Article

Mitochondria Play Essential Roles in Intracellular Protection against Oxidative Stress—Which Molecules among the ROS Generated in the Mitochondria Can Escape the Mitochondria and Contribute to Signal Activation in Cytosol?

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Abstract: Questions about which reactive oxygen species (ROS) or reactive nitrogen species (RNS) can escape from the mitochondria and activate signals must be addressed. In this study, two parameters, the calculated dipole moment (debye, D) and permeability coefficient (Pm) (cm s^{-1}), are listed for hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), superoxide ($O_2^{\bullet-}$), hydroperoxyl radical ($HO_2\bullet$), nitric oxide ($\bullet NO$), nitrogen dioxide ($\bullet NO_2$), peroxynitrite ($ONOO^-$), and peroxynitrous acid ($ONOOH$) in comparison to those for water (H_2O). $O_2^{\bullet-}$ is generated from the mitochondrial electron transport chain (ETC), and several other ROS and RNS can be generated subsequently. The candidates which pass through the mitochondrial membrane include ROS with a small number of dipoles, i.e., H_2O_2, $HO_2\bullet$, $ONOOH$, $\bullet OH$, and $\bullet NO$. The results show that the dipole moment of $\bullet NO_2$ is 0.35 D, indicating permeability; however, $\bullet NO_2$ can be eliminated quickly. The dipole moments of $\bullet OH$ (1.67 D) and $ONOOH$ (1.77 D) indicate that they might be permeable. This study also suggests that the mitochondria play a central role in protecting against further oxidative stress in cells. The amounts, the long half-life, the diffusion distance, the Pm, the one-electron reduction potential, the pK_a, and the rate constants for the reaction with ascorbate and glutathione are listed for various ROS/RNS, $\bullet OH$, singlet oxygen ($^1O_2$), H_2O_2, $O_2^{\bullet-}$, $HO_2\bullet$, $\bullet NO$, $\bullet NO_2$, $ONOO^-$, and $ONOOH$, and compared with those for H_2O and oxygen (O_2). Molecules with negative electrical charges cannot directly diffuse through the phospholipid bilayer of the mitochondrial membranes. Short-lived molecules, such as $\bullet OH$, would be difficult to contribute to intracellular signaling. Finally, $HO_2\bullet$ and $ONOOH$ were selected as candidates for the ROS/RNS that pass through the mitochondrial membrane.

Keywords: mitochondria; reactive oxygen species; dipole moment; cell signaling; signal transduction; Nrf2/Keap1

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) consist of both radical and nonradical molecules and are reactive species that have different degrees of oxidizing potential in biological systems [1]. Many chronic diseases, such as cancer, alcoholic liver disease, Crohn's disease, rheumatoid arthritis, diabetes, muscular dystrophy, cystic fibrosis, septic shock, premature babies, atherosclerosis, infertility, cataracts, aging, hepatitis, ARDS, ischemia, neuronal degeneration, etc., are recognized as oxidative-stress-related diseases (OSDs) [2]. A major source of ROS in cells is the mitochondria [3]. The electron transport chain (ETC) consists of Complexes I, II, III, and IV. Oxidative phosphorylation is the process of the coupling between the ETC and ATP production in Complex V. Mitochondrial DNA (mtDNA) encodes 13 proteins inside the mitochondrial matrix, and those proteins are parts of Complexes I, III, IV, and V. [4]. Overall, 2~3% of electrons leak from the ETC and oxygen captures them, resulting in the production of superoxide anions ($O_2^{\bullet-}$). It is well known that mitochondria are the major site of ATP production, but they also produce $O_2^{\bullet-}$, which mainly leaks from Complexes I and III [2]. Impairment of the ETC caused by chemicals or mtDNA damage can cause an increase in the generation of $O_2^{\bullet-}$ and subsequent ROS [3]. These impairments are closely related to the cause of OSDs [4,5]. Hydroperoxyl radical ($HO_2\bullet$) is the protonated form of $O_2^{\bullet-}$, but whether its amount could be affected by the pH gradient across the mitochondrial inner membrane is uncertain [6]. There is evidence of nitic oxide ($\bullet NO$) formation in the mitochondria, although whether mitochondrial nitric oxide synthase (NOS) exists is still controversial [7]. Singlet oxygen ($^1O_2$) can be generated endogenously through different mechanisms [8], but its formation in the mitochondria has only been addressed in one study [9].

