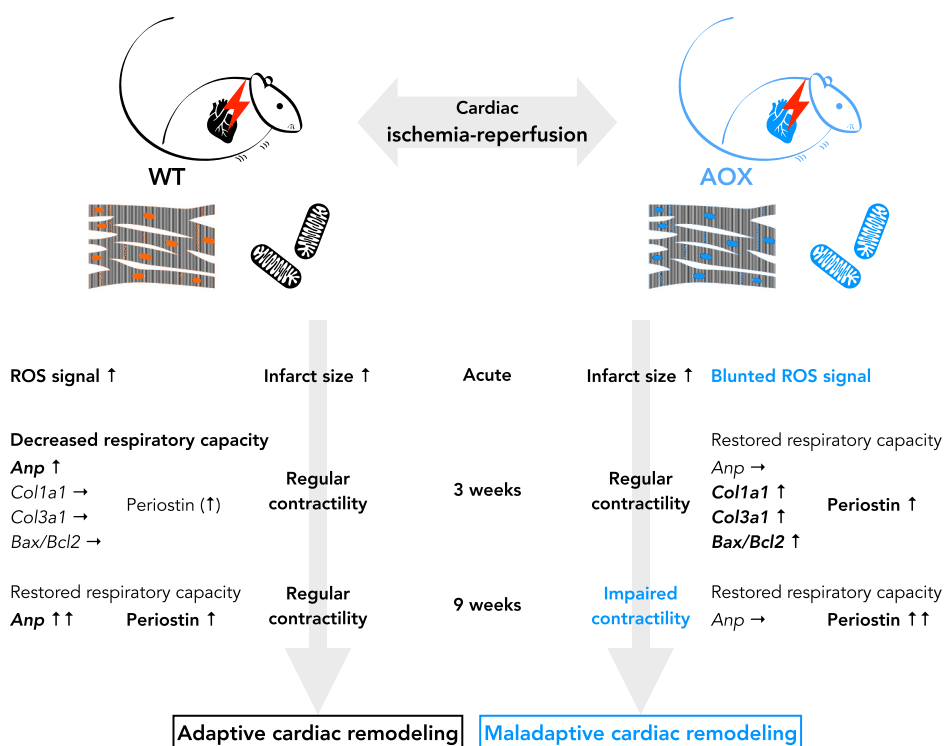


Fig. 4. Differential cardiac regenerative response after ischemia-reperfusion (IR). Expression of AOX prevents expression of the classic hypertrophy marker *Anp*, decreases mitochondrial ROS production, restores mitochondrial respiratory capacity, but worsens cardiac contractility in the long term (9 weeks after ischemia-reperfusion). Arrows correspond to the degree of induction of the depicted marker, with one arrow indicating a moderate increase and two arrows indicating a strong increase [44].

phenotype and shortened lifespan with cardiomyocyte-specific AOX expression clearly argues for its catalytic engagement. This deleterious effect, however, may be masked when AOX is ubiquitously expressed in all cardiac cells including the macrophages that infiltrate MCP1-expressing hearts in large numbers. Exactly how mitochondrial respiratory chain function affects the interplay between contractile and noncontractile cells, however, remains dubious.

3.5. AOX use in mouse models of lung disease

The role of the mitochondrial respiratory chain was also tested in pathologies of non-contractile organs, in particular the lung [42,71]. Initially it was tested if AOX could prevent the development of cigarette smoke-induced emphysema [42]. The rationale for this study was that cigarette smoke contains amongst the more than 5000 often toxic and oncogenic compounds [95] at least two prominent inhibitors of cIV, *i.e.*, carbon monoxide and hydrogen cyanide. Indeed, xenotopic expression of CiAOX in the mouse did mitigate the development of emphysema following cigarette smoke exposure, a condition characterized by thinning and rupturing of the inner walls of alveoli thereby decreasing the surface area for gas exchange (Fig. 5). Furthermore, AOX decreased mitochondrial ROS production and increased cell viability upon treatment of cultured fibroblasts with cigarette smoke condensate, a surrogate substance to test cigarette smoke toxicity *in vitro* (Fig. 5) [42].

It should be noted, however, that the use of AOX^{Rosa26} mice also resulted in some unexpected outcomes. For instance, it was previously proposed that the initial event priming the mammalian lung for adverse remodeling upon cigarette smoke exposure is the infiltration of neutrophils and macrophages. Such an infiltration event, however, was seen in both cigarette smoke-exposed wildtype and AOX^{Rosa26} mice to a strikingly similar extent [42]. Importantly, the pro-inflammatory cytokine signature measured in the fluid obtained by bronchoalveolar lavage (BAL) was not altered by AOX expression (Fig. 5). In particular, BAL was tested for the presence of (i) KC, a mouse-specific functional homolog of human IL-8 typically correlated COPD severity [96]; (ii) MIP2, which together with KC belongs to the CXC class of chemokines which stimulate chemotaxis of neutrophils [97,98]; (iii) IL-6, an inflammatory marker often elevated during COPD; and (iv) matrix metalloproteinase 9 (MMP-9), which is known to contribute to tissue remodeling during disease exacerbation [99]. All these markers were comparably and significantly upregulated in both AOX^{Rosa26} and wildtype littermates [42] suggesting that either the acute phase of tissue response does not relate to an impairment of the ETC or that AOX is not catalytically engaged in certain subsets of lung cells.

