Biochemical basis and metabolic interplay of redox regulation

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A B S T R A C T

Accumulated evidence strongly indicates that oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) production and antioxidants in favor of oxidants, plays an important role in disease pathogenesis. However, ROS can act as signaling molecules and fulfill essential physiological functions at basal levels. Each ROS would be different in the extent to stimulate and contribute to different pathophysiological effects. Importantly, multiple ROS generators can be activated either concomitantly or sequentially by relevant signaling molecules for redox biological functions. Here, we summarized the current knowledge related to chemical and biochemical features of primary ROS species and corresponding antioxidants. Metabolic pathways of five major ROS generators and five ROS clearance systems were described, including their ROS products, specific ROS enriched tissue, cell and organelle, and relevant functional implications. We provided an overview of ROS generation and induction at different levels of metabolism. We classified 11 ROS species into three types based on their reactivity and target selectivity and presented ROS homeostasis and functional implications in pathological and physiological status. This article intensively reviewed and refined biochemical basis, metabolic signaling and regulation, functional insights, and provided guidance for the identification of novel therapeutic targets.

1. Introduction

Oxidative stress, an imbalance between reactive oxygen species (ROS) production and antioxidants in favor of oxidants leading to cell damages [1], is an important feature of cardiovascular disease (CVD) and other diseases. Case-control studies showed a strong link between increased oxidative stress biomarkers, such as F2-isoprostanes and 8-epi-PGF2α [2,3], and CVD [4] (atherosclerosis [5], myocardial infarction [6], heart failure, and stroke [7]), metabolic disorders (obesity [8], diabetes [8], hyperlipidemia [9], hyperhomocysteinemia (HHcy) [10]) and cancer [11,12]. Cohort studies found that high intake of antioxidants, such as vitamin A, C, and E, was associated with a lower rate of CVD [13] and cancer [14,15], although the majority of large scale clinical trials yielded negative results for oral antioxidant supplementation [16,17]. Moreover, experimental studies provided considerable evidence supporting the hypothesis that oxidative stress was the common mechanism for cancer [18] and CVD [19]. In addition, inhibiting ROS-generating enzymes using pharmacologic or genetic approaches reduced ROS levels and disease phenotype in experiment disease models [20–22]. However, the negative findings in clinical trials may be due to three major reasons. Firstly, the targeted participants in clinical trials might not be as specific as models used in preclinical studies [16]. For example, the index disease would be concomitant with other disorders due to recruiting broad study populations into a trial. Secondly, the excessive ROS might be inadequate to be removed [16,17]. It has been reported that the rate constant between general scavengers with ROS is lower than the rate constants between ROS-targeted biomolecules with ROS. Nitric oxide (NO$^\cdot$) is a targeted biomolecule of superoxide radical anion ($O_2^{\cdot-}$). The rate constant between vitamin C with $O_2^{\cdot-}$ is 10^5 times lower than the rate constant between NO$^\cdot$ with $O_2^{\cdot-}$ [23]. The required concentration of vitamin C within the cell needs to be extremely high in order to compete with $O_2^{\cdot-}$. Finally, conventional antioxidants lack the specificity of sites or target [17,24], which indiscriminately interferes with a number of ROS

protective processes. ROS act as signaling molecules involving cell survival and death via modulation of transcription factors and epigenetic pathways. The physiological level of H_2O_2 is essential to regulate basic biological processes such as cell proliferation/differentiation and metabolic homeostasis [25]. General antioxidants may reduce ROS levels to below "normal range". Additionally, redox signaling also is associated with time-dependent ROS metabolism process and organelle function, that is, the spatiotemporal differentiation of Redox [26]. Therefore, it is in great need to better understand the biochemical and metabolic regulation of ROS homeostasis and to develop more specific and effective strategies for oxidative stress in human disease. A future therapeutic strategy would be to modulate the oxidative stress without severely disturbing general redox homeostasis and less harmful to healthy tissues/cells.

2. Chemical features of major ROS (Table 1)

ROS is a term for chemically reactive species containing oxygen. Another term such as reactive nitrogen species (RNS) was used for ROS containing nitrogen. Because ROS and RNS are closely interrelated, we use "ROS" as the general term representing both. Basing on their chemical features, ROS can be divided into two classes, free radicals, and non-radicals (Table 1). The free radicals usually refer to ROS containing one unpaired reactive electron in the outer orbit and belong to one-electron oxidants. The major free radicals include nitric oxide ($NO^•$), superoxide radical anion ($O_2^{•-}$), hydroxyl radical ($OH^•$), carbonate radical anion ($CO_3^{•-}$), nitrogen dioxide ($NO_2^•$), and alkoxyl/alkyl peroxyl ($RO^•/ROO^•$). The non-free radicals do not contain unpaired electron and belong to two-electron oxidants. Hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$)/peroxynitrous acid ($ONOOH$), and hypochlorous acid ($HOCl$) are the major non-radical species. The presence of unpaired electron confers to some shared properties among free radicals that are distinct from non-radicals. In general, free radicals are more unstable and reactive than non-radicals. Their reactions with targeted molecules favor a chain-reacting behavior, which consists of an initiation step, a propagation step, and a termination step. Typically, the initiation step is the generation of starting free radicals from initiators via thermal, radiation or electron transfer (redox) processes. Within cells, usually, oxygen (O_2) is the initiator. The electron transfer process catalyzed by various endogenous ROS generators accounts for the production of

starting radicals. There is a net increase of free radical in this step. Once a starting radical is formed, it can react with a stable molecule without unpaired electrons to form a covalent bond. The targeted molecule then loses an electron and becomes the new radical species. This process is called propagation in which there is no net increase of free radicals. This propagation continues until the radicals react with phenolic antioxidants such as vitamin E, flavonoids and ascorbic acid, or with another free radical to form a covalent bond, a step called termination which leads to a net decrease of free radicals. Relative reactivity of free radicals can be simply ranked according to the one-electron reduction potential (E^O) which reflect the tendency of a chemical species to receive electrons and to be reduced [27] and is graded in the range from one plus (+) to five plus in Table 1. Relative reactivity of non-radical is based on their second order rate constant (k), a chemical kinetic parameter for reaction rate with glutathione (GSH) [27]. As indicated in Table 1, the lifetime of ROS ranges from 10^{-9} to 1 s, and diffusion distance ranges from 10^{-12} to 10 m. ROS can target a large scale of molecules, including protein residues (Cys, Met, Tyr, and Trp), Fe–S, ROS and any macromolecules (DNA, RNA, protein, and lipids), which leads to various pathological changes. The major analytical approaches to measure ROS are spectroscopic and electrochemical methods, which were described in highly comprehensive reviews [28–32]. Spectroscopic methods include direct measurement of adducts formed between ROS and endogenous target molecules (mass spectrometry), exogenous fluorescent probe (fluorescence) or spin traps (electron paramagnetic resonance spectroscopy), and indirect measurement of byproducts formed between ROS and other chemical species (chemiluminescence). Electrochemical methods directly measure ROS using selective biosensors and are technically challenging.

2.1. Radicals

$NO^•$ — $NO^•$ is a very weak oxidant with the $E^O \sim 0.80$ V, with the longest life among identified ROS ($\sim 1s$). It has high solubility, being able to diffuse across a membrane freely up to 10 m. This property makes it difficult to define its cellular origin because the detected $NO^•$ can be from another cell or even different tissues. $NO^•$ tends to be oxidized instead of to be reduced and is relatively inert to most biomolecules. It prefers reacting with free radicals and metals. Reaction with different free radicals results in the different biological effect.

Table 1
Chemical features of major ROS.

Species	Reactivity	Lifetime	Diffusion distance	Preferred Target	Measurement
Free-Radical:					
Nitric oxide ($NO^•$)	+	$\sim 1s$	10 m	Metals	Fe-DETC, DAF-2DA
Superoxide radical anion ($O_2^{•-}$)	+ +	~ 50 ms	320 nm	Fe–S cluster, $NO^•$	DMPO, DHE
Hydroxyl radical ($OH^•$)	+ + + + + +	$\sim 10^{-9}$s	4.5 nm	Any macromolecules	HPLC or GC-MS (indirect)
Carbonate radical anion ($CO_3^{•-}$)	+ + + + +	$\sim 10^{-6}$s	152 nm	Tyr, Trp, thiols, guanine	Continuous flow EPR
Nitrogen dioxide ($NO_2^•$)	+ + +	NA	188 nm	Thiols, Tyr	NA
Alkoxyl radical ($RO^•$)	+ + + +	NA	NA	Organic compounds	Low temperature EPR
Peroxyl radical ($ROO^•$)	+ + +	NA	NA	Organic compounds	Low temperature EPR
Non-radicals:					
Hydrogen peroxide (H_2O_2)	+ +	~ 1 ms	1.5 mm	Thiols, metals	PG1/PC1
Peroxynitrite ($ONOO^-$/$ONOOH$)	+ + + +	$\sim 15\mu$s	50 μm	CO_2, Cys, Trp, Met, metals	JH-PN4, HKGreen-1 boronate based probe1, HPLC
Hypochlorous acid (HOCl)	+ + + +	NA	NA	Amino, thiols	HKOCl-1
Singlet oxygen ($^1O_2$)	+ +	$\sim 45\mu$s	60–268 nm	Guanine, unsaturated lipids, amino acids	TMPyP

ROS are a chemically reactive species containing oxygen which is classified to free radical and non-radical species. The table describes chemical features of major ROS including reactivity, lifetime, diffusion distance, preferred target, and measurement methodology. Reactivity is defined according to one electron reduction potential (E^o) at pH7, or the reaction rate with glutathione (GSH) for radicals and non-radicals, and provided with a relative description of + and + + (Low), + + + (mediate), and $\geq$ + + + + (high). Lifetime equals the natural log2 divided by the sum of the products of rate constant and concentrations for all molecules that ROS react with. Diffusion distance is calculated by using the formula x = $(6Dt)^{1/2}$ (x, D, t stand for diffusion distance, diffusion coefficients and lifetime, respectively). **Abbreviation:** Cys, Cysteine; DAF-2DA, 5-diaminofluorescein diacetate; DHE, dihydroethidium; DMPO, 5,5-dimethylpyrroline-N-oxide; EPR, Electron paramagnetic resonance; Fe-DETC, Iron-diethyldithiocarbamate; Fe–S, Iron (II) sulfide; GC-MS, Gas chromatography-mass spectrometry; HPLC, High performance liquid chromatography; Met, Methionine; PG1, Peroxygreen 1; PC1, Peroxycrimson 1; ROS, Reactive oxygen species; TMPyP, 5,10,15,20-tetrakis(N-methyl-4-pyridyl)-21H, 23H-porphine; Tyr, Tyrosine; Trp, Tryptophan; NA, Not Available. Unpaired electron denoted by a superscript dot to the right preceding the charge symbol.

When it reacts with the lipid-free radicals such as lipid oxy (LO$^\bullet$) or peroxyl radical (LOO$^\bullet$), LONO/LOONO will be formed and the lipid peroxidation will be terminated (protective). When it reacts with $O_2^{\bullet-}$, more potent oxidant ONOO$^-$ will be generated and oxidative stress is induced (deleterious). When it reacts with protein radicals, a nitrosylated protein will be generated. NO$^\bullet$ targets on metals (heme iron, non-heme iron, Fe–S cluster, and copper compound) and results in nitrosyl-metal complex formation, metal oxidation, and metal reduction [33]. The best example of a nitrosyl-metal complex is the nitrosyl-heme complex within the soluble guanylate cyclase (sGC), leading to the alternation of sGC configuration and subsequent sGC activation. Activated sGC then converts guanosine triphosphate (GTP) to 3′, 5′-cyclic guanosine monophosphate (cGMP) leading to vessel relaxation [34]. The nitrosyl-heme complex formation has also been reported in mediating the reversible inhibition of cytochrome P450, catalase (CAT), lipoxygenase (LPO), myeloperoxidase (MPO) and nitric oxide synthase (NOS) [35,36]. NO$^\bullet$ is commonly detected by using fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) [37] or spin trap iron diethyldithiocarbamate (Fe-DETC) [38].

