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# Mitochondrial reactive oxygen species and inflammation: Molecular mechanisms, diseases and promising therapies

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**Running title:** Mitochondrial ROS and inflammation

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***Abstract***

Over the last few decades, many different groups have been engaged in studies of new roles for mitochondria, particularly the coupling of alterations in the redox pathway with the inflammatory responses involved in different diseases, including Alzheimer's disease, Parkinson's disease, atherosclerosis, cerebral cavernous malformations, cystic fibrosis and cancer. Mitochondrial dysfunction is important in these pathological conditions, suggesting a pivotal role for mitochondria in the coordination of pro-inflammatory signaling from the cytosol and signaling from other subcellular organelles. In this regard, mitochondrial reactive oxygen species are emerging as perfect liaisons that can trigger the assembly and successive activation of large caspase-1-activating complexes known as inflammasomes. This review offers a glimpse into the mechanisms by which inflammasomes are activated by mitochondrial mechanisms, including reactive oxygen species production and mitochondrial $Ca^{2+}$ uptake, and the roles they can play in several inflammatory pathologies.

**Keywords:** mitochondrial dysfunction, inflammatory response, ROS, inflammasome, inflammation-related diseases

***Introduction***

Over the last few years, it has been demonstrated that perturbations in mitochondrial activities are sufficient to activate innate immune responses (Randow and Youle, 2014; Tschopp, 2011; van der Burgh and Boes, 2015; Wellen and Hotamisligil, 2005), suggesting the elegant hypothesis that cells use intracellular stress responses to initiate innate immunity programs when pathogens or environmental stresses perturb cell functions. In this regard, mitochondrial reactive oxygen species (ROS) are emerging as the perfect liaisons.

Mitochondria are essential organelles in living cells and are characterized by a double membrane and a circular double-stranded DNA molecule. The double membrane is composed of a outer layer (mitochondrial outer membrane; OMM), which allows the passage of ions (particularly $Ca^{2+}$) and metabolites, and the more selective inner membrane (IMM), which is characterized by invaginations known as cristae (Cogliati et al., 2016). Mitochondrial DNA (mtDNA) encodes 13 proteins, all of which are known components of the mitochondrial electron transport chain (mETC) (Anderson et al., 1981; Bibb et al., 1981). Due to their biosynthetic capacities, mitochondria play a central role in supplying the large amount of energy required for many different cellular functions,

as reviewed in (Braschi and McBride, 2010). Substrates derived from other intracellular processes, such as glycolysis or fatty acid metabolism, are converted to acetyl-CoA, which enters the tricarboxylic acid cycle (TCA), and its complete degradation is coupled with the production of NADH and $FADH_2$, which are the effective electron donors for the mETC. The energy is stored as an electrochemical gradient across the IMM, which explains the presence of the negative mitochondrial membrane potential ($\Delta\Psi$). F1Fo-ATP synthase allows $H^+$ to cross the IMM and reenter the matrix, coupling the energy derived from the proton gradient with the phosphorylation of ADP to produce ATP (Johnson and Ogbi, 2011). The new ATP molecules are then ready to leave the mitochondria.

Although the roles of mitochondria and the endoplasmic reticulum (ER) are classically distinct, accumulating evidence indicate a privileged interplay and cooperation with the ER, which is essential for several mitochondrial functions, such as lipid metabolism, modulation of $Ca^{2+}$ signaling, selective autophagy, apoptotic death and inflammation (Contreras et al., 2010; Giorgi et al., 2015; Lopez-Crisosto et al., 2015; Marchi et al., 2014; Raturi and Simmen, 2013; Vance, 2014). ER-mitochondria $Ca^{2+}$ transfer modulates mitochondrial bioenergetics (Glancy and Balaban, 2012), mitophagy (segregation and elimination of the damaged mitochondria) (Rimessi et al., 2013) and cell fate (Rimessi et al., 2008), which significantly affects the mitochondrial ROS production capacity.

Over the last few decades, many different groups have been engaged in studies to comprehend new roles for the mitochondria, particularly in coupling the alterations in the redox pathway with the inflammatory responses involved in different pathological conditions, such as neurodegenerative diseases, motor neuron disorders, genetic diseases, aging and cancer.

The aim of the present review is to discuss the role of the mitochondria in coordinating pro-inflammatory signaling from the cytosol and signaling from other subcellular organelles.

***Mitochondrial ROS production systems***

For years, mitochondria were considered the main source of intracellular ROS in both physiology and pathology. In the 1970s, Chance et al. first proposed that 1-2% of the cellular oxygen used in oxidative phosphorylation, which accounts for 90-95% of the total cellular oxygen consumption, can be converted to anion superoxide ($O_2^{\bullet-}$) as a result of electron leakage from the respiratory chain (Chance et al., 1979). Although it was propagated through the literature, this percentage was not accurate, as it was valid for only a particular set of experimental conditions (Murphy, 2009). The greatest criticism against this dogma was presented in 2012 by Brown and

Borutaite, who presented a list of examples demonstrating that mitochondria do not seem to be the main source of ROS under physiological conditions (Brown and Borutaite, 2011). Other authors who used different experimental settings estimated that approximately 0.1% of the cellular oxygen can be converted to $O_2^{\bullet-}$ in the mitochondrion, leaving open the possibility that the ER and peroxisomes may have greater capacities to produce ROS than the mitochondria, at least in the liver (Fridovich, 2004). Many different sites of ROS production have been identified in mammalian mitochondria, including complex I and complex III of the mETC and the dihydrolipoamide dehydrogenase enzyme (Kudin et al., 2008; Mailloux et al., 2013; Murphy, 2009; Quinlan et al., 2013). Complex I produces superoxide in two ways: a) a reduced flavin mononucleotide (FMN) site on complex I (a high ratio of $NADH/NAD^+$) and b) reverse electron transfer from the coenzyme Q (CoQ) pool back to complex I.

Under physiological conditions, ROS production by complex III is much lower than ROS production by complex I. However, the role of complex III in superoxide production is much more important when it is inhibited. Complex II (succinate dehydrogenase) was not considered a ROS producer *per se*; however, its contribution to ROS formation is related to reverse electron transfer, the process by which electrons are transferred from succinate to ubiquinone via complex II and then back to complex I, where ROS are produced (Liu et al., 2002; Yankovskaya et al., 2003).

