MINI-REVIEW

The role of mitochondrial ROS in antibacterial immunity[†]

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Abstract

Reactive oxygen species (ROS) are essential participants of various innate immune cell responses against microorganisms and are also involved in many cellular regulatory pathways. It was believed that the main pool of ROS in the innate immune cells is generated by the NADPH oxidase enzymatic complex. However, it was discovered recently that mitochondrial ROS (mtROS) are equally important for the functioning of the immune system. mtROS play an important role in the development of the antimicrobial innate immune responses. The present mini-review summarizes the most recent data on the role of mtROS in the antibacterial immunity. The principles of mtROS formation and possible mechanisms of their generation under the activation of innate immunity are highlighted in this review. We also speculate on the possibilities of using activators of mtROS production in clinical practice. This article is protected by copyright. All rights reserved

Keywords: mitochondria; mitochondrial reactive oxygen species; electron-transport chain;

phagocytosis; antibacterial immunity.

Abbreviations

Acad9, acyl-CoA dehydrogenase family member 9;
Bcl-2, B-cell lymphoma 2;
CGD, chronic granulomatous disease;
CIA30, Complex I intermediate-associated protein 30;
CuZnSOD, copper-zinc superoxide dismutase;
ECSIT, Evolutionarily Conserved Signaling Intermediate in Toll pathway;
fMLP, N-formyl-methionyl-leucyl-phenylalanine;
FMN, flavin mononucleotide;
IFNγ, interferon gamma;
IRAK4, interleukin-1 receptor-associated kinase 4;
LPS, lipopolysaccharide;
MEKK-1, mitogen-activated protein kinase kinase kinase-1;
MnSOD, manganese superoxide dismutase;
Mst1/2 kinases, mammalian sterile 20-like kinases 1 and 2;
mtROS, mitochondrial reactive oxygen species;
MyD88, myeloid differentiation primary response gene (88);
NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase;
NDUFAF1, NADH:ubiquinone oxidoreductase complex assembly factor 1;
NEMO protein, NF-kB essential modulator;
NETosis, neutrophil extracellular trap formation;
NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells;
NLR, NOD-like receptor;
NOD, nucleotide binding oligomerization domain;
PAMPs, pathogen-associated molecular patterns;
PGC-1α, Peroxisome Proliferator-Activated Receptor γ coactivator-1α;
PKCα, protein kinase Cα;
PPARγ, Peroxisome Proliferator-Activated Receptor γ;
PRRs, pattern-recognition receptors;
Rac2, Ras-related C3 botulinum toxin substrate 2;
SK channel, small conductance calcium-activated potassium channel;
SkQ1, 10-(6'-methylplastoquinonyl)decyltriphenylphosphonium;
TGF-β, transforming growth factor-β;
TLR, Toll-like receptor;
TMEM126B, transmembrane protein 126B;
TNF, tumor necrosis factor;
TRAF6, tumor necrosis factor receptor-associated factor 6;
UCP2, mitochondrial uncoupling protein 2;

Introduction

The main function of mitochondria is the production of ATP in the process of oxidative phosphorylation. The main byproduct that is formed in the course of electron transport in the respiratory chain is superoxide anion radical. Superoxide emerges as a result of one-electron reduction of molecular oxygen in various parts of the respiratory chain, including Complex I (NADH: ubiquinone oxidoreductase), Complex II (succinate dehydrogenase), Complex III (ubiquinol: cytochrome oxidoreductase), and mitochondrial matrix dehydrogenases [Grivennikova and Vinogradov, 2013]. Subsequently, the superoxide gives rise to various reactive oxygen species (ROS). Normally, only 1-2% of the oxygen consumed by the cells are used for ROS formation in mitochondria (mtROS) [Turrens, 2003, Inoue et al., 2003].

Until recently, it was believed that mtROS predominantly play a destructive role causing cell damage and accelerating aging [Harman, 2003]. These views are exemplified by the ideas that are expressed in the work of Huang and co-workers [1997]. It was established that mice with a knockout of cytosolic CuZn-dependent superoxide dismutase gene are viable, while mice with a nonfunctional mitochondrial Mn-dependent superoxide dismutase gene do not survive. Recently, it has been established that mtROS formation is mandatory for the functioning of virtually all organs and tissues, being involved in numerous metabolic pathways and in the regulation of signaling processes [Dan Dunn et al., 2015].

Importantly, mtROS are essential for the functioning of the immune system. However, their influence may be either positive or negative, depending on the quantity of the mtROS involved. From the immunological viewpoint, the positive effects of mtROS are primarily based on their activating influence on antimicrobial immunity, while their negative effects, caused by their excessive production, result in the induction of autoinflammatory [van der Burgh and Boes, 2015] and autoimmune processes [Lee et al., 2016; Lood et al., 2016]. The formation of mtROS plays an important role in the development of the antiviral immunity including the production of proinflammatory cytokines by immune system cells [Kim et al., 2015]. This review is concerned with the analysis of the mtROS role in terms of antibacterial innate immunity.