$O_2^{\bullet-}$ — $O_2^{\bullet-}$ is also a weak radical ($E^O \sim 0.94$ V). This is in part due to its anionic charge that prevents its access to electron-rich centers. It has a relatively short lifetime (~ 50 ms) because it can be rapidly dismutated to H_2O_2 spontaneously or by superoxide dismutase (SOD). The anionic charge limits its diffusion distance at ~ 320 nm $O_2^{\bullet-}$ prefers to react with NO$^\bullet$ or the transition metals particularly the Fe–S cluster. When it reacts with NO$^\bullet$, because of the much shorter lifetime and diffusion distance than NO$^\bullet$, the reaction mostly occurs at the site of $O_2^{\bullet-}$ generation. When it reacts with Fe–S clusters, Fe–S clusters will be oxidized into unstable high valent clusters which decompose and then releases free Fe^{2+}. For example, the SoxR, a transcriptional factor in *E. coli* to regulate resistance genes to prevent oxidative damage, is active only when its Fe–S clusters is oxidized by $O_2^{\bullet-}$ to high valent cluster [39]. $O_2^{\bullet-}$ is usually detected by fluorescent probe dihydroethidium (DHE) or spin trap 5,5-dimethylpyrroline-N-oxide (DMPO) [30].

OH$^\bullet$ — OH$^\bullet$ is the most reactive radical ($E^O \sim 2.31$ V). It can indiscriminately react with most of the biomolecules including lipids, proteins, carbohydrates, and DNA, at a rate close to diffusion limit. Because of its extremely high reactivity and ability to rapidly react with a target at its site of formation, it has a very short lifetime at $\sim 10^{-9}$s and the shortest diffusion distance at ~ 4.5 nm. OH$^\bullet$ reacts with its targets through hydrogen (H) abstraction, addition, and electron transfer mechanism. OH$^\bullet$ can easily attract the H atoms from organic compounds (proteins and lipids) particularly those with weak bond, generating a carbon center free radical (R$^\bullet$). Isoprostanes are also produced through hydroxyl radical attack on arachidonic acid and are used as clinical markers of oxidative stress for various conditions [40]. For the aromatic compounds (DNA/RNA), OH$^\bullet$ can be added directly to produce hydroxylated radical adducts. These stable adducts such as 8-Oxo-deoxyguanosine [41,42] can be detected at extremely low concentration and used as a biomarker of OH$^\bullet$. When reacting with anions, OH$^\bullet$ takes an electron, producing a corresponding new radical. Hydroxylated radical adducts can be detected by HPLC electrochemical (HPLC-EC) detector and by GC-MS analysis of trimethylsilyl derivatives [43].

$CO_3^{\bullet-}$ — $CO_3^{\bullet-}$ is a highly reactive radical ($E^O \sim 1.78$ V) with a very short lifetime ($\sim 10^{-6}$s) and very short diffusion distance at ~ 152 nm $CO_3^{\bullet-}$ can oxidize biothiols, metalloproteins, protein residues (Trp, Cys, Tyr, Met, and histidine) and guanine moieties of DNA/RNA to the corresponding radicals [44]. CO3$^{\bullet-}$ has been proposed as a key mediator of the ONOO$^-$ oxidative damage [45]. Like OH$^\bullet$, $CO_3^{\bullet-}$ oxidizes these targets through H abstraction or electron transfer mechanism. Unlike OH$^\bullet$, $CO_3^{\bullet-}$ oxidation might not produce or probably form radical adducts that have a short lifetime to be detected. Therefore, $CO_3^{\bullet-}$ cannot be measured by HPLC-EC detector or GC-MS analysis. $CO_3^{\bullet-}$ can be directly detected by continuous flow EPR, but requiring high instantaneous concentrations [46].

$NO_2^\bullet$ — $NO_2^\bullet$ is a moderately reactive radical ($E^O \sim 1.04$ V) and has

short diffusion distance (~ 188 nm). It reacts with its target predominantly through electron transfer mechanism [47]. It oxidizes the thiols to thiyl free radicals, which subsequently react with NO$^\bullet$ to generate nitrosothiol (RSNO). It can oxidize the tyrosine residue to tyrosyl radical, but the reaction is slower than its reaction with thiols. $NO_2^\bullet$-mediated tyrosine nitration is likely relying on pre-existing tyrosine free-radicals induced by $CO_3^{\bullet-}$. Biological nitration of tyrosine residues is also largely affected by the nanoenvironment of the tyrosine such as neighboring acidic residues (e.g. aspartate) that can cause partial abstraction of the proton of the phenolic OH-group in tyrosine [48]. In addition, metal enzymatic catalysis also confers specificity for nitration of nearby tyrosine residues (eg. the nitration of tyrosine 430 in prostacyclin synthase [49]).

RO$^\bullet$/ROO$^\bullet$ — RO$^\bullet$/ROO$^\bullet$ are moderately reactive radicals ($E^O > 1.78$ V). Its reactivity can be affected by the substituents at the α-carbon. An electron-withdrawing group increases the reactivity while electron-donating groups decrease it. In lipid peroxidation, LO$^\bullet$/LOO$^\bullet$ can rapidly abstract the H from nearby lipid, generating new lipid free radicals and propagating the chain reaction. RO$^\bullet$/ROO$^\bullet$ can be detected by lower temperature EPR [50]. In addition, products of oxidative stress such as isoprostanes can be used for indirect detection RO$^\bullet$/ROO$^{\bullet 40}$.

2.2. Non-radicals

H_2O_2 – H_2O_2 is a mild non-radical with a relatively long lifetime (~ 1 ms) and with a long diffusion distance at ~ 1.5mm. H_2O_2 can react with Fe–S cluster, loosely bound metals, glutathione, free cysteine (Cys) and methionine (Met) residues but at a relatively slower rate than with Cys residues. Its reaction rate with Cys residue depends on the ionization state of Cys residue. Thiolate anion form (Cys–S-) of Cys residue favors a faster reaction than the protonated form (Cys–SH) of Cys residue. In physiological condition, Cys residue usually exists in the form of Cys–S-. Low levels of H_2O_2 can oxidize the Cys–S- to the sulfenic form (Cys–SOH). Cys–SOH can be reduced back to Cys–S- by thioredoxin (TRX) and GSH. High levels of H_2O_2 can further oxidize Cys–SOH to the irreversible form of sulfinic (Cys–SO_2H) or sulfonic (Cys–SO_3H). Despite of a fast reaction with Cys–S, its reactions with seleno, thiol or heme peroxidases are much faster. H_2O_2 can be quickly converted to H_2O by these surrounding peroxidases before reaching Cys–S-. Therefore, even though H_2O_2 is highly diffusible, it is usually restricted within a small range from its site of generation. This compartmentation allows the H_2O_2 to selectively target the thiols protein such as protein tyrosine phosphatases (PTPs) that are restricted within the same compartment. When H_2O_2 reacts with some heme peroxidases like MPO or reduced transition metal centers, more reactive free-radicals like OH$^\bullet$ or non-free-radicals like HOCl will be produced. The Amplex Red probe can be used to detect and quantitate H_2O_2 because of its high sensitivity [29].

ONOO$^-$ — ONOO$^-$ is a highly reactive non-radical with a short lifetime (~ 10–20 ms) [51,52] and a relatively long diffusion distance > 50 μm (an average cellular length). ONOO$^-$ can react with its favored targets located in the same or adjacent cells. In most cases, ONOO$^-$ oxidizes a wide variety of biomolecules through its derivatives $CO_3^{\bullet-}$, $NO_2^\bullet$ and OH$^\bullet$ generated in RNS pathway (Fig. 1). By its own, it can selectively react with few molecules including CO_2, thiols, selenium compounds and metal centers. Reaction with CO_2 results in the generation of more toxic free-radicals $CO_3^{\bullet-}$ and $NO_2^\bullet$ (Fig. 1) which account for the most oxidative and nitrative effect of ONOO$^-$. Reaction with thiols results in the generation of the corresponding disulfide. In contrast, its derivatives $CO_3^{\bullet-}$ and $NO_2^\bullet$ will oxidize thiol to thiyl free-radicals. Its reaction with the thiol peroxidase (PDRX) has the highest second-rate constants. This makes PDRX the most efficient ONOO$^-$ scavengers. Rapid reaction with selenium peroxidase (GPX) is another efficient way for scavenging ONOO$^-$. Reaction with the metal centers can cause disruption of the active site and loss of enzyme activity. ONOO$^-$ can be directly detected by boronate-based probe [28,30].
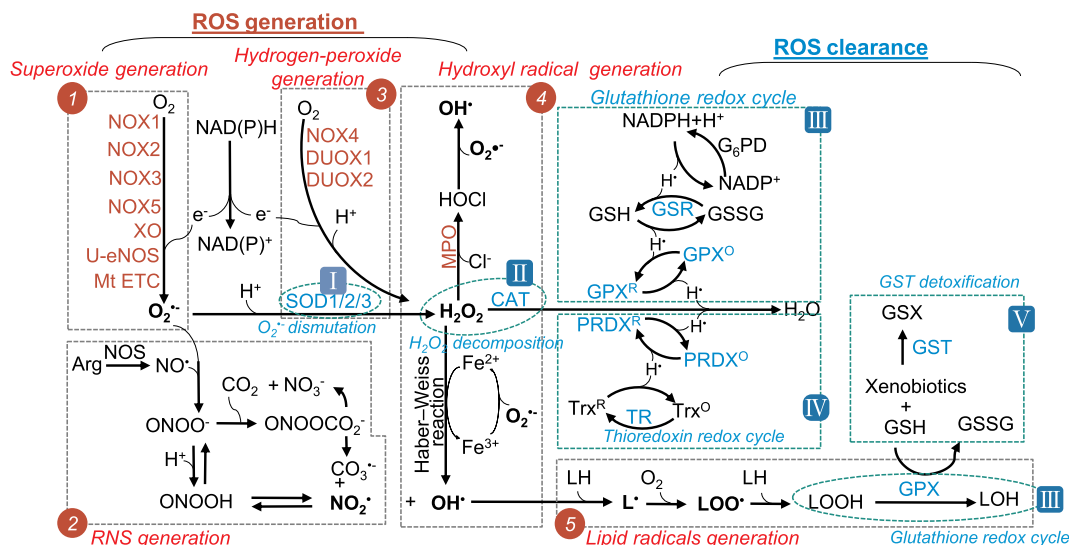
Fig. 1. Reactive oxygen species (ROS) metabolism. A. ROS generation: Cellular ROS is generated by 5 pathways. 1) Superoxide generation: $O_2^{\cdot-}$ is derived from oxygen (O_2) after receiving an electron from various oxidases or from mitochondrial electron transport chain (ETC). 2) Reactive nitrogen species generation: $O_2^{\cdot-}$ reacts with nitric oxide ($NO^{\cdot}$) to form peroxynitrite ($ONOO^{-}$). At physiological pH (pKa = 6.8), $ONOO^{-}$ is in equilibrium with peroxynitrous acid (ONOOH). In the aqueous phase, $ONOO^{-}$ rapidly reacts with carbon dioxide (CO_2) to generate carbonate radical anion ($CO_3^{\cdot-}$) and nitrogen dioxide ($NO_2^{\cdot}$). In the hydrophobic phase, ONOOH can undergo homolytic fission to generate hydroxyl radical ($OH^{\cdot}$) and $NO_2^{\cdot}$. 3) Hydrogen peroxide generation: $O_2^{\cdot-}$ is dismutated spontaneously or by superoxide dismutase (SOD) into H_2O_2. NOX4 and DOX1/2 can also dismutate O_2 directly to H_2O_2. 4) Hydroxyl radical generation: $OH^{\cdot-}$ can be derived from ONOOH as described in pathway 2. It can also be generated from H_2O_2 through Haber–Weiss reaction in the presence of transition metals, such as Fe^{2+}, Cu^{2+}, Zn^{2+}. H_2O_2 can be converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO), and then react with $O_2^{\cdot-}$ to form $OH^{\cdot}$. 5) Lipid radical generation: Lipid peroxidation is usually initiated by $OH^{\cdot-}$ abstracting an allylic hydrogen (H) from unsaturated lipid and forming the carbon-centered lipid radical ($L^{\cdot}$). Generated $L^{\cdot}$ then rapidly reacts with O [2] to form a lipid peroxyl radical ($LOO^{\cdot}$), which abstracts a H from another unsaturated lipid (LH) molecule to form lipid hydroperoxide (LOOH). B. ROS clearance: There are five major ROS clearance pathways, including I) Superoxide dismutation: Two $O_2^{\cdot-}$ can be converted to one H_2O_2 and O_2 by SOD1-3. II) Hydrogen peroxide decomposition: Two H_2O_2 molecules can be decomposed by catalase (CAT) into H_2O and O_2. III) Glutathione redox cycle: H_2O_2 can be reduced by glutathione peroxidase (GPX) or Peroxiredoxin (PRDX)1/4/6 isoforms into H_2O using GSH as the electron donor. Oxidized GSSG will be reduced back to GSH by glutathione reductase (GSR) after receiving a H from NADPH. GPX can also reduce LOOH to become LOH. IV) Thioredoxin redox cycle: H_2O_2 can also be reduced by PRDX1-5 into H_2O using reduced Thioredoxin (Trx^R) as an electron donor. Oxidized Thioredoxin (Trx^O) will be reduced back to Trx^R by Thioredoxin reductase (TR). V) glutathione-S-transferase detoxification: Some electrophilic compounds, such as xenobiotics, can be conjugated with GSH by glutathione-S-transferase (GST) forming Glutathione conjugates (GSX). Abbreviation: Arg, L-arginine; CAT, Catalase; DUOX1/2, Dual oxidase1/2; DHFR, Dihydrofolate reductase; eNOS, Endothelial nitric oxide synthase; ETC, Electron transport chain; GPX, glutathione peroxidase; GSH, Glutathione; GSSG, Glutathione disulfide; GSR, glutathione reductase; G6PD, Glucose-6-phosphate dehydrogenase; GST, glutathione-S-transferase; GSX, Glutathione conjugates; $H^{\cdot}$, Hydrogen atom; H^{+}, Hydrogen cation; HOCl, Hypochlorous acid; LH, Unsaturated lipid; $L^{\cdot}$, Lipid radical; $LOO^{\cdot}$, Lipid peroxyl radical; LOOH, Lipid peroxide; MPO, Myeloperoxidase; Mt, Mitochondria; $NADP^{+}$, Nicotinamide adenine dinucleotide phosphate; NOS, Nitric oxide synthase; NOX1-5, NADPH oxidase complex1-5; ONOO-, Peroxynitrite; PRDX, Peroxiredoxin; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; SOD, superoxide dismutase; TR, Thioredoxin reductase; Trx^R, Reduced Thioredoxin; Trx^O, Oxidized Thioredoxin; U-eNOS, uncoupled endothelial nitric oxide synthase; XO, xanthine oxidase.