In addition to the above-mentioned mitochondrial respiratory chain complexes, other mitochondrial proteins also participate in ROS production. Mitochondrial enzymes, such as acyl-CoA dehydrogenase and glycerol α-phosphate dehydrogenase (both flavoproteins), are involved in ROS generation in tissues during the oxidation of lipid-derived substrates (Lambertucci et al., 2008; St-Pierre et al., 2002). Other enzymes, such as pyruvate and α-ketoglutarate dehydrogenase, which both contain the flavoenzyme dihydrolipoyl dehydrogenase subunit, are additional mitochondrial ROS sources (Starkov et al., 2004). In addition, monoamine oxidase and dihydroorotate dehydrogenase are other documented sources of ROS in the mitochondria (Cadenas and Davies, 2000; Lenaz, 2001). Other examples of mitochondrial enzymes that are involved in superoxide production include the adrenodoxin reductase-adrenodoxin-cytochrome P450scc (cholesterol side chain cleavage) system, which is coupled with the NADPH pool in the mitochondrial matrix (Hanukoglu et al., 1993). In addiction, anion superoxide may react with other radicals, including nitric oxide (NO), producing reactive nitrogen species (RNS) (Radi et al., 2002). The RNS interact with mitochondrial components, leading to a range of biological responses spanning from modulation of mitochondrial respiration to apoptotic cell death. In particular, NO is a signaling molecule that plays a key role in the pathogenesis of inflammation, as a toxic agent towards infectious organisms or as immunoregulator (Bogdan et al., 2000; Brunet, 2001). NO functions as a

pro-inflammatory mediator at low concentrations by inducing vasodilatation and neutrophil recruitment, whereas at high concentrations, it down-regulates adhesion molecules, suppresses activation and induces apoptosis of inflammatory cell (Albina et al., 1991; Lu et al., 1996; Shin et al., 1996). NO is a mediator of Natural Killer (NK) cell killing of target cells and regulates NK cell function (Cifone et al., 2001); it inhibits mast cell activation and can enhance or inhibit neutrophil activation, depending on its concentration (Armstrong, 2001; Bidri et al., 2001; Forsythe et al., 2001). NO induces vasodilatation in the cardiovascular system and is involved in immune responses by cytokine-activated macrophages (Coleman, 2001).

Another protein, p66Shc, binds cytochrome *c* (cyt *c*) when it is translocated to the mitochondrial inter membrane space (IMS) and then subtracts electrons from the mitochondrial respiratory chain and acts as a redox-enzyme, generating $H_2O_2$ (Giorgio et al., 2005).

A more detailed description of the sites where different ROS are produced is presented in Table 1.

### *The multitarget effects of mitochondrial ROS: mtDNA, mitochondrial membranes (lipids) and pivotal mitochondrial proteins*

Excessive ROS levels may be generated by mechanisms that produce ROS in a nonregulated fashion, including ROS production by the mETC, the most quantitatively important source of ROS in higher organisms. Thus, mitochondrial structures are particularly susceptible to oxidative damage, as evidenced by mtDNA mutations, lipid peroxidation and protein oxidation (Cadenas and Davies, 2000).

*Mitochondrial DNA*

The mitochondrial genome displays an interesting feature. The subunits of the complexes in the mETC are produced partially from nuclear DNA transcription and partially from mtDNA transcription. However, mitochondrial transcription is coupled to mtDNA replication; therefore, a high mtDNA copy number and frequent replication are essential to maintaining the integrity of the mETC and a high level of ATP production (Kelly et al., 2012; Moyes et al., 1998; Trounce, 2000). The integration of the majority of mitochondrial proteins into the nuclear DNA reduced the size of mtDNA; the double-stranded closed circular mitochondrial genome is 16 kb and encodes only 13 of the subunits of the mETC, along with 22 tRNAs and 2 rRNAs (Anderson et al., 1981; Bibb et al., 1981). In addition, mtDNA displays a regulatory non-coding region, the displacement loop (D-loop), which primes DNA transcription and replication (Kucej and Butow, 2007) and which is a target of nuclear-encoded proteins.

The reduced dimensions and absence of histones allow the mtDNA to rapidly respond to replicative stimuli; however, its close proximity to the IMM, where the majority of ROS are generated, makes it very susceptible to oxidative damage when ROS production exceeds the antioxidant defenses (Brondani et al., 2012).

The hydroxyl radical represents the most effective mtDNA damaging radical, as it is able to directly react with all components of DNA, such as purine and pyrimidine bases and the deoxyribose sugar backbone.

The D-loop region can be highly vulnerable compared with the rest of the mtDNA (Tewari et al., 2012a). Oxidative damage to this promoter region may reduce the number of mtDNA copies (Tewari et al., 2012b) and subsequently decrease the transcription of the mETC gene set, resulting in structural alteration of the multi-protein complexes and increased free radical production. When these ROS exceed the antioxidant defenses, a vicious cycle of DNA damage, impaired protein translation, and increased ROS production may begin.

*Lipid alterations*

As the mETC is the major ROS producer in mitochondria, the phospholipids present in mitochondrial membranes are particularly prone to ROS-induced oxidative attack. Their oxidation may result in the rearrangement of lipids from a fluid lamellar phase to different structures (Sankhagowit et al., 2016). In turn, these alterations in membrane fluidity and permeability may affect all of the functions associated with the IMM, including the mETC, the mitochondrial permeability transition pore (mPTP) and ATP synthase activities and the mitochondrial $Ca^{2+}$ buffering capacity. Consistently, when Szeto-Schiller peptides coupled with an antioxidant have been used to target cardiolipin (CL) in the IMM (Szeto, 2014b), the prevention of CL oxidation optimizes cristae architecture, improves mitochondrial bioenergetics, and reduces ROS production.

Under hydroxyl radical attack, polyunsaturated fatty acids may undergo fragmentation to produce several reactive by-products, two of which are the reactive aldehydes malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Uchida, 2003). MDA and HNE covalently react with the side chains of histidine, cysteine, and lysine residues (Schaur, 2003), resulting in the attachment of a free carbonyl to the protein or the formation of inter- or intraprotein crosslinks at lysine side chains (Bruenner et al., 1995). These modifications can occur at the enzyme active site and can inactivate protein function. Alternatively, these modifications can create hydrophobic patches that mediate the interactions between oxidized proteins and determine the formation of protein aggregates (Levine et al., 1994). In particular, MDA and HNE interact with and inactivate mETC components, including complexes I (a rate-limiting step for the mETC), III and IV (Musatov et al., 2002; Paradies et al., 2002).

Cyt *c* is a small hemoprotein residing in the IMS and is an essential component of mETC, where it carries one electron. As a member of the mETC, cyt *c* is often associated with the external side of the IMM but can be released to the cytosol as part of the intrinsic apoptotic signaling pathway. In addition to its electron transfer activity, cyt *c* can catalyze several reactions, such as hydroxylation and peroxidation. In particular, peroxidative activity is greatly increased after oxidation of its $Met_{80}$ residue by the action of $H_2O_2$ and lipid hydroperoxides (Chen et al., 2002); subsequently, the binding of CL further increases peroxidase activity (Kagan et al., 2005). Peroxidation is principally exerted on CL itself, generating CL hydroperoxides.

