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Review article

ROS systems are a new integrated network for sensing homeostasis and alarming stresses in organelle metabolic processes

Yu Sun [a], Yifan Lu [a], Jason Saredy [b], Xianwei Wang [b], Charles Drummer IV [a], Ying Shao [a], Fatma Saaoud [a], Keman Xu [a], Ming Liu [a], William Y. Yang [b], Xiaohua Jiang [a,b], Hong Wang [b], Xiaofeng Yang [a,b,*]

[a] Centers for Cardiovascular Research and Inflammation, Translational and Clinical Lung Research, USA
[b] Metabolic Disease Research and Cardiovascular Research and Thrombosis Research, Departments of Pharmacology, Microbiology and Immunology, Lewis Katz School of Medicine at Temple University, Philadelphia, PA 19140, USA

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ABSTRACT

Reactive oxygen species (ROS) are critical for the progression of cardiovascular diseases, inflammations and tumors. However, the mechanisms of how ROS sense metabolic stress, regulate metabolic pathways and initiate proliferation, inflammation and cell death responses remain poorly characterized. In this analytic review, we concluded that: *1)* Based on different features and functions, eleven types of ROS can be classified into seven functional groups: metabolic stress-sensing, chemical connecting, organelle communication, stress branch-out, inflammasome-activating, dual functions and triple functions ROS. *2)* Among the ROS generation systems, mitochondria consume the most amount of oxygen; and nine types of ROS are generated; thus, mitochondrial ROS systems serve as the central hub for connecting ROS with inflammasome activation, trained immunity and immunometabolic pathways. *3)* Increased nuclear ROS production significantly promotes cell death in comparison to that in other organelles. Nuclear ROS systems serve as a convergent hub and decision-makers to connect unbearable and alarming metabolic stresses to inflammation and cell death. *4)* Balanced ROS levels indicate physiological homeostasis of various metabolic processes in subcellular organelles and cytosol, while imbalanced ROS levels present alarms for pathological organelle stresses in metabolic processes. Based on these analyses, we propose a working model that ROS systems are a new integrated network for sensing homeostasis and alarming stress in metabolic processes in various subcellular organelles. Our model provides novel insights on the roles of the ROS systems in bridging metabolic stress to inflammation, cell death and tumorigenesis; and provide novel therapeutic targets for treating those diseases. (Word count: 246).

1. Introduction

Reactive oxygen species (ROS) are defined as oxygen-containing reactive species. It is well established that increased ROS play a vital role in promoting cardiovascular disease [1–3] such as hypertension, aortic aneurysm, hypercholesterolemia, atherosclerosis [4], diabetic vascular complication, cardiac ischemia reperfusion injury, myocardial infarction [5], heart failure, and cardiac arrhythmias [6], chronic kidney disease [7–11], hyperhomocysteinemia [12–14], metabolic syndrome [15], induction of regulatory T cells (Treg) and T cell-mediated inflammation [16,17], cigarette smoking [18], metabolically healthy obesity [19,20] and obesity [21], and tumorigenesis [22]. Additionally, ROS have been studied as the therapeutic targets for these disease progression and complications [23]. However, two important issues remain unknown, whether and how the ROS system senses danger associated molecular pattern (DAMP) [24] and conditional DAMP [25,26] signals in metabolic diseases [27] and connects danger signals to proliferative, inflammatory and cell death-related responses.

ROS are generated by oxidant enzymes and scavenged by scavenging systems, which include enzymatic or non-enzymatic reactions. Imbalances between generation and scavenging ROS systems increase ROS levels and represent alarming stresses. ROS are generated in almost every subcellular organelle in cell [28], including plasma membrane [29], cytosol [30], mitochondria [31], nucleus [31], peroxisome [32],

* Corresponding author. Centers for Cardiovascular Research, Inflammation, Translational and Clinical Lung Research, Metabolic Disease Research and Thrombosis Research, Lewis Katz School of Medicine at Temple University, 3500 North Broad Stress, Philadelphia, PA 19140, USA.
E-mail address: xfyang@temple.edu (X. Yang).

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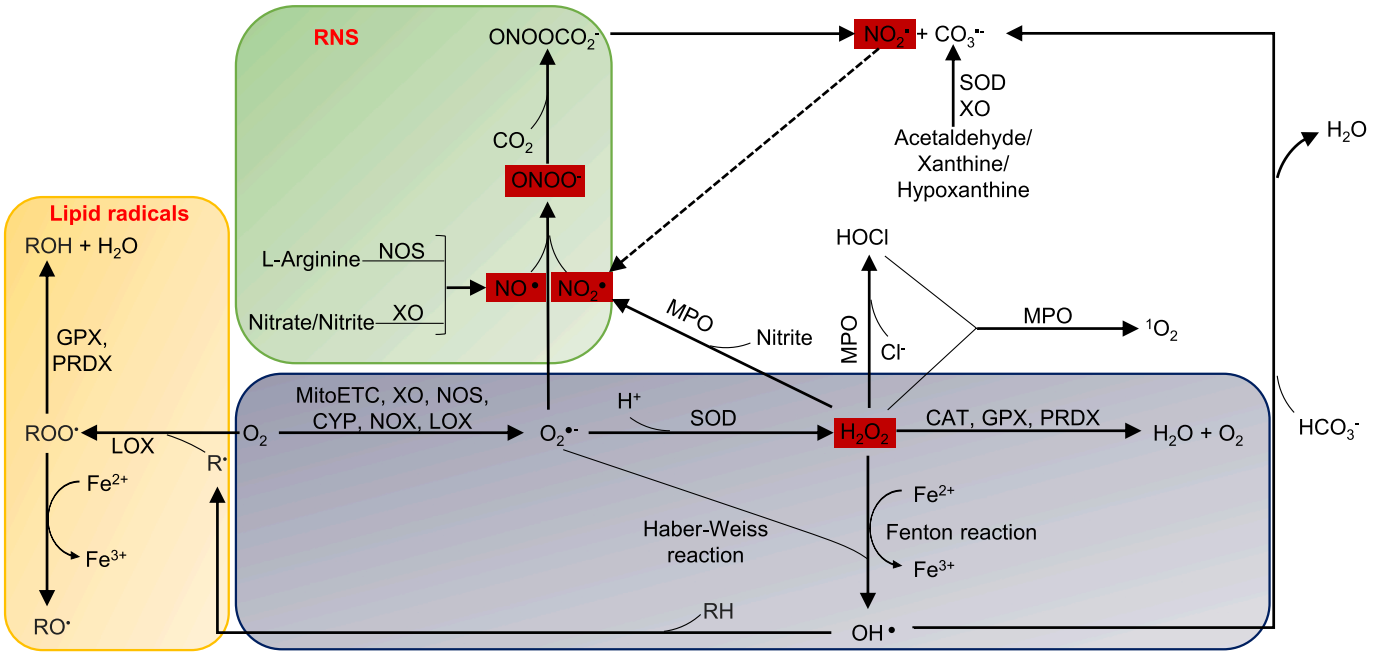

Fig. 1. Four types of ROS (red box) that are generated by nitrogen and oxygen are communication ROS, which are, connected with different types of ROS systems. NO■, NO2 ■, H2O2 and ONOO− have the ability to cross lipid membrane and are classified as communication ROS. Three types of nitrogen-containing ROS are communication ROS.

Abbreviations: O2■−—superoxide, NO■—nitric oxide radical, NO2 ■—nitrogen dioxide, OH■—hydroxyl radical, ROO■—peroxyl radical, RO■— alkoxyl radical, CO3■−—carbonate radical, H2O2—hydrogen peroxide, ONOO−—peroxynitrite, 1O2—singlet oxygen, HOCl—hypochlorous acid, ONOOCO2 −—nitrocarbonate, MitoETC: Mitochondrial electron transport chain, XO—xanthine oxidase, NOS—nitric oxide synthase, CYP— cytochrome P450, NOX—NADPH oxidase, LOX—lipoxygenase, SOD—superoxide dismutase, MPO—myeloperoxidase, MAO— monoaminoxidase. GPX—glutathione peroxidase, PRDX—peroxiredoxin, CAT—catalase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

endoplasmic reticulum (ER) [33], Golgi [29], and others. Of note, 41 human enzymes involved in generating H_2O_2 and O_2^- listed in the Supplemental Table 1 demonstrated the proof of principle that various metabolic processes contribute to generation of ROS [34]. Studies found that mitochondrial ROS production promotes endothelial cell activation and monocyte migration [35–42]; and cytosolic ROS production via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activation contributes to innate immunity. These findings suggest that while each organelle can generate ROS, they differ in sensing danger signals [43,44]. Current studies have found that 2801 metabolic pathways(https://metacyc.org/) exist in various organelles [45] with efficient metabolic requirements for space and compartmentalization [46,47], as we discussed in our recent paper [48].

However, several important questions remain: *1)* are there sensing and monitoring systems for each of 2801 metabolic pathways [49], in one-to-one basis, or an integrated sensing system for all the metabolic pathways compartmentalized in all the subcellular organelles; *2)* it is non-evolutionally economic and efficient to have one-to-one sensing system for each of 2801 metabolic pathways. An example of an evolutionally efficient sensing system is the humoral immune system, where only four groups of IgG subtypes [50] as backbone structures are needed in order to recognize millions of antigens. So, how can it be possible for danger-associated molecular pattern receptors (DAMP-receptors) [24,27,36] and conditional DAMP receptors [25], including plasma membrane-localized Toll-like receptors, and cytosolically localized caspase-1 [4,5,51–57]/canonical and caspase-4 (humans)/caspase-11 (mice) non-canonical inflammasome pathways [58,59], to monitor the two cellular status, physiological homeostasis and pathologic abnormality of metabolic processes in all the organelles, in an evolutionarily efficient manner; and *3)* is there any currently known sensing system capable of: *i)* localizing in all the subcellular organelles; *ii)* trafficking across the membranes of organelles; *iii)* utilizing a universal process shared by all the metabolic pathways, such as being sensitive to any abnormalities of electron donation and electron acceptance as elucidated in our previous reviews [37–40] and reports [41,42,60,61]; and *iv)* converging and activating inflammasomes [62]; and *v)* regulating gene expression, damaging DNAs and determining cell proliferation and cell death [44].

To address those significant issues, we analyzed recent progress in the ROS field. ROS levels are tired to generally every reaction via reception and donation of electrons and participate in various intra-organelle signaling pathways. Thus, ROS might act as an evolutionarily efficient monitoring system for cellular metabolic status. However, whether the functions of ROS in organelles other than mitochondria are connected still needs to be elucidated. Superoxide ($O_2^{\bullet-}$) [30], nitric oxide radical (NO^-) [63] and hydrogen peroxide (H_2O_2) [64] are the most studied ROS subtypes, but other subtypes of ROS are not mentioned in many studies. Increased ROS are damaging to lipids, proteins and DNA; and some of these ROS can cross lipid membranes. However, the functional differences between ROS subtypes remain unknown. Furthermore, studies found that ROS are not only by-products of metabolic reactions, but also participate in physiological signaling [65–71], while increased ROS levels contribute to various metabolic outcomes via regulating metabolic processes and pathways [43,72–75]. Therefore, some antioxidant therapies fail because of the undesirable disruption of physiological functions caused by ROS. Therefore, the therapeutic window is important in order to maintain the ROS in normal levels. In addition to the regulatory mechanisms of gene transcription, mRNA splicing, mRNA stability with microRNA modulation, translation efficiency, and post-translation modifications, activations of different metabolic enzymes are also affected by metabolic processes. These enzymes alter the ROS production and scavenging, which qualify the ROS system for their metabolic sensing functions.

In this review, we analyzed ROS literature extensively regarding ROS generation, scavenging, subcellular localization, physiological and pathological signaling and outcomes. Since ROS are functionally

Table 1A
Reactive oxygen species (ROS) include seven radical- and four non-radical ROS; the generation enzymes and subcellular locations are presented. Among radical ROS, superoxide and nitric oxide are the dominant radicals which are generated by 10 enzymes and 4 enzymes, respectively. Among non-radical ROS, hydrogen peroxide, singlet oxygen and peroxynitrite are major ROS which are generated by 11 and 8 reactions, respectively.