$ONOO^{-}$ can be indirectly detected by measuring its biomarker 3-nitrotyrosine (3-NT) with HPLC/MS [53].

ONOOH — ONOOH is the protonated form of $ONOO^{-}$ in solution. In the literature, peroxynitrite is referred to both $ONOO^{-}$ and ONOOH. Compared to $ONOO^{-}$, ONOOH was reported to have a much higher reactivity closed to $OH^{\cdot}$.

HOCl — Hypochlorous acid (HOCl) is moderately reactive. It can react with amino and thiol groups, DNA/RNA, fatty acid groups and cholesterol [54]. Thiols and amino groups are the major targets of HOCl. Oxidation of thiol leads to the formation of disulfide bonds that can result in crosslinking and aggregation of proteins. Oxidation of amino groups yields organic chloramines, which retain some oxidative capacity, leading to increased oxidative damage. HOCl also reacts with unsaturated bonds of fatty acid and cholesterol, generating chlorohydrin that disrupts and increases the permeability of lipid bilayers. Its reaction rate with DNA/RNA is relatively slow. It has been reported that DNA exposed to HOCl leads to decreased viscosity of DNA similarly caused by heat denaturation. HOCl can be directly detected by a recently developed HKOCl-1 probe [55].

$^{1}O_2$ — Singlet oxygen ($[1]\Delta_g$) is a strong biological oxidant generated via light-dependent and independent processes [56]. The light-independent $^{1}O_2$-produced reaction refers to the chemiexcitation catalyzed by enzymes such as peroxidases and oxygenases. Light-dependent $^{1}O_2$ generation occurs when the endogenous sensitisers interact with the UVA component of solar radiation in the presence of molecular oxygen. Main oxidation reactions initiated by $^{1}O_2$ can result in modifications within key cellular targets including guanine for nucleic acids, unsaturated lipids, and targeted amino acids, which would be implicated in various diseases [56,57]. Most recently, $^{1}O_2$ also can regulate vascular tone via the tryptophan-derived hydroperoxide as a signaling molecule inducing arterial relaxation [58].

3. Reactive oxygen species metabolism (Fig. 1)

ROS Generation: ROS is generated in a sequence of reactions where one type of ROS product is used for generating a new type of ROS. ROS generating cascade consists of 5 major pathways, including the generation of $O_2^{\cdot-}$, H_2O_2, RNS, $OH^{\cdot}$ and $L^{\cdot}$/$LOO^{\cdot}$.

$O_2^{\cdot-}$ Generation — $O_2^{\cdot-}$ is usually the first ROS generating cascade. It is generated from the coupling of O_2 with an electron (e^{-}) from e^{-} donor, which usually are reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) in mammalian cells. This one e^{-} reduction of O_2 produces a charged ionic species with a single unpaired electron and a net negative charge of -1 ($\cdot\ddot{O}{:}\ddot{O}\cdot\ + e^{-} \rightarrow {:}\ddot{O}{:}\ddot{O}\cdot^{-}$). The coupling of the e^{-} with O_2 can be catalyzed by various oxidases including NADPH oxidase (NOX1/2/

3/5 family), uncoupled NOS, xanthine oxidase (XO) and complex I/II/III/IV of mitochondria (Fig. 1, **pathway 1**).

RNS generation — RNS are the derivatives of $NO^\bullet$ which is generated from L-arginine (L-Arg) and catalyzed by NOS. $NO^\bullet$ can rapidly react with $O_2^{\bullet-}$ to form $ONOO^-$. The second order rate constant between $NO^\bullet$ with $O_2^{\bullet-}$ is nearly 10 times faster than SOD-catalyzed dismutation of $O_2^{\bullet-}$ ($1.9 \times 10^{10}\,M^{-1}\,s^{-1}$ vs $2.0 \times 10^9\,M^{-1}\,s^{-1}$) [23,59]. However, due to the high containment of cellular SOD (1000 times more than intracellular $NO^\bullet$ level) at physiological conditions, $O_2^{\bullet-}$ is eliminated before encountering $NO^\bullet$. In addition, the alternative pathway of exogenous NO_3^--NO_2^--NO is enhanced when the production of endogenous NO from L-arginine is inhibited due to hypoxia or ischemia [60]. $ONOO^-$ can be produced via the reaction between NO and $O_2^{\bullet-}$ only in pathological conditions [52]. In a biological system, $ONOO^-$ is in equilibrium with its neutral form peroxynitrous acid (ONOOH) which has a pKa = 6.8 [61]. At physiological pH (7.4), the estimated proportion of $ONOO^-$ and ONOOH is ~80% and ~20%, respectively. In aqueous phase, $ONOO^-$ predominantly reacts with carbon dioxide (CO_2) to form a transient adduct nitrosoperoxycarbonate ($ONOOCO_2^-$), 2/3 of which is decomposed into CO_2 and a relatively inert nitrate anion (NO_3^-), and 1/3 of which is decomposed to toxic $CO_3^{\bullet-}$ and $NO_2^\bullet$. ONOOH can undergo homolysis fission to generate the $OH^\bullet$ and $NO_2^\bullet$. However, the reaction rate is very slow in the aqueous phase and can greatly speed up when ONOOH goes into lipid membrane (Fig. 1, **pathway 2**).

H_2O_2 generation — H_2O_2 is generated from the dismutation of $O_2^{\bullet-}$ which is mostly catalyzed by superoxide dismutase (SOD) (:$\ddot{O}$:$\ddot{O}^-$ + :$\ddot{O}$:$\ddot{O}^-$ + $2H^+$ → $\ddot{O}$:$\ddot{O}$ + H:$\ddot{O}$:$\ddot{O}$:H). A small portion of $O_2^{\bullet-}$ dismutation can occur spontaneously. $O_2^{\bullet-}$ can react with some reduced transition metal such as $[4Fe–4S]^{2+}$, resulting in reduction of $O_2^{\bullet-}$ to H_2O_2 and oxidation of $[4Fe–4S]^{2+}$ to $[4Fe–4S]^{3+}$ ($\ddot{O}$:$\ddot{O}^-$ + $2H^+$ + $[4Fe–4S]^{2+}$ → $[4Fe–4S]^{3+}$ + H:$\ddot{O}$:$\ddot{O}$:H). Some oxidases like NOX4 and DUOX1/2 oxidases are equipped with dismutase activity, and can directly convert O_2 to H_2O_2 instead of $O_2^{\bullet-}$ (Fig. 1, **pathway 3**).

$OH^\bullet$ generation — $OH^\bullet$ can be generated from homolysis fission of ONOOH (Fig. 1, **pathway 2**). Most $OH^\bullet$ is generated from H_2O_2 and $O_2^{\bullet-}$ via a metal ion (iron or copper) catalyzed by Habor-Weiss reaction (Fig. 1, **pathway 4**). Habor-Weiss is divided into two steps. The first step involves the reduction of Fe^{3+} to Fe^{2+} by $O_2^{\bullet-}$. The second step involves the reaction of Fe^{2+} with H_2O_2 to generate $OH^\bullet$ and OH^- (Fenton reaction). In diseases accompanied with iron accumulation, which is observed in atherosclerotic lesion [62] or in sickle cell patients [63], $OH^\bullet$ mediated oxidative stress may be a major underlying mechanism. $OH^\bullet$ can also be generated from the MPO-catalyzed reaction between HOCl and $O_2^{\bullet-}$ (Fig. 1, **pathway 4**).

$L^\bullet$/$LOO^\bullet$ generation — When lipid is attacked by $OH^\bullet$, a hydrogen atom will be abstracted and a carbon-centered lipid radical ($L^\bullet$) will be formed. $L^\bullet$ can react rapidly with O_2 to generate lipid peroxyl radical ($LOO^\bullet$), which is a moderate oxidant capable of abstracting the H from nearby lipid to generate lipid hydroperoxide (LOOH) and a new $L^\bullet$. The reaction propagates until the lipid radicals react with scavenging antioxidants or other free-radicals to form a covalent bond (Fig. 1, **pathway 5**). In addition, $L^\bullet$/$LOO^\bullet$ can exist during a reaction process in which lipoxygenases catalyze the addition of dioxygen to polyunsaturated fatty acids to form hydroperoxides [64,65].