Principles of ROS formation in the mitochondria

The most important source of mtROS is Complex I, which is the largest component of the electron transport chain. The Complex I consists of 14 core and 31 accessory subunits [Zhu et al., 2016]. Formation of ROS including superoxide and hydrogen peroxide in Complex I can result from NADH oxidation, ubiquinone reduction, and the energy-consuming reverse

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electron transfer that causes NAD^+ reduction and requires a high transmembrane electrical potential [Murphy, 2009; Koopman et al., 2010, Vinogradov and Grivennikova, 2016]. ROS formation in Complex I presumably involves flavin coenzyme FMN that directly catalyzes NADH oxidation. In Complex II [Grivennikova et al., 2017] and III [Ksenzenko et al., 1983], superoxide generation is associated with ubiquinone (coenzyme Q) oxidation that yields the semiquinone radical.

Respiratory chain complexes are apparently organized in such a way as to minimize the "leakage" of electrons that results in ROS formation. However, under unfavorable conditions, the structure and conformation of these enzymes can be disrupted, and this entails increased ROS production. For instance, point mutations in some subunits of Complex I may result in a significant increase in mtROS production. This contributes to the development of a large number of "mitochondrial diseases" such as Leber's hereditary optic neuropathy, Leigh syndrome, and some myopathies [Hayashi and Cortopassi, 2015; Carelli et al., 2009]. Presumably, modifying Complex I via phosphorylation and S-nitrosylation also enhances the production of mtROS [Dröse et al., 2016]. Dismantling Complex II by acidifying the cytoplasm and accumulating Ca^{2+} in mitochondria results in a drastic increase in ROS formation [Hwang et al., 2014]. The molecular target in Complex III is probably the cluster of easily oxidizable, tightly associated molecules of cardiolipin [Dibrova et al., 2013]. Oxidation of this phospholipid can disrupt the enzyme's function and stimulate ROS formation. The feasibility of such a scenario is confirmed by the fact that ROS production drastically increases under the influence of antimycin A, a specific inhibitor of Complex III. Antimycin A stabilizes the semiquinone form of coenzyme Q in the active center of the enzyme [Ksenzenko et al., 1983].

An important regulatory role in terms of mtROS production is played by supercomplexes, which include stoichiometric amounts of the respiratory chain complexes, the ATP synthase (complex V) and the carrier of adenine nucleotides. The most stable supercomplexes are formed by Complexes I and III, and cardiolipin molecules play an essential role in their formation [Lenaz et al., 2016]. The disruption of supercomplexes can cause the inhibition of oxidative phosphorylation and enhanced mtROS formation. An example is provided by neurons and astrocytes. In neurons, where supercomplexes are stable, low amounts of mtROS are normally produced, whereas in astrocytes, where a significant part of the respiratory chain enzymes are in a free state, much more mtROS are formed [Lopez-Fabuel et al., 2016].

Since the excess of mtROS formation is potentially dangerous for the cell, mitochondria

use a number of antioxidant systems. These systems include the aforementioned Mn-dependent superoxide dismutase, catalase, glutathione peroxidases, peroxiredoxin and glutaredoxin systems. In addition, uncoupling family proteins (UCP) can be involved in the regulation of mtROS formation. In particular, UCP2 protein inhibits mtROS formation by decreasing the membrane potential. Besides, UCP2 catalyzes the exchange of intramitochondrial four-carbon intermediates such as oxaloacetate, malate and aspartate for phosphate and a proton across the mitochondrial membrane [Vozza et al., 2014]. By exporting these intermediates from mitochondria, UCP2 negatively regulates the oxidation of acetyl-CoA-producing substrates via the Krebs cycle, lowering the redox pressure on the mitochondrial respiratory chain and ROS production [Vozza et al., 2014]. UCP2 gene knockout in mice results in oxidative and metabolic disorders in various tissues, and, particularly, enhances inflammation in diabetes, cardiovascular and neurodegenerative diseases [Mattiasson and Sullivan, 2006]. At the same time, excessive activation of inflammation in UCP2-deficient mice stimulates a protective antimicrobial response during acute infections (see below) [Arsenijevic et al., 2000; Bai et al., 2005; Rousset et al., 2006].

An important mechanism of cell protection from excess mtROS is based on inducing the selective autophagy of mitochondria (mitophagy). Mithophagy results in the isolation and subsequent degradation of damaged mitochondria that may be the main source of ROS [Fivenson et al., 2017; Kulikov et al., 2017]. Impairment of autophagy processes causes accumulation of damaged cell structures (in particular, mitochondria), ROS buildup in the cell, and, as a consequence, inflammatory processes and fibrosis in adjacent tissues [Sun et al., 2017; Lee et al., 2016].