In mammalian cells, there are three superoxide dismutase (SOD) isoenzymes: copper–zinc SOD (CuZnSOD), or SOD1 [10]; manganese SOD (MnSOD), or SOD2 [11]; and extra-

cellular SOD (ECSOD), or SOD3 [12]. SOD catalyzes the dismutation of two superoxide radicals into hydrogen peroxide and oxygen. MnSOD is an enzyme localized in the mitochondrial matrix. Okado-Matsumoto and Fridovich showed that CuZnSOD is localized in the intermembrane space of the mitochondria [13]. It has been recognized that increases in the generation of ROS from the mitochondria can cause lipid oxidation and apoptosis. MnSOD could protect against these processes [14].

How do antioxidant systems, which are intracellular defense systems, work? MnSOD generates one hydrogen peroxide (H_2O_2) from two superoxide radicals ($O_2^{\bullet-}$). MnSOD may also reduce the formation of hydroxyl radicals ($\bullet OH$) from superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) through the Haber–Weiss reaction under the catalysis of iron ions [15–17]. However, H_2O_2 from MnSOD could be quickly detoxified by mitochondrial glutathione peroxidase (mtGPx) by reducing it to water [14,18]. This reaction could be accompanied by glutathione, of which the level for most cells is ~5 mM, an excess amount for the reaction [14,18]. Furthermore, GPx4 knockout (KO) is known to cause acute renal failure and death [19,20], suggesting that GPx4 plays an essential role as an antioxidant in mitochondria. Due to the emergence of the role of nitric oxide ($\bullet NO$) in OSDs, reactive nitrogen cascades are sometimes included in reactive oxygen cascades. $O_2^{\bullet-}$ and $\bullet NO$ can be easily bound and produce peroxynitrite ($ONOO^-$) with $k = 5 \times 10^9$ M^{-1} s^{-1}; however, in the opposite reaction, $k = 0.023$ s^{-1} [21]. ONOOH produces $\bullet NO_2$ and $\bullet OH$ with $k = 0.35$ s^{-1}, indicating that the decomposition of $ONOO^-$ and ONOOH is not straightforward [21]. Kissner et al. (2003) suggested that, regarding peroxynitrite formation under physiological conditions, when 10 nM $\bullet NO$ and 10 μM SOD, $ONOO^-$ formation/$O_2^{\bullet-}$ dismutation is 1/125, while with 2 μM $\bullet NO$ and 2 μM SOD, $ONOO^-$ formation/ $O_2^{\bullet-}$ dismutation is 8/1 [22], suggesting that $ONOO^-$ formation is dependent on intracellular $\bullet NO$ concentration.

Mitochondrial ROS (mtROS) might be related to an increase in signal transduction and may control anti-oxidative-stress-related molecular defense mechanisms. Redox states could thus represent essential pathways to maintain homeostasis. The importance of this subject, the mitochondrial ROS come out from mitochondria and initiate the signal transduction inside cells, has been hypothesized by many researchers [23–33]. The role of mitochondrial ROS in initiating signal transductions in the cell cytosol has been the subject of discussion [34]. Indo et al. showed that manganese superoxide dismutase (MnSOD) transfection decreases the expression levels of GATA 1, 3, 4, and 5, which are nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) regulating genes [34]. The results showed that MnSOD transfected cells revealed a decrease in expression compared to those in the control. We previously demonstrated that mtROS causes intracellular signaling, and we published a paper entitled "Evidence of Nrf2/Keap1 Signaling Regulation by Mitochondrial-Generated Oxygen Species in RGK1 cells" in a Special Issue of *Biomolecules* entitled "The Physiological and Pathological New Function of Mitochondrial ROS and Intraorganellar Cross-Talks" in 2023 (https://www.mdpi.com/journal/biomolecules/special_issues/0XTJ2MAYET, accessed on 7 November 2023) [35]. They transfected MnSOD gene-contained vectors in a gastric mucosal tumorized cell line, RGK1 cells. They examined the expression levels of NF-E2-related factor 2 (Nrf2), Kelch-like ECH-associated protein1 (Keap1), heme oxygenase-1 (HO-1) and 2, MnSOD, glutamate-cysteine ligase (GCL), glutathione S-transferase (GST), and NAD(P)H Quinone oxidoreductase 1 (NQO1), which are all Nrf2-Keap1 regulating gens. The results of immunocytochemistry staining showed a decrease in those expressions in the MnSOD transfected RGK1 cells compared to those in the control. The transfected MnSOD gene should decrease the mitochondrial ROS levels, so after MnSOD transfection, all decreased expression was shown, suggesting mtROS levels control the levels of Nrf2-Keap1 regulating genes. However, the question of which ROS go out from mitochondria and contribute to intracellular signaling remains unclear.