ROS Clearance: In order to maintain the ROS at a steady state, there are five major ROS clearance pathways (Fig. 1): **I**) $O_2^{\bullet-}$ dismutation to H_2O_2 by SOD; **II**) H_2O_2 decomposition to H_2O and O_2 by catalase (CAT); **III**) Glutathione redox cycle: H_2O_2 and LOOH decomposition by glutathione peroxidase (GPX) using GSH as electron donor; **IV**) Thioredoxin redox cycle: H_2O_2 reduction into H_2O by Peroxiredoxin (PRDX) 1-5 using reduced Thioredoxin (TrxR) as the electron donor; **V**) Xenobiotic detoxification by glutathione transferase (GST).

4. Major antioxidants (Table 2)

ROS metabolic clearance is involved with antioxidants. The aerobic organisms evolved an efficient antioxidant system which can be classified into two categories: enzymatic antioxidants and non-enzymatic antioxidants.

4.1. Enzymatic antioxidants

SOD — SOD is responsible for the dismutation of $O_2^{\bullet-}$ (Fig. 1, **clearance pathway I**). In human, there are three isoforms of SOD: SOD1 (Cu/Zn), SOD2 (Mn) and SOD3 (Cu/Zn) [66]. All three isoforms rely on their containing metals for their dismutation activity. SOD1 is widely distributed in all tissues and cells. It is the major isoform for clearance of $O_2^{\bullet-}$ generated in the cytosol and intermembrane space of mitochondria. Within the mitochondria, it interacts with BCL2 and prevents apoptosis of spinal cord neurons. SOD1 mutation is implicated in amyotrophic lateral sclerosis [67]. SOD2 is also widely distributed in most tissues and cells. It is the major isoform for the clearance of $O_2^{\bullet-}$ within the mitochondrial matrix, conferring strong protection against apoptosis of cells, in particular neurons, myocytes, and cardiomyocyte (CM) [66]. SOD2 deficiency is highly linked with neurodegenerative disease, skeletal muscle disease, and cardiac dysfunction. Unlike SOD1 and SOD2 which are ubiquitous, SOD3 has the highest expression in vessel, lung, and kidney. Moreover, SOD3 is secreted to extracellular spaces (ExC), anchored to the extracellular matrix and is the only isoform protecting against the extracellular $O_2^{\bullet-}$.

CAT — CAT is an iron-containing peroxidase to catalyze two H_2O_2 into two H_2O and one O_2 (Fig. 1, **clearance pathway II**). The second order rate constant of reaction with peroxide is $8 \times 10^6\,M^{-1}\,s^{-1}$ [68]. In mammals, it is predominately expressed in liver and erythrocytes [69], and located primarily in peroxisomes where H_2O_2 is formed during the breakdown of various substrates by flavoprotein oxidases. The role of CAT in cytosolic or nucleic H_2O_2 control is not so important. It does this job only when the H_2O_2 level is far above the physiological levels such as in oxidative stress. CAT is mainly designed for ethanol metabolism to turn alcohol to acetaldehyde. CAT is far less expressed than GPX and PRDX, which are mainly designed for H_2O_2 decomposition. Unlike GPX or PRDX, CAT does not require any reducing agent to recover its reductive state and the activity.

GPX — GPX is a family of selenium-containing peroxidases (except GPX5) transforming the H_2O_2 to H_2O, or converting the organic hydroperoxide (ROOH) to corresponding alcohol (ROH). The second order rate constant of reaction with hydrogen peroxide is ~$4.0 \times 10^7\,M^{-1}\,s^{-1}$ [70]. In the glutathione redox cycle, GPX reduces H_2O_2 or ROOH and become oxidized (GPX^O) and inactive. GPX^O can be converted back to its reduced form (GPX^R) by GSH. GSH becomes glutathione disulfide (GSSG), which is reduced back to GSH after a hydride (H-) is transferred from NADPH catalyzed by glutathione reductase (GSR) (Fig. 1, **clearance pathway III**). Unlike CAT, GPX senses small elevation of H_2O_2. In human, eight isoforms of GPX (GPX1-8) have been identified, which vary by distribution, ROS targets and biological functions [71,72] (Table 2). GPX1 is the most abundant isoform and ubiquitously expressed in most tissue, mostly found in the cytosol and mitochondrial matrix, and mainly used for H_2O_2 clearance. GPX2 is restricted to gastrointestinal epithelium, detected in EC, mainly located at ExC and mainly targeting H_2O_2. GPX3 is primarily found in plasma, highly expressed in kidney and lung, mainly located in the cytosol, and targeting on H_2O_2. GPX4 is a phospholipid hydroperoxidase. It is ubiquitously expressed, found in the cytosol, mitochondria, and nucleus, and targets peroxidized lipid (ROOH). GPX5 is specifically expressed in the epididymis, critical for protecting spermatozoa against lipid peroxidation. GPX6 is restricted to in embryo and olfactory epithelium and mainly targeting on H_2O_2. GPX7 is found in Leydig cell, hepatocyte, and adrenal gland. GPX7 is involved in detoxification of H_2O_2 in esophageal epithelia. GPX8 is found in ExC and

Table 2
Features and functions of major antioxidants.

Antioxidants	Targeted ROS	Enriched tissue/cell	Enriched organelle	Functions
Enzymatic antioxidants:				
SOD1(Cu/Zn)	$O_2^{\cdot-}$	Ubiquitous	Cyto, Mt IM-space	Cytosol, Mt $O_2^{\cdot-}$ dismutation; Spinal neural antiapoptosis
SOD2 (Mn)	$O_2^{\cdot-}$	Ubiquitous	Mt matrix	Mt $O_2^{\cdot-}$ dismutation, antiapoptosis
SOD3 (Cu/Zn)	$O_2^{\cdot-}$	Vessel, lung, kidney	ExC	ExC $O_2^{\cdot-}$ dismutation
CAT	H_2O_2	Liver, erythrocyte	Peroxisome, Hepatocyte Mt, Erythrocyte Cyto	H_2O_2 detoxification, Ethanol metabolism
GPX1	H_2O_2	Ubiquitous	Cyto, Mt matrix	Inflammation, glucose hemostasis
GPX2	H_2O_2	Epithelium of GI tract	ExC	Prevent inflammation-mediated carcinogenesis
GPX3	H_2O_2	Plasma, kidney, lung	Cyto	Prevent inflammation-mediated carcinogenesis
GPX4	ROOH	Ubiquitous	Mt, Cyto, Nu	Sperm maturation, Eicosanoid biosynthesis, Embryonic development, Cell survival
GPX5	ROOH	Epididymis	ExC, Cyto, Mt	Protecting spermatozoa, Preventing premature acrosome reaction
GPX6	H_2O_2	Embryos, olfactory epithelium	ExC, ER	H_2O_2 detoxification
GPX7	H_2O_2	Leydig cells, hepatocyte, adrenal gland	ExC, ER	Protect esophageal epithelia from H_2O_2
GPX8	H_2O_2	SMC	ExC, ER	Prevent ER H_2O_2 leakage, involved in Linoleic acid metabolism.
GSR	GSSG	Ubiquitous	Mt, Cyto, PM	Reducing GSSG to GSH
Typical 2-cys PRDX:				
PRDX1	H_2O_2, ROOH	Ubiquitous	Cyto	Cell cycle regulation Preventing Akt-driven tumorigenesis
PRDX2	H_2O_2, ROOH	Erythrocyte	Cyto	Antiviral activity of CD8(+) T cells, GF signaling, TNFα signaling
PRDX3	H_2O_2, ROOH	Ovary	Mt, endosome	Cell proliferation and differentiation
PRDX4	H_2O_2, ROOH	Plasma	ExC	Protecting EC from ROS, Spermatogenesis
Atypical 2-cys PRDX				
PRDX5	H_2O_2, ROOH ONOO$^{\cdot-}$	Tendon fibroblasts	Mt, Cyto, Nu	Tendon fibroblast apoptosis, tendon Degeneration. ONOO$^{\cdot-}$ clearance
1-cys PRDX:				
PRDX6	H_2O_2, PLPCOOH	Plasma, thyroid gland, kidney, trachea (Clara cells)	Cyto, liposome	Phospholipid turnover, regulation of NOX1 activity
Non-enzymatic antioxidants:				
GSH	GPX, PRDX1/4	Ubiquitous	Ubiquitous	GPX and PRDX1/4 reduction xenobiotic detoxification
TRX1	PPDX1-5	Ubiquitous	Cyto, ExC	PPDX1-5 reduction
TRX2	PPDX1-5	Ubiquitous	Mt	PPDX1-5 reduction
Uric acid	OH$^{\cdot}$, HOCl, ROO$^{\cdot}$	Plasma	ExC	Major plasma ROS scavenger, Within the cell as pro-oxidant
Vitamin E	OH$^{\cdot}$, LOO$^{\cdot}$	Plasma	Membrane	Membrane lipid peroxidation termination
Vitamin C	TO$^{\cdot}$	Plasma	ExC	Vitamin E reduction
Bilirubin	$O_2^{\cdot-}$, H_2O_2, ONOO$^{\cdot-}$	Plasma, spleen	Membrane	Protect lipids from oxidation

Antioxidants consist of two classes: enzymatic and non-enzymatic. This table describes the antioxidant distribution, target and function. **Abbreviation:** Akt, Protein kinase B; CAT, catalase; Cys, Cysteine; Cyto, Cytosol; ExC, Extracellular space; ER, Endoplasmic reticulum; GF, Growth factor; GI, Gastrointestinal; GPX1-8, glutathione peroxidase1-8; GSH, Glutathione; GSR, glutathione reductase; GSSG, Glutathione disulfide; GST, Glutathione-S-transferase; H_2O_2, Hydrogen peroxide; IM, Intermembrane space; LOO$^{\cdot}$, Lipid peroxyl radical; Mt, Mitochondria; NOX1, NADPH oxidase complex1; Nu, Nucleus; O2$^{\cdot-}$, Superoxide radical anion; OH$^{\cdot}$, Hydroxyl radical; ONOO-, Peroxynitrite; PM, Plasma Membrane; PRDX1~6, Peroxiredoxin1~6; PCOOH, phosphatidylcholine hydroperoxide; ROO$^{\cdot}$, Peroxyl radical; ROOH, Organic peroxide; ROS, Reactive oxygen species; SMC Smooth muscular cell; SOD1~3, Superoxide dismutase1~3; TNFα, Tumor necrosis factor alpha; TRX, Thioredoxin; TO•, Vitamin E radical.

endoplasmic reticulum (ER), targeting on H_2O_2 and involved in Linoleic acid metabolism.