The role of mtROS in phagocytosis

In the immune system, mtROS formation is mainly associated with the activation of innate immunity. Bacteria are major activators of innate immunity. Their pathogen-associated molecular patterns (PAMPs) represent the evolutionarily conserved structures that are common to a number of taxonomically related microbial groups. Bacterial PAMPs are recognized by surface pattern-recognition receptors (PRRs) of innate immune cells. The surface PRRs include the Toll-like receptors TLR1, TLR2 and TLR4, which recognize lipopeptides, lipoteichoic acid and lipopolysaccharides (LPS), respectively.

Apparently, the main interaction of bacterial PAMPs with PRRs occurs in the phagosome of neutrophils and monocytes/macrophages that have engulfed bacteria during phagocytosis. Interestingly, the TLR2$^{-/-}$TLR4$^{-/-}$ double knock-out macrophages phagocytose

much fewer bacteria than the wild type (WT) macrophages either in a serum-free or in a serum-containing medium. At the next stage of phagocytosis, the phagosomes of WT macrophages fuse with the lysosomes to form phagolysosomes. Under the influence of lysosomal enzymes, bacteria are degraded, that results in the liberation of PAMPs. The phagosomes of TLR2^{-/-} TLR4^{-/-} macrophages that have engulfed a certain number of bacteria poorly attract lysosomes and poorly fuse with them. As a result, there is reduced degradation of bacteria and liberation of PAMPs [Blander and Medzhitov, 2004; 2006]. PAMPs that have been formed through the degradation of bacteria are recognized in the phagosome by the TLRs present in the internalized cytoplasmic membrane forming the phagosome. However, the interaction of bacterial PAMPs with TLRs can also occur on the cell surface in cases of extracellular degradation of bacteria or recognition of the outer membrane vesicles (OMVs) containing PAMPs [Thay et al., 2014; Chu et al., 2016].

The binding of PAMPs with TLRs causes the activation of a number of signaling pathways, which leads to significant changes in the functional activity of the cell. In terms of the subject of this review, these changes are manifested in the activation of serine-threonine kinases Mst1 and Mst2, activation of the adapter proteins TRAF6 and ECSIT, activation of NADPH oxidase, and induction of synthesis of pro-inflammatory cytokines [West et al., 2011a; Geng et al., 2015] (see, Figure).

The TRAF6 protein (TNF receptor-associated factor 6) was originally identified in the cytoplasm as the agent involved in the IL-1-receptor-mediated activation of transcription factor NF-κB. In addition, TRAF6 is implicated in the signaling pathways downstream of TNF receptors, TLR, T-cell receptors, TGF-β receptors, etc. An important property of TRAF6 is its ubiquitin ligase activity [Walsh et al., 2015]. In intact macrophages, TRAF6 is associated with the inactive GDP-bound form of the Rac protein, which occupies the site required for subsequent binding of the Evolutionarily Conserved Signaling Intermediate in Toll pathways (ECSIT) protein [Geng et al., 2015].

ECSIT is present in the cytoplasm and in the mitochondria of virtually all cells. Originally, this protein was found to function as a specific mediator in the Toll/IL-1 pathway and the activator of the MEKK-1 kinase that stimulates NF-κB phosphorylation [Kopp et al., 1999]. Subsequently, it was established that, by interacting in mitochondria with the chaperone NDUFAF1 [Vogel et al., 2007] and proteins CIA30, Acad9 and TMEM126B, ECSIT forms an association that enables assembly of Complex I [Heide et al., 2012].

Importantly, ECSIT contains a site that interacts with TRAF6. After bacterial ligands bind to PRRs in innate immune cells, TRAF6 is transferred to mitochondria, where it binds to

the fraction of the ECSIT protein that is located in the outer mitochondrial membrane. In association with TRAF6, ECSIT is ubiquitinated. This results in disruption of Complex I, decelerated oxidative phosphorylation and accelerated superoxide anion radical and hydrogen peroxide formation in macrophage mitochondria [West et al., 2011a]. Interestingly, when activated by TLR4, the TRAF6-ubiquitinated ECSIT protein can bind to the p65/p50 components of NF-κB and stimulate the expression of proinflammatory cytokines [Mi Wi et al., 2015].