*Protein carbonylation*

In contrast to the oxidation of -SH residues, which can be reversed by glutathione peroxidase and peroxiredoxin activities, protein carbonylation and other aldehydic modifications cannot be repaired; consequently, the modified proteins and aggregates must be degraded by selective proteolysis (Kastle and Grune, 2011). When these modified proteins and aggregates are not properly removed, they can significantly impair cellular functions and can contribute to cellular dysfunction. However, proteasomes are not present in mitochondria. The essential function of removing oxidized proteins is handled by three ATP-stimulated proteases in mitochondria: Lon protease, Clp-like protease and AAA protease. The first two proteases are located in the matrix, and the third is localized to the IMM. Together, these proteases maintain oxidized proteins at the lowest possible levels (Bota et al., 2005; Escobar-Henriques and Langer, 2006).

***Mitochondrial ROS detoxification systems***

$O_2^{\bullet-}$ is a highly reactive state of oxygen; it exhibits a short half-life and is present at low concentrations in mitochondria (Giorgio et al., 2007). $O_2^{\bullet-}$ can be detoxified through the action of the mitochondrial manganese superoxide dismutase (MnSOD), a matrix-abundant and highly efficient enzyme that can convert superoxide to hydrogen peroxide under physiological conditions at a rate faster than the rate at which the $O_2^{\bullet-}$ can oxidize its potential targets. $H_2O_2$ is the more stable and less reactive form of oxygen radical; consequently, $H_2O_2$ has a longer diffusion radius and can exit the mitochondrion and enter other subcellular organelles, such as the nucleus, to act on DNA (Candas and Li, 2014). The increased half-life and concentration of $H_2O_2$ make it a suitable second messenger, although it becomes a proapoptotic/necrotic agent when it exceeds a threshold amount (Giorgio et al., 2007). In the mitochondrial IMS, $O_2^{\bullet-}$ dismutation is predominantly performed by the cytosolic copper-zinc-SOD (Okado-Matsumoto and Fridovich, 2001).

$H_2O_2$ can be broken down by catalases (Kirkman and Gaetani, 2007). Catalase is a Fe-heme-containing enzyme; in its tetrameric form, it exhibits one of the highest turnover numbers among all enzymes. This property makes catalase a non-saturable enzyme or, in other words, an enzyme whose reaction rate is limited only by substrate diffusion. Catalase is an oxidoreductase that catalyzes the following reaction: $H_2O_2 + H_2R \rightarrow 2H_2O + R$. Catalase can decompose two molecules of $H_2O_2$ to water and oxygen ($R=O_2$); alternatively, it can use $H_2O_2$ to oxidize various metabolites and toxins. Catalase is expressed at higher levels in peroxisomes than in the mitochondria, where other enzymes, such as glutathione peroxidases (GPx) and peroxiredoxins (Prx) (Koopman et al., 2010; Murphy, 2009), cooperate with catalase to detoxify $H_2O_2$. GPx uses glutathione (GSH) as a cofactor and electron source to directly reduce $H_2O_2$ to water. GSH is produced in the cytosol and is then transported into the mitochondrial matrix, where 2 molecules of GSH can be oxidized to GSSG through the formation of an intermolecular disulfide bridge. GSH is regenerated from GSSG by the action of glutathione reductase, which requires NADPH. Prx is another antioxidant enzyme that is present at high levels in the mitochondria (Chang et al., 2004). The conversion of $H_2O_2$ into $H_2O$ requires Prx oxidation, rendering it inactive; Prx requires the donation of electrons from reduced thioredoxin to restore its catalytic activity. In turn, the latter requires NADPH and the action of thioredoxin reductase-2 to be regenerated.

Decomposition of $H_2O_2$ can also be achieved through a non-enzymatic mechanism known as the Fenton reaction, which requires the participation of metal ions (iron or copper) as a catalyst. This reaction uses $H_2O_2$ as a reactant; it can use $O_2^{\bullet-}$ or other electron donors to reduce the metal to its active form and results in the degradation of $H_2O_2$ to produce water and a highly reactive, non-selective oxidant, the hydroxyl radical ($OH^{\bullet}$) (Giorgio et al., 2007). This molecule displays the highest reactivity and, consequently, the lowest half-life and concentration among the ROS. Unlike $O_2^{\bullet-}$ and $H_2O_2$, $OH^{\bullet}$ cannot be scavenged through enzymatic reactions; its detoxification occurs through the actions of a wide number of antioxidants, which terminate the oxidative action of the radical that is subsequently regenerated by the actions of other antioxidants. Among these antioxidants, GSH plays a pivotal role because it not only directly neutralizes the lipid radicals formed by the hydroxyl radical attack but also restores the reduced form of hydrosoluble antioxidants, such as ascorbic acid, and lipid-soluble reductants, including tocopherols, tocotrienols, carotenoids, flavonoids and lipoic acid (Valko et al., 2007). When these compounds are partitioned into the mitochondrial membranes, they are able to trap and scavenge lipid peroxyl radicals, thereby preventing the propagation of lipid peroxidation (Smith et al., 1999).

Although all these antioxidant molecules have shown great potential for mitochondria protection *in vitro*, many extensive clinical trials using conventional antioxidants such as vitamin E

or vitamin C did not confirm the expectations (Bjelakovic et al., 2008; Cocheme and Murphy, 2010). The hypothesis of a nonselective biodistribution, with only trace amount of drugs being taken up by mitochondria, can represent a reasonable explanation of these unexepected results. Therefore, new classes of mitochondrial ROS scavengers have been developed to specifically target biologically active molecules to mitochondria. Szeto-Schiller peptides spontaneously target and accumulate at the IMM, where they bind CL and exert antioxidant activity (Szeto, 2014a). Prevention of CL oxidation has been shown to optimize cristae architecture, improving mitochondrial bioenergetics and reducing ROS production.

Alternatively, coupling a lipophilic triphenylphosphonium moiety to several antioxidants, such as coenzyme Q and vitamin E, allows these antioxidants to be able to be taken up by and enriched in the mitochondria by several hundred fold (Smith and Murphy, 2011), greatly improving their antioxidant capacities in several pathologies. These compounds protect the mitochondria from oxidative damage induced by iron/ascorbate far more effectively than vitamin E itself, whereas mitochondria-targeted ubiquinone (MitoQ) can reduce cardiac ischemia/reperfusion injury (Adlam et al., 2005). Using MitoQ, inflammatory cytokine production was abolished following LPS stimulation in cells from patients with TNF receptor-associated periodic syndrome, an autoinflammatory disorder associated with enhanced innate immune responsiveness in which mutations of the TNF receptor-1 gene lead to aberrant mitochondrial ROS production (Simon et al., 2010).