The plasma membrane consists of both lipids and proteins. The fundamental structure of the membrane is the phospholipid bilayer, which forms a stable barrier between two aqueous compartments [36]. Most biologically important solutes require protein carri-

ers to cross cell membranes, via a process of either passive or active transport. Active transport requires the cell to expend energy to move the materials, while passive transport can be performed without using cellular energy [37]. Certain substances easily pass through the membrane through passive diffusion, such as O_2 and CO_2, along with small relatively hydrophobic molecules, fatty acids, and alcohols [37]. Mitochondria possess double membranes, and the inner membrane contains cardiolipin. Cardiolipin is not the main lipid that forms a phospholipid bilayer but fulfills other functions (e.g., stabilization of protein complexes), because it contains four fatty acid residues, and is a non-bilayer forming phospholipid [38,39]. It is known that cardiolipin is oxidized in mitochondria by X-irradiation [40]. If the ROS are related to cell defense signal transduction, ROS must pass through the membranes and exist in the cytosol to activate signal transduction. In this study, in the mitochondria, we study which ROS can pass through the mitochondrial membrane.

In this paper, we try to clarify which ROS are responsible for signal activation in cytosol through calculations and examination of the literature: •OH, singlet oxygen ($^1O_2$), HO_2•, •NO, •NO_2, $ONOO^-$, ONOOH. The dipole moments of ROS and RNS are calculated using density functional theory (DFT) calculations. Possible candidates of ROS which pass through the mitochondrial membrane and enter the cytosol to activate the signal transduction pathway are estimated using the calculated dipole moment and experimental permeability coefficient. In addition, the lifetime of each molecule is listed, and ROS that escape from the mitochondria and act as initiators to activate signal transduction in the cytosol are taken into consideration.

2. Materials and Methods

2.1. Theoretical Calculations of Dipole Moments for ROS and RNS

The dipole moments [41] were calculated according to the dipole information (Table 1). The DFT calculations were performed using Gaussian 09 (Revision A.02, Gaussian, Inc., Wallingford, CT, USA) [42]. The calculations were performed on a 32-processor QuantumCube^{TM} (Parallel Quantum Solutions, Fayetteville, AR, USA) at the B3LYP/6-311++G(3df,3pd) level of theory [43–45] with a keyword "polar" to output the molecular polarity (electric dipole moment in D (debye)) [46]. Graphical outputs of the computational results were generated using the GaussView software program (ver. 3.09) developed by Semichem, Inc., Shawnee, KS, USA [47]. The dipole moments were calculated for various ROS and RNS; we calculated the dipole moments of major ROS and RNS that exist in the mitochondria (Table 1): hydroxyl radical (•OH), superoxide ($O_2^{•-}$), hydroperoxyl radical (HO_2•), nitric oxide (•NO), nitrogen dioxide (•NO_2), peroxynitrite ($ONOO^-$), and peroxynitrous acid (ONOOH). We also listed the number of molecules of water (H_2O) and hydrogen peroxide (H_2O_2).