Serine-threonine kinases Mst1 and Mst2 were shown to play major roles in the signaling pathways that control cell growth and apoptosis. Lack of these kinases results in a combined immunodeficiency that is fraught with the development of bacterial and viral infections, neutropenia and lymphopenia [Abdollapour et al., 2012]. A study by Geng and co-workers [2015] demonstrated that Mst1/2 kinases are involved in the accumulation of ROS and in ROS-dependent destruction of bacteria in phagolysosomes. The Mst1/2 kinases are activated upon triggering of surface TLRs and activation of the downstream MyD88-dependent signaling pathway. Interestingly, stimulation of the strictly endosomal TLRs such as TLR3, TLR7, TLR8 and TLR9, for reasons yet unknown, does not result in the activation of Mst1/2 [Geng et al., 2015]. When activated, the Mst1/2 kinases activate protein kinase Cα (PKCα), which phosphorylates and inactivates the LyGDI protein, an inhibitor of a small GTPase Rac. The inactive GDP-bound form of Rac transforms into the active GTP-bound form. The latter immediately undergoes TRAF6-dependent ubiquitination, which results in dissociation of Rac from TRAF6. Consequently, the ECSIT-binding site in the TRAF6 molecule becomes accessible for the interaction with ECSIT [Geng et al., 2015].

In addition, Mst1/2 activation promotes the recruitment of mitochondria to phagolysosomes. In monocytes and alveolar macrophages, within one hour after the phagosome/phagolysosome formation, mitochondria are directed towards the phagolysosome and come into the juxtaposition with it [Horwitz, 1983; West et al., 2011a]. Up to 80% of the phagolysosomes that are formed in Chinese hamster ovary cells and in U937-derived macrophages are associated with one or more mitochondria [Chong et al., 2009].

Presumably, the purpose of the migration of mitochondria towards phagolysosomes and integration of these organelles into one functional complex is the summation of the bactericidal effects of the ROS generated by NADPH oxidase in the phagolysosome and the mitochondrial ROS.

The mitochondrial movement towards phagolysosomes is carried out by actin cytoskeleton which is reorganized upon activation of the small GTPase, Rac2

[Dharmawardhane and Bokoch, 1997; Williams et al., 2000]. Activation of Rac2 by the Mst1/2 kinases seems to be the trigger that launches the movement of mitochondria. The assembly of the TRAF6-ECSIT complex stimulates mitochondrial movement as well [Geng et al., 2015]. It seems likely that mtROS are also involved in regulating this process. In mice lacking both Mst1 and Mst2 in their hematopoietic cells, mitochondria do not move towards phagolysosomes and ROS formation is inhibited. This disrupts phagosomal killing of bacteria, attenuating the antimicrobial protective system and significantly increasing the infection-dependent mortality of these mice [Geng et al., 2015].

Along with TLRs, NLRs (NOD-like receptors) play an important role in recognizing pathogen-associated molecular patterns. The NOD2 receptor in human macrophages significantly promotes the inflammatory response and suppresses the intracellular growth of *Mycobacterium tuberculosis* [Brooks et al., 2011]. It has been shown recently that muramyl dipeptide, a classical activator of NOD2 receptor, stimulates mtROS formation in skeletal muscle cells [Maurya et al., 2015]. Presumably, NOD2-dependent signaling in macrophages is also associated with mtROS formation.

mtROS are involved in the regulation of innate immune responses

In addition to the bactericidal effect, mtROS perform important regulatory functions in innate immune cells. It has been established that mtROS promote the activation of NADPH oxidase, which produces ROS in phagosomes and in the extracellular milieu, being responsible for antimicrobial protection in many respects. It was revealed that the NADPH oxidase-derived ROS can enter the cytoplasm and stimulate ROS formation in mitochondria. Thus, there is a continuous interaction or crosstalk between the NADPH oxidase and mitochondria, which plays an essential role in the vital activities of living cells [Dikalov, 2011; Kröller-Schön et al., 2014].

Most studies on the crosstalk between mitochondria and the NADPH oxidase were conducted with non-lymphoid cells [Dikalov, 2011]. Recently, we have shown for the first time that this interaction occurs in human neutrophils [Vorobjeva et al., 2017]. Using the mitochondria-targeted antioxidant SkQ1 [Antonenko et al., 2008a; 2008b], we demonstrated that mtROS are involved in a variety of neutrophil effector functions, such as the oxidative burst, degranulation, and apoptosis. Inhibition of the fMLP-induced respiratory burst by SkQ1 indicated a significant role of mtROS in the receptor-dependent activation of NADPH oxidase [Vorobjeva et al., 2017]. It was shown that mtROS are required for the fMLP-dependent exocytosis of both primary (azurophilic) and secondary (specific) granules [Vorobjeva et al.,

2017]. Degranulation of neutrophils plays an important role in antimicrobial defense and does not seem to depend on NADPH oxidase activity. Neutrophils from patients with chronic granulomatous disease (CGD), an illness caused by genetic defects in the NADPH oxidase complex, showed enhanced fMLP-dependent (as well as spontaneous) degranulation [Pak et al., 2007]. However, signaling pathways that lead to the activation of NADPH oxidase and to the degranulation of neutrophils have not been elucidated and their mtROS-sensitive mediators have not yet been identified.