PRDX — PRDX is a family of thiol peroxidases also catalyzing the reduction of H_2O_2 to H_2O, or ROOH to ROH. Their second order rate constant of reaction with peroxide is $\sim 10^{6-7} M^{-1} s^{-1}$ [73]. In the thioredoxin redox cycle, PRDX reduces H_2O_2 or ROH and become oxidized (PRDX^O) and inactive. PRDX^O can be converted back to its reduced form (PRDX^R) by TRX^R. TRX^R is oxidized to TRX^O (Fig. 1, **clearance pathway IV).** In mammals, six isoforms (PRDX1-6) have been identified [74]. All isoforms contain a conserved cysteine residue (C^P), which can be oxidized by H_2O_2 or ROOH into sulfenic acid (C^P–SOH). C^P-SOH then reacts with another semi-conserved Cys residue (C^R) to form a disulfide bond. Based on the location or absence of the C^R, PRDX is classified as typical 2-Cys PRDX (PRDX1-4), atypical 2-Cys PRDXs (PRDX5) and 1-Cys PRDXs (PRDX6). Both typical 2-cys PRDXs and atypical 2-cys PRDXs are heterodimers containing both C^P and C^R

in each subunit. For typical 2-cys PRDXs, C^P-SOH reacts with the CR from another subunit to form an intersubunit disulfide bond. For atypical 2-Cys PRDXs, C^P–SOH reacts with C^R from the same subunit. For the 1-Cys PPRDXs, C^P-SOH reacts with C^R from other molecules. PRDX isoforms vary in their distribution and biological function. In general, PRDX1 is ubiquitously expressed, found in the cytosol, and involved in cell cycle control and Akt-driven tumorigenesis [75]. PRDX2 is also ubiquitously expressed but with especially high expression in erythrocyte, found in the cytosol and involved in regulating the antiviral activity of CD8$^+$ T cells, growth factor, and TNFα signaling [76]. PRDX3 is mostly expressed in ovary, located in mitochondria and involved in cell proliferation and differentiation regulation. PRDX4 exists predominantly in plasma, anchored to the extracellular space (ExC) of EC and protecting the EC from oxidative damage. PRDX5 is highly expressed in tendon fibroblast, found at mitochondria, cytosol, and nucleus, and is involved in inhibiting p53-induced ROS generation,
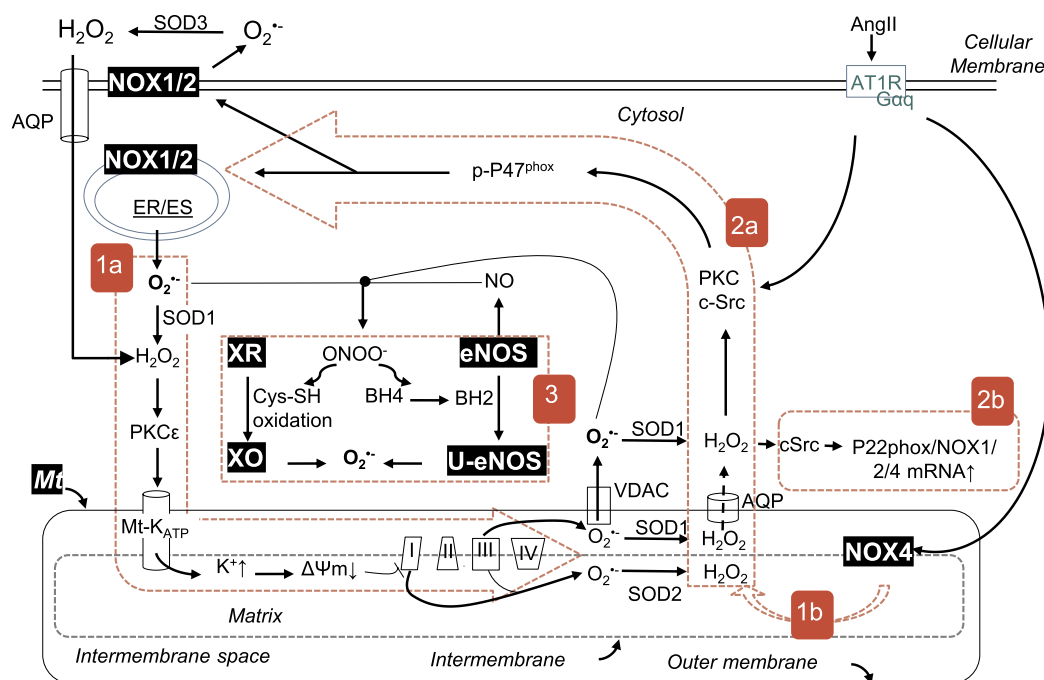
Fig. 2. Metabolic interplay between various ROS generators. ROS generator interplay is summarized in three major pathways: 1) NOX-ROS promoted Mt-ROS generation. In response to activation signaling, such as AngII, NOX1/2 located in PM/ER/ES can be activated via PKC-induced P47phox phosphorylation. Extracellular $O_2^{\cdot-}$ generated from PM-located NOX1/2 can be dismutated by SOD3 to form H_2O_2 which then diffuses into the cytosol via AQP, while intracellular $O_2^{\cdot-}$ generated from ER/ES-located NOX1/2 can be dismutated by SOD1 to form H_2O_2. 1a) ROS produced by Mt ETC. NOX1/2-derived H_2O_2 can activate Mt-KATP via PKC-ε signaling, leading to the K^+ influx, decrease of Mt membrane potential ($\Delta\Psi m$), inhibition of Mt complex I (Com I) and finally the increase of Mt $O_2^{\cdot-}$ production. 1b) ROS produced by NOX4 in Mt. In certain cell types such as CM and EC, NOX4 isoform is detected at the Mt. Increase expression of NOX4 by activation signaling such as AngII/Gαq pathway can directly contribute to the increase of Mt ROS. 2) Mt-ROS activated NOX-ROS generation. In Mt, $O_2^{\cdot-}$ generated by Com I is mainly released to Mt matrix and dismutated by SOD2 to H_2O_2, while $O_2^{\cdot-}$ generated by Com III can be released to either Mt matrix or intermembrane space and dismutated by SOD1. A small amount of Mt $O_2^{\cdot-}$ can be transported to the cytosol by VDAC directly and then dismutated by SOD1 to form H_2O_2. Mt-generated H_2O_2 can be leaked to the cytosol through water channel aquaporin (AQP). Mt-derived H_2O_2 then further activates NADPH oxidase by two pathways: 2a) The p47phox phosphorylation via activating PKC/cSrc; 2b) The increased expression of P22phox/NOX1/2/4 probably via c-Src activation. 3) NOX/Mt-ROS regulated eNOS/XR-ROS. Cytosolic $O_2^{\cdot-}$ from Mt or NADPH oxidase can react with NO to form $ONOO^-$, resulting in eNOS uncoupling via depletion of BH4, or XR to XO transformation via oxidation of critical thiols in Cys535/992. $O_2^{\cdot-}$ is generated by XO and uncoupled eNOS. Abbreviation: AngII, Angiotensin II; AT1R, Angiotensin II receptor type 1; AQP, Aquaporin; BH2, dihydrobiopterin; BH4, Tetrahydrobiopterin; eNOS, Endothelial nitric oxide synthase; ER, Endoplasmic reticulum; ES, Endosome; ETC, Electron transportation chain; H_2O_2, hydrogen peroxide; Mt-KATP, Mitochondrial adenosine triphosphate (ATP)-sensitive potassium channel; NO, nitric oxide; NOX, NADPH oxidase; $ONOO^-$, Peroxynitrite; p-P47phox, phosphorylated P47phox; PKC-ε, PKC-epsilon; PM, plasma membrane; SOD, Superoxide dismutase; U-eNOS, uncoupled eNOS; VSMC, Vascular smooth muscle cells; VDAC, Voltage-dependent anion channel; XO, Xanthine oxidase; XR, Xanthine reductase; $\Delta\Psi m$, Mitochondria membrane potential.

preventing apoptosis of tendon fibroblast and tendon degeneration [77]. PRDX5 is the most efficient antioxidant for $ONOO^-$ removal. PRDX6 can be found in plasma, thyroid gland, kidney, and trachea (Clara cells), and mainly located in the cytosol. PRDX6 is also equipped with Phospholipase A2 activity, involved in the turnover of Phospholipid and regulation of NOX1 activity [41].

4.2. Non-enzymatic antioxidants

GSH — GSH is the most abundant (5–10 mM) intracellular antioxidant ubiquitously expressed, with the highest concentration in the liver. It is the major antioxidant maintaining the whole body's redox status and often referred to as the body's master antioxidant. It is a tripeptide composed of three amino acids: Cys, glycine, and glutamate. The sulfhydryl (SH) group on Cys residue accounts for its strong e−donating feature. As described above in Fig. 1, GSH can be used as the reductant of GPX^O (**clearance pathway III**) or used by glutathione S-transferases (GST) for detoxification of xenobiotics (**clearance pathway V**). In addition, GSH serves as a reductant of a variety of oxidized antioxidants—such as vitamin C and Vitamin E.

TRX — TRX is also ubiquitously expressed. In the mammal cell, there are two types of TRX: TRX1 is a cytosolic and extracellular enzyme while TRX2 is the mitochondrial specific enzyme. Both TRX1/2 serve as e− donor for reducing the oxidized PRDX1-5.

Uric acid — Uric acid is the final oxidation product of purine metabolism. In healthy human plasma, the uric acid level is ~300 μM. In plasma, uric acid functions as antioxidant by its direct scavenging of ROS or by modifying the activity of xanthine oxidase (XO). It was claimed that more than half of antioxidant capacity comes from uric acid in human blood. The scavenging ability of uric acid highly depends on the environment. In the aqueous phase, uric acid is a powerful scavenger for $R^{\cdot}$, $ROO^{\cdot}$, $OH^{\cdot}$, OHCL and $ONOO^-$, but not for $O_2^{\cdot-}$. In the hydrophobic phase, uric acid greatly loses antioxidant ability.

Vitamin E — Vitamin E is lipid soluble and the major antioxidant found in the lipid phase of membrane. It reacts with lipid radicals leading to the termination of membrane lipid peroxidation.

Vitamin C — Vitamin C is water-soluble and capable of reacting with a wide variety of ROS, including $OH^{\cdot}$, $R^{\cdot}$, $ROO^{\cdot}$, $TO^{\cdot}$, $O_2^{\cdot-}$ and $ONOO^-$. Ascorbate can donate electrons to highly reactive free radicals such as hydrogen peroxide and is converted to the ascorbyl radicals via monodehydroascorbate reductase [78]. Monodehydroascorbate radical is rapidly dismutated into ascorbate and dehydroascorbate with catalyzation of NADH-dependent semidehydroascorbate reductase. Dehydroascorbate can be converted to ascorbate by dehydroascorbate reductase [79,80]. Vitamin C helps the reduction of oxidized Vitamin E during lipid peroxidation. Ascorbate was shown to stabilize the eNOS

cofactor tetrahydrobiopterin for increasing NOS enzyme activity in vitro and vivo [81,82]. However, it also steadily increased eNOS activity via increased phosphorylation at eNOS-Ser[1177] and concomitantly decreased phosphorylation at eNOS-Thr[495], which is independent of its effects on BH4 stabilization [83]. In some situation, Vitamin C can be a prooxidant, as it can reduce the high valent transition metal to low valent form which will drive the Fenton reaction and increase the generation of toxic $OH^•$ radical.

Bilirubin — Bilirubin is the end product of heme metabolism by two sequential reactions catalyzed by heme oxygenase and biliverdin reductase [84,85]. The lipophilic bilirubin can act as antioxidants to protect lipids from oxidation [86]. Mice with deletion of heme oxygenase-2 or biliverdin reductase display greater lipid than protein oxidation. Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection against ROS because bilirubin but not biliverdin increased expression of the protective protein GTP cyclohydrolase in ECs [87].

5. Major ROS generators, distribution and function (Table 3)

Mitochondrial ETC — ROS detected in mitochondria can be generated by the Mitochondrial-localized oxidases [22]. The vast majority of Mitochondrial ROS is generated from the mitochondrial electric transport chain (ETC), especially from complex I (Com I) and Com III (Fig. 2, **pathway 1a**). Their relative contribution to overall mitochondrial $O_2^{•-}$ production is tissue specific, dependent on pathophysiological conditions, respiring state and redox potential of Mitochondrial ETC. In the normal condition, Com I was reported as the primary source of Mitochondrial ROS in brain, while Com III contributed to the major Mitochondrial ROS in the heart and lung [88,89]. In pathological conditions like ischemia and cell apoptosis, Com IV can be inhibited and then $O_2^{•-}$ was mainly released from Com III. The importance of Mitochondrial ROS in CVD pathogenesis is emphasized by the observation that animals with deficiency of the Mitochondrial specific $O_2^{•-}$ scavenger SOD2 can result in severe cardiac dysfunction [90]. SOD2 deficiency increased mitochondrial oxidative stress and aggravate age-dependent vascular endothelial dysfunction due to ROS-mediated mitochondrial DNA damage [91]. Overexpression of a mitochondrial specific H_2O_2 scavenger peroxiredoxin-3 (PRDX3) can prevent myocardial infarction (MI)-induced heart failure in mice [92]. Besides, Mitochondrial ROS induced by various CVD risk factors are involved in EC activation/dysfunction, SMC proliferation/migration, dendritic cell differentiation, atherosclerosis, and other inflammatory disease [93].