Category	Name	Formula	Classification	Generating system	Intracellular organelle	PMID	Ref #
Radicals	Superoxide	$O2^{\bullet -}$	ROS	Complex I	Mt	24481843	[122]
				Complex II	Mt	24481843	[122]
				Complex III	Mt	24481843	[122]
				XO	Mt, Px, Cyto	15475499	[30]
				eNOS	Golgi, PM	24180388	[229]
				iNOS	Px	24180388	[229]
				nNOS	SR	24180388	[229]
				CYP	ER, Mt	12605691	[33]
				NOX	NOX1 (Caveolae), NOX2 (Phagosome, PM), NOX3/5 and Duox1/2 (PM), NOX4 (Mt, ER, Nuc)	15475499	[30]
				LOX	Cyto, Nuc	15475499	[30]
Radicals	Nitric oxide	$NO^{\bullet}$	RONS	eNOS	Golgi, PM, Mt	22178243, 20388537, 11729179	[29,63, 230]
				iNOS	Px	22178243, 12085352	[29,88]
				nNOS	SR	22178243	[29]
				XO	Px	22178243	[29]
Radicals	Nitrogen dioxide	$NO2^{\bullet}$	RONS	MPO	Nuc, Cyto, Mt	19298861	[89]
Radicals	hydroxyl radical	$OH^{\bullet}$	ROS	Fenton reaction	Mt, Cyto, Nuc, Px	24987008	[31]
				Haber-Weiss Reaction	Mt, Cyto	24987008	[31]
Radicals	peroxyl radical	$ROO^{\bullet}$	ROS	LOX	Microsome, Mt, Px, PM	15728540	[96]
Radicals	alkoxyl radical	$RO^{\bullet}$	ROS	LOX	Microsome, Mt, Px, PM	15728540	[96]
Radicals	Carbonate radical	$CO_3^{\bullet -}$	ROS	SOD1	Px	17505962	[97]
				XO	Px	17505962	[97]
Nonradicals	hydrogen peroxide	H_2O_2*	ROS	SOD	SOD1 (Cyto, Mt, Nuc, Px), SOD2 (Mt matrix),	17505962	[97]
				MAO	Mt	24987008	[31]
				XO	Mt, Px, Cyto	25678748	[64]
				p66shc	Cyto, Mt	27925481	[39]
				acyl-CoA oxidases	Px	25906193	[32]
				urate oxidase	Px	25906193	[32]
				D-amino acid oxidase	Px	25906193	[32]
				D-aspartate oxidase	Px	25906193	[32]
				L-pipecolic acid oxidase	Px	25906193	[32]
				L-α-hydroxyacid oxidase	Px	25906193	[32]
				polyamine oxidase	Px	25906193	[32]
Nonradicals	peroxynitrite	$ONOO^-$	RONS	–	–	17505962	[97]
Nonradicals	singlet oxygen	$^1O_2$	ROS	Photooxygenation	Mt, Cyto	22266568	[106]
				MPO	Cyto	27042259	[105]
				LOX	Px	169247	[104]
				dioxygenase	Px	169247	[104]
				lactoperoxidase	Px	169247	[104]
				CYP	ER	29081894	[107]
				Cytochrome C	Mt	29081894	[107]
				Fenton reaction	Px	26070643	[108]
Nonradicals	hypochlorous acid	HOCl	ROS	MPO	Mt, Cyto, Nuc	27042259, 26632272	[105, 111]

Abbreviations: XO—xanthine oxidase, NOS—nitric oxide synthase, CYP—cytochrome P450, NOX—NADPH oxidase, LOX—lipoxygenase, SOD—superoxide dismutase, eNOS—endothelial NOS, iNOS—inducible NOS, nNOS—neuronal NOS, MPO—myeloperoxidase, MAO—monoaminoxidase, Mt—mitochondria, Px—peroxisome, Cyto-cytosol, PM—plasma membrane, ER–endoplasmic reticulum, Nuc—Nucleus, SR–Sarcoplasmic reticulum, ECM—extracellular matrix.

* 41 specific generation enzymes for hydrogen peroxide and superoxide are also studied in Suppl. Table 1.

communicated each other between organelles, we propose a new working model that ROS is an integrated network for sensing homeostasis and alarming stress in metabolic processes in various subcellular organelles. Based on increased ROS levels, ROS participated in various metabolic pathways that can relay the metabolic stress to inflammasomes or branch out and cross-talk to other cell stress and stress-sensing pathways (also see section 5) to initiate corresponded reactions such as proliferation, inflammation and cell death [76].

2. ROS serve as the new sensing network for homeostasis and stress of metabolic processes in various subcellular organelles

ROS are generated by oxidant enzymes localized in different subcellular organelles; and ROS scavengers also contribute to maintaining redox homeostasis through enzymatic or non-enzymatic mechanisms. Oxidative stress, as a concept formulated in 1985, is induced when ROS generation is increased, or ROS scavengers are dysregulated [77]. Since oxidative stress is strongly correlated with inflammation and cell death [74,78,79], the imbalance of ROS generation and scavenging systems is an alarming signal for cellular metabolisms. In addition, mitochondrial ROS convey information to the nucleus and modulate the expression of nuclear genes accordingly in both physiological [80] and pathological states [66]. Based on the studies we mentioned above, ROS work as a sensor of organelles' metabolic homeostasis and alarming stress, additionally, ROS transduce signals among organelles and contribute to the determination of cellular responses [34,81]. To better understand the functions of ROS in physiological and pathological conditions, we classify eleven of ROS based on their chemical structures. However, only

Table 1B
ROS scavengers and subcellular localization are indicated, and the scavengers include enzymatic- and non-enzymatic- scavengers. Among the 11 types of ROS, superoxide, peroxyl radical, carbonate radical, hydrogen peroxide and peroxynitrite can be scavenged by enzymes; and the rest six types of ROS are found to be scavenged by non-enzymatic scavengers but not enzymatic scavengers.

Formula	Enzymatic scavenger	Localization (enzymatic-scavenger)	PMID	Ref #	Non-enzymatic scavenger	PMID	Ref#
$O2^{\bullet-}$	SOD	SOD1 (Cyto, Mt, Nuc, Px), SOD2 (Mt matrix), SOD3 (ECM)	23442817	[83]	Bilirubin	31400697	[131]
NO•							
NO2•					Urate	22101009	[231]
					Ferrocyanide	22101009	[231]
OH•					Vitamin E	31400697	[131]
					Uric acid	31400697	[131]
					NDGA	20415502	[232]
					Carotenoids	23015774	[233]
					NAC	21118657	[234]
					Vitamin C	9090754	[235]
ROO•	GPX4/5	Cyto, Nuc, Mt	22178243	[29]	Carotenoids	23015774	[233]
	PRDX1/2/3/4/5	Cyto, Nuc, Mt, Px	31400697	[131]	Vitamin E	31400697	[131]
					Uric acid	31400697	[131]
RO•					Vitamin C	31400697	[131]
					Edaravone	17115906	[236]
$CO_3^{\bullet-}$	CAT	Px, Mt, Cyto, Nuc	17505962	[97]	Melatonin	16153306	[237]
					AMK	16153306	[237]
H_2O_2	CAT	Px, Mt, Cyto, Nuc	31467634, 22178243, 25646037	[29,93, 98]	Bilirubin	31400697	[131]
	GPX1/2/3/6/7/8	Cyto, ECM, Mt, ER	31819197	[238]			
	PRDX1/2/3/4/5/6	Cyto, Nuc, Mt, Px	22178243	[29]			
$ONOO^-$	GPX	Cyto, ECM			Bilirubin	31400697	[131]
	PRDX5	Cyto, Nuc, Mt, Px	22178243	[29]	Uric acid	9435251	[239]
$^1O2$					Sodium azide	21491580	[240]
					Vitamin E	9119263	[241]
					Carotenoids	23015774	[233]
					Edaravone	21871447	[242]
HOCl					Uric acid	31400697	[131]
					Carotenoids	23015774	[233]
					Bilirubin	2542140	[243]
					NAC	21118657	[234]
					5-HT/Serotonin	26699077	[244]

Abbreviations: GPX—glutathione peroxidase, PRDX—peroxiredoxin, CAT—catalase, NDGA– nordihydroguaiaretic acid, AMK– N^1-acetyl-5-methoxykynuramine, NAC– N-acetylcysteine, 5-HT/Serotonin– 5-Hydroxytryptamine.

three types of ROS, including superoxide ($O_2^{\bullet-}$), nitric oxide radical ($NO^{\bullet}$) and hydrogen peroxide (H_2O_2), are mostly studied and characterized. In addition, even though the important signaling transducing roles of ROS have been found, the subcellular localizations and functions of different types of ROS are less studied and discussed.

As we mentioned above, eleven types of ROS are classified into two groups, radical ROS and non-radical ROS. Radical ROS include seven types such as superoxide ($O_2^{\bullet-}$), nitric oxide radical ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$) and carbonate radical ($CO_3^{\bullet-}$). Non-radical ROS includes four types such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl). In addition, three other types of ROS are less studied, such as hydroperoxyl radical ($HO_2^{\bullet}$), nitrocarbonate ($ONOOCO_2^-$), and ozone (O_3), and are not discussed in this review. Reactive oxygen and nitrogen reactive species (RONS) are generally discussed as ROS in this review including $NO^{\bullet}$, $NO2^{\bullet}$ and $ONOO^-$ [82]. As showed in Fig. 1, among the eleven types of ROS, four types of ROS such as $NO^{\bullet}$, $NO_2^{\bullet}$, H_2O_2 and $ONOO^-$ have the ability to cross lipid membrane and transduce signaling across organelles and cells, which we classify as organelle communication ROS in our new working model based on their functions in new integrated sensing and alarming system for metabolic stress. For the other types of ROS, they can hardly pass through lipid membrane due to limited half-life or its physical property, but they contribute to the production of other

Table 1C
The subcellular organelles of ROS generation and scavenging system are organized as follows. Among the twelve subcellular localizations, mitochondria, peroxisome, plasma membrane, cytosol and nucleus cause more than five types of ROS.

Organelle	Mt		Px		PM	Cyto		Nuc		Golgi	Caveolae	ER		Phagosome	Microsome	SR	ECM
$O2^{\bullet-}$	+	–	+	–	+	+	–	+	–	+	+	+		+			–
NO•	+		+		+					+						+	
NO2•	+					+		+									
OH•	+		+			+		+									
ROO•	+	–	+	–	+		–		–						+		
RO•	+		+		+										+		
$CO_3^{\bullet-}$		–	+	–			–		–								
H_2O_2	+	–	+	–		+	–	+	–				–				–
$ONOO^-$		–		–			–		–								–
$^1O2$	+		+			+						+					
HOCl	+					+		+									

+ Indicates relative ROS generation enzyme subcellular localization and - indicates relative ROS scavenging enzyme subcellular localization.

aggressive ROS or biological molecule damages [83], which contribute to activation of other stress pathways [44]. Nitrogen-containing ROS mostly are communication ROS. Lipid radicals are dominant in peroxisome. The ROS, which are generated by hydrogen and oxygen, such as $HO^{\bullet}$ and H_2O_2, are connected with different types of ROS systems, and are functionally classified as the chemical connecting ROS in our new working model.

The enzymes generated and subcellular localization of eleven types of ROS are listed in Table 1A. The following results in Table 1A are highlighted: *first*, among seven radical ROS, $O_2^{\bullet-}$ and $NO^{\bullet}$ are the dominant radicals, which are generated by 10 enzymes and 4 enzymes, respectively. *Second*, among non-radical ROS, H_2O_2 and $^1O_2$ are major ROS, which are generated by 11 and 8 enzymes or reactions, respectively. Notably, cell type information related to those ROS are under-investigated. Enzymatic and non-enzymatic scavenging systems and organelle localization of eleven types of ROS are listed in Table 1B. Among the 11 types of ROS, five types of ROS such as $O_2^{\bullet-}$, $ROO^{\bullet}$, $CO_3^{\bullet-}$, H_2O_2 and $ONOO^-$ can be scavenged by enzymes; and the rest are found to be scavenged by non-enzymatic scavengers but not enzymatic scavengers, suggesting that they are modulated *in*directly by gene product regulations. We integrated the subcellular localization of each type of ROS in Table 1C, and the following results are easily found: *1)* more than five types of ROS are found to be generated and functioned in the metabolic intensively active organelles, including mitochondria, peroxisome, plasma membrane, cytosol and nucleus. *2)* the generation and scavenging enzymes of ROS are both found in some organelles. However, for some types of ROS only generation or scavenging system is found in the organelle. For example, $O_2^{\bullet-}$ was found to be generated in Golgi and ER, but the corresponded scavenging enzymes have not been reported in these organelles, indicating that $O_2^{\bullet-}$ related metabolic stress can either relay to other types of ROS or branch out to other stress pathways [84]. $ROO^{\bullet}$ scavenging enzymes are found to be expressed in the nucleus, whereas it is currently unknown if $ROO^{\bullet}$ is generated in the nucleus. Further studies are needed to determine the subcellular location of $ROO^{\bullet}$ synthesis.

To clarify, the ROS generated by enzymes in the plasma membrane are released into the cytosol and are scavenged by intracellular systems. For example, $ROO^{\bullet}$ is generated by enzymes located on the plasma membrane, and the scavenge enzymes are localized in the cytosol. To better illustrate the functions of these ROS, the biochemical methods and specific inhibitors are indicated in supplemental (S.) Tables 2 and 3. Based on the roles of different types of ROS in relaying the metabolic stress signals from 2801 metabolic pathways in organelles to a final end in activating inflammasomes, we classify various types of ROS into seven functional groups in our new working model: *1)* metabolic stress-sensing (initiation), *2)* chemical connecting (one type ROS converted/dismutated into another type of ROS), *3)* organelle communication (organelle membrane crossing RNAs and capable of relaying metabolic stress to mitochondrial ROS central hub and/or nuclear ROS converging hub), *4)* stress branch-out (non-inflammasome activating and crosstalk to other stress pathways (also see section 5) such as misfolded proteins for endoplasmic reticulum (ER) stress [84], DNA damage for nuclear stress [44], RNA damage for RNA degradation [85]), *5)* inflammasome-activating (final stop for this sensing system), *6)* dual functions, and *7)* triple functions. We will explain the rationale for this functional categorization of each type of ROS in the following paragraphs.