AOX^{Rosa26} mice were also used to test the involvement of the mitochondrial respiratory chain in pulmonary oxygen sensing [71]. Acute hypoxic pulmonary vasoconstriction (HPV) is a vital response

mechanism of the pulmonary vasculature that diverts blood flow away from poorly ventilated to well-ventilated lung alveoli to ultimately optimize arterial oxygenation [100,101]. This is a process that is found to depend on a central oxygen sensor within the mitochondrial respiratory chain. Hypoxia has been shown to cause hyperpolarization of the mitochondrial membrane, concomitant with an increase in mitochondrial ROS production, which subsequently inhibits cellular potassium channels and causes cell membrane depolarization. Finally, membrane depolarization then activates voltage-gated calcium channels, ultimately leading to HPV [101–104]. The use of AOX^{Rosa26} mice confirmed that electron flux through the mitochondrial respiratory chain is essential and at the top level in the hierarchy of oxygen sensing and signaling in the pulmonary vasculature [71]. In contrast to acute oxygen sensing, the role of mitochondria in chronic oxygen sensing appears to be unrelated to mitochondrial respiratory functions and/or mitochondrial ROS production. Indeed, chronic exposure of AOX^{Rosa26} mice and wildtype littermates to hypoxia revealed no differences between the two [71]. As noted above, the use of AOX to test disease paradigms *in vivo* was essential to unmask different molecular mechanisms underlying conditions that are seemingly only triggered by similar events.

4. What can we learn from xenotopic AOX expression?

The promising first effects observed in mammalian cells [26,28] including the metabolic phenotype of $\rho 0$ cells [65] partially contrast with later findings collected in some model organisms in which some support the promotion of AOX-based therapies [42,69,82] whilst others do not [41,43]. This is presumably related to the increased complexity in organisms with systemic signals that come into play during adaptive organ remodeling but are absent in cell culture. The results also highlighted another important limitation of xenotopic AOX expression, *i.e.*, AOX failed to ameliorate the phenotypes due to mtDNA maintenance and/or expression, as demonstrated by the lack of rescue in fly mutants defective for Poly, TWINKLE, or in the translation machinery. Thus, AOX seems to fail under conditions when more respiratory complexes than those of the cytochrome segment are involved, likely because of cI deficiency associated with these conditions.

Similarly, in isolated defects in the OXPHOS system, AOX expression also displayed some limitations as it was unable to improve the phenotype of complete knockouts of subunits of cIV whereas it effectively corrected milder defects associated with knockdown of some subunits in a tissue-specific manner. Such conflicting results suggest either that AOX facilitates very different effects depending on the tissue or that it is generally more effective in milder pathologies. This seems to be supported by the findings observed in the COX15-KO mouse myopathy model where AOX expression exacerbated the phenotype [41] whereas its expression in a global cIII mutation in the GRACILE model

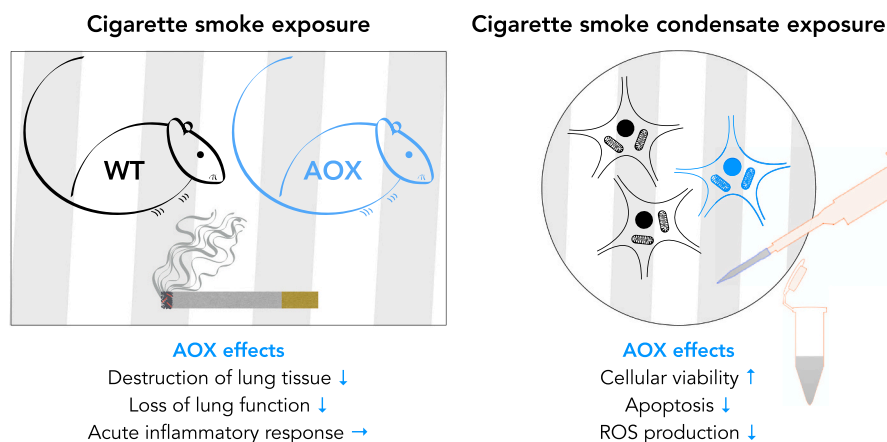


Fig. 5. AOX in cigarette smoke-induced organ and cell damage.