***The role of mitochondrial ROS in inflammation-related diseases***

Deviation of the mitochondrial biochemical status quo triggers activation of the inflammatory response. In general, oxidative stress can incite inflammation, and excess inflammation can cause oxidative stress, inducing excessive cell and tissue damage and ultimately leading to the destruction of normal tissue and chronic inflammation. This feedback loop is also accrued by NLRP3 inflammasome activation, leading to mitochondrial damage and mitophagy inhibition (Yu et al., 2014). The accumulation of damaged mitochondria is responsible for ROS production and increased interleukin-1β (IL-1β) secretion (Nakahira et al., 2011), although this mechanism can work in the opposite direction, as some autophagy proteins are also necessary for IL-1β release (Zhang et al., 2015). Defects in mitophagy have been suggested to play roles in neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Osellame and Duchen, 2013; Wong and Holzbaur, 2015). Both of these pathologies are characterized by the accumulation of toxic proteins and their aggregates in mitochondria, leading to

energy deficits, excessive ROS generation, mutations in both the mtDNA and the proteins regulating mitochondrial homeostasis, and impaired mitochondrial dynamics. Together, these effects result in neuronal damage as well as constant activation of microglia and astrocytes (Witte et al., 2010). A direct link between the NLRP3 inflammasome and the development of AD has been shown in transgenic mice that are deficient in both NLRP3 and caspase-1 and that develop chronic amyloid-β deposition. These mice display reduced chronic amyloid-β secretion, neuronal inflammation and cognitive impairment, in addition to skewed numbers of microglial cells (Heneka et al., 2013). In PD, the neurons contain aggregated inclusions that are primarily composed of α-synuclein (Shulman et al., 2011), a protein that is able to activate the inflammasome by inducing caspase-1 activation and mature IL-1β production. This pathway requires phagocytosis, cathepsin B, and ROS production, which are thought to lie upstream of NLRP3 activation (Codolo et al., 2013).

The evidence gathered from animal and human studies points to central roles for inflammation and the mitochondria in the initiation and development of multiple sclerosis (MS). MS is considered a prototypic autoimmune disease and is characterized by demyelination, inflammation, gliosis and axonal damage (Kidd, 2001). Mitochondrial abnormalities, such as changes in the number and shape of the mitochondria and in the levels of components of the respiratory chain complex and markers of oxidative stress, drive the inflammatory processes in MS (Bonora et al., 2014; Kalman and Leist, 2003). Furthermore, impaired mitochondrial complex I activity in chronic active plaque zones was associated with oxidative damage to the mtDNA (Lu et al., 2000) and constitutive mitochondrial energy loss as a cause of the intermittent demyelination and profound central nervous system symptoms that mimic MS (Powell et al., 1990). The released cytokines, particularly tumor necrosis factor-α (TNF-α), induce metabolic changes driven by mitochondrial impairments, ROS production and AMP-activated protein kinase (AMPK) activation, resulting in the inhibition of oligodendrocyte progenitor cell differentiation (Bonora et al., 2014). NLRP3 expression is increased in the spinal cord during the progression of experimental autoimmune encephalomyelitis; NLRP3-deficient mice have a dramatically delayed course and reduced severity of this disease (Gris et al., 2010; Inoue et al., 2012). In addition, a study using the cuprizone model of MS demonstrated that NLRP3-deficient mice exhibited delayed demyelination and oligodendrocyte loss (Jha et al., 2010). The mitochondria-MS connection was further reinforced by the observation of an increased incidence of some LHON (Leber's hereditary optic neuropathy, a mitochondrial disease) mutations in patients with MS (Kalman et al., 1995).

Friedreich ataxia (FA), a mitochondrial disease, is associated with neuroinflammation, neurodegeneration, cardiomyopathy and diabetes (Durr and Brice, 2000; Ristow et al., 2003). This

pathological etiology derives from mutations in the frataxin gene, causing reduced expression of the mitochondrial protein and oxidative damage (Campuzano et al., 1997). Frataxin is involved in the biogenesis of iron-sulfur clusters, and defects in its expression cause an increase in ROS production by decreasing thiol-dependent antioxidant protection and increasing free iron and redox cycling (Vaubel and Isaya, 2013). In neural Schwann cells, the loss of frataxin expression induces explicit inflammation, oxidative stress, and cell death (Lu et al., 2009). The neuroinflammatory and neurodegenerative consequences are mediated by a decrease in antioxidant protection (such as peroxiredoxins, glutaredoxins, and glutathione S-transferase) and an induction of prostaglandin synthases, specifically cyclooxygenase 2 (COX2) (Hayashi et al., 2014; Shan et al., 2013).

Chronic inflammation plays an essential role in the initiation and progression of metabolic disorders such as atherosclerosis. The development of atherosclerosis is associated with excessive mitochondrial ROS production within the vasculature. Specific mitochondrial antioxidant enzymes, such as MnSOD and Trx2, are known to protect against the endothelial dysfunction induced by atherosclerotic lesions in ApoE-deficient mice (Ohashi et al., 2006; Zhang et al., 2007). Similarly, in the same mouse model of atherosclerosis, the lack or blockade of IL-1β significantly decreased the sizes of the atherosclerotic lesions (Bhaskar et al., 2011; Kirii et al., 2003). Indeed, data demonstrated that nitric oxide in ischemic conditions mediates cardioprotection after ischemia/reperfusion. The mechanism involves the inhibition of mitochondrial complex I by S-nitrosation, leading to a subsequent decrease in mitochondrial ROS generation, limiting apoptosis and cytotoxicity at reperfusion (Shiva et al., 2007). ROS production also plays a role in ischemia-reperfusion injury. Although apparently conflicting, convincing evidence indicates that excessive ROS production can mediate post-ischemic injury. In fact, an increase in ROS seems to be dependent on the integrity of respiratory supercomplexes (Rosca et al., 2008); consequently, hypoxic conditions, which lead to mitochondrial fusion, membrane potential impairment and supercomplex disassembly, could be responsible for the paradoxical observation associating a low [$O_2$] to an increase in ROS production (Baracca et al., 2010; Genova et al., 2008). The detection of increased levels of ROS and lipid peroxidation products in post-ischemic tissues, such as the protective effect of antioxidants against reperfusion injury, support the involvement of ROS in ischemia/reperfusion injury. Moreover, hypertension is also associated with increased ROS production, which contributes to blood pressure regulation (Harrison and Gongora, 2009). Angiotensin II-induced activation of NADPH oxidase further increases mitochondrial dysfunction and mitochondrial ROS production (Doughan et al., 2008). NO may interfere with ROS generation by NADPH oxidase, suppressing its activity by S-nitrosylation (Selemidis et al., 2007).

Importantly, transgenic mice that overexpress Trx2 resist the development of angiotensin II-induced hypertension and endothelial dysfunction (Widder et al., 2009).