2.1. Superoxide ($O_2^{\bullet-}$)

Up to 1–4% of oxygen is reduced into $O_2^{\bullet-}$, which is the first formed ROS. We classify $O_2^{\bullet-}$ as the dual function ROS with metabolic stress-sensing and chemical connecting. $O_2^{\bullet-}$ is generated in most organelles such as mitochondria, peroxisome, cytosol, plasma membrane, ER and nucleus. It could be generated by eight types of oxidant enzymes, including mitochondrial electron transport chain complex I, complex II, complex III, as well as xanthine oxidase (XO), NO synthases (NOS), cytochrome p 450 enzymes (CYP), NADPH oxidases (NOX) and lipoxygenase (LOX). $O_2^{\bullet-}$ is unstable in aqueous solutions because of its short half-life. $O_2^{\bullet-}$ can be scavenged or converted in three pathways: 1) $O_2^{\bullet-}$ is quickly dismutated to H_2O_2 after generation; 2) if the production level of $O_2^{\bullet-}$ is low (picomolar range), $O_2^{\bullet-}$ reacts with $NO^{\bullet}$ to form $ONOO^-$, which process is even faster than dismutation into H_2O_2 [30]; and 3) at high levels of $O_2^{\bullet-}$, it can react with H_2O_2 to produce highly reactive radical $OH^{\bullet}$ via protein iron-sulfur center reaction and iron release [30]. Of note, it has been reported that endogenously produced $O_2^{\bullet-}$ and H_2O_2 primarily contribute to NLRP3 inflammasome formation and activation [86] in mouse glomeruli resulting in glomerular injury or consequent sclerosis during hyperhomocysteinemia [87]. Therefore, $O_2^{\bullet-}$ and H_2O_2 are also functionally classified as the inflammasome activating ROS.

2.2. Nitric oxide ($NO^{\bullet}$)/nitrogen dioxide ($NO2^{\bullet}$)

$NO^{\bullet}$ is also the first formed ROS and the triple-function ROS with metabolic stress-sensing, chemical connecting and organelle communication, which is produced by a family of NO synthases (NOS), including neuronal NOS (NOS1, nNOS), inducible NOS (NOS2, iNOS) and endothelial NOS (NOS3, eNOS). In addition to $NO^{\bullet}$, studies found that NOS also produces $O_2^{\bullet-}$ when NOS is not coupled with its cofactors or substrate; and this process is called NOS uncoupling [88]. However, other publications also reported that NOS also generates $O_2^{\bullet-}$ in coupled conditions [88]. Besides, $NO^{\bullet}$ is also generated by xanthine oxidase (XO) via reducing nitrates and nitrites [29]. Nitrites reacts with H_2O_2 to generate $NO2^{\bullet}$ via the function of enzyme myeloperoxidase (MPO). $NO_2^{\bullet}$ also reacts with $O_2^{\bullet-}$ and produces $ONOO^-$ [89]. They both can cross the lipid membrane, which are responsible for communication between organelles and cells.

2.3. Hydroxyl radical ($OH^{\bullet}$)

As mentioned above, $OH^{\bullet}$ is generated from H_2O_2 and $O_2^{\bullet-}$ through the Haber-Weiss reaction or Fe^{3+}-mediated decomposition of H_2O_2 through Fenton reaction [31]. However, no enzymes have been identified for $OH^{\bullet}$ generation [90]. In addition, there are no existing enzymatic systems to scavenge $OH^{\bullet}$ [90]. These findings suggest that $OH^{\bullet}$ generation and conversion overcome no energy barriers and do not require the enzymes. Furthermore, $OH^{\bullet}$ has the capability to damage different cellular components via lipid peroxidation, protein damage, DNA bases damage, and membrane destruction [31]. Therefore, the generation and accumulation of $OH^{\bullet}$ lead to cell death. However, based on recent understanding, the organelles that contain $O_2^{\bullet-}$ and H_2O_2 generation enzymes can produce $OH^{\bullet}$. Since $OH^{\bullet}$ does not convert into other types of ROS, and does not activate nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain-containing 3 (NLRP3) inflammasome [87] but damage and "branch out oxidative stress" to other molecules and pathways, therefore, $OH^{\bullet}$ is functionally classified as the stress-branch out ROS.

2.4. Peroxyl radical ($ROO^{\bullet}$)/Alkoxyl radical ($RO^{\bullet}$)

$ROO^{\bullet}$ is generated as intermediates in the lipid peroxidation reactions [91]. Lipid peroxidation is a chain reaction process resulting in the generation of $ROO^{\bullet}$, which is characterized by repetitive hydrogen abstraction [92] and addition of oxygen to $R^{\bullet}$. 0.3% of cytosolic $O_2^{\bullet-}$ is in the protonated form as $HOO^{\bullet}$, which is the simplest form of $ROO^{\bullet}$ [93]. $ROO^{\bullet}$ nearly attacks all major classes of biomolecules, mainly the polyunsaturated fatty acids (PUFA) of cell membranes [94] and DNA bases [95]. $RO^{\bullet}$ is generated form $ROO^{\bullet}$ via a tetroxide. $RO^{\bullet}$ is a less aggressive ROS than $HO^{\bullet}$, but more reactive than $ROO^{\bullet}$ [29]. Studies reported that LOX contributes to lipid peroxidation and is localized in cytoplasm and nucleus [96], which indicating the subcellular

localization of $ROO^{\bullet}$. They are the most dominant ROS in peroxisome, which may have critical sensing functions in peroxisome. Therefore, $ROO^{\bullet}$ is functionally classified as the triple function ROS with metabolic stress sensing, chemical connecting and ROS-converting and stress-branch-out.

2.5. Carbonate radical ($CO_3^{\bullet-}$)

$CO_3^{\bullet-}$ is a negatively charged radical in physiological and pathological conditions, and is formed in the following four reactions: *first*, superoxide dismutase 1 (SOD1) peroxidase activity. *Second*, XO-mediated oxidation of acetaldehyde, xanthine and hypoxanthine [97]. *Third*, hydrogen abstraction from bicarbonate by $OH^{\bullet}$. *Fourth*, nitrocarbonate is converted into $NO2^{\bullet}$ and $CO_3^{\bullet-}$ [97]. $CO_3^{\bullet-}$ is less oxidizing than $OH^{\bullet}$, but it has larger ranges of oxidative actions because of its longer half-life in comparison to $OH^{\bullet}$ [97]. $CO_3^{\bullet-}$ could rapidly oxidizes DNA guanine residues and protein residues [97]. However, $CO_3^{\bullet-}$ is unable to produce stable adducts, which makes it difficult to prove its production compared with other radicals. $CO_3^{\bullet-}$ is functionally classified as the stress-branch out ROS.

2.6. Hydrogen peroxide (H_2O_2)

H_2O_2 is generated via SOD-mediated dismutation of $O_2^{\bullet-}$ [31]. There are three isoforms of SOD. *First*, Cu, Zn-dependent SOD (SOD1) converts $O_2^{\bullet-}$ into H_2O_2 in mitochondrial intermembrane space (IMS) and cytosol. *Second*, Mn-dependent SOD (SOD2) converts $O_2^{\bullet-}$ into H_2O_2 in the mitochondrial matrix (MM). *Third*, Cu, Zn-dependent SOD (SOD3) converts $O_2^{\bullet-}$ into H_2O_2 in extracellular space [83]. Monoaminoxidase (MAO) resides in the outer mitochondrial membrane and generates H_2O_2 [31,98]. XO is one of the most studied ROS generation enzymes in the cytosol [83,99] and mediates H_2O_2 production through the process of hypoxanthine and xanthine catalyzation [64]. H_2O_2 is also generated in peroxisomes via various enzymes from the normal catalytic cycle [100]. These enzymes are mainly flavoproteins, including acyl-CoA oxidases, urate oxidase, D-amino acid oxidase, D-aspartate oxidase, L-pipecolic acid oxidase, L-α-hydroxyacid oxidase, polyamine oxidase, and XO [32]. The adaptor and ROS-regulating protein P66shc normally resides in the cytosol and translocates into the mitochondria under oxidative stress via a protein kinase C (PKC)-dependent manner. After translocation, p66shc oxidizes cytochrome c, and generates H_2O_2 [101]. H_2O_2 is more stable than $O_2^{\bullet-}$, and the concentrations of H_2O_2 are also 100 times higher than $O_2^{\bullet-}$ in mitochondria. These properties render H_2O_2 as an ideal signaling molecule in cells [83]. H_2O_2 is released via aquaporin 8 formed channel from cell membrane. As discussed above, H_2O_2 is functionally classified as the dual function ROS with organelle communication and inflammasome activating.

To demonstrate the proof of principle that how ROS generating enzymes are connected to metabolic pathways, we also list the metabolic functions of 41H2O2-generating enzymes and O2- generating enzymes, which suggests that many metabolic pathways contribute H2O2 generation and O2- generation, which are further connected to inflammasome activation and other cellular stress pathways [102]. The enzymes participate in various metabolic processes and pathways as shown in Supplemental Table 1. For example, by searching the metabolomics database (https://hmdb.ca/metabolites/HMDB0004062) using the method that we reported [11], we can find that the enzyme aldehyde oxidase 1 is functional in amino acid valine, leucine and isoleucine degradation, tyrosine metabolism, tryptophan metabolism, vitamin B6 metabolism and nicotinate and nicotinamide metabolism.

2.7. Peroxynitrite ($ONOO^-$)

As mentioned above, the powerful oxidant $ONOO^-$ is produced from the rapid reaction between $O_2^{\bullet-}$ and $NO^{\bullet}$ [97]. $ONOO^-$ causes DNA single-strand breakage and activates poly-ADP-ribose polymerase (PARP) [103]. $ONOO^-$ also induces nitration and S-nitrosylation of proteins and lipids [103]. It is capable to cross lipid membranes and thus has the potential to communicate cellular status between organelles. $ONOO^-$ is functionally classified as the dual function ROS with organelle communication and stress branch-out.

2.8. Singlet oxygen ($^1O_2$)

$^1O_2$ is formed via photooxygenation and enzymatic reactions. The enzymatic reactions for $^1O_2$ production is mediated by several peroxidases in lipid peroxidation, including lipoxygenase (LOX), dioxygenase and lactoperoxidase [92,104]. Oxidation of H_2O_2 and HOCl mediated by MPO [105] contributes to the production of $^1O_2$ in a chemical system [104,106]. In addition, $^1O_2$ is also formed by CYP 2E1 and cytochrome c in the ER and mitochondria, respectively [107]. Fenton-type reactions may also give rise to the production in peroxisomes of $^1O_2$. Thus, $^1O_2$ could be a new player in the peroxisome-derived signaling network [108]. Furthermore, studies also reported that $^1O_2$ causes mitochondrial ETC proteins oxidation. In addition, studies found that $^1O_2$ generation after light stimulation significantly affects the activities of mitochondrial ETC complex I, complex III, complex IV but except complex II [109]. Studies found that the secondary generation of $O_2^{\bullet-}$ and H_2O_2 are induced in $^1O_2$ caused dysfunctional mitochondrial respiration [109,110]. Thus, we classify $^1O_2$ as the triple function ROS with metabolic sensing, chemical connecting, and stress branch-out.

2.9. Hypochlorous acid (HOCl)

MPO catalyzes the formation of HOCl from H_2O_2 and chloride ion [105]. MPO is found to localize to the nucleus, mitochondria and cytoplasm, which may explain the subcellular localization of HOCl seen in neutrophil and monocytes [111]. However, it remains unclear whether HOCl roles in our integrated sensing and alarming system for metabolic stress.

3. Mitochondrial ROS systems serve as the central hub for connecting ROS systems with inflammasomes, trained immunity and immunometabolic pathways

Mitochondria consume the greatest amount of oxygen (80–95%) to allow oxidative phosphorylation (OXPHOS) for energy generation [112]. OXPHOS produces 95% of cell energy by coupling tricarboxylic acid (TCA) cycle and mitochondrial electron transport chain (ETC) in most cell types [113], except for endothelial cells (both tip cells and non-tip cells) [114], which mostly take use of glycolysis for energy generation [115]. During OXPHOS, mitochondrial ROS is generated at mitochondria ETC [38]. In addition, studies found that mitochondrial ROS production [38] accounts for 2% of total oxygen consumed in mitochondria in physiological condition, and fluctuates from 0.25% to 11% depending on the animal species and respiration rates in pathophysiological conditions [31]. Studies found that mitochondrial ROS (mtROS) are connected with ROS systems in other organelles, and initiated inflammatory [78,116] or cell death signals [117]. Therefore, as the most oxygen consuming and ROS generation organelle, mitochondria serves as the central hub for connecting ROS systems in other organelles (upstream) with the downstream DAMP sensing pathways such as inflammasomes [27], trained immunity [42,48,118] and immunometabolic pathways [119]. At the central hub, the metabolic homeostasis, and alarming stress in each organelle are sensed and bridged to DAMP sensing pathways in the downstream into the cytosol (for example, inflammasomes).

MtROS are generated at mitochondrial ETC and by other mitochondrial oxidant enzyme systems. Mitochondrial ETC includes five complexes from complex I to complex V [120]. The complexes except complex II are assembled crossing both inner and outer mitochondrial membranes, while complex II expresses on mitochondrial inner

Table 2
Extra-mitochondrial ROS modulate mtROS production, and two types of mtROS modulate extra-mitochondrial ROS production.