An important aspect of neutrophil activation is the suppression of spontaneous apoptosis, which is otherwise characteristic of these cells. It was revealed in our studies that neutralization of mtROS with SkQ1 enhances spontaneous apoptosis and cancels the anti-apoptotic effect of fMLP, a classical neutrophil activator [Vorobjeva et al., 2017]. The main mechanisms that counteracts neutrophil apoptosis depend on the transcription factor NF-κB, which controls the expression of anti-apoptotic proteins of the Bcl-2 family [François et al., 2005]. It had been shown earlier that in endothelial cells, mtROS are necessary for TNF-induced NF-κB activation [Zinovkin et al., 2014]. Furthermore, stimulation of human neutrophils with CpG DNA, a TLR9 agonist, induces pro-survival signaling in neutrophils, which is mediated via NF-κB-dependent Mcl-1 mRNA transcription and/or stabilization and subsequent Mcl-1 translocation to the mitochondria [El Kebir et al., 2014]. Thus, activation of NF-κB-dependent signaling is likely to mediate the anti-apoptotic effect of mtROS in neutrophils.

An additional mechanism of antimicrobial defense is based on the formation of neutrophil extracellular traps (NETosis). It has been shown that the activation of NETosis depends on ROS accumulation in neutrophils [Fuchs et al., 2007; Kirchner et al., 2012; Vorobjeva and Pinegin, 2016]. Recent studies on granulocytes from patients with systemic lupus erythematosus have revealed that spontaneous NETosis of low-density neutrophils depends on mtROS production [Lood et al., 2016]. A dependence of NETosis on mtROS was also detected in the neutrophils of patients with CGD [Lood et al., 2016]. Similar to neutrophil degranulation, NETosis may not be dependent on the activity of NADPH oxidase.

The mtROS involvement in antibacterial defense is also exemplified by mice with a knockout of regulatory protein, UCP2. It was revealed that large amounts of mtROS are produced in the bone marrow of such mice, while the level of ATP synthesis is reduced [Kretzschmar et al., 2016]. The knockout of UCP2 resulted in ROS-dependent activation of the transcription factor NF-kB and increased synthesis of proinflammatory cytokines and chemokines. This is indicative of the general activation of the immune system in the absence of

infection [Bai et al., 2005]. The macrophages of these mice produce much more mtROS and kill *T.gondii* much more efficiently than normal immune cells [Arsenijevic et al., 2000]. In addition, UCP2$^{-/-}$ mice infected with *L.monocytogenes* are characterized by an increased survival rate. This is due to the elevated levels of ROS [Rousset et al., 2006].

A decrease in mtROS production results in disruption of the antimicrobial defense system that eliminates pathogens. If the TRAF6 and ECSIT proteins in mouse macrophages are knocked down, mtROS formation is significantly reduced and the mechanism of *S. typhimurium* elimination is disrupted. Mice that are deficient in TRAF6 and ECSIT contain 2-3 times more *Salmonella* in the liver and in the spleen than wild type mice. In addition, such mice are characterized by increased mortality rates [West et al., 2011b]. Apart from microorganisms and their components, pro-inflammatory cytokines synthesized upon cell activation can also induce mtROS formation. In macrophages activated with IFN-γ or TNF, formation of mtROS is increased. This enhances their ability to kill the intracellular bacteria *L.monocytogenes* and *M. tuberculosis* [Sonoda et al., 2007; Roca and Ramakrishnan, 2013]. However, in tuberculosis, the increased bactericidal activity of macrophages is observed only at the first stage of the infection. Subsequently, macrophage necrosis, release of mycobacteria into the environment and the infection of surrounding tissues occur under the influence of TNF and excess of mtROS radicals. This results in exacerbation of the infectious process [Roca and Ramakrishnan, 2013].

Activators of mtROS formation

The formation of mtROS can be regulated by endogenous and exogenous activators. As noted above, the basic sites for mtROS formation in mitochondria are Complexes I and III of the electron-transport chain. However, dehydrogenase complexes, such as alpha-ketoglutarate dehydrogenase (2-oxoglutarate dehydrogenase) and pyruvate dehydrogenase with dihydrolipoamide dehydrogenase (DLDH) as the main component may be the predominant source of mtROS under certain conditions [Grivennikova and Vinogradov, 2013]. In the mitochondria of skeletal muscles, these dehydrogenases are able to produce 8 and 4 times more ROS, respectively, than Complex I in the presence of alpha-ketoglutarate or pyruvate [Quinlan et al., 2014]. Dihydrolipoamide dehydrogenase activity increases multifold in the presence of ammonium ions, and this enables an additional increase in ROS formation [Grivennikova and Vinogradov, 2013]. Maximum ROS production in Complex I is achieved by the reverse transfer of electrons from ubiquinone to NAD$^+$. However, the rate of this process is limited by the low content of succinate which serves as the main reductant of ubiquinone. Addition of an

exogenous analog of succinate that penetrates the cell membrane caused an increase in ROS levels and IL-1 production in macrophages, promoting the manifestation of the M1 phenotype [Mills et al., 2016]. Thus, modification of metabolic pathways enables to substantially increase ROS formation, and, consequently, to enhance the bactericidal properties of neutrophils and monocytes/macrophages.