Mitochondria are indispensable for energy metabolism, cell signaling and apoptosis regulation. The mitochondria in malignant cells differ structurally and functionally from those in normal cells and are characterized by ROS overproduction, which promotes metabolic reprogramming and genomic instability, modifies gene expression and participates in signaling pathways that induce cancer development (Rimessi et al., 2015b). Oncogene hyperactivation has long been associated with elevated mitochondrial ROS levels. The expression of oncogenic H-RAS and K-RAS promotes mitochondrial changes that lead to ROS overproduction by and damage to mitochondria (Hu et al., 2012; Rimessi et al., 2014). Ectopic MYC overexpression induced mitochondrial ROS production and concomitantly increased oxidative DNA damage (K et al., 2006). The contributions of ROS and the inflammasomes that are induced by mitochondria in cancer cells are controversial; they can positively affect cell-autonomous death pathways and anticancer immunosurveillance, but they may also stimulate autocrine or paracrine processes that favor carcinogenic inflammation, tumor growth, metastasis and angiogenesis (Zitvogel et al., 2012). A protective role for NLRP3 has been described in hepatocellular carcinoma (Wei et al., 2014); NLRP3 and caspase-1 null mice are more susceptible to azoxymethane/dextran sulfate sodium-induced carcinogenesis (Allen et al., 2010; Zaki et al., 2010). Based on this evidence, the discovery of NLRC4 as a downstream transcriptional target of p53 constituted promising evidence for the anti-tumorigenic functions of NLR (Sadasivam et al., 2005). Moreover, the lack of the NLRC4 inflammasome has been associated with the attenuation of p53-mediated cell death, which is indicative of a protective role for NLRC4 during tumor development. Given the ambiguity of the roles of inflammasomes in cancer, which are strictly dependent on the neoplasm type and stage, the cell type recruited and the environmental conditions, it is not possible to formulate an unequivocal set of indications to stimulate or inhibit inflammasomes in the context of therapy.

The pathophysiological importance of the mitochondrial redox status, inflammation and apoptosis regulation was also taken into consideration during the study of cerebral cavernous malformations (CCMs) (Goitre et al., 2010). CCM1 is an autosomal dominant disease caused by mutations in the Krev Interaction Trapped 1 (KRIT1) gene and characterized by multiple brain lesions that often result in intracerebral hemorrhage, seizures, and neurological deficits. Emerging evidence shows that inflammation and the immune response play roles in CCM1 pathogenesis and may be used as predictors of disease severity (Choquet et al., 2014). Krit1 ablation leads to a significant increase in intracellular ROS levels due to modulation of the expression of the mitochondrial antioxidant MnSOD, a drastic decrease in mitochondrial energy metabolism and

autophagy suppression, and a subsequent increase in the susceptibility to oxidative damage (Goitre et al., 2010; Marchi et al., 2015; Marchi et al., 2016). The inflammatory cytokine genes are involved in the pathogenesis of brain vascular disease, as observed in patients with brain arteriovenous malformations and in CCM1 subjects with intracerebral hemorrhage (Choquet et al., 2014; Fontanella et al., 2012). Multiple genetic polymorphisms in inflammatory cytokines have been reported to act as modifying factors in numerous diseases, including the severity of pulmonary disease in patients with cystic fibrosis (CF) (Pasaniuc et al., 2011). Chronic airway infection by *Pseudomonas aeruginosa* (*P. aeruginosa*) is a common pathological manifestation in patients with CF and is associated with an excessive inflammatory response characterized by the accumulation of large amounts of cytokines, including IL-1β (Levy et al., 2009). IL-1β levels are increased in the bronchoalveolar lavage fluid (BALF) of patients with CF, and *IL-1β* gene polymorphisms have been associated with varying degrees of disease severity in patients with CF (Douglas et al., 2009; Levy et al., 2009). Recently, we demonstrated that the degree and quality of the inflammatory response in CF are supported by *P. aeruginosa*-dependent mitochondrial perturbations, in which the mitochondrial $Ca^{2+}$ uniporter (MCU) is a signal-integrating organelle that mediates mitochondrial ROS-dependent inflammasome activation (Rimessi et al., 2015a). Manipulation of the MCU has indicated a link between mitochondrial $Ca^{2+}$ signaling and *P. aeruginosa*-dependent inflammasome activation in CF, demonstrating that the exacerbated inflammatory response in CF is sustained by the recruitment of both the NLRP3 and NLRC4 inflammasomes (Fig. 1). This result suggests that the inflammasome is a highly dynamic macromolecular platform that is able to recruit different Nod-like receptors (NLRs), as also shown by *Salmonella* infection that simultaneously activates NLRC4 and NLRP3 in an apoptosis-associated speck-like protein ring-like structure (Man et al., 2014).

***Inflammation-associated or sensing proteins: Inflammasomes***

Nod-like receptors are an evolutionarily conserved family of receptors that reside in the cytoplasm and recognize pathogen- and danger-associated molecular patterns (PAMPS and DAMPs) to activate pro-inflammatory responses through specific intracellular signaling pathways (MacMahon, 1991). Certain NLRs induce the assembly of a large caspase-1-activating complex known as the inflammasome, which leads to the processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18 (Martinon et al., 2002). To date, four inflammasomes have been well characterized and defined according to the NLR protein that they contain: NLRP1, NLRC4, NLRP3

and AIM2 (absent in melanoma 2). Among them, the best-characterized inflammasome coupled to mitochondria is NLRP3 (Martinon et al., 2006).

Several DAMPs, such as extracellular ATP, alum hydroxide, silica crystals, urea crystals, nigericin, and bacteria, viruses, and fungal infections, activate the NLRP3 inflammasome (Anand et al., 2011). Longstanding questions in this field include how NLRP3 recognizes these different ligands and whether a common signal converges downstream of PAMPs and DAMPs to activate NLRP3. NLRP3 activation requires two signals: a priming signal that is required to upregulate NLRP3 and pro-IL1β and an activation signal that prompts NLRP3 to assemble the inflammasome complex (Fig. 1). The requirement for a second signal to activate NLRP3 may constitute a fail-safe mechanism to ensure that potent inflammatory responses are induced only in the presence of a *bona fide* stimulus. The *bona fide* stimuli include potassium efflux out of the cell, the generation of mitochondrial ROS, the translocation of NLRP3 to the mitochondria, the release of mitochondrial DNA or CL, and the release of cathepsins into the cytosol following lysosomal destabilization (Lamkanfi and Dixit, 2014). The role of ROS in activating the inflammasome is supported by studies using a variety of different inflammasome signaling modulators (reviewed in (Martinon, 2010; Tschopp and Schroder, 2010)). There are examples of specific inflammasome-activating signals that are associated with the mitochondria and ROS, such as silica (Hu et al., 2007), ATP (Cruz et al., 2007) and bacterial infection (Rimessi et al., 2015a), which result in NLRP3 activation and pro-inflammatory cytokine release.