Table 1. Calculated dipole moment and experimental permeability coefficient of ROS and RNS.

	ROS or RNS	Calculated Dipole Moment/D	Permeability Coefficient/cm s^{-1}
H_2O	Water	1.89	2.3×10^{-3} [48]
H_2O_2	Hydrogen peroxide	0.00 (permeable)	6.1×10^{-3}, 6.6×10^{-4} [49]
•OH	Hydroxyl radical	1.67	
$O_2^{•-}$	Superoxide	0	1×10^{-6} (pH 7.3, 25 °C) [50] $(7.6 + 0.3) \times 10^{-8}$ [51]
HO_2•	Hydroperoxyl radical	2.23	4.9×10^{-4} [51]
•NO	Nitric oxide	0.14 (permeable)	93 (20 °C) [52]
•NO_2	Nitrogen dioxide	0.35	[53] and discussion in the text
$ONOO^-$	Peroxynitrite	2.14	Through anion exchanger [54] 8.0×10^{-4} [55]
ONOOH	Peroxynitrous acid	1.77	$4–13 \times 10^{-4}$ [56–58]

2.2. Predictive Performance of Mitochondria-Originating Reactive Oxygen Species

The predictive performance of mitochondria-originating reactive oxygen species included the following parameters: the intracellular amount (amount/cell); the half-life; the diffusion distance (μm); permeability coefficients (Pm) (in cm s^{-1}); the one-electron reduction potential (E^o) (in V vs. NHE, NHE: normal hydrogen electrode) at pH 7.4; pK_a; and the rate constants for the reaction with ascorbate (AscH$^-$) (k (AscH$^-$)/M^{-1} s^{-1}) and glutathione (GSH) (k (GSH)/M^{-1} s^{-1}) for various ROS and RNS. We focused on ROS generated from the mitochondrial electron transport chain (mtETC). The ROS studied included $\bullet$OH, singlet oxygen ($^1O_2$), $O_2^{\bullet-}$, HO$_2\bullet$, $\bullet$NO, $\bullet$NO$_2$, ONOO$^-$, ONOOH, alkoxyl radicals (RO$\bullet$), and peroxyl radicals (ROO$\bullet$). The H$_2$O, oxygen (O$_2$), and H$_2$O$_2$ were also listed. Those radicals were initiated from $O_2^{\bullet-}$, starting from electron leakage from the ETC and then binding with O$_2$. Then, the $O_2^{\bullet-}$ changed form to become other ROS, such as $\bullet$OH, singlet oxygen ($^1O_2$), HO$_2\bullet$, $\bullet$NO, $\bullet$NO$_2$, ONOO$^-$, and ONOOH, in the mitochondria [2]. $\bullet$OH and $\bullet$NO$_2$ are constructed by the binding of $O_2^{\bullet-}$ and $\bullet$NO. This information was collected from the literature listed in the Table 2 references. To exit the mitochondrial membrane into the cytosol, the ROS should be present in an appropriate amount and have a long half-life, long diffusion distance, large Pm and E^o, and relatively small rate constants for the k (AscH$^-$)/M^{-1} s^{-1}) and k (GSH)/M^{-1} s^{-1}).

Table 2. Predictive performance of mitochondria-originating reactive oxygen species.

	ROS or RNS	Half-Life Time	Amount/Cell	Diffusion Distance (μm)	Permeability Coefficients (P_m) (cm s^{-1})	$E^{0'}$; One-Electron Reduction Potential (V) at pH 7	pK_a	k (AscH$^-$)/M^{-1} s^{-1}	k (GSH)/M^{-1} s^{-1}
H_2O	Water	—	—	—	3.3×10^{-3} (EYPC) [59]	-2.87 [60] -2.87 [61]	15.7 [62]	—	—
O_2	Oxygen	—	—	—	12 (DMPC) [59] 125 (DMPC) [59] 114 (DOPC) [59] 157 (POPC) [59] 50 (EYPC: 30% Chol) [59] 38 (RBC human) [59] 21 (CHO cells) [59] 42 (CHO cells) [59]	-0.18 (pH 7) [60] -0.33 [61] -0.16 [63] -0.18 (pH 7, 25 °C) [64]	—	—	—
•OH	Peroxynitrous acid	10^{-9} s [65] 10^{-9}~10^{-6} s (diffusion-controlled reactivity) [66] 10^{-10} s [67] 10^{-9} s (1 M, 37 °C) [68] 10^{-9} s [69]	—	3 Å [70] A large flux of hydroxyl radicals would be required to inactivate a substantial fraction of any biological target [70] 0.02 (GSH+) [71]	—	$+2.32$ (pH 7) [60] $+2.31$ [61] $+2.31$ (pH 7, 25 °C) [64] $+2.31$ [72] $+2.31$ (pH 7) [73]	11.9 [62] 11.6 [74]	1.1×10^{10} (pH 7.4) [61]	1.0×10^{10} [72] $1.64 \pm 0.01 \times 10^{10}$ [74] 1×10^{9} [75] 8.8×10^{9} (pH 1.0) [76] 9.0×10^{9} (pH 7.6) [77] 1×10^{10} [78] 1.1×10^{10} (oxidized GSH) [79] 1.4×10^{10} (reduced GSH) [79] $1.4 \pm 0.1 \times 10^{10}$ (pH 7.8) [80] $4.4 \pm 0.5 \times 10^{10}$ (pH 10.6) [80] 2.3×10^{10} [81] $4.4 \pm 0.5 \times 10^{10}$ (pH 10.6) [82]
$^1O_2$	Singlet oxygen	10^{-6} s [65] 10^{-6} s [67] 10^{-6} s (solvent, 37 °C) [68] 10^{-5} s [69] 10^{-9}~10^{-6} s [83]	—	—	—	$+0.81$ (pH 7, 25 °C) [64]	—	3.2×10^{8} [83] 1.8×10^{8} [84]	$9.39 \pm 0.07 \times 10^{8}$ [74]
H_2O_2	Hydrogen peroxide	Stable [65] Stable) Stable, decomposed by catalase and GSH peroxidase and by EDTA and ADP [68] Enzymatic [69] 18.1 ± 2.7 min [82]	Physiological condition (proliferation/differentiation/migration/angiogenesis): 0.001~0.1 μM) Stress responses/adaptation (e.g., NRF2): 0.05~5.0 μM [85] Inflammation/fibrogenesis/tumor growth/metastasis: 0.01~10.0 μM [85] Growth arrest/cell death: 1.0~10.0 μM [85]	1600 (GSH+) [78]	6×10^{-4} (RBC horse) [59] 3×10^{-3} (peroxisome rat liver) [59] 1.2×10^{-2} (RBC rat) [59] 2×10^{-4} (Jurkat T cells) [59] 3.6×10^{-4} (*Chara coralina*) [59] 1.6×10^{-3} (*Escherichia coli*) [59] 4×10^{-4} (PC12 cells) [59] 1.6×10^{-3} (HUVEC cells) [59] 1.1×10^{-3} (IMR-90 cells) [59] 4.4×10^{-4} (HeLa cells) [59]	$+0.39$ (pH 7) [60] $+0.32$ [61] $+1.77$ [72] $+1.8$ [78] $+0.39$ (pH 7, 25 °C) [86]	11.6 [62] 11.75 (pH 7.2) [78]	—	9×10^{-1} [72] 9×10^{-1} [73] 9×10^{-1} (pH 7.4, 37 °C) [78] 8.7×10^{-1} [81]
$O_2{}^{\bullet-}$	Superoxide	10^{-6} s [65] 1 s (pH 10) [66] 10^{-6} s (diffusion-controlled reactivity) [66] 10^{-6} s [67] The lifetime of superoxide in a cellular environment in water would be expected to be very short, too short to permit diffusion for great distances [68] Enzymatic [69] 3000 ms (10^{-6} M) [87] 175 ms (10^{-6} M + SOD 10^{-9} M) [87] hours (10^{-9} M) [87] 175 ms (10^{-9} M + SOD 10^