Exogenous activators of mtROS can use a number of mechanisms of action. Various electron acceptors in the respiratory chain can be oxidized by molecular oxygen to form ROS. The most well-known compound of this type is Methylene Blue (MB) that belongs to the group of phenothiazines. MB as the first synthetic drug of this kind was developed in 1886 by Paul Gutman and Paul Ehrlich to fight malaria. Recently, the interest in MB has been rekindled because it has been successfully used as a remedy for malaria and microbial infections. Another example of the phenothiazines action is the stimulation of antimicrobial defense by a well-known neuroleptic substance, thioridazine, which protects mice from the infection caused by *Salmonella enterica* (*Typhimurium*) and promotes the destruction of this bacterium in the phagolysosomes of macrophages [Dasgupta et al., 2010]. In addition, thioridazine was efficient in treating antibiotic-resistant myxoplasmosis and toxoplasmosis [Amaral and Molnar, 2014]. Brilliant green and similar dyes of the triarylmethane group use a similar mechanism of mtROS stimulation. Despite the known bactericidal properties of these substances, their practical potential in terms of stimulating innate immunity responses still remains to be explored.

Another approach to researching the stimulatory effect of mtROS is based on investigating changes in ion transport processes. It was demonstrated that diazoxide, a vasodilator and an activator of ATP-sensitive potassium channels in the mitochondria of endothelial cells, substantially increases mtROS formation in these cells [Dikalov, 2011]. Diazoxide also stimulates the expression of adhesion molecules, such as P-selectin and ICAM-1, which enhances the rolling and adhesion of neutrophils [Dal-Secco et al., 2008]. An important function with regard to neutrophil activation is performed by another type of potassium channels, calcium-dependent channels of low conductivity (SK-channels). Opening these channels stimulates the neutrophil-dependent elimination of pathogens [Segal, 2005]. Fay and co-workers [2006] demonstrated that the artificial activator of SK channels, 1-ethyl-2-benzimidazoline, causes NADPH-independent ROS formation in neutrophils. mtROS formation in conjunction with the activation of SK channels (in particular, SK3-channels) stimulates NETosis. This process does not depend on the NADPH oxidase either [Douda et al., 2015].

Recently, extensive research and development projects concerning mitochondria-targeted drugs have been carried out [Antonenko et al., 2008a; 2008b; Vorobjeva et al., 2017; Lood et al., 2016]. Inhibition of mtROS production or their neutralization by means of mitochondria-targeted antioxidants is considered as a high-priority project. It is assumed that such drugs will be efficient in treating autoimmune diseases and suppressing excessive inflammatory processes that accompany various pathological conditions. Conversely, the activators of mtROS production discussed above can be envisaged as potential activators of the bactericidal effects of neutrophils and monocytes/macrophages, the key cell types of antibacterial immunity. Apart from this potentially useful strategy, specific activators of mtROS formation can be used currently in clinical practice.

Prospects of using the mtROS activators in clinical practice

Potentially, mtROS activators could be successfully used in treating patients with defective NADPH oxidase, which are at an increased risk of infectious morbidity. These deficiencies can be primary and secondary. Primary deficiencies are associated with mutations in the genes that encode the subunits of the NADPH oxidase (gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, Rac2). In this situation, the NADPH oxidase virtually does not make ROS [Nauseef, 2008]. Such mutations entail the development of CGD. Children with CGD suffer from recurrent bacterial and fungal infections since the first days of their life [Roos, 2016]. In addition to the increased infectious morbidity, children with CGD are prone to autoinflammatory and autoimmune processes [Holland, 2010]. Gene expression profiling of neutrophils from patients with CGD revealed a constitutively increased expression of the genes that encode inflammatory mediators and innate immunity-associated receptors [Kobayashi et al., 2004]. Such patients can reach the mature age if treated with antimicrobial drugs. However, they suffer from granulomas formed in the skin and urogenital tract, Crohn's disease and other chronic inflammatory processes [Riber et al., 2012].