The link between NLRP3 and mitochondria is strengthened by its subcellular localization and through mitochondrial antiviral signaling protein (MAVS). Under resting conditions, the NLRP3 protein is localized to the ER, and upon inflammasome stimulation, it relocates to the mitochondria-associated ER membranes (Zhou et al., 2011), a "hot spot" for intracellular signaling from important pathways (Giorgi et al., 2015). MAVS is an adaptor protein located on the OMM that regulates signal transduction from cytosolic RNA sensors. MAVS activity depends on mitochondrial dynamics and function, which promotes mitochondrial recruitment and the pathophysiological activity of NLRP3 through the assembly of a large signaling complex on the mitochondria (Koshiba et al., 2011). The administration of NLRP3 activators generates $O_2^{\bullet-}$, which is sequestered by mitochondria-specific autophagy (mitophagy) to suppress inflammasome activation (Zhou et al., 2011). Notably, extracellular ATP causes a rapid pulse of ROS production in alveolar macrophages via purinergic receptors (P2X7 receptor) (Cruz et al., 2007), but it leads to the loss of intracellular $K^+$ in human and murine neutrophils, inducing NLRP3 activation (Karmakar et al., 2016). Indeed, NLRP3 activation was highly impaired in macrophages in which mitochondrial activity was reduced by depletion of the mtDNA or by inactivation of the OMM

protein voltage-dependent anion channel (VDAC) (Nakahira et al., 2011; Zhou et al., 2011). VDACs are the major channels for the exchange of metabolites and ions (*i.e.*, $Ca^{2+}$) between the mitochondria and the ER. In cells with diminished VDAC expression, caspase-1 activation was considerably impaired upon the addition of NLRP3 activators.

It has been recently proposed that $Ca^{2+}$ is a novel molecular activator of the NLRP3 inflammasome. In support of the intimate correlation between $Ca^{2+}$ signaling and the inflammasome, Lee G.S. et al. showed that a murine $Ca^{2+}$-sensing receptor activated NLRP3 by increasing the intracellular $Ca^{2+}$ concentration, independent of the P2X7 receptor (Lee et al., 2012). Indeed, Murakami and colleagues showed that several NLRP3 activators mobilized $Ca^{2+}$, whereas blocking the $Ca^{2+}$ signal inhibited NLRP3 activation (Murakami et al., 2012). Additional evidence of the contribution of $Ca^{2+}$ signaling to NLRP3 activation, particularly in mitochondria, is sustained by the role of the MCU. Specifically, the loss of the MCU blunts NLRP3 activation induced by both the complement membrane attack complex in human lung epithelial cells (Triantafilou et al., 2013) and *P. aeruginosa* in airway epithelial cells in patients with CF (Rimessi et al., 2015a), thus preserving mitochondrial dysfunction and limiting ROS production (Fig. 2).

A recent study demonstrated that NLRC4 could also be activated by mitochondrial dysfunction (Jabir et al., 2015). Pathogen infection resulted in mitochondrial damage with increased ROS production and mtDNA release. The mtDNA directly activated the NLRC4 inflammasome; its oxidation by ROS enhanced this effect, whereas macrophages lacking mitochondria failed to activate NLRC4 following infection.

Based on these findings, it is clear that mitochondria integrate distinct signals and relay the information to the inflammasomes for recruitment and activation through a dangerous mix of its constituents: $Ca^{2+}$, ROS and mtDNA.

***Conclusions***

The identification of potential drugs that directly target the inflammasome would be a major achievement in research and would be beneficial to many people suffering from certain inflammation-related diseases. Currently, the developed drugs that target the inflammasome dissociate such molecular scaffolds by directly interacting with the inflammasome or inhibiting the ATPase activity of the NLR (Duncan et al., 2007) (Fig. 1). Opsona Therapeutics has developed cytokine release inhibitory drug 3 (CRID3, also known as CP-456773 or MCC950), which targets ASC oligomerization during NLRP3 and AIM2 activation (Coll et al., 2011). Taking advantage of

this mechanism, isoliquiritigenin (Honda et al., 2014), a simple chalcone-type flavonoid, exhibits antioxidant, anti-inflammatory, and anti-tumor activities (Jung et al., 2014).

The NF-kappaB inhibitory compound parthenolide and the synthetic I kappaB kinase-beta inhibitor Bay 11-7082 are both inhibitors of the ATPase activity of NLRP3 (Juliana et al., 2010). Both compounds selectively inhibit NLRP3 activity in macrophages *in vitro*, independent of their inhibitory effect on NF-kappaB activity. In contrast, TherimuneX Pharmaceuticals has produced a NLRP3 inflammasome activator, an acetylated 18-mer peptide (acALY18) that is used to enhance inflammasome-mediated pathogen clearance and that is beneficial as a broad-spectrum anti-infective drug (Thacker et al., 2012). The actual limitation in the discovery of drugs targeting the inflammasome is the complexity of the activation pathway. Targeting the post-translational modifications of inflammasome components could be an alternative strategy useful for modulating inflammasome activation. NLRP3 activation is mediated by a two-step deubiquitination mechanism identified as an early priming event that is initiated by Toll-like receptor signaling and occurs in response to stimulation involving ROS production (Juliana et al., 2012; Wen et al., 2012). Therapies that specifically promote NLRP3 ubiquitination or that antagonize the deubiquitination mechanism could mitigate NLRP3-dependent pathologic inflammation, promoting NLRP3 degradation by proteosome. Another alternative is represented by the many reagents that target the inflammasome products IL-1β and IL-18, specific antagonist antibodies (IL-1β antibody Canakinumab or anti-IL-18 receptor monoclonal antibody) or proteins (the recombinant IL-1 receptor antagonist Anakinra or the IL-18-binding protein) that neutralize the released cytokines and their receptors with promising therapeutic results. The use of all these therapeutic approaches must be pondered inasmuch as not all inflammasome activation can be considered harmful, *e.g.*, for the host response to microbial pathogens. Thus, therapeutic inhibition of inflammasome activation needs to be balanced against its beneficial contribution.

However, over the last few years, we have moved into a new research area of intervention for inflammation-related diseases: mitochondria-targeting medicine. Increasing evidence confirms the roles of mitochondria and mitochondrial ROS in triggering and regulating the amplitude of the inflammatory response in different pathologies. It is now apparent that the mitochondria have become an area of interest to industry; companies will focus more on investigating direct drug-induced mitochondrial protection or dysfunction, with the outcome of controlling the inflammatory response based on a mitochondrial end-point.