Category	Triggering ROS	Pivotal proteins	Findings	PMID	Ref #
Extra-mt ROS induced Mt ROS	$O_2^{\bullet-}$	IMAC	Extra-Mt $O_2^{\bullet-}$ induced opening of IMAC which depolarized ΔΨm; $O_2^{\bullet-}$ was produced in ETC complex III.	12930841 24657720	[128,129]
	$H_2O_2/NO^{\bullet}$	Mt-K_{ATP}	Extra-Mt H_2O_2 activated Mt-K_{ATP} via PKC-ε leading to the increase of K^+ influx and production of $O_2^{\bullet-}$	18586884 31400697	[130,131]
	HOCl	ETC enzymes	HOCl–oxLDL leads to Mt ROS generation via decreasing activities of ETC dehydrogenase and reductases	19843872 18282575	[132,133]
	$^1O_2/H_2O_2$	MPTP	Mt ROS was triggered by external ROS (possibly $^1O_2$) and linked to MPTP opening and calcium sparks.	11015441 28279675	[134,135]
Mt ROS induced extra-mt ROS	$ONOO^-$	Zinc cluster or BH4	$O_2^{\bullet-}$ reacts with $NO^{\bullet}$ to form $ONOO^-$ which causes uncoupling of eNOS via oxidation of zinc cluster or BH4	11901190 14656731 31400697	[131,136, 137]
		Cysteine of XDH	$O_2^{\bullet-}$ reacts with $NO^{\bullet}$ to form $ONOO^-$ which induces disulfide formation between two cysteines of XDH	22657349 31400697	[131,138]
	H_2O_2	c-Src	Activation of c-Src by mitochondrial H_2O_2 can activate cytoplasmic NOX or increasing its mRNA levels, increasing cytosolic $O_2^{\bullet-}$ production	24053613 24759683 31400697	[131,139, 140]

Abbreviation: ΔΨm, Mitochondrial membrane potential; BH4, Tetrahydrobiopterin; c-Src, Proto-oncogene tyrosine-protein kinase Src; eNOS, Endothelial nitric oxide synthase; ETC, Electron transport chain; H_2O_2, Hydrogen peroxide; HOCl, Hypochlorous acid; IMAC, Inner membrane anion channel; Mt, Mitochondrion; Mt-K_{ATP}, Mitochondrial adenosine triphosphate (ATP)-sensitive potassium channel; MPTP, mitochondrial permeability transition pore; NO, Nitric oxide; NOX NADPH oxidase; $O_2^{\bullet-}$, Superoxide radical anion; $^1O_2$, Singlet oxygen; $ONOO^-$, Peroxynitrite; oxLDL, Oxidized low-density lipoprotein; PKC-ε, Protein kinase C-epsilon; ROS, Reactive oxygen species; XDH, xanthine dehydrogenase.

membrane. TCA cycle products such as nicotine adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) donating electrons at complex I and complex II, respectively, and during which process, $O_2^{\bullet-}$ is generated and released into mitochondrial matrix from complex I and complex II. The electrons then pass through complex III and ultimately reduce O_2 to water at complex IV (cytochrome C); and $O_2^{\bullet-}$ is also generated and released into both mitochondrial intermembrane space (IMS) and mitochondrial matrix (MM) through this process at complex III. Meanwhile, positively charged protons (H^+) from complex I, III and IV, but not complex II, are actively being pumped from the mitochondrial matrix into the intermembrane space, and thus creating a mitochondrial membrane potential (Δψm) across the inner mitochondrial membrane. This proton-motive force allows complex V– ATP synthase (ATPase) to generate adenosine triphosphate (ATP) when protons re-enter the mitochondrial matrix via the complex V enzyme [83]. However, increased mtROS generation is uncoupled from ATP production and promoted by proton leaks, which means the reactions between complex I to complex IV is accelerated and uncoupled from ATP generation as we reported [37,39,40,60,121]. Interestingly, due to the low levels of complex II substrate—succinate in physiological conditions, the mtROS generation of complex II draws less attention [31]. However, recent studies also found that complex II also contributes to $O_2^{\bullet-}$ generation in pathological conditions [122]. For example, tumor necrosis factor- α (TNF-α) induced mtROS production is mediated by complex II but not complex I and complex III in retinal pigment epithelium (RPE) cells [78]. mtROS production rates are higher by using complex II substrate—succinate in comparison to pyruvate, glutamate and malate [101]. Additionally, complex II mediated $O_2^{\bullet-}$ release is uncoupled with proton, which is unlike complex I and III as mentioned above.

MtROS is mostly produced by the mitochondrial ETC; however, several other oxidant enzymes also contribute to mtROS generation, including four ETC associated enzymes (glycerol 3-phosphate dehydrogenase, Q oxidoreductase, pyruvate dehydrogenase, and 2-oxoglutarate dehydrogenase) [83], XO, CYP, NOX4, eNOS, MPO, LOX and MAO (Table 1A). $O_2^{\bullet-}$ is the first formed ROS, and subsequently, $O_2^{\bullet-}$ is quickly dismutated to H_2O_2 by SOD2 in the mitochondrial matrix (MM) and SOD1 in intermembrane space (IMS) (Table 1B). Therefore, the most studied mtROS are $O_2^{\bullet-}$ and H_2O_2; however, nine of the eleven types of ROS are found to exist in mitochondria except $CO_3^{\bullet-}$ and $ONOO^-$. But $ONOO^-$ may also be localized in mitochondria because of the rapid interactions of $O_2^{\bullet-}$ and $NO^{\bullet}$. Even though other types of ROS are harmful to lipid, protein and DNA in mitochondria and cells (stress branch-out function), there has not been much published on the determination of their functions and signals. One of the reasons may be the limited detection methods of different types of ROS. Furthermore, mitochondrial DNA (mtDNA) encodes seven subunits of complex I, one subunit of complex III, three subunits of complex IV and two subunits of complex V [123]. However, mtDNA is not protected by histones as nuclear DNA, and the rates of mtDNA damage are higher than that of nuclear DNA [123]. MtDNA damage includes mtDNA mutations and decreased mtDNA copy numbers [124]. Studies reported that mtDNA damage and dysfunction are susceptible to increased cytosolic and mitochondrial ROS levels in metabolic disease and age-related disease [124–126]. Although mtRNA is one of mitochondrial DAMPs [127], however, the relationship between mtDNA damages and nuclear ROS levels is not clear.

MtROS is connected to ROS systems of other organelles; and the signals are transduced between organelle. Studies found that extra-mitochondria ROS could transduce the signals to mitochondria and induce the production of mtROS (Table 2). Extra-mitochondria $O_2^{\bullet-}$ induce opening of inner membrane anion channel (IMAC) and depolarize Δψm; and $O_2^{\bullet-}$ is produced at complex III [128,129]. In addition, extra-mitochondrial H_2O_2 activate mitochondrial ATP sensitive potassium channel via protein kinase C (PKC)-ε pathway, thus potassium influx and mtROS generation are increased [130,131]. Moreover, extra-mitochondrial ROS– HOCl, H_2O_2 and $^1O_2$, all triggers mtROS production via affecting ETC enzyme activities [132,133] or mitochondrial permeability transition pore (MPTP) opening [134,135]. On contrast, mitochondrial generated $ONOO^-$ and H_2O_2 cause eNOS uncoupling and activation of NOX, which lead to extra-mitochondrial ROS generation [131,136–140]. After the communications between mitochondria and other organelles, imbalanced ROS participates and regulates various metabolic pathways to respond, especially mtROS via a feedback mechanism. MtROS are important signal transducer and regulates downstream inflammatory and cell death pathways (Fig. 2A–a). For examples, mtROS promotes inflammation via NLRP3-inflammasome/caspase-1 activation [78,116,141], promotes cell proliferation or migration via the key inflammatory transcription factor NF-kB [142] or Rac1 (a small G-protein in the Rho family) pathway [143] in inflammasome-dependent or independent manners, and promotes apoptosis via caspase-3 activation [117]. In addition, a new conditional DAMP [25], pro-atherogenic stimuli lysophosphatidylcholine (LPC) [144]-induced increased mtROS production contributes to trained immunity via histone 3 lysine 14 acetylation [145].

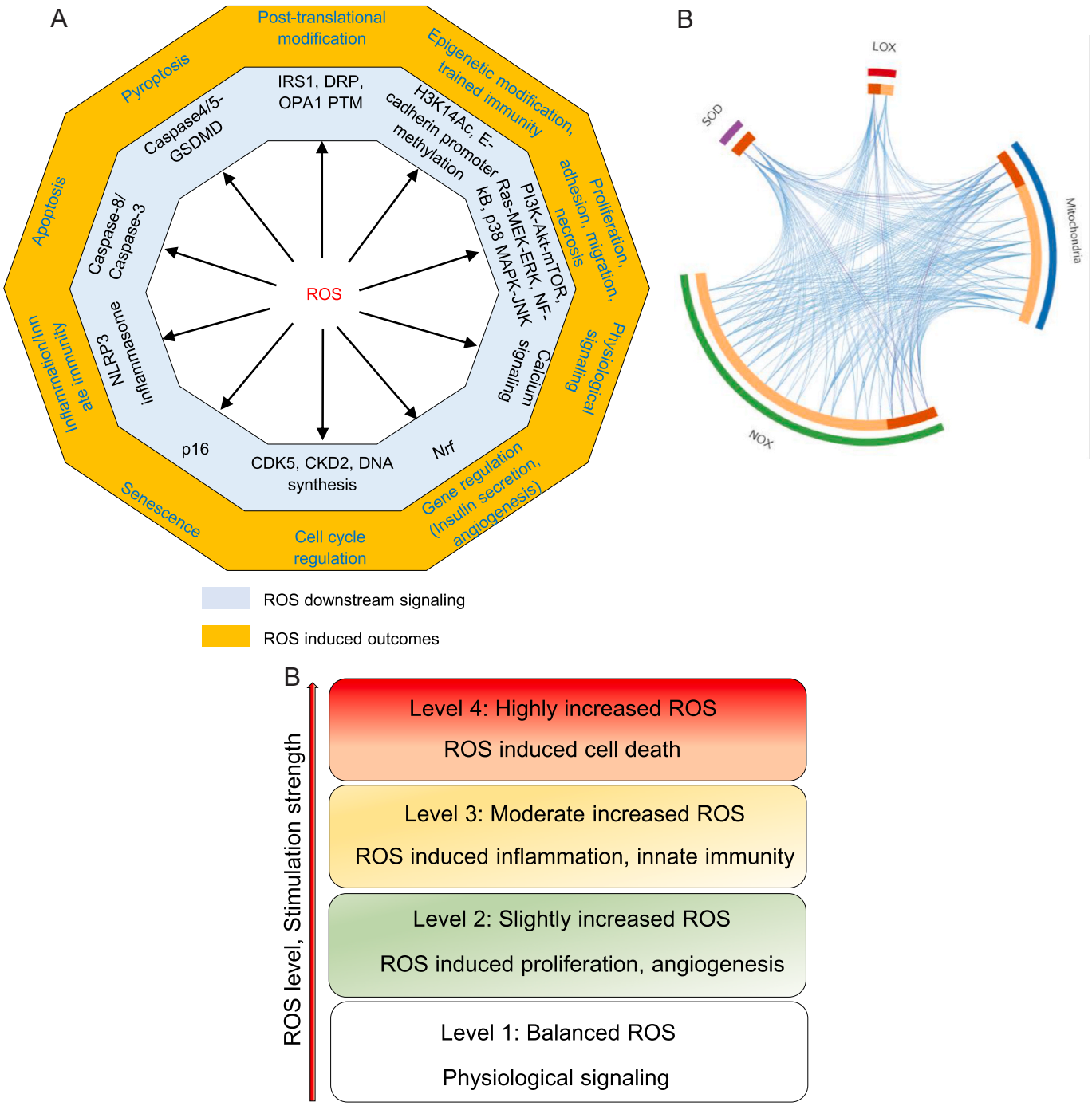

Fig. 2. A. Left panel (a): ROS induces ten types of cell functions (yellow) via various specific downstream pathways (grey). The ten cell functions include: 1) physiological signaling, 2) proliferation, 3) gene regulation, 4) cell cycle regulation, 5) epigenetic modification, 6) post-translational modification, 7) inflammation/innate immunity, 8) pyroptosis, 9) apoptosis, and 10) senescence. Right panel (b): The circus plot indicates that four major ROS generating and anti-ROS regulatory systems have both shared genes and unique genes. On the outside, each arc represents the identity of each gene list。On the inside, each arc represent a gene list, where each gene has a spot on the arc. Dark orange color represents the genes that appear in multiple lists and light orange color represents genes that are unique to that gene list. Purple lines link the same gene that are shared by multiple gene lists. Blue lines link the different genes where they fall into the same ontology them. (also see the detailed information in a supplemental table). Detailed information of Fig. 2A-b see supplemental Table 4.

Abbreviations: IRS1—insulin receptor substrate 1, DRP–dynamin-related protein, OPA1–optic atrophy 1, PTM—post-translational modification, H3K14Ac—histone 3 lysine 14 acetylation, PI3K–phosphatidylinositol-3-kinase, Akt–protein kinase B, mTOR–mammalian target of rapamycin, Ras—GTPase, MEK–MAPK kinase, ERK–extracellular signals-regulated kinase, NF-kB–nuclear factor kappa-lightchain- enhancer of activated B cells, MAPK– mitogen-activated protein kinases, JNK– MAPK-Jun N-terminal kinase, Nrf– Nuclear factor erythroid2-related factor, CDK5–cyclin dependent kinase 5, CKD2– cyclin dependent kinase 2, NLRP3– NOD-, LRR- and pyrin domain containing protein 3, GSDMD—gasdermin D, SOD– superoxide dismutase, LOX–lipoxygenase, NOX–NADPH oxidase. B. The ladder indicates different levels of ROS contribute to different cellular functions. In redox homeostasis state, ROS contributes to physiological signaling. Low ROS increase contributes to proliferation and angiogenesis. Moderated ROS increase contributes to inflammatory response and innate immunity. High ROS increase contributes to various types of cell death. C. Seven types of stimulations promote ROS production, including cytokines, growth factors, hormone/neurotransmitters, hypoxia, high glucose, DAMPs and extracellular ROS.