Secondary defects in ROS formation are not associated with the mutations of NADPH oxidase, but are due to defects in metabolic pathways [de Oliveira-Junior et al., 2011]. Defects in the enzyme IRAK4 (the early key kinase of TLR/IL-1R/IL-18R signaling pathway), the IκB protein (the inhibitor of transcription factor NF-κB), and the NEMO protein (the modulator of factor NF-κB activity) cause a decrease in NADPH oxidase activity. The consequences of this downregulation include a decline in ROS formation and an increase in infectious morbidity that is associated with a CGD-like syndrome and a propensity for the development of autoinflammatory processes [Nishikomori et al., 2004; Ku et al., 2007; Luengo-Blanco et al.,

2008]. Profound inhibition of NADPH oxidase function and ROS formation can result from a defect in the glucose-6-phosphate dehydrogenase, the key regulatory enzyme of the pentose phosphate pathway. Patients with this defect develop recurrent infections resembling CGD [Roos et al., 1999].

In all cases of increased infectious morbidity associated with both primary and secondary deficiencies of NADPH oxidase, it is reasonable to use drugs that stimulate mtROS formation. In our opinion, thiazolidinedione drugs, which are Peroxisome-Proliferator Activated Receptor γ (PPARγ) transcription factor agonists, hold particular promise for the future. PPARγ belongs to the superfamily of nuclear hormone receptors that regulate reproduction, metabolism, development and the immune response [Feige et al., 2006; Kostadinova et al., 2005]. PPARγ is detected in all cells of the immune system including neutrophils and monocytes/macrophages. Natural PPARγ agonists are prostaglandins (e.g., 15-deoxy-delta-12,14-prostaglandin J2), while artificial agonists are synthetic compounds such as thiazolidinediones (cigilitazone, rosiglitazone, troglitazone, and pioglitazone). Thiazolidinediones are permitted for medical use as antidiabetic drugs due to their ability to increase the sensitivity of cells and tissues to insulin [Fernandez-Boyanapalli et al., 2010].

It is known that thiazolidinediones stimulate phagocytosis and reduce the microbial load of the organism [Aronoff et al., 2007; Kielian et al., 2008; Gautier et al., 2012]. This is related to the ability of thiazolidinediones to stimulate mtROS formation. Using mitochondrial fluorescent dyes, pioglitazone was shown to stimulate ROS formation in the mitochondria of neutrophils and monocytes in mice with a knockout of gp91, the main component of NADPH oxidase. The phagocytes of gp91$^{-/-}$ mice fed with pioglitazone were capable of killing *St. aureus,* in contrast to the phagocytes of untreated mice. This effect was dependent on PPARγ and was ablated by PPARγ inhibitors. Interestingly, both in macrophages and neutrophils, pioglitazone-induced ROS formation was completely inhibited by rotenone and, therefore, was associated with the NAD$^+$ reduction in Complex I of respiratory chain [Fernandez-Boyanapalli et al., 2015]. It is known that PPARγ together with PGC-1α (PPARγ coactivator-1α) stimulates the expression of mitochondrial proteins and the biogenesis of mitochondria. However, the enhanced formation of mtROS is likely not associated with this effect. Moreover, it has been shown that in many cases, PGC-1α stimulates the expression of antioxidant enzymes and inhibits ROS formation. This effect probably underlies the anti-inflammatory and antidiabetic action of thiazolidinediones [Olefsky and Glass, 2010]. However, elucidating the mechanism of stimulation of ROS formation in the mitochondria of neutrophils and macrophages under the action of PPARγ agonists requires further research efforts.

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The feasibility of application the thiazolidinediones in the NADPH oxidase-deficient system is due to the following two important features of these drugs. Along with the ability to reduce the microbial cargo in the organism, thiazolidinediones possess anti-inflammatory properties. They inhibit the production of a number of LPS-induced proinflammatory mediators, such as NO, TNF-α, IL-1β, IL-6, IL-12, IL-8, CCL20, MCP-1, MIP-1α, and also suppress Th1 and Th17 cells. However, they concomitantly promote the synthesis of anti-inflammatory cytokines such as IL-10 [Olefsky and Glass, 2010; Qiu and Li, 2015].

Thus, the application of thiazolidinediones is expected to produce therapeutic and prophylactic effects in systems with primary or secondary deficiencies of the NADPH oxidase. Presumably, this area of pharmacological research will be of paramount importance if gene therapy proves unsuccessful, and will help to ameliorate disease course in children with CGD or CGD-like syndromes.

Nonetheless, therapeutic application of mtROS activators has certain limitations because of their toxic effects on the immune cells per se and on adjacent tissues. Using the model of tuberculosis infection of macrophages, it was demonstrated that macrophage necrosis is largely due to an mtROS-dependent increase in the contents of cyclophilin D, a component of the mitochondrial pore (mitochondrial permeability transition pore), and of ceramide which is a powerful inducer of the pore. The opening of the mitochondrial pore causes an increase in the permeability of the mitochondrial membrane, mitochondrial swelling, and cell death. The inhibition of cyclophilin D and ceramide synthesis in macrophages with alisporivir and desipramine, respectively, prevents macrophage necrosis while maintaining their enhanced bactericidal properties [Roca and Ramakrishnan, 2013]. In this regard, one of the directions in the therapeutic application of mtROS activators is the elaboration of methods aiming to limit their toxic effects on the host cells while preserving their bactericidal effects. We believe that this approach can significantly expand the possibilities of treating a number of chronic infectious and inflammatory processes.