Mice chronically (9 months) exposed to cigarettes smoke show lung damage/remodeling which is attenuated by AOX expression. Fibroblasts cultured in the presence of cigarette smoke condensate (CSC) show improved viability, decreased expression of apoptosis markers, and decreased production of ROS when AOX is expressed [42].

largely resulted in improvement [82]. These considerations highlight, on the one hand, the complexity of mitochondrial disorders but on the other possibly reflect our limited understanding of both the pathogenetic mechanisms underlying mitochondrial defects and the mechanisms by which AOX becomes catalytically engaged.

It should be stressed that the vast majority of studies to date have been carried out using *Ci*AOX, which has some peculiar features not present in other AOXs. In fact, although the AOX family contains a highly conserved active site, striking differences are seen in ubiquinol oxidation activities. Of those AOXs which have been purified to date, AOX from *Trypanosoma brucei* (TAO) has the highest V_{\max} ($601 \pm 27.0 \mu\text{mol}/\text{min}/\text{mg}$) [49], whilst *Ci*AOX possesses a much lower V_{\max} ($\sim 3 \mu\text{mol}/\text{min}/\text{mg}$) [55]. Another important observation is that although various small-molecules (such as pyruvate or α -keto acids) seem to act as activators of plant AOXs [54], purified AOX proteins are insensitive to pyruvate, and the structural models across the AOX family indicate that the features of the hydrophobic cavity itself may well regulate the access to specific metabolites [55]. This implies that the presence or accumulation of cell-specific metabolites may regulate AOX activity in a tissue-specific way. For instance, the residues lining the tunnel leading to the active site are primarily hydrophobic in TAO, with hydrophilic residues facing the active site. This arrangement facilitates the correct orientation of ubiquinol [105]. In other AOXs, including *Ci*AOX, the cavity is smaller thus restricting the access of CoQ to the active site [55].

It should also be noted that *C. intestinalis* is adapted to live in relatively cold water around 20 °C or less, whilst *T. brucei* is adapted to the human blood temperature, where its role is to maintain a high glycolytic rate even in the absence of a functional respiratory chain. All the above considerations suggest that *Ci*AOX may not necessarily be the best choice to develop therapies for all mitochondrial and other human diseases and AOXs from other organisms may be better suited for different pathologies. Choosing an AOX based on its high catalytic activity alone, however, may also have its drawbacks since it could have dramatic side-effects such as reducing ROS production to below the basal level required for signaling. Overall, such considerations warrant further research to establish if AOX from alternative sources other than *C. intestinalis*, such as the trypanosomal AOX, may be more effective and thus more suitable for the treatment of human pathologies. A possible way to solve this conundrum may also lie in the development of synthetically engineered AOX proteins, tailored to specific applications, for instance, through increasing or decreasing sensitivity to specific metabolites or inhibitors/activators [55].

Another relevant aspect is related to the use of transgenic mice expressing AOX throughout development. In fact, xenotopic expression of AOX upon genomic integration may rather provide insights into disease developments and/or the prevention of a disease but not reflect a therapeutic value. The latter should be investigated by transient expression of AOX in different disease models. Such transient expression can be achieved by using AAV-based gene therapies, the therapeutic use of recombinant proteins, or mRNA species. The appropriate formulation for such deliveries is critical and to date largely unexplored. The applicability of AOX treatments by using codon-optimized AOX-encoding chemically modified RNA species (cmRNA), however, has been tested in cultured cells, including immortalized mouse embryonic fibroblasts (iMEFs), human lung carcinoma cells (A549) and primary mouse pulmonary arterial smooth muscle cells (PASMCs) [106]. Given the explosion of RNA-based technologies in recent years, these results open the realistic possibility of using RNA species for transient AOX expression. Such an approach could be exploited at least in some conditions such as acute intoxications or life-threatening inflammatory conditions. However, these exciting data warrant further investigations to assess their applicability *in vivo*.

Declaration of competing interest

The authors declare the following financial interests/personal

relationships which may be considered as potential competing interests:

Carlo Viscomi reports financial support was provided by Telethon Foundation. Carlo Viscomi reports financial support was provided by AFM-Telethon. Carlo Viscomi reports financial support was provided by Associazione Luigi Comini Onlus. Anthony L. Moore reports financial support was provided by Biotechnology and Biological Sciences Research Council. Anthony L. Moore reports a relationship with AlternOx Scientific Ltd. that includes: board membership. Marten Szibor reports a relationship with ALTOX Oy that includes: equity or stocks. Anthony L. Moore has patent “AOX inhibitors as phytopathogenic and anti-parasitic inhibitors” licensed to AlternOx Scientific Ltd..

Data availability

No data was used for the research described in the article.

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