Abbreviations: TNF-α–tumor necrosis factor- α, IFN-γ– interferon gamma, IL-1–interleukin 1, IL-2–interleukin 2, IL-4–interleukin 4, IL-13– interleukin 13, IL-27–

interleukin 27, IL-10–interleukin 10, IL-35–interleukin 35, EGF– epidermal growth factor, PDGF–Platelet derived growth factor, VEGF– vascular endothelial growth factor, IGF-1–Insulin-like growth factor 1, NE—Norepinephrine, AngII—angiotensin II, AGE– AGEsadvanced glycation end products, AOPP– advanced oxidation protein products, ATP– adenosine triphosphate, LPS—lipopolysaccharide, HS– Hemodynamic strain, PS– Pulmonary stretch.

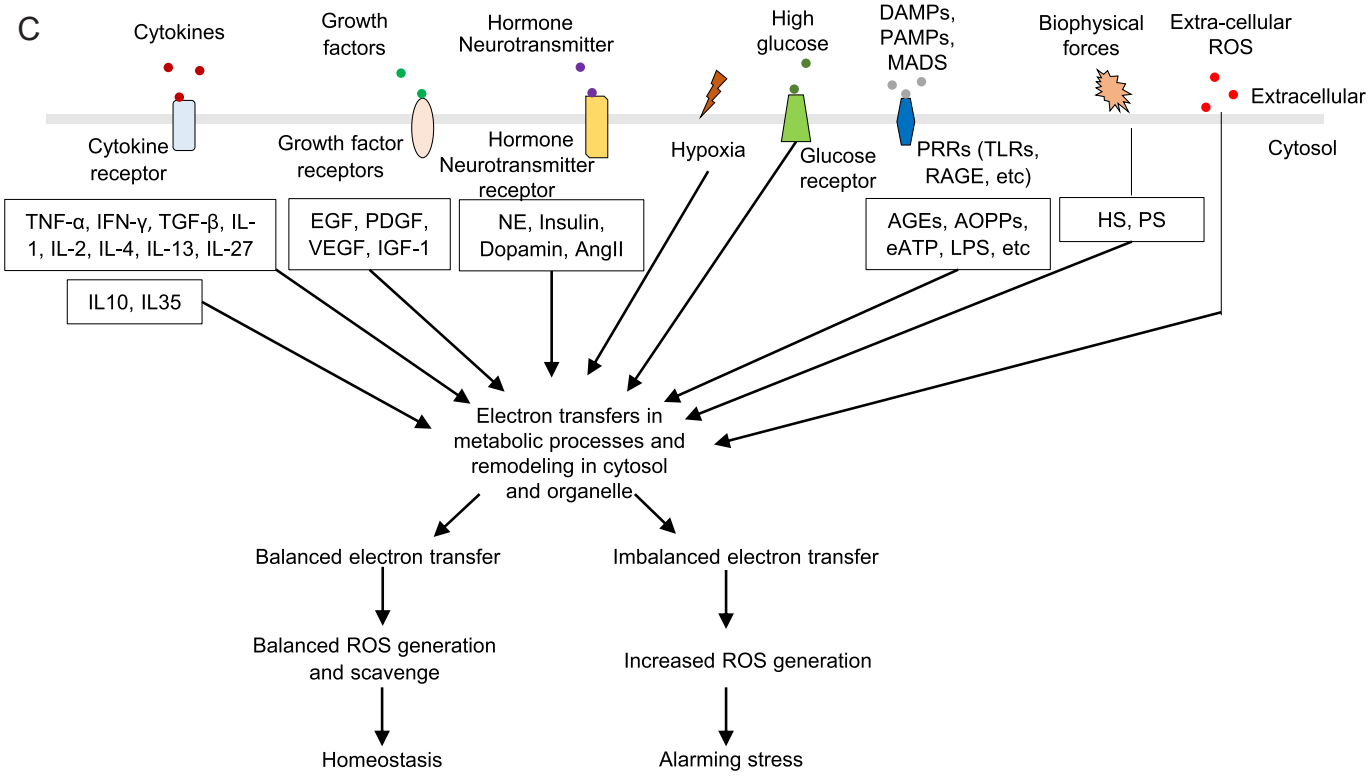

Fig. 2. (*continued*).

Furthermore, studies found that anti-inflammatory cytokine interleukin 35 (IL-35) inhibits endothelial cell activation via inhibiting the increase of mtROS [146]. In addition, we analyzed the target enzymes of various treatments in Fig. 4 and genes of their downstream pathways by circus plot (Fig. 2A and b; also see the Supplemental Table 4 for the details). Various stimuli have different ROS generation enzymes and downstream signaling, however, their signaling pathways crosstalk with each other.

Nucleotide-binding oligomerization domain (NOD)-like receptor-containing pyrin domain 3 (NLRP3) inflammasome is a multiprotein complex, which is composed of NLRP3, apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD) (ASC, PYCARD) and pro-caspase 1 [27]. Increased ROS levels contribute to NLRP3 inflammasome activation. Among various subtypes of ROS, $ONOO^-$, $O_2^{\bullet-}$, and H_2O_2 are reported to be involved in inflammasome activation [147]. Upon activation by ROS, NLRP3 inflammasome promotes secretion of pro-inflammatory cytokines such as IL-1β and IL-18 and an inflammatory form of cell death [4]. Inhibition of NOX activity, p22phox subunits and $ONOO^-$ production significantly prevented caspase-1 activation and IL-1β production [147]. The structure of disulfide bond that connects the PYD domain and the nucleotide-binding site domain in NLRP3 is highly sensitive to ROS [147]. The inactive form of NLRP3 resides on ER and translocates on mitochondrial outer membrane upon activation [148–151]. NLRP3 located in mitochondria interacts with mitochondrial ASC to assemble inflammasome or with mitochondrial antiviral signaling protein (MAVS) [152]. MAVS is a mitochondrial adaptor protein which mediates the translocation and association of NLRP3 to mitochondria. MAVS facilitates NLRP3 binding with ASC and pro-caspase 1 [147,153]. In addition, ER and mitochondria associated membrane are found to be the potential site for NLRP3 assembly [154]. Reports of subcellular localization of ASC varies significantly [53], including mitochondria [155], cytosol [150] and nucleus [156]. Upon inflammasome activation, ASC is especially observed in cytosol, which is correlated with the subcellular localization of assembled NLRP3 inflammasome. Furthermore, ROS activate NLRP3 inflammasome via direct and indirect ways. Studies found that increased ROS level induced mitochondrial DNA damage is also an indirect pathway for NLRP3 inflammasome activation by ROS [152,154]. In high level of ROS, thioredoxin-interacting protein (TXNIP) is dissociated with TRX and activates NLRP3 inflammasome [157]. Therefore, mitochondrial ROS systems serve as the central hub for connecting ROS systems in other organelles (upstream) with the downstream DAMP sensing pathways such as inflammasomes, trained immunity, immunometabolic pathways, cell proliferation, migration and cell death.

4. Nuclear ROS systems serve as a convergent hub and decision-makers to connect unbearable and alarming metabolic stresses from intracellular organelles to inflammation and cell death

Our previous reports showed that proatherogenic lipids (lysophosphatidylcholines, LPC)-induced mitochondrial ETC-generated ROS upregulate endothelial adhesion molecule intercellular adhesion molecule-1 (ICAM-1) expression and human aortic endothelial cell (HAEC) activation [42,60] via increasing histone 3 lysine 14 acetylation (H3K14ac) and increasing transcription factor AP-1 binding to ICAM-1 promoter in the nucleus [41]. In addition, we also found that LPC activates HAEC by increasing the enzyme expression of trained immunity (innate immune memory) [118]-related three metabolic pathways such as glycolysis, acetyl-CoA generation and mevalonate pathways via H3K14ac-mediated epigenetic innate immune memory mechanisms [48]. Our results have demonstrated that mitoROS and metabolic pathways are connected to an epigenetic histone modification and gene expression in the nucleus via so-called mito-nuclear communication [158], which is functional as a form of newly characterized innate immune memory (also termed trained immunity) [118,159]. Moreover, we

Table 3
Extracellular ROS induce intracellular ROS generation and promote different signaling and functions.

	ROS	Target	Concentration	Function	PMID	Ref #
ONOO-	Increase		100uM	Angiogenesis	18309287	[192]
	Increase			Apoptosis	9020024	[218]
H2O2	Increase		1-4uM	Insulin secretion	17400930	[70]
	Increase	NOX4	10 uM	Proliferation, Migration	25315297	[191]
	Increase		10-20uM	Proliferation	15109912	[193]
	Increase	Cyto/Nuc ROS	50uM	Nuclear Calcium signaling	20393594	[71]
	Increase		100uM		26461342	[142]
	Increase		200uM	Apoptosis	29061494	[187]
	Increase		300uM	Tumor progression	18801366	[198]
	Increase		1 mM	Caspase-independent apoptosis	20084055	[69]

found that intracellular organelle-generated stress can be converged into the nucleus and regulate DNA damage and DNA repair pathways [44]; and presumably cytosol-located inflammasome-caspase-1 activation [5,55,56,160] can regulate gene expression in caspase-1 processed IL-1β-, IL-18-, and sirtuin-1-, independent manners [52]. Taken together, our reports have demonstrated that in addition to mitochondria, cytosol stress and organelle stress are also converged into the nucleus in inflammasomes-dependent or independent manners [53].

The nucleus is responsible for epigenetic and genomic regulation of gene expression, cell differentiation, proliferation, senescence, and cell death [161]. Studies found that the nuclear membrane also contains cytochrome oxidases and electron transport systems, and may contribute to nuclear ROS production [162]. The basal levels of ROS in the nucleus are higher than those in the cytosol at resting state [163],

Table 4A
Eight cytokines, four growth factors, three out of four hormone/neurotransmitters promote ROS production, and anti-inflammatory cytokines IL-10 and Il-35 inhibit ROS production. IGF-1, NE and Dopamin also decrease ROS production in certain condition.

		ROS	Sensor	Target	Concentration	Function	PMID	Ref#
Cytokine	TNF-α	Increase	TNFR	5-lipoxygenase	10 ng/ml	Inflammation	10934206	[72]
		Increase	TNFR1	Complex II, ΔΨm	20 ng/ml	Inflammation, Apoptosis	17765224, 20203691	[78,117]
	IFN-γ	Increase	IFN-γR	Complex II/ NOX	20 U/ml	Inflammation	17765224	[78]
		Increase	IFN-γR	Duox2	100 U/mL	Innate immunity	16111680	[174]
	TGF-β	Increase	TGFBR	NOX, Complex I	10 ng/ml	Fibronectin secretion/EMT	15677311	[245]
		Increase	TGFBR	NOX4	50 ng/ml	Angiogenesis	25315297	[191]
	IL-1	Increase	IL1R		10 ng/ml	Angiogenesis	26811540	[173]
		Increase	IL1R	NOX	20 ng/ml	Inflammation	17765224	[78]
	IL-2	Increase	IL2R		100 U/ml	Angiogenesis	18309287	[192]
	IL-4	Increase	IL4R	Duox1	10 ng/ml	Inflammation, Innate immunity	16249002, 16111680	[174,206]
	IL-13	Increase	IL13R	Duox1	10 ng/ml	Innate immunity	16111680	[174]
		Increase	IL13R	NOX	1 μg/μl	Cell death/damage	19752235	[175]
	IL-27	Enhance	IL27R	NOX2	100 ng/ml	Inflammation	28240310	[79]
	IL-10	Decrease	IL10R		10 ng/ml	Inhibit endothelial cell activation	31731100	[146]
	IL-35	Decrease	IL12R/ IL27R	Mt	10 ng/ml	Inhibit endothelial cell activation	29371247, 31731100	[146,208]
Growth factor	EGF	Increase	EGFR		5 ng/ml	Adhesion, Migration, Proliferation	19635476	[209]
		Increase	EGFR	NOX	10 ng/ml	Proliferation	25122478	[188]
		Increase	EGFR		40 ng/ml	Angiogenesis	17045920	[194]
		Increase	EGFR		100 ng/ml	Necrosis	10854274	[73]
	PDGF	Increase	PDGFR		1 ng/ml	Proliferation	15109912	[193]
		Increase	PDGFR		15 ng/ml	Senescence	16081426	[220]
		Increase	PDGFR		20 ng/ml	Inflammation	23774581	[246]
	VEGF	Increase	VEGFR	mtROS	50 ng/ml	Migration	31653897	[143]
	IGF-I	Decrease	IGF-1R		100 ng/ml	Survival	20084055	[69]
		Increase	IGF-1R	NOX4	200 ng/ml	Migration	18567639	[210]
Hormone/ Neurotransmitter	NE	Decrease			10uM	Protect DNA damage	26167254	[185]
	Insulin	Enhanced	IR		10–100 nM	Enhance insulin sensitivity	19808019	[183]
	Dopamine	Increase		MAO	0-50uM	Physiological signaling	20547771	[67]
		Increase		mtROS (SOD)	1uM	Physiological signaling	23994527	[68]
		Increase		MAO	100μM, 500uM	Cell death	20547771	[67]
		Decrease	D5R	NOX		Protect from hypertension	23425954	[186]
	Serotonin	Increase	5-HT1BR	NOX1	1uM	Post-translational oxidative modification of protein, proliferation	28473438	[247]
					10-100uM	Caspase-3 dependent apoptosis	16286591	–
	AngII	Increase	AT1R	NOX4 (Nuc/ Cyto)	1 nM	Gene regulation, Protect from cell damage	19409874, 19948986	[169,176]
		Increase	AT1R	NOX	100 nM	maintain arterial tone	29472601	[177]
		Increase	ET-1AR	NOX	100 nM	Proliferation	14642698	[178]
		Increase	AT1/2R	NOX	100 nM	Apoptosis, Necrosis, Senescence	12606818, 21270817	[179,180]
		Increase	AT1R	NOX	1uM	Impair insulin signaling	16982630	[181]