NOS — NOS belongs to the family of enzymes that catalyzed the production of NO from L-Arg. In mammalian cells, it consists of three isoforms: eNOS (EC, CM), iNOS (VSMC, MC) and nNOS (neuron, skeletal muscle) [94]. Within ECs, eNOS localizes at the periphery of the golgi complex and cholesterol-rich microdomains of the plasma membrane (PM) such as caveolae, as homodimers. Deficiency of cofactor BH4 or substrate L-Arg leads to the electron transferring directly to free O_2 for the formation of $O_2^{•-}$, a phenomenon called "NOS uncoupling". Another direct redox-regulatory pathway for eNOS function is the oxidative disruption of the zinc-sulfur complex in the binding region of the eNOS dimer, caused by peroxynitrite and hypochlorous acid, resulting in the loss of functional dimers [95,96]. NOS uncoupling can reduce NO generation and increase $O_2^{•-}$ production. NO is a cardio-protective RNS while $O_2^{•-}$ is toxic to the cell in most case. The uncoupling process transforms eNOS from a cardio-protective enzyme to an oxidative stress contributor in CVD [97]. Interestingly, BH4 is highly sensitive to oxidant, particularly $ONOO^-$, and often oxidized to BH2, suggesting that oxidative stress-mediated BH4 depletion contributes greatly to NOS uncoupling (Fig. 2, **pathway 3**). NOS uncoupling can also be induced by other mechanisms [98], such as oxidative stress-mediated S-glutathionylation of NOS. eNOS may interact with mitochondrial outer membrane porin and modulate mitochondrial ROS production. Blocking this interaction decreased NO release and

increased mitochondrial ROS levels in the fetal pulmonary artery endothelial cells [99].

XOR — XOR is a purine degrading enzyme converting hypoxanthine to xanthine and finally to uric acid. It is widely distributed, with the highest expression/activity in liver, intestine, and mammary epithelial cells and capillary EC [100]. XOR consists of two inter-convertible forms, xanthine oxidase (XO) and xanthine oxidoreductase /dehydrogenase (XOR/XDH) (Fig. 2, **pathway 3**). XOR can be converted to XO either irreversibly by proteolysis or reversibly by oxidation of Cys [101]. Like NOS uncoupling, $ONOO^-$ can promote the Cys oxidation and subsequent XDH/XO transformation. XDH isoform is able to oxidize NADH under acidic conditions (pH ~6.5) to produce $O_2^{•-}$ in the early stage of ischemia-reperfusion (IR) [102]. In the late stage, XO isoform may be the quantitatively important source of $O_2^{•-}$. XOR as a source of ROS contributes to oxidative stress in a variety of disease, particularly IR injury [103]. Moreover, IR injury induces the production of inflammatory cytokines which can transcriptionally regulate XDH and increase XO activity [102]. XOR-derived ROS also seem to play a more important role in XO enriched tissue such as liver and intestine than some tissue lacking XO activity such as heart [102,104,105]. However, it is premature to conclude the exact effect of XO-derived ROS in CVD, because most of the findings are based on the use of allopurinol/oxypurinol for the inhibition of XOR.

NOX — unlike other ROS generating enzymes in which ROS are byproducts, NADPH oxidase directly catalyzes $O_2^{•-}$ or H_2O_2 production [106]. It is a membrane-bound enzyme complex consisting of seven members in mammalian cells, named by their core catalytic subunit: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2.[107]. NOX2 family is the prototype of NADPH oxidase first described in phagocytes for its critical role in microbial killing via generating oxidative burst [108]. It was found to be widely expressed in the cardiovascular system including EC, VSMC from small arteries, CM, and adventitial fibroblasts [109,110]. NOX1 family was first identified in colon epithelial cells [111]. It was also detected in the cardiovascular system including EC, VSMC, and CM. NOX3 family is expressed almost exclusively in the inner ear, playing an important role in the perception of balance and gravity [112]. NOX4 family was originally identified in meningeal cells of kidney [113]. It was also highly expressed and as a major NOX isoform in MC /Mφ and nearly all vascular cells including EC, VSMC, and CM. NOX5 family was first discovered in human spleen, testis, and kidney [114]. It was the only NOX isoform missing in rodents. In the human vasculature, NOX5 was found expressed in EC, VSMC, and CM, but not in adventitial fibroblasts [115,116]. NOX5 was also found constitutively expressed in MC/Mφ [117]. DUOX1/2 family is discovered in thyroid epithelial cells and has its function as oxidase to generate ROS [118,119]. But there is a paucity of information about the expression profile of DUOX1/2 as well as their role in CVD.

Myeloperoxidase (MPO) — It is a tetrameric, heavily glycosylated and heme-containing protein which is a member of the heme peroxidase superfamily. The mature enzyme consists of two identical disulfide-linked dimers each of which possesses light and heavy chains [120,121]. MPO contained in azurophilic granules is released from stimulated phagocytes [122,123] and uniquely can convert chlorides into hypochlorous acids that further involves in the generation of other ROS such as $OH^•$ and NO_2Cl [122,124]. Roles of MPO mainly focus on the tyrosine chlorination, pathogen killing, and the endothelial dysfunction in the vasculature [125,126], which are related to actions of its catalytic activity and relevant downstream products. MPO also was identified as a NO oxidase to regulate NO bioavailability in which NO was catalytically consumed by radicals generated by MPO [127]. Notably, MPO independent of its catalytic properties can cause a collapse of the endothelial glycocalyx via physically interacting with heparan sulfate side chains [128].

Table 3
Major intracellular ROS generator, distribution and function.

ROS generator	ROS products	Enriched tissue/cell	Enriched organelle	Functions
Mt ETC	$O_2^{\cdot-}$	Ubiquitous	Mt	ATP generation
eNOS	NO, $O_2^{\cdot-}$	EC	PM, Golgi, Cyto	Vasodilation
XOR	$O_2^{\cdot-}$	Liver, intestine, Mammal gland	Cyto, ExC	Purine metabolism
NOX1	$O_2^{\cdot-}$	Colon, VSMC, EC	PM	VSMC proliferation, migration
NOX2	$O_2^{\cdot-}$	Phagocyte, lymphocyte, neurons	PM, ER, ES	Bacteria killing, redox signaling
NOX3	$O_2^{\cdot-}$	Inner ear, fetal kidney & spleen	PM	Inner ear development
NOX4	H_2O_2	Kidney, EC, VSMC	ER, Mt(CM), FA(VSMC, MC)	VSMC migration/differentiation
NOX5	$O_2^{\cdot-}$	Lymphoid, testis, thyroid, airway, EC	PM	Thyroid hormone biosynthesis
DUOX1/2	H_2O_2	Epithelia	PM	Thyroid hormone biosynthesis
MPO	HOCl	Phagocyte	PM	Bacteria killing, protein nitrosylation

Major intracellular ROS generators are described for their distributions, products, and relevant functions. **Abbreviation**: ATP, Adenosine Triphosphate; CM, Cardiomyocytes; Cyto, Cytosol; DUOX1/2, Dual oxidase 1/2; EC, Endothelial cells; ER, Endoplasmic reticulum; ES, Endosome; EXC, Extracellular space; eNOS, Endothelial nitric oxide synthase; ETC, Electron transport chain; FA, Focal adhesion; HOCl, Hypochlorous acid; H_2O_2, hydrogen peroxide; Mt, mitochondria; MPO, Myeloperoxidase; MC, Monocyte; NOX1 ~ 5, NADPH oxidase complex 1–5; NO, Nitric oxide; $O_2^{\cdot-}$, Superoxide radical anion; PM, Plasma membrane; ROS, Reactive oxygen species; VSMC, Vascular smooth muscle cells; XOR, xanthine oxidoreductase.

6. Metabolic interplay between ROS generators (Fig. 2)

In most cases, cellular oxidative stress involves concomitant activation of multiple ROS generators. The cross talk between the major ROS generators (Mitochondria-ETC, NOX and NOS/XOR) could lead to further activation of each other, and result in positive feedback (a vicious cycle) which augments the ROS generation and oxidative stress [129,130]. We define the metabolic interplay or cross-talk between ROS generators as three pathways, 1) NOX-ROS promoted Mitochondria-ROS generation, 2) Mitochondria-ROS activated NOX-ROS generation, and 3) NOX/Mitochondria-ROS regulated eNOS/XR-ROS.

NOX-ROS promoted Mitochondria-ROS generation — a well-described NOX activation signaling is AngII stimulation. AngII binds to its receptor AT1R leading to the activation of NOX1/2 located in PM/ER/endosome and $O_2^{\cdot-}$ generation [131]. Extracellular $O_2^{\cdot-}$ generated by PM-located NOX1/2 can be dismutated by SOD3 to form H_2O_2 which then diffuses into the cytosol via water channel aquaporin. While intracellular $O_2^{\cdot-}$ generated from ER/ES-located NOX1/2 can be dismutated by SOD1 to form H_2O_2 which activates PKC-ε/Mitochondria-KATP signaling [132], leading to the increase of K^+ influx, decrease of mitochondrial membrane potential ($\Delta\Psi$m), inhibition of mitochondrial Com I and finally the increase of mitochondrial $O_2^{\cdot-}$ production [133] (Fig. 2, **pathway 1a**). Mitochondria-ROS generation can be prevented by blocking the NOX1/2 or mitochondrial K_{ATP} [134], or by inhibiting NOX1/2 or PKCε activity [135]. Mitochondria-ROS can also be produced by NOX4 in mitochondrial intermembrane in certain cell types such as CM and EC which can be activated by AngII/Gαq signaling [136](Fig. 2, **pathway 1b**). In addition, NOX-ROS can be generated by stimulation of advanced glycation end-products, lipopolysaccharide or cytokines, and results in Mitochondria-ROS production in rat renal cells [137] or glioma cells respectively [138].

Mitochondria-ROS activated NOX-ROS — Mitochondria-ROS generated by Com I and NOX4 is mainly released to the mitochondrial matrix and dismutated by SOD2 to H_2O_2, while $O_2^{\cdot-}$ generated by Com III can be released to either mitochondrial matrix or intermembrane space and dismutated by SOD1. A small amount of mitochondrial $O_2^{\cdot-}$ can be transported to the cytosol by voltage-dependent anion channel (VDAC) directly and then dismutated by SOD1 to form H_2O_2. Mitochondria-generated H_2O_2 can be leaked to the cytosol through water channel aquaporin (AQP). Mitochondria-derived H_2O_2 then further activates NOX [134,139,140] by two pathways: PKC/cSrc activation and p47phox phosphorylation [135,141] (Fig. 2, **pathway 2a)** and cSrc mediated P22phox/NOX1/2/4 phosphorylation [142,143] (Fig. 2, **pathway 2b**). Mitochondria-ROS was found responsible for NOX1/2

activation [144–146] and NOX1/2/4 induction [142,143] as mitochondrial TEMPO can significantly attenuate these effect. NOX activity can be regulated by expression of mitochondrial-specific SOD2 which modulates AngII-enhanced mitochondrial ROS [147].