The mitocans are one of many examples of drugs that have been developed and designed to target the mitochondria. The mitocans are vitamin E analogs that selectively target cancer cell mitochondria to induce cell death by triggering ROS production (Neuzil et al., 2007). The vitamin E

(α-tocopherol) analogs (VEA) alpha-tocopheryl succinate (α-TOS) and alpha-tocopheryloxyacetic acid (α-TEA) have not only been examined for their anti-tumor activities but have also recently been shown to have immunomodulatory properties (reviewed in (Hahn et al., 2013)). The α-TOS and α-TEA analogs can suppress the growth of established tumors and can reduce the incidence of spontaneous metastases when combined with cancer immunotherapy via dendritic cell vaccination, causing immunogenic tumor cell death. The produced or released ROS are danger signals that promote an immune reaction and reinforce the response against the cancer, resulting in inflammasome activation and the release of pro-inflammatory mediators. In particular, malignant cells produce more $O_2^{\bullet-}$ than normal cells and thus are more vulnerable to further inflammasome activation and damage by ROS-generating agents (Hileman et al., 2004).

To reduce mitochondrial ROS production, mitochondria-targeted antioxidants have been developed (Oyewole and Birch-Machin, 2015), and preclinical and clinical studies have been performed to test their therapeutic effects in the treatments of inflammatory diseases (Jin et al., 2014; McManus et al., 2011). The antioxidant MitoQ was used several phase I and II studies. Among these studies, two double-blind trials, placebo-controlled studies involving patients with PD (NCT00329056) and patients with chronic hepatitis C virus (HCV) who are unresponsive to the conventional HCV treatments (NCT00433108) were completed and discussed in published reports (Smith and Murphy, 2010). In the same manner, the mitochondrial-targeted Szeto-Schiller peptide SS-31 (the tetrapeptide D-Arg-dimethylTyr-Lys-Phe-NH2, drug name Bendavia, or MTP-131) was analyzed in 13 phase I and II clinical studies, one of which (NCT01572909) was led in patients with acute coronary events, to assess its capacity to reduce reperfusion injury (Chakrabarti et al., 2013). With the exclusion of the study on patients with HCV, which suggested that MitoQ could selectively affect the liver damage associated with HCV infection, other clinical trials demonstrated that targeting mitochondria-associated antioxidants did not significantly changed the disease progression. This unexpected result may have different explanations: reduced bioavailability in the target organs (Snow et al., 2010) or a preexisting predominant lesion (for patients with PD, irreversible dopaminergic neuron loss). In contrast, in the time range explored, both MitoQ and Bendavia did not show any relevant sign of toxicity. This finding sustains not only the feasibility and safety of further investigations but also the possibility to widen the testing to other inflammation-related pathologies. The need to refine the approach to the mitochondria-targeted therapy has also led to the development of newer drugs, such as SkQ compounds, molecules that contain a quinone antioxidant moiety that is covalently conjugated to a lipophilic cation via alkyl chains (Izyumov et al., 2010). Among these drugs, SkQ1 (plastoquinonyl-decyl-triphenylphosphonium) shows the highest membrane-penetrating capacity and potent antioxidant

activity (Antonenko et al., 2008). Consistent with the oncogenic role of ROS, SkQ1 compounds are effective at preventing cancer and as anticancer therapies (Bazhin et al., 2016).

To date, the mitochondrial dysfunction/ROS/inflammasome axis is increasingly considered a druggable line of action to counteract some inflammation-related diseases. Such drugs may not always resolve the pathology but are often useful in preserving a functional mitochondrial network pivotal for cell viability, preventing impairments in the processes that they regulate. A better understanding of the role played by mitochondria in inflammation will help to reveal additional therapeutic targets and to increase the activity and selectivity of mitochondria-targeted drugs.

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***Figure legends***

**Figure 1. The signals required for inflammasome activation: The mitochondrial signals**

NLRP3 activation requires two signals: a priming signal (signal 1) that is required to upregulate NLRP3 and pro-IL1β expression and an activation signal (signal 2) that prompts NLRP3 to assemble the inflammasome complex. The requirement for mitochondrial signals for NLRP3 activation constitutes a fail-safe mechanism to ensure that the induction of potent inflammatory responses occurs only in the presence of a *bona fide* stimulus. This figure shows the discussed drugs that directly target the inflammasome and mitochondrial ROS production.