Conclusions

Taken together, the data presented here highlight the essential role of mtROS in innate immunity. mtROS formation in phagocytizing neutrophils and monocytes/macrophages can be promoted using endogenous metabolites and exogenous agents including widely used drugs and immunostimulators. This strategy can be used to enhance the bactericidal effect of these cells and to more effectively eliminate pathogens in the organism. Activators of mtROS formation can be used for the therapy and prevention of chronic infections and inflammatory

diseases that develop in settings of primary or secondary NAPDH oxidase deficiencies.

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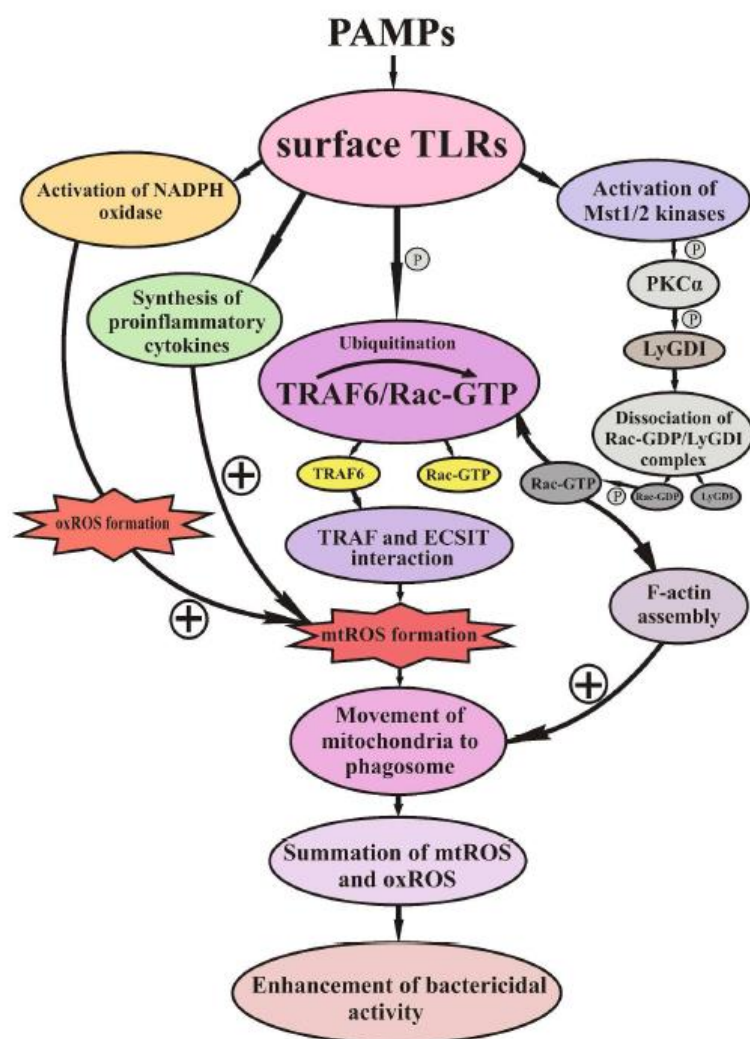
Figure. A scheme illustrating the role of mtROS in the enhancement of bactericidal activity.

The pathway resulting in the enhanced mtROS production involves the adapter protein and ubiquitin ligase TRAF6, serine-threonine kinases Mst1 and Mst2 as well as G-proteins, Rac1 and Rac2, of the Rho family GTPases. Upon interaction of ligands with surface TLRs, TRAF6 and Mst1/2 are activated. Mst1/2 activates its downstream target, PKCα kinase, which phosphorylates the inhibitor LyGDI resulting in the dissociation of the Rac-GDP/LyGDI complex, liberation and activation of Rac proteins. Activated Rac-GTP are ubiquitinated by and dissociate from TRAF6. This enables interaction of TRAF6 with ECSIT, which entails reduced oxidative phosphorylation and enhanced mtROS formation. Rac-GTP also activates F-actin assembly, which facilitates movement of mitochondria towards the phagosome and consolidation of bactericidal effects of mitochondrial and cytoplasmic ROS. Enhanced formation of mtROS can also be induced by pro-inflammatory cytokines and by NADPH-oxidase-derived ROS [West et al. 2011b; West and Shadel, 2017; Geng et al., 2015].