NOX/Mitochondria-ROS regulated eNOS/XR-ROS — It is reported that oxidative stress is a major priming event for NOS uncoupling or XR to XO transformation. Cytosolic $O_2^{\cdot-}$ from mitochondria or NOX can react with NO to form $ONOO^-$, resulting in eNOS uncoupling via depletion of BH4 or oxidative disruption of the zinc-sulfur complex, or XR to XO transformation via oxidation of critical thiols in Cys. XO and eNOS uncoupling further produce cytosolic $O_2^{\cdot-}$ and exacerbate intracellular oxidative stress (Fig. 2, **pathway 3**). $O_2^{\cdot-}$ would be incorporated into pathway 1a to be dismutated by SOD1 to form H_2O_2 which initiate or enhance PKC-ε/Mitochondria-KATP. p47phox knockout abolished iNOS induction due to the significant reduction of the $O_2^{\cdot-}$ production [148]. Inhibition of eNOS using L-NAME resulted in $O_2^{\cdot-}$ reduction [149]. It is reported that NOX inhibition prevents XR to XO transformation and further prevented $O_2^{\cdot-}$ production in endothelial cells under the oscillatory shear stress [150].

Taken together, the interplay or cross-talk between mitochondrial ETC, NOX and NOS/XOR provide a network of intracellular redox regulation. Since NOX family members are the key members of this metabolic interplay, have convenient accessibility and are highly regulable, it seems the compatible target for intervention of the vicious oxidative stress circle.

7. Phenotypes of ROS-related gene knockout in mice (Table 4)

Table 4 summarizes phenotypes of ROS relevant gene knockouts involving with ROS-scavenging/producing enzymes or other ROS regulators. Detrimental consequence occurs with either excessive or deficient ROS generation. SOD2 deficiency in mice is lethal with excessive ROS generation whereas a deficiency in SOD1 and SOD3 is not but with multiple pathological changes or various diseases [151]. Additionally, deficiency of Nox/Duox-derived $O_2^{\cdot-}$ and H_2O_2 also would lead to diverse phenotypes such as hypothyroidism, CGD-like immune defect and a balance disorder [152–154]. Voltage-dependent anion channels (VDACs) is a mitochondrial porin which controls the release of $O_2^{\cdot-}$ from mitochondria. Knockout of VDAC3 increases ROS levels and alters renal sodium transport, leading to hypertension [155].

8. ROS homeostasis and functional implications

(Fig. 3)Cellular ROS levels undergo consistent changes in redox

Table 4
Phenotypes of representative redox regulatory gene knockout mice.

Gene	Genotype	ROS level	Phenotype	Reference (PMID#)
Anti-oxidants:				
SOD1	SOD1$^{-/-}$	↑ROS	Hepatocarcinogenesis, bone fragility, progressive distal motor axonopathy	15531919, 21056548, 21963651
	SOD1$^{\pm}$	↑ROS	Enhanced motor neuron death following axonal injury	8673102
SOD2	SOD2$^{-/-}$	↑ROS	Early neonatal death	7493016, 11796207,8790408
	SOD2$^{\pm}$	↑ROS	Cardiomyocyte apoptosis, increased cancer incidence	11514315,14679299
SOD3	SOD3$^{-/-}$	↑$O_2^{\cdot-}$	Reduced survival under hyperoxia, angiotensin II-induced or Renovascular hypertension	7603981, 12933702
CAT	CAT$^{-/-}$	↑ROS	Pre-diabetic status, renal fibrosis	27939935, 16105032
GPX1	GPX1$^{-/-}$	↑ROS	Increased hearing loss	11545230
PRDX4	PRDX4$^{-/y}$	↑ROS	Elevated spermatogenic cell death	19105792
ROS generators:				
XOR	XOR$^{\pm}$	↑H_2O_2	Obesity with insulin resistance	24158515
NOX3	Nox3$^{-/-}$	↓$O_2^{\cdot-}$	Complete lack of otoconia resulting in balance problem	15014044
DUOX2	Duox$^{-/-}$	↓H_2O_2	Hypothyroidism, dwarfism, and hearing impairment	17440044
p22^{phox}	p22^{phox} $^{-/-}$	↓$O_2^{\cdot-}$	CGD-like immune defect and a balance disorder	18292807
NOXO1	Noxo1$^{-/-}$	↓$O_2^{\cdot-}$	Head slant	16431374
DUOXA	Duoxa$^{-/-}$	↓H_2O_2	Hypothyroidism	22301785
eNOS	eNOS$^{-/-}$	↑ROS	Cardiomyocyte hypertrophy	17075027
iNOS	iNOS$^{-/-}$	NA	Liver fibrosis in steatohepatitis, contextual fear conditioning	25881230, 25618404
nNOS	nNOS$^{-/-}$	NA	Impaired cognitive performance	15158692
Other ROS regulators:				
VDAC3	VDAC3$^{-/-}$	↑$O_2^{\cdot-}$	Hypertension due to alteration of renal sodium transport	29180450
UCP3	UCP3$^{-/-}$	↑ROS	Skeletal muscle mitochondrion coupling	10748196
NRF2	NRF2$^{-/-}$	↑ROS	Age-related disruption of spermatogenesis	20692336

The table summarizes phenotypes of mouse gene knockout associated with anti-oxidants, ROS generators, and other ROS regulators. **Abbreviation**: CAT, catalase; DUOX, Dual oxidase; DUOXA, Dual oxidase activator; eNOS, Endothelial nitric oxide synthase; GPX, glutathione peroxidase; H_2O_2, hydrogen peroxide; iNOS, inducible nitric oxide synthase; NA, Not available; nNOS, Neuronal nitric oxide synthase; NOX3, NADPH oxidase complex 3; NOXO1, NADPH oxidase organizer 1; NRF2, Nuclear Factor-Erythroid 2-Related Factor; $O_2^{\cdot-}$, Superoxide radical anion; PRDX, Peroxiredoxin; ROS, Reactive oxygen species; SOD, Superoxide dismutase; UCP3, Uncoupling protein 3; VDAC3, Voltage dependent anion channel type 3; XOR, xanthine oxidoreductase.
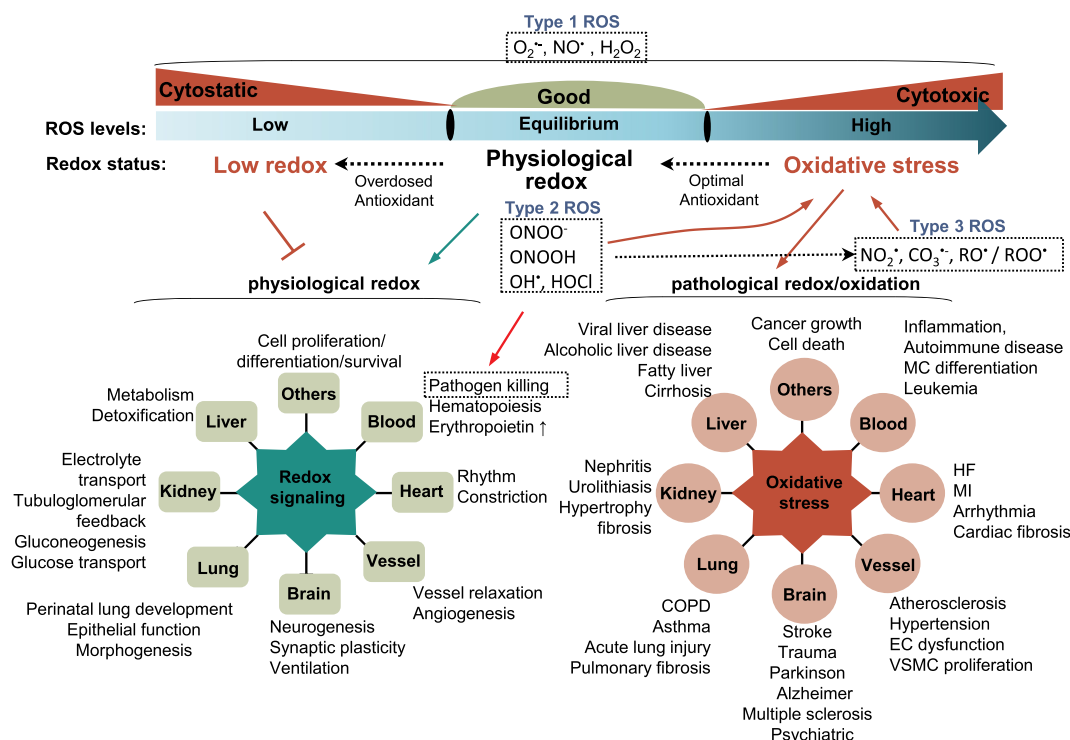

Fig. 3. Cellular ROS homeostasis and their pathophysiological effects. Cellular ROS levels undergo consistent changes in redox status. In physiological condition, ROS is maintained at equilibrium levels to facilitate physiological redox signaling as described in the green radial network on the left. Impaired ROS production causes low redox status and suppresses physiological redox signaling. In the case of high ROS status or oxidative stress, excessive ROS would initiate pathological redox signaling and induce cellular damage and various diseases as indicated in the red radial network on the right. Type 1 ROS ($O_2^{\cdot-}$, NO$^{\cdot}$, H_2O_2) is firstly generated and has essential physiological functions. Type 2 ROS (ONOO$^-$, ONOOH, OH$^{\cdot}$, HOCl) and type 3 ROS (NO2$^{\cdot}$, CO3$^{\cdot-}$, RO$^{\cdot}$/ROO$^{\cdot}$) are subsequently products of Type 1 ROS and play important role in oxidative stress. ROS reactivity is defined based on descriptions in Table 1 legend. Abbreviation: CO3$^{\cdot-}$, Carbonate radical anion; COPD, Chronic obstructive pulmonary disease; EC, Endothelial cells; HF, Heart failure; OH$^{\cdot}$, Hydroxyl radical; H_2O_2, Hydrogen peroxide; MC, Monocytes; MI, Myocardial infarction; NO$^{\cdot}$, Nitric oxide radical; NO2$^{\cdot}$, Nitrogen dioxide radical; O2$^{\cdot-}$, Superoxide radical anion; ONOO$^-$, Peroxynitrite; RO$^{\cdot}$, Alkoxyl radical; ROO$^{\cdot}$, Peroxyl radical; ROS, Reactive oxygen species; VSMC, Vascular smooth muscle cells.

Table 5
Features of three ROS types.

	Species	Sequential Products	Reactivity	Selectivity
Type 1 ROS	$O_2^{\cdot-}$, H_2O_2, $NO^\cdot$	Initial	Low $(+ \sim ++)$	Oxidation of cysteine residue by H_2O_2, activation of autophagy by $O_2^{\cdot-}$, nitrosyl-metal complex formation
Type 2 ROS	$OH^\cdot$, HOCl, $ONOO^-$/ONOOH	Secondary	High $(\geq ++++)$	Indiscriminately reacting with DNA, lipids, and proteins
Type 3 ROS	$NO_2^\cdot$, $CO_3^{\cdot-}$, $RO^\cdot$/$ROO^\cdot$	Tertiary	Mediate $(+++)$/high	Reacting with most of biomolecules leading to deteriorating oxidative stress

Type 1 ROS ($O_2^{\cdot-}$, $NO^\cdot$, H_2O_2) is firstly generated and has essential physiological functions. Type 2 ROS ($ONOO^-$, ONOOH, $OH^\cdot$, HOCl) and type 3 ROS ($NO_2^\cdot$, $CO_3^{\cdot-}$, $RO^\cdot$/$ROO^\cdot$) are subsequently products of Type 1 ROS and play important role in oxidative stress. ROS reactivity is defined based on descriptions in Table 1 legend. **Abbreviation**: $CO3^{\cdot-}$, Carbonate radical anion; $OH^\cdot$, Hydroxyl radical; H_2O_2, Hydrogen peroxide; $NO^\cdot$, Nitric oxide radical; $NO_2^\cdot$, Nitrogen dioxide radical; $O_2^{\cdot-}$, Superoxide radical anion; $ONOO^-$, Peroxynitrite; $RO^\cdot$, Alkoxyl radical; $ROO^\cdot$, Peroxyl radical; ROS, Reactive oxygen species.

status. It is recognized that ROS can be signaling molecules and have essential physiological functions at basal levels. ROS is a double edged sword which can also result in oxidative stress.