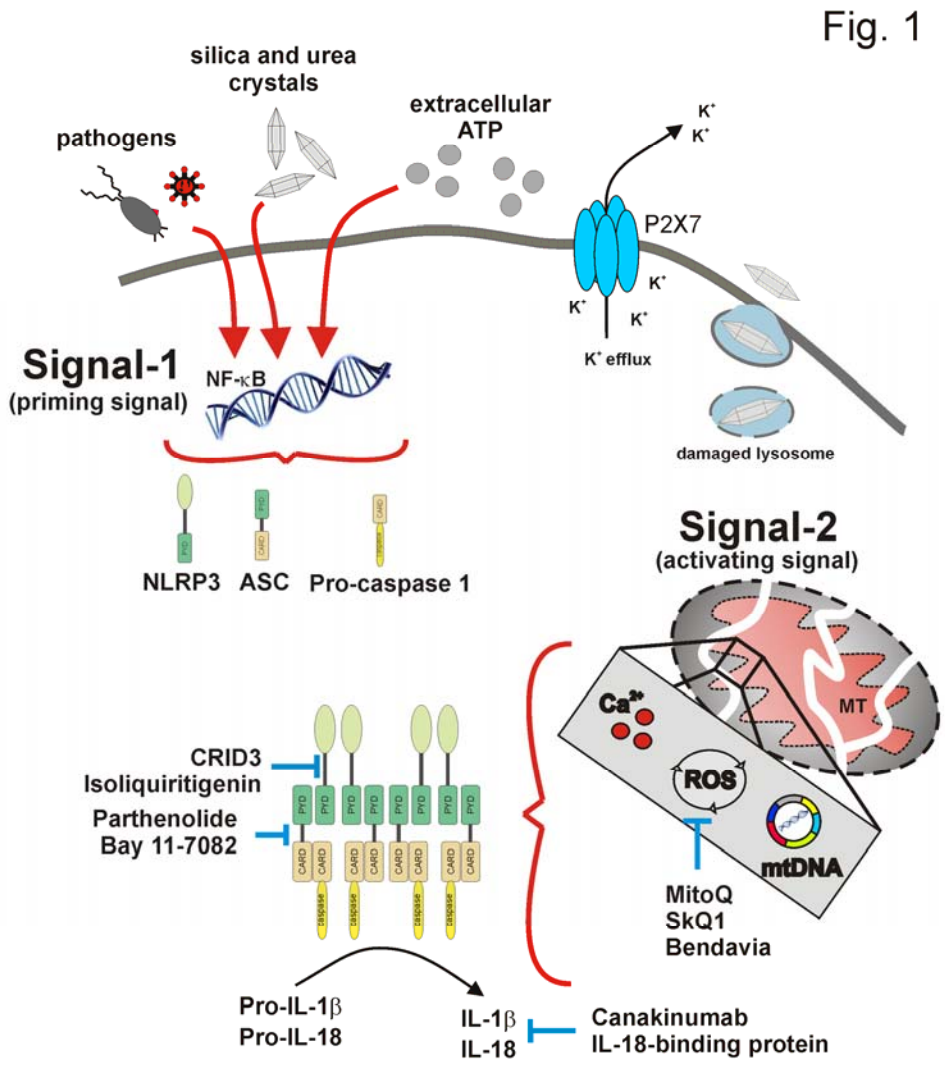

**Figure 2. MCU induces NLRP3 inflammasome activation**

The proposed models show the role of the MCU in inducing NLRP3 activation by producing mitochondrial dysfunction in the complement membrane attack complex in human lung epithelial cells and by *Pseudomonas aeruginosa* (*P. aeruginosa*) infection in the lung epithelial cells of patients with CF. In both cases, the complement cascade and the lack of expression of the CFTR channel promote intracellular $Ca^{2+}$ influx. These effects cause $Ca^{2+}$ release from the ER via the ryanodine and inositol-triphosphate receptors (IP3R). Mitochondrial $Ca^{2+}$ uptake occurs via the MCU, leading to mitochondrial dysfunction, loss of membrane potential, ROS production and oxidative damage.

Fig. 2

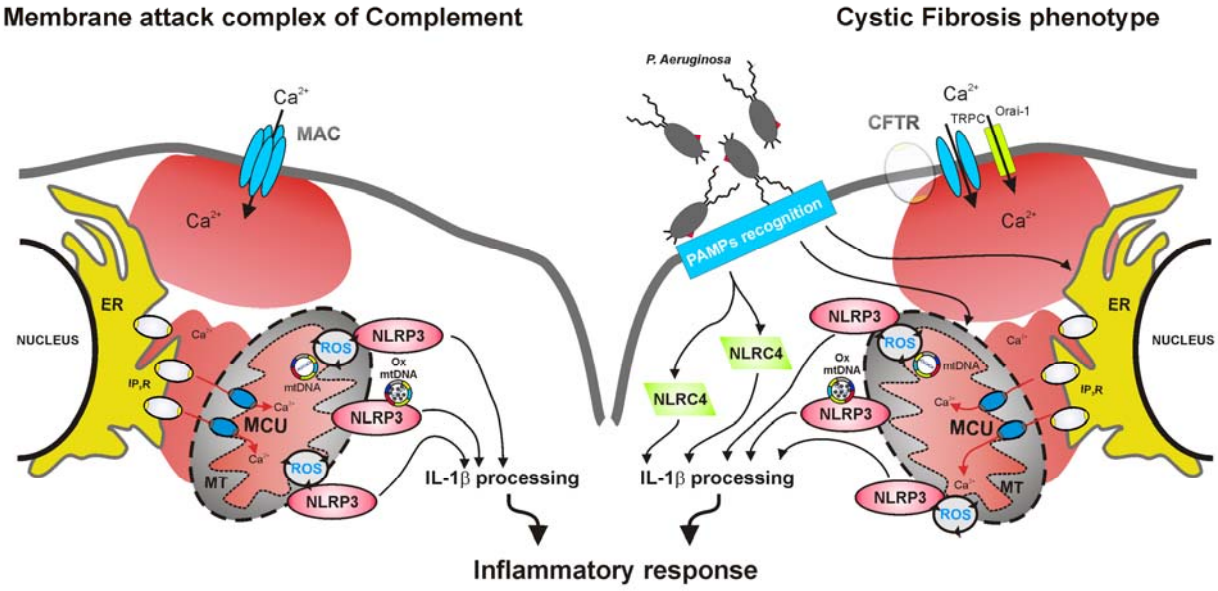

**Table 1.** Detailed description of the types of ROS generated at different sites in the mitochondrion.

| Protein/complex | Mitochondrial compartment | Type of ROS produced | Site of ROS production | Ref. | Role in pathogenicity/ inflammation |
|---|---|---|---|---|---|
| Mitochondrial cytochrome b5 reductase | OMM | $O_2^{\bullet-}$ | cytosol or IMS | (Whatley et al., 1998) | (Lund et al., 2015) |
| Monoamine oxidases (MAO-A and MAO-B) | OMM | $H_2O_2$ | cytosol | (Kunduzova et al., 2002) | (Chaaya et al., 2011; Vuohelainen et al., 2015) |
| Apoptosis-inducing factor (AIF) | IMS | $O_2^{\bullet-}$ | cytosol and IMS | (Miramar et al., 2001) | (Thornton and Hagberg, 2015) |
| p66 Shc | IMS | $H_2O_2$ | IMS | (Giorgio et al., 2005) | (Tomilov et al., 2010; Yang et al., 2016) |
| Zn-Cu superoxide dismutase (SOD1) | IMS | $H_2O_2$ | IMS | (Jezek and Hlavata, 2005) | (Li et al., 2011; Ni et al., 2016) |
| Dihydroorotate dehydrogenase (DHODH) | IMM | $H_2O_2$ and $O_2^{\bullet-}$ | IMS | (Forman and Kennedy, 1975) | (Fitzpatrick et al., 2010; Leban and Vitt, 2011) |
| Glycerol-3-Phosphate Dehydrogenase (mGPDH) | IMM | $H_2O_2$ | IMS | (Mracek et al., 2009(Mracek et al., 2009) | (Raja Gopal Reddy et al., 2016) |
| NADH: ubiquinone oxidoreductase (C.I) | IMM | $O_2^{\bullet-}$ | matrix | (Muller et al., 2004) | (Huang et al., 2007; Kelly et al., 2015) |
| Ubiquinol: cytochrome c oxidoreductase (C.III) | IMM | $O_2^{\bullet-}$ | IMS and matrix | (Jezek and Hlavata, 2005; Muller et al., 2004) | (Aguilera-Aguirre et al., 2009) |
| α-ketoglutarate dehydrogenase complex (α-KGDHC) | matrix/IMM | $O_2^{\bullet-}$ and $H_2O_2$ | matrix | (Starkov et al., 2004; Tahara et al., 2007) | |
| Pyruvate dehydrogenase complex (PDC) | matrix | $O_2^{\bullet-}$ and $H_2O_2$ | matrix | (Tahara et al., 2007) | (Meiser et al., 2016; Xu et al., 2015) |
| Aconitase | matrix | OH• | matrix | (Vasquez-Vivar et al., 2000) | (Talib and Davies, 2016) |
| Mn superoxide dismutase (SOD2) | matrix | $H_2O_2$ | matrix | (Jezek and Hlavata, 2005) | (Ishihara et al., 2015; Majolo et al., 2015; McCarthy et al., 2013) |
| Electron transfer flavoprotein | matrix | $O_2^{\bullet-}$ | matrix | (Jezek and Hlavata, 2005) | (Hussain et al., 2006; Salomone et al., 2014) |
| Electron transfer flavoprotein quinone oxidoreductase | matrix | $O_2^{\bullet-}$ | matrix | (St-Pierre et al., 2002) | |