but the potential role of nuclear ROS have rarely been studied or discussed. On the other hand, an important question remains unknown whether nuclear ROS are generated in the nucleus or due to the trafficking from increased cytosolic ROS. In Table 1A, ROS generation enzymes are found to be expressed in the nucleus, which presents strong evidence for ROS production in the nucleus. In addition, comparing increased ROS levels in various organelles including cytosol, mitochondria, endosome, Golgi and nucleus [164], increased nuclear ROS significantly promote cell death compared to that of other organelles; in contrast, increased ROS in the ER does not always affect cell viability [164]. Therefore, nuclear ROS serves as a convergent hub and decision-makers to connect unbearable and alarming metabolic stresses from intracellular organelles to inflammation or cell death [165]. In addition, nucleus ROS connected with ROS systems from extracellular space or extra-nucleus also regulate inflammatory or cell death responses [76]. Theoretically, there are at least two means for microenvironmental ROS to affect cellular homeostasis via an impact on the cell nucleus [165]. For example, extracellular H_2O_2 (50 μmol/L) stimulation induces sustained increase of nuclear ROS and regulates nuclear calcium signaling (Table 3); and the antioxidant glutathione (GSH) [166] could reverse the increase of nuclear ROS [163]. In addition, the nuclear envelope membranes contain several G protein-coupled receptors, including prostaglandin E2 (EP3R) and endothelin-1 (ET-1) receptors. Activation of EP3R increases endothelial nitric oxide synthase (eNOS) RNA expression in nuclei. eNOS and inducible NOS (iNOS) are reported to also be present at the nuclear levels. Moreover, stimulation with NO donor sodium nitroprusside [167] results in an increase of intranuclear calcium that is dependent on guanylate cyclase activation, but independent of mitogen-activated protein kinases (MAPK) [168]. H_2O_2 and ET-1 increase both cytosolic and nuclear ROS in human endocardial endothelial cells and in human aortic vascular smooth muscle cells [163]. Furthermore, pro-hypertension hormone angiotensin II (Ang II) (1 nM) stimulation activates Ang II type 1 receptor (AT1R), which is highly expressed in nuclei, and increases ROS production via NADPH oxidase 4 (NOX4) activation, which has 65% expression in nuclei [169]. Based on these studies, nuclear ROS senses the status of cell conditions. The imbalanced ROS levels contribute to cell inflammation and cell death. Studies on nuclear ROS remain less than that of other organelles, and further studies are needed to clearly uncover the mechanisms underlying death signaling and nuclear ROS generation and scavenging. One of the reasons for this may be due to the fact there is not a simple, convenient and specific method for nuclear ROS detection except for ROS staining with fluorescent ROS probe carboxy H2DCFDA (DCF) and co-stained with live nucleic acid stain Syto-11 [163].

In our new model proposed here, we emphasize the mitochondria/organelle-to-nucleus retrograde communications [41]. Actually, recent progresses include nucleus-to-mitochondria (anterograde) and mitochondria-to-nucleus (retrograde) communication, mito-nuclear feedback signaling and proteostasis (pathways that control the biogenesis, folding, trafficking and degradation of proteins) regulation, the integrated stress response and non-cell-autonomous communication [170].

5. Balanced ROS levels indicate a physiological homeostasis of various metabolic processes in subcellular organelles while imbalanced ROS levels present alarms for pathological organelle stresses in metabolic processes

ROS systems are found to play important roles in promoting the progression in metabolic diseases, such as cardiovascular disease (CVD), chronic kidney disease, inflammations, diabetes and tumors. However, the detailed mechanisms of how ROS play the critical roles remain poorly characterized. We propose that ROS system serves as the sensing network for cellular homeostasis and alarming stress in metabolic processes. Therefore, ROS levels are increased in metabolic disorders and participate in disease progression. Balanced ROS contribute to physiological signals and functions after sensing metabolic homeostasis, while imbalanced ROS participate in metabolic processes regulating proliferation, inflammation or cell death in pathological conditions. Studies found that lightly increased ROS (1.1 fold changes) contribute to cell proliferation, angiogenesis and metastasis, moderate increased ROS (1.1–20 fold change) reach the maximum inflammatory responses, and highly increased ROS (more than 20 fold change) promote apoptosis, necroptosis, autophagy, pyroptosis and ferroptosis [171,172] (Fig. 2B). However, cell types and stimuli cause variations for fold differences in ROS change effects. Thus, the criteria of increased ROS levels may be used in the same stimulation conditions and cell types, and it is not suitable for comparing across different experiments. Importantly, lightly increased ROS can be recognized as a complimentary signal to rescue the imbalanced ROS system and alarming stress and promote cell survival. Therefore, ROS system is a network connecting various DAMPs/conditional DAMPs, including cytokines, growth factors, hormones/neurotransmitters, hypoxia, and high glucose, and other disease risk factors, to the initiation of metabolic processes (Fig. 2C). In Table 4A, even though the fold changes do not match the criteria mentioned above, the response severity and scales are dose-dependently increased according to the stimulation strength, such as interleukin 1 (IL-1) [78,173], IL-13 [174,175] and Ang II [169,176–181]. Here, we attempted to collect the evidences to determine whether ROS systems are functionally qualified in sensing metabolic stress in organelles. One requirement for this function is the ROS system must have the capacity to sense the strengths of stress with different functional consequences to activate inflammasomes and cross-talk to different stress pathways in inflammasomes-dependent, or independent manners.

Balanced ROS levels are critical for cell signaling and homeostasis in physiological condition. In physiological homeostasis conditions, ROS initiate diverse cellular responses including triggering signaling pathways and regulating cell protection besides to activate inflammasome pathways. In addition, ROS participates in coordinating activation of mitochondrial fission fusion and mitophagy to optimize clearance of abnormal mitochondria and cell [31]. These process prevent the damage signals to spread to neighboring mitochondria or cells [31]. In addition, ROS regulate various metabolic processes in cell cycle and survival via calcium signaling in physiological conditions [67,68,177,182]. Moreover, lightly increased ROS complementarily rescue the imbalanced ROS system and alarming stress via regulating metabolic processes. For example, slightly increased ROS enhance insulin sensitivity via phosphatidylinositol-3-kinase (PI3K)-protein kinase B (Akt)-mammalian target of rapamycin (mTOR) pathway [183] and protein synthesis via Ras (a small GTPase)-MAPK kinase (MEK)-extracellular signals-regulated kinase (ERK) pathway [184], protect from DNA damage [185] and increase cell survival [69,186,187]. Additionally, ROS are slightly increased to induce cell proliferation [178,188–191] and angiogenesis [173,191,192] via regulating p38 MAPK-Jun N-terminal kinase (JNK) pathway [193], hypoxia induced factors alpha (HIF-α) stabilization [194] and signal transducer and activator of transcription 3 (Stat3) activation [195]. Studies found that NLRP3 inflammasome could be activated via PI3K-Akt pathway [196] and p38 MAPK pathway [197]. ROS serve as a bridge to connect metabolic stress and inflammasome activation and regulate metabolic pathways in a feedback mechanism. On the other hand, ROS contribute chronically to tumorigenesis or metastasis [198,199]. In addition to ROS regulation of certain signaling pathways above discussed, it remains poorly defined whether ROS in physiological conditions regulate a functional status of vascular cell type-endothelial cells, which we recently classified as innate immune cells [36,200]. To address the issue, we examined human aortic endothelial cells (HAECs). Both mtROS and ATP are produced as a result of electron transport chain activity [38], but it remained enigmatic whether mitochondrial ROS (mtROS) could be generated independently from ATP synthesis. Our report shed light on this important question and found that, during endothelial cell (EC) activation, mtROS could be upregulated in a proton leak-coupled, but ATP

Table 4B
Hypoxia, hyperglycemia and eighteen types of DAMPs promote ROS production, and hypoxia can also decrease ROS generation in certain conditions.

		ROS	Sensor	Target	Concentration	Function	PMID	Ref #
Hypoxia		Increase		NOX4	1% O_2	Angiogenesis, Migration	26297045, 29123322	[195, 205]
		Increase		SOD	5% O2	Proliferation	16624953	[189]
		Decrease			1% O_2	Protect from apoptosis	29061494	[187]
Hyperglycemia	High glucose	Increase	GLUT	H2O2	20 mM	Insulin secretion	17400930	[70]
		Increase	GLUT	NOX	33 mM	Angiogenesis	23274526	[248]
DAMPs	LPA	Increase	Edg	NOX	10 nM	Proliferation	20934509	[190]
		Increase	LPAR5		1uM	pro-inflammatory cytokine and chemokine	29258556	[202]
	LPC	Increase		mtROS	40uM	Trained immunity	31153039	[48]
	AGEs	Increase	RAGE		300μg/ml	Cytokine secretion, Caspase-dependent apoptosis	31587010, 22944044	[212, 223]
		Increase	Galectin-3, CD36, SR-AI and RAGE	NOX	400μg/ml	Liver fibrosis	20133001	[249]
	AOPPs	Increase		NOX4	50uM	Cytokine secretion	31539804	[211]
		Increase		NOX1/4	150uM	S-phase arrest	29032312	[250]
		Increase	RAGE	NOX2	50μg/ml	Apoptosis	23453926	[224]
		Increase			200μg/ml	Apoptosis	28000869	[225]
	HSA	Increase			10 mg/ml	Cell death	25713411	[74]
	Fatty acid	Increase		Mt		Mitochondrial fission	29092894	[251]
	eATP	Increase	P2X7		130uM	Cell death	23431238	[252]
		Increase	DORN1	NOX	200uM	Bacterial defense	29273780	[253]
	Zymosan	Increase	TLR2	NOX			24121038	[254]
	Concanavalin A	Increase	TLR2/4/9	NOX			24121038	[254]
	dsDNA (NCS)	Increase		NOX1	0.5ug/ml	Apoptosis	22237206	[75]
	Thrombin	Increase	GPIbα, PAR4	FAK/NOX1	1–2U/ml		29569550	[255]
		Increase		NOX	20U/4ul	Neurodegeneration	15843610	[256]
	Integrin	Increase	PRR (β2 integrin Mac-1)	NOX2		Innate immunity (Phagocytosis)	29544096	[43]
		Increase	RTKR	LOX, NOX		Cell adhesion	12796479	[204]
	ET-1	Increase	ET1R	eNOS	1 nM	Nuclear Calcium homoeostasis	20393594	[71]
		Increase	ET1R	NOX	10 nM	Protein synthesis	15203192	[184]
		Increase	ETAR	NOX		Cardiac hypertrophy	16107552	[257]
	Methionine	Increase		Mt			29679893	[258]
	Homocysteine	Increase		Mt	500uM	Caspase-1 inflammation activation	27006445	[259]
PAMPs	RSV	Increase	TLR2			Cytokine secretion	22295065	[141]
	DENV	Increase	TLR9	Mt		Innate immunity	29880709	[116]
	Shiga toxin	Increase	CD77	Caspase-4/ GSDMD	200 ng/ml	Pyroptosis	30404007	[221]
	LPS	Increase	CD14/TLR4	Caspase-5	10 ng/ml	Inflammation	26508369	[213]
		Increase	TLR4	NOX	10μg/ml	Monocyte adhesion	23153039, 24121038	[203, 254]
Biophysical forces	Hemodynamic strain	Increase	N/A	N/A	Average strain (12%)	Adhesion molecule expression, Monocyte adhesion	9449403, 19186986	[214, 215]
		Increase	Integrins	Mito complex I	Uniaxial cyclic stretch (20%)	Membrane spreading	23738008	[216]
	Pulmonary strech	Increase	Integrin, EC receptor and ion channel	NOX, XO, eNOS	N/A	Pulmonary hypertension and lung injury	30623676	[217]

Abbreviations: EGF-Epidermal growth factor; PDGF-platelet-derived growth factor; VEGF-vesicular epithelial growth factor; IGF-I-insulin-like growth factor; LPA-lysophosphatidic acid; LPC–lysophosphatidylcholine; LPS-lipopolysaccharide; AGEs-advanced glycation end products; AOPPs-advanced oxidation protein products; HAS-human serum albumin; eATP-extracellular ATP; RSV-respiratory syncytial virus; DENV-dengue RNA virus; dsDNA-double-stranded DNA; endothelin-1—ET1; RIRR-ROS-induced ROS release; TBE cell-Tracheobronchial epithelial cells; EC-Endothelial cell; EPC-Endothelial progenitor cell; HCC-hepatocellular carcinoma cell; PPP-pattern recognition receptors; EMT-epithelial mesenchymal transition; ΔΨm, Mitochondrial membrane potential; NE-Norepinephrine; LPC-lysophosphatidylcholine.

synthesis-uncoupled manner [60]. As a result, EC could upregulate low dose mtROS production for physiological EC activation without compromising mitochondrial membrane potential and ATP generation, and consequently without causing mitochondrial damage and EC death. Thus, a novel pathophysiological role of proton leak [37,40] in driving mtROS production is uncovered for low grade EC activation, patrolling immunosurveillance cell (for example, non-classical monocytes [201]) trans-endothelial migration and other signaling events without compromising cellular survival. This new working model explains how mtROS could be increasingly generated independently from ATP synthesis and endothelial damage or death. Mapping the connections among mitochondrial metabolism, physiological EC activation, patrolling cell migration, and pathological inflammation is significant towards the development of novel therapies for inflammatory diseases and cancers [39].

Moderate increased ROS promote cytokine secretion [202], inflammatory cell adhesion [203,204] and migration [205]. Studies found that six proinflammatory cytokines cause increased cytosolic and mitochondrial ROS generation, including TNF-α [72,78,117], interferon gamma (IFN-γ) [78,174], interleukin 1 (IL-1) [78,173], IL-4 [174,206], IL-13 [174,175] and IL-27 [79] (Table 4A). The increased ROS generation promote inflammatory responses and innate immunity via regulating cyclin dependent kinase 5 (CDK5)-related cell cycle [207], histone 3 lysine 14 acetylation [208], caspase-1 inflammasome activation [116], NF-kB pathway [142] and expressions monocyte chemoattractant protein-1 (MCP1) [206]. In addition, anti-inflammatory cytokines, IL-10 [146] and IL-35 [146,208], are found to inhibit endothelial cell activation via decreasing mtROS production in the

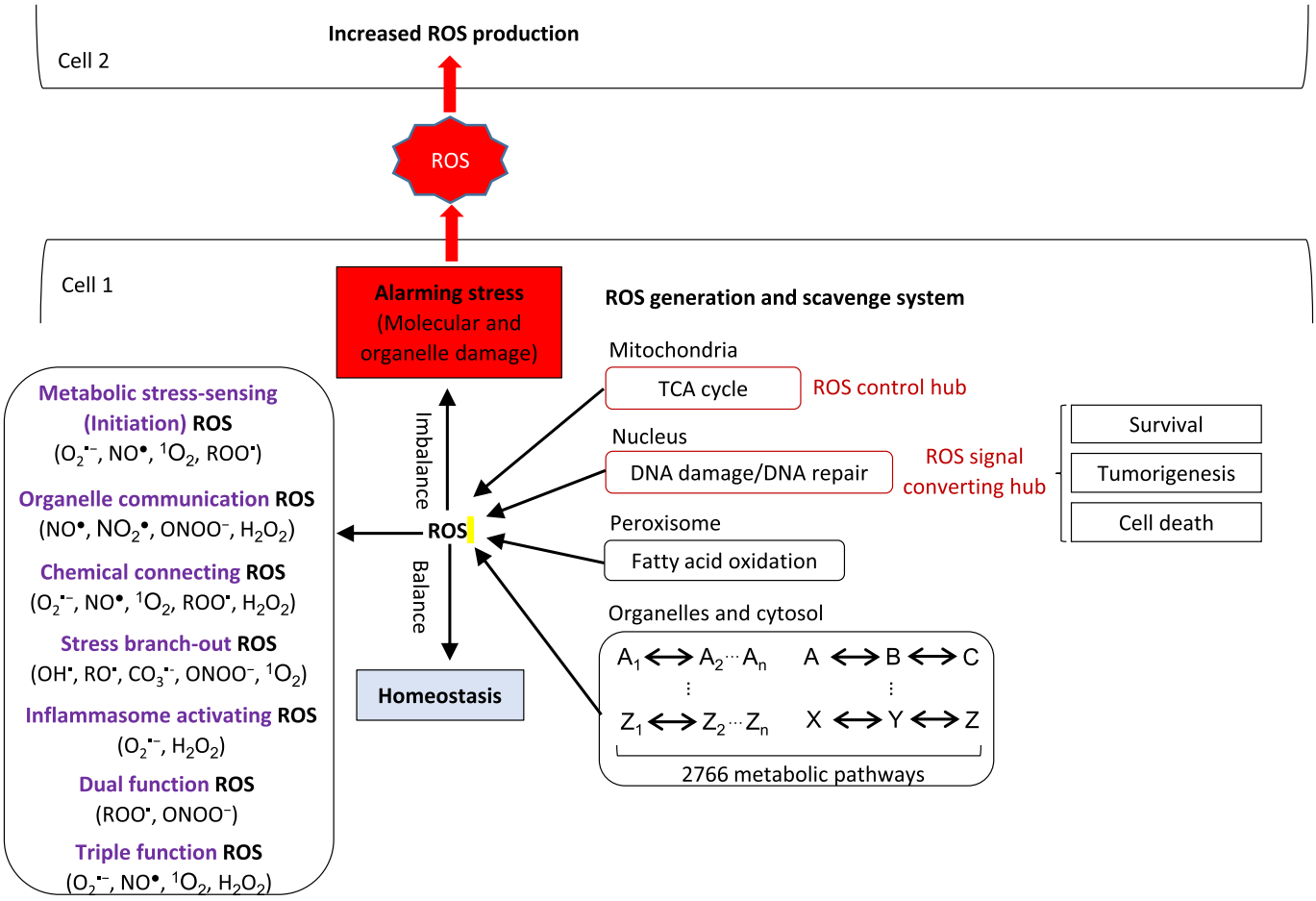

Fig. 3. A novel working model: 1) ROS are a novel integrated network for sensing homeostasis and alarming stress in organelle- or cytosolic-metabolic stresses; and 2) ROS also serve as cellular communication signaling to increase neighboring cell ROS production.
Abbreviations: O2■−—superoxide, NO■—nitric oxide radical, NO2 ■—nitrogen dioxide, OH■—hydroxyl radical, ROO■—peroxyl radical, RO■— alkoxyl radical, CO3■−—carbonate radical, H2O2—hydrogen peroxide, ONOO−—peroxynitrite, 1O2—singlet oxygen, HOCl—hypochlorous acid, TCA: tricarboxylic acid cycle.

presence of pro-inflammatory stimulation. Growth factors, epidermal growth factor (EGF) [209] and vascular endothelial growth factor (VEGF) [143,210], induced cell migration via increasing ROS production and matrix metalloproteinases (MMP)2/9 expression [143] (Table 4A). DAMPs stimulation, such as advanced oxidation protein products (AOPPs) [211], AGEs-advanced glycation end products (AGEs) [212], respiratory syncytial virus (RSV) [141], lipopolysaccharide (LPS) [213] and integrin [43], induce ROS generation via NOX activation, therefore, promote cytokine secretion and innate immunity (Table 4B). Furthermore, cells are exposed to several types of biophysical forces (hemodynamic strain and pulmonary stretch) in physiological and pathological condition [214–217]. Studies found that these biophysical forces also induce higher level of cytosolic and mitochondrial ROS which promote the inflammatory signaling and disease progression (Table 4B).

Highly increased ROS significantly contributes to different types of cell death, including apoptosis, pyroptosis (inflammatory cell death), necrosis and senescence [76]. TNF-α induced ROS generation by affecting mitochondrial membrane potential contributes to apoptosis [117]. IL-13 leads to cell death via NOX activation and ROS generation in microglia [175]. A high concentration of extracellular ROS [187], such as $ONOO^-$ and H_2O_2, promotes apoptosis via DNA fragmentation [218], potentially NLRP3 inflammasome-DNA damage pathway [219], and caspase-independent pathway [69]. Epidermal growth factor (EGF) induced H_2O_2 generation promotes cell necrosis and senescence via regulating Ras-MEK-ERK pathway [73,220]. In addition, Shiga toxin 2 increases ROS generation via caspase-4 activation/presumably noncanonical inflammasome pathway [59] and then promotes cell pyroptosis [221]. Moreover, mitochondrial membrane potential (MMP) collapse and ROS generation induce NLRP3 inflammasome activation. The elimination of ROS alleviates the cleavage of Gasdermin D (GSDMD) carried out by activated caspase-1/caspases-4/caspase-11 and non-canonical cytokine secretory pathways mediated by N-terminal Gasdermin D-protein pores on the plasma membrane [59]. Hydrogen peroxide treatment augments the cleavage of GSDMD by caspase-1. Four amino acid residues of GSDMD are oxidized under oxidative stress in macrophages, suggesting that GSDMD oxidation serves as a *de novo* mechanism, by which mitochondrial ROS promote NLRP3 inflammasome-dependent pyroptosis [222]. Other DAMPs, including AOPPs, serotonin and AGEs [223], accelerate cell death [75] via increased ER stress [74], p38 MAPK-JNK pathway [224,225] and Gasdermin family [59] (Table 4B).

6. Our new working model: ROS systems are a new integrated network for sensing homeostasis and alarming stress in metabolic processes in various subcellular organelles

The key knowledge gap that this analytic review attempted to address is how an integrated system can sense homeostasis and stress in 2801 metabolic pathways localized in various intracellular organelles, cytosol and nucleus. We proposed that ROS are a new integrated network for sensing homeostasis and alarming stress in metabolic processes in various subcellular organelles. The following evidence are found: *first*, ROS are generated in most organelles during metabolic

processes and are participating in cellular signaling; *second*, the electron transfer process existed in most metabolic reactions is the most convenient cue, which could be sensed and monitored in the 2801 complex metabolic pathways. Indeed, we classify a few types of ROS that are functional in sensing metabolic stress; *third*, ROS systems make use of the most general chemical resources in cytosol and organelles—oxygen, which is also generally used in metabolic processes; *Fourth*, four types of ROS are our newly classified organelle communication ROS that are able to cross lipid membrane which is responsible for the communication of cellular metabolic status between organelles and cells, including $NO^{\bullet}$, $NO_2^{\bullet}$, H_2O_2 and $ONOO^-$ (Fig. 3). In addition, increased levels of ROS can activate inflammasome pathways in cytosol and are harmful to DNA, protein and lipid, that are newly classified stress branch-out (cross-talking) ROS. Moreover, some ROS can be converted into another ROS, which we classify as chemical converting ROS as mentioned in Section 2 in details. *Fifth*, after sensing the imbalance of metabolism in metabolic disease in the form of electron transfer imbalance, ROS activate inflammation initiation pathways such as capase-1 inflammasomes in the cytosol to initiate inflammation, which we classify as inflammasome-activating ROS. Furthermore, mitochondria consume 80–95% oxygen and generates mtROS, thus mitochondria are the ROS control hub for connecting cell status and metabolic processes. Increased nuclear ROS significantly increase cell death compared to other organelles. Therefore, the nucleus is the ROS signal converting hub in retrograde, which determines the functions of survival, tumorigenesis and various types of cell death. In homeostasis status, balanced ROS initiate physiological signaling, while imbalanced ROS in organelles as an integrated network promote pathological signals after sensing alarming stress [226]. The increased ROS cross organelle lipid membrane to share signals between organelles or plasma membrane to spread danger signals to neighboring cells via exosomes [227,228]. Our model provides novel insights on the roles of ROS system in bridging metabolic stress to inflammation, cell death and tumorigenesis; and provide novel therapeutic targets for treating those diseases.

Declaration of competing interest

None.

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YS carried out the primary literature search and drafted the manuscript. Others provided material input and helped revise the manuscript. XY supervised the study, data analysis, and manuscript writing. The authors are very grateful to Dr. Christ Kevil at Louisiana State Univ. Health Sciences Center Shreveport, Dr. Yabing Chen and Dr. Rakesh P. Patel at Univ. of Alabama at Birmingham for invitation and advices. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101696.

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Supplemental table 1
The 41 enzymes which are regulating superoxide and hydrogen peroxide are actively functioned and regulated in metabolic processes.

Name[c]	Abbreviation	Function
Aldehyde oxidase	AOX1	Valine, leucine and isoleucine degradation, Tyrosine metabolism, Tryptophan metabolism, Vitamin B6 metabolism and Nicotinate and nicotinamide metabolism
Amine oxidase (flavin- containing) A	AOFA	Deamination of 5-hydroxytryptamine, norepinephrine and epinephrine.
Amine oxidase (flavin- containing) B	AOFB	Deamination of benzylamine and phenylethylamine
D- Amino acid oxidase	OXDA	Regulate the level of neuromodulator D-serine and dopamine synthesis
L- Amino acid oxidase	OXLA	Lysosomal antigen processing and presentation
D- Aspartate oxidase	OXDD	Deamination of D-aspartate and its N-methylated derivative, N-methyl D-aspartate
Amiloride- sensitive amino oxidase (copper containing)	AOC1	Degradation of putrescine, histamine, spermine and spermidine
Cytochrome P450 3A4	CP3A4	Metabolism of sterols, steroid hormones, retinoids and fatty acids
Cytochrome P450 2D6	CP2D6	Metabolism of fatty acids, steroid and retinoids
Cytochrome P450 2E1	CP2E1	Metabolism of saturated fatty acids and xenobiotics
Cytochrome P450 4A11	CP4AB	Metabolism of fatty acids and their oxygenated derivatives (oxylipins)
ERO1- like protein- α	ERO1A	Involved in disulfide bond formation in ER and reoxidizes P4HB/PDI
ERO1- like protein- β	ERO1B	Involved in disulfide bond formation in ER and reoxidizes P4HB/PDI
FAD- linked sulfhydryl oxidase ALR	ALR	Regenerate the redox-active disulfide bonds in CHCHD4/MIA40
Hydroxyacid oxidase 1	HAOX1	Active on 2-carbon substrate glycolate and 2-hydroxy fatty acids
Hydroxyacid oxidase 2	HAOX2	Oxidation of L-alpha-hydroxy acids and L-alpha-amino acids
Membrane primary amine oxidase	AOC3	Semicarbazide-sensitive (SSAO) monoamine oxidase activity
Peroxisomal N1- acetylspermine/ spermidine oxidase	PAOX	Oxidation of N1-acetylspermine to spermidine and putrescine, and polyamine back-conversion
Peroxisomal acyl- CoA oxidase 1	ACOX1	Desaturation of acyl-CoAs to 2-trans-enoyl-CoAs
Peroxisomal acyl- CoA oxidase 3	ACOX3	Oxidizes the CoA-esters of 2-methyl-branched fatty acids
Peroxisomal sarcosine oxidase	SOX	Metabolizes sarcosine, L-pipecolic acid and L-proline
Prenylcysteine oxidase 1	PCYOX	Degradation of prenylated proteins and cleavage of thioether bond of prenyl-L-cysteines
Prenylcysteine oxidase- like	PCYXL	Prenlcysteine oxidase activity
Protein- lysine 6- oxidase	LYOX	Post-translational oxidative deamination of peptidyl lysine residues and regulate Ras expression
Pyridoxine 5′- phosphate oxidase	PNPO	Oxidation of pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate
Retina- specific copper amine oxidase	AOC2	Oxidation of 2-phenylethylamine and tryptamine
Spermine oxidase	SMOX	Oxidation of spermine and metabolism of N1-acetylspermine and spermidine
Sulfhydryl oxidase 1	QSOX1	Oxidation of sulfhydryl groups in peptide and protein thiols

(*continued on next page*)

Supplemental table 1 (*continued*)

Name[c]	Abbreviation	Function
Sulfhydryl oxidase 2	QSOX2	Oxidation of sulfhydryl groups in peptide and protein thiols
Sulfite oxidase, mitochondrial	SUOX	Metabolism of sulfur
Xanthine dehydrogenase/oxidase	XDH	Purine degradation; oxidation of hypoxanthine and xanthine
NADPH oxidase 1	NOX1	Regulate cellular pH and mediate proton currents
NADPH oxidase 2	NOX2 (CY24B)	Regulate cellular pH and mediate proton currents
NADPH oxidase 3	NOX3	Biogenesis of otoconia/otolith
NADPH oxidase 4	NOX4	Inhibit phosphatase and regulate KCNK3/TASK-1 patassium channel
NADPH oxidase 5	NOX5	calcium-dependent proton channel and may regulate redox-dependent processes in lymphocytes and spermatozoa
Dual oxidase 1	DUOX1	Synthesis thyroid hormone, contribute to thyroid peroxidase/TPO and lactoperoxidase/LPO activity
Dual oxidase 2	DUOX2	Synthesis thyroid hormone, contribute to thyroid peroxidase/TPO and lactoperoxidase/LPO activity
Superoxide dismutase [Cu–Zn]	SOD1	Form fibrillar aggregates in the absence of intramolecular disulfide bond
Superoxide dismutase [Mn], mitochondrial	SOD2	Transcription of RNA polymerase Ii and release of cytochrome C
Extracellular superoxide dismutase [Cu–Zn]	SOD3	Response to copper ion and hypoxia

[c] **The 41 enzymes are generated from PMID:** 32231263 [102]. The function of enzymes are collected from Human metabolome database (https://hmdb.ca/) and Uniport database (https://www.uniprot.org/).

Supplemental table 2
MitoSOX Red and Amplex Red are used for mtROS detection *in vitro;* and mitoB is used for mtROS detection *in vivo*.

Category	Method	Reagent	Off-target response	Comment	PMID	Ref #
ROS, RNS		ESR		Specific, sensitive, expensive	24862270	[260]
Extracellular $O_2^{\bullet-}$	Fluorescent probe	HPr+		Membrane impermeable	29790367	[261]
General ROS	Fluorescent probe	DCFH-DA			24862270, 16297980, 27909341	[260,262,263]
General ROS	Fluorescent probe	H_2DCF			23665586	[264]
General ROS	Fluorescent probe	CM-H2DCFDA			24862270, 27302663	[260,265]
$O_2^{\bullet-}$	Fluorescent probe	HE/DHE	OH■, H2O2, OONO-	Cell permeable, intensity quantifiable, low specificity	24862270, 16297980, 27909341	[260,262,263]
$O_2^{\bullet-}$	Fluorescent probe	DPBF			16297980	[262]
$O_2^{\bullet-}$	Fluorescent probe	2-(2-Pyridil)-benzothiazoline			16297980, 27909341	[262,263]
$O_2^{\bullet-}$	Chemiluminescent probe	L-012			24862270	[260]
$O_2^{\bullet-}$	Chemiluminescent probe	Lucigenin	ONOO-	Cell permeable, low selectivity/sensitivity	24862270	[260]
$O_2^{\bullet-}$	Spectrophotometry	NBT			24862270, 20387054	[260,266]
$O_2^{\bullet-}$	Fluorescent probe	cpYFP			24862270, 27288722	[260,267]
$OH^{\bullet}$	Fluorescent probe	CHD			16297980, 27909341	[262,263]
$OH^{\bullet}$	Fluorescent probe	3-CCA			16297980, 26516887	[262,268]
$OH^{\bullet}$	Fluorescent probe	APF	HOCl		16297980, 26516887	[262,268]
$OH^{\bullet}$	Fluorescent probe	HPF	HOCl		16297980, 16168507	[262,269]
$OH^{\bullet}$	Fluorescent probe	FL			16297980, 27909341	[262,263]
ROO■	Fluorescent probe	C11-BODIPY$^{581/591}$	HO•, RO•, ONOO−	Lipid peroxidation sensor	16297980, 21396693	[262,270]
ROO■	Fluorescent probe	DPPP		Lipid peroxidation sensor	16297980	[262]
H_2O_2	Histochemical staining	DAB		In Situ imaging of ROS	24862270, 20387054	[260,266]
H_2O_2	Fluorescent probe	DCFH	CO3•−, NO2•, HO•		16297980, 27909341	[262,263]
H_2O_2	Fluorescent probe	HyPer			24862270, 27302663	[260,265]
ONOO-	Fluorescent probe	DHR	HOCl		16297980	[262]
$^1O_2$	Fluorescent probe	DMA			16297980	[262]
$^1O_2$	Fluorescent probe	DPAX			16297980, 19794917	[262,271]
$^1O_2$	Fluorescent probe	DMAX			16297980, 19794917	[262,271]
HOCl	Fluorescent probe	BODIPY-based			26043093	[272]
Mt $O_2^{\bullet-}$	Fluorescent probe	MitoHE			23668959	[273]
Mt $O_2^{\bullet-}$	Fluorescent probe	MitoSox Red			24862270	[260]
Mt H_2O_2		MitoB		Analyzed by mass spectrometry ex vivo	23726990	[274]
$O_2^{\bullet-}$, H_2O_2	Fluorescent probe	Amplex Red		High sensitivity, low background fluorescence	24862270, 16297980, 27909341	[260,262,263]

(*continued on next page*)

Supplemental table 2 (*continued*)

Category	Method	Reagent	Off-target response	Comment	PMID	Ref #
$O_2^{\bullet-}$, H_2O_2	Chemiluminescent probe	Luminol	OH■, ONOO-		24862270, 29780884	[260, 275]
ONOO-, H_2O_2	Spectrophotometry	Boronates			24862270, 23665586	[260, 264]
Redox status changes	Fluorescent probe	roGFP		Slow in reaction, non-sensitive, real-time, cell friendly but restriction in receptor cells	24862270, 27306519	[260, 276]
Redox status changes	Fluorescent probe	rxYFPs			24862270, 27306519	[260, 276]
Redox status changes	Fluorescent probe	rxRFP			24862270. 27208426	[260, 277]

ESR: electron spin resonance; DCFH-DA: 2,7-Dichlorodihydrofluorescein diacetate; H_2DCF: 2′,7′-dichloro-dihydrofluorescein; DHE/HE:dihydroethidium; DPBF: 1,3-Diphenylisobenzofuran; L-012: luminol analogue 8-amino-5-chloro-2,3-dihydro-7-phenylpyrido; NBT: Nitroblue tetrazolium; CHD: 1,3-Cyclohexanedione; 3-CCA: Coumarin, coumarin-3-carboxylic acid; SECCA:N-succinimidyl ester of coumarin-3-carboxylic acid; HPF: 2-[6-(4′-Hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoic acid; APF: 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl] benzoic acid; FL: Fluorescein; DMA: 9,10-Dimethylanthracene; DPAXs: 9-[2-(3-Carboxy-9,10-diphenyl)anthryl]-6-hydroxy-3H-xanthen-3-ones; DMAX: [2-(3-Carboxy-9,10-dimethyl)anthryl]-6-hydroxy-3H-xanthen-3-one; CM-H2DCFDA: 5-(and 6)-chloromethyl-2′,7′-dichlorohydrofluorescein diacetate; scopoletin: 7-hydroxy-6-methoxy-coumarin; HVA: Homovanillic acid (4-hydroxy-3-methoxy-phenylacetic acid; DHR: Dihydrorhodamine 123; Amplex Red:10-acetyl-3,7-dihydroxyphenoxazineDAB: Diaminobenzidine; C11-BODIPY581/591: 4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; DPPP: Diphenyl-1-pyrenylphosphine; mitoHE: mito-hydroethidine; HPr+: hydropropidine.

Supplemental table 3
ROS generation Inhibitors.

	Target	Drug	Cell type	Concentration	PMID	Ref #
General ROS		NAC	RPE cell	1 mM	17765224	[78]
			Epithelial cell	30 mM	15109912	[193]
			Fibroblast	20 mM	16081426	[220]
			INS-1 cell	0.4 mM	17400930	[70]
		PDTC	RPE cell	50uM	17765224	[78]
		Vitamin C	Endothelial cell	10uM	10828488	[278]
		Vitamin E	Endothelial cell	10uM	10828488	[278]
		Tempol	Astrocyte	3 nM	21270817	[180]
		MnTBAP	Astrocyte	50uM	20547771	[67]
Mitochondrial ROS	complex I	rotenone	RPE cell	2.5uM	17765224	[78]
			NIH-3T3 fibroblast	5uM	12796479	[204]
	complex II	TTFA	RPE cell	10uM	17765224	[78]
		Atpenin A5	Cardiomyocyte	100 nM-1uM	19242645	[279]
		Malonate	Heart Mt	25-100uM	18433712	[280]
	Complex III	Myxothizol	Liver Mt	0.56 mg/kg	24661880	[281]
		Antimycin A	hepa1-6,Huh-7 cell	1ug/ml	26461342	[142]
			RPE cell	100 ng/ml	17765224	[78]
	Compled IV	Sodium azide	Motoneurone	2 mM	14660707	[282]
	ATPase	Oligomycin			20107899	[283]
	Non-selective	Mito-Tempo	HAEC	1 mM	27127201	[60]
	Non-selective	Mito-Vit-E	HUVEC	1uM	31653897	[143]
NOS	Non-selective	L-NAME	L6 myotube	1 mM	16982630	[181]
	nNOS	NOSIP			19298861	[89]
	eNOS	NMMA			28904082	[284]
NOX	Non-selective	apocynin	SMC	50uM	29472601	[177]
			VSMC	100uM	23774581	[246]
		DPI	RPE cell	5uM	17765224	[78]
			Endothelial cell	15uM	16249002	[206]
			HT-29 cell	10uM	25122478	[188]
			Fibroblast	20uM	16081426	[220]
			NIH-3T3 fibroblast	5uM	12796479	[204]
		AEBSF		0.9uM	23251382	[285]
	NOX1	ML171	SMC	1uM	29472601	[177]
	NOX1/4	GKT136901			24319690	[286]
LOX		NDGA	Epithelial cell		19635476	[209]
			NIH-3T3 fibroblast	10uM	12796479	[204]
MPO		ABAH			23251382	[285]
MAO		Selegiline	Astrocyte	20uM	20547771	[67]

NAC– N-acetyl-L-cysteine, PDTC– pyrrolidene-dithiocarbamate, MnTBAP– Manganese III tetrakis, TTFA– 2-thenoyltrifluoroacetone, L-NAME—N omega-Nitro-L-arginine methyl ester hydrochloride, NMMA– NG-monomethyl-arginine, NOSIP– nitric oxide synthase interacting protein, DPI– diphenylene iodonium, SEBSF– 4-(2-aminoethyl)-benzenesulfonyl fluoride, NDGA– nordihydroguaiaretic acid, ABAH– 4-aminobenzoic acid hydrazide.

Supplemental Table 4
The targets in various treatments in Figure 4 (MitoETC and membrane potential, NOX, SOD and LOX) and genes of their downstream pathways are analyzed by circus plot (see details in Fig. 2A and b).

Target	Mitochondria ETC and membrane potential	NOX	SOD	LOX
Downstream signaling genes	ROMO1	CDK5	MAPK3	PTK2
	BCL2L1	MAPK1	MAPK1	PLA2G4A
	CASP3	MAPK2		
	CASP8	MAPK3		
	NFKB1	NOS3		
	MAPK1	PIK3CB		
	MAPK2	AKT1		
	MAPK3	HMOX1		
	RAC1	NFKB1		
	AKAP1	MMP2		
	UTRN	MMP9		
	OPA1	PRRT2		
	NLRP3	CDKN2A		
	PYCARD	IRS1		
	CASP1	NFE2L2		
		NOS2		
		MAP1LC3A		
		PTK2		
		STAT3		
		VEGFA		
		MAPK3		
		MAPK1		
		CTNNB1		
		CDK2		
		MAPK8		
		MAP2K7		
		VCAM1		

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