In physiological condition, ROS is maintained at equilibrium levels to facilitate physiological redox signaling. Normal level of type 1 ROS is required for cell metabolism [156], survival [157], proliferation and differentiation [158], angiogenesis [159], neurogenesis [160], hematopoiesis [161], morphogenesis [162], immune defense [163], etc (Fig. 3, **green radial network**). It needed to be noticed that the normal level of ROS differs in cell types. Proliferation cells like progenitor cells usually have a higher basal level of ROS. The effect of ROS/RNS also depends on the duration of exposure. But impaired ROS production causes low redox status and suppresses physiological redox signaling.

In the case of high ROS status or oxidative stress, excessive ROS would initiate pathological redox signaling and induce cellular damage and various disease. In pathological conditions when cellular ROS/RNS generation shifts to more toxic ROS/RNS species of type 2 or 3, oxidative stress can be induced. For instance, in a disease with iron accumulation, H_2O_2 can be converted to the extremely toxic $OH^\cdot$ via the Haber-Weiss reaction (also called Fenton reaction). Even mild elevation of these species within the cells will cause oxidative stress and cytotoxicity. Higher level of ROS causes stem cell premature exhaustion [164], tumorigenesis [165], autoimmunity [166], inflammation [167], EC dysfunction [168], VSMC proliferation [169], multiple sclerosis [170], cell death [171], etc (Fig. 3, **red radial network**).

The functional implication of ROS is determined by their inherent chemical features, including cellular concentration, time of exposure, and compartmentation [172]. Based on their functional implication, reactivity, and target selectivity, we classified ROS into three types as summarized in Table 5.

Type 1 ROS — Type 1 ROS are firstly generated and have essential physiological functions, including $O_2^{\cdot-}$, $NO^\cdot$ and H_2O_2. Type 1 ROS have relatively low reactivity ($+$ and $++$), are relatively selective to their molecular targets, and play both physiological and pathological roles. They are initial ROS species and serve as essential redox signaling molecules regulating physiological functions [173]. Nanomolar NO concentration can serve as an antioxidant and essential redox signaling molecules for vessel relaxation, thrombosis, and immune system hemostasis, whereas micromolar NO concentration will react with $O_2^{\cdot-}$ to generate cytotoxic peroxynitrite causing oxidative stress and cell injury [174,175]. Likewise, low level of H_2O_2 serves as an important mediator of growth factor-induced receptor tyrosine phosphorylation by inhibition of protein tyrosine phosphatases (PTPs) via oxidizing its Cys to reversible sulfenic form Cys–SOH, while high level H_2O_2 can lead to damage of PTPs and hyperactive growth signaling via further oxidizing reversible Cys–SOH to irreversible form of sulfinic or sulfonic [173]. $O_2^{\cdot-}$ mediated the major epigenetic progress via DNA methylation, histone methylation, and histone acetylation, and also regulated autophagy [176,177].

Type 2 ROS — Type 2 ROS ($ONOO^-$, ONOOH, $OH^\cdot$, HOCl) are subsequent products of Type 1 ROS and play an important role in oxidative stress. Type 2 ROS are mostly pathological relevant, have relatively high reactivity ($\geq +++$), and low selectivity to their molecular targets. Hydroxyl radical can indiscriminately react with its targets resulting in lesions or instability of DNA [178]. $ONOO^-$/ONOOH can oxidize or nitrate a wide range of macromolecules. HOCl produced by MPO in neutrophil is involved in oxidization or chlorination of many biological molecules such as thiols, cholesterol, and unsaturated lipid, DNA, ascorbate and urate [54]. When they are generated in the peroxisome, they are efficient tools for bacteria killing. However, within another intercellular organelle, they can cause irreversible oxidation of most biomolecules. Only when ROS generation overwhelms the antioxidant defense, they cause oxidative stress and cellular damages.

Type 3 ROS — Type 3 ROS ($NO_2^\cdot$, $CO_3^{\cdot-}$, $RO^\cdot$/$ROO^\cdot$) are also subsequent products of Type 1 ROS and powerful oxidative stress inducer.

Their reactivity is mediate or high but selectivity is relatively low for oxidizing substrates. Type 3 ROS form the downstream metabolites of type 2 ROS and react with most of the biomolecules leading to cellular damages and deteriorating oxidative stress. For instance, peroxynitrite-mediated protein tyrosine nitration is further promoted by type 3 ROS of $NO_2^\bullet$, $CO_3^{\bullet-}$ and lipid peroxyl radicals arising from peroxynitrite [45].

ROS compartmentation — ROS metabolism has strong intracellular compartmentation. The redox code recently comes out as a methodology to study the spatiotemporal organization of redox systems, especially for activation/deactivation cycles involving O_2 and H_2O_2[26]. ROS are usually accumulated in a certain cellular compartment, where the antioxidant surrounding ROS generator therefore quickly remove ROS and prevent its diffusion. For example, H_2O_2 is able to diffuse throughout the cell and react with its target. However, H_2O_2 generated from NADPH oxidase NOX4 in the ER only oxidize protein tyrosine phosphatase 1B located nearby NOX4 [179]. Oxidation of cysteine residue by H_2O_2 within the ER compartment induces disulfide bond formation which is required for protein folding and secretion. Additionally, H_2O_2 was formed earlier in mitochondria following monoamine oxidase activation as compared to the cytosol [172]. The spatiotemporal manner of ROS generation indicates that the mitochondria is the initial organelle in which endogenous oxidative stress occurs. Subsequent signaling cascades in other compartments would be triggered by mitochondrial ROS generations. Superoxide generated from cytosol has outcome difference with that in the mitochondrial matrix in which the high content of iron-sulfur cluster exist [173]. Oxidation occurring in the cytosol is involved with translocation of proteins to other compartments such as nuclei for their signal transduction. Cysteine residues of transcription factors need to remain the reduced and functional status to maintain zinc-finger DNA binding motifs, in which GSH and Trx 1 play a pivotal role in the nuclei compartment [172]. ROS generation is also heterogeneous and varies in different microdomains of cellular organelles in response to different stimuli [172]. For instance, H_2O_2 would be formed in the endosomes and the ER membrane under activation of the epidermal growth factor receptor, but would be formed at the plasma membrane with stimulation of platelet-derived growth factor receptor. Different NOX component can be activated in the specific compartment which account for the differential ROS formation [172]. Obviously, ROS compartmentation contributes to its functional specificity.

9. Concluding remarks

As highlighted in this review, it is particularly vital to understand major pathways of ROS generation and clearance, the interplaying network between various ROS generators, and roles of each ROS type in cellular redox biology and oxidative stress. The normal level of ROS is required for specific signaling transduction in normal cell function and survival, which maintains redox homeostasis. However, excess ROS generation would disturb the physiological function of redox balance, inducing oxidative stress and resulting in the cell or tissue damages. ROS serves as critical mediators in cell signaling regulation for both physiological and pathophysiological status. This article provides intensive knowledge about the biochemical basis of redox regulation and metabolic interplay, and important guidance for future studies involving redox modification.

Conflicts of interest

The authers have no competing interests to disclose.

Disclosures

None.

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Nonstandard Abbreviations and Acronyms

AngII: Angiotensin II
AQP: Aquaporin
Arg: L-arginine
AT1R: Angiotensin II receptor type 1
ATP: Adenosine Triphosphate
BH2: dihydrobiopterin
BH4: Tetrahydrobiopterin
CAT: Catalase
cGMP: cyclic guanosine monophosphate
CM: Cardiomyocytes
CO_2: carbon dioxide
$CO_3^{\cdot-}$: Carbonate radical anion
COPD: Chronic obstructive pulmonary disease
C^P-SOH: sulfenic acid
C^R: Cys residue
CVD: cardiovascular disease
Cys: cysteine
Cys–S-: Thiolate anion form
Cys–SH: Thiolate protonated form
Cys–SO_2H: sulfinic form
Cys–SO_3H: sulfonic form
Cys–SOH: sulfenic form
Cyto: Cytosol
DAF-2 DA: 4,5-diaminofluorescein diacetate
DAF-2DA: 5-diaminofluorescein diacetate
DHE: dihydroethidium
DHFR: Dihydrofolate reductase
DMPO: 5,5-dimethylpyrroline-N-oxide
DUOX1/2: Dual oxidase1/2

EC: Endothelial cells
eNOS: Endothelial nitric oxide synthase
EPR: Electron paramagnetic resonance
ER: Endoplasmic reticulum
ES: Endosome
ETC: Electron transport chain
ExC: Extracellular space
FA: Focal adhesion
Fe-DTC: Iron-dithiocarbamate
Fe–S: Iron (II) sulfide
G6PD: Glucose-6-phosphate dehydrogenase
GC-MS: Gas chromatography-mass spectrometry
GF: Growth factor
GI: Gastrointestinal
GPX: glutathione peroxidase
GPX1-8: glutathione peroxidase1-8
GSH: Glutathione
GSR: glutathione reductase
GSSG: Glutathione disulfide
GST: glutathione-S-transferase
GSX: Glutathione conjugates
GTP: guanosine triphosphate
H+: Hydrogen cation
H˙: Hydrogen atom
H_2O_2: Hydrogen peroxide
HF: Heart failure
HOCl: Hypochlorous acid
HPLC: High performance liquid chromatography
HPLC-EC: HPLC electrochemical
IFN: interferon
IL: interleukin
IM: Intermembrane space
IR: ischemic reperfusion
L˙: Lipid radical
L-Arg: ʟ-arginine
LH: Unsaturated lipid
LO˙: lipid oxy
LOO˙: Lipid peroxyl radical
LOOH: Lipid peroxide
LPO: lipoxygenase
MC: Monocyte
Met: Methionine
MI: Myocardial infarction
MPO: Myeloperoxidase
Mt: Mitochondria

Mt-KATP: Mitochondrial adenosine triphosphate-sensitive potassium channel
Mφ: macrophage
NADH: nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NO: Nitric oxide
NO_3^-: nitrate anion
NOS: Nitric oxide synthase
NOX1-5: NADPH oxidase complex1-5
NOXA1: NADPH oxidase activator 1
NOXO1: NADPH oxidase organizer 1
Nu: Nucleus
$O_2^{˙-}$: Superoxide radical anion
OH˙: Hydroxyl radical
$ONOO^-$: Peroxynitrite
PC1: Peroxycrimson 1
PCOOH: phosphatidylcholine hydroperoxide
PG1: Peroxygreen 1
PKC: Protein kinase C
PKC-ε: PKC-epsilon
PM: Plasma Membrane
PRDX1-6: Peroxiredoxin1-6
PTP: protein tyrosine phosphatase
RNS: Reactive nitrogen species
ROO˙: Peroxyl radical
ROOH: Organic peroxide
ROS: Reactive oxygen species
RSNO: nitrosothiol
sGC: soluble guanylate cyclase
SMC: Smooth muscular cell
SOD1-3: superoxide dismutase1-3
TNFα: Tumor necrosis factor alpha
TO˙: Vitamin E radical
TR: Thioredoxin reductase
Trp: Tryptophan
TRX: Thioredoxin
Trx^O: Oxidized Thioredoxin
Trx^R: Reduced Thioredoxin
Tyr: Tyrosine
U-eNOS: uncoupled endothelial nitric oxide synthase
VDAC: Voltage-dependent anion channel
VSMC: Vascular smooth muscle cells
XO: xanthine oxidase
XOR: xanthine oxidoreductase
XR: Xanthine reductase
ΔΨm: Mitochondria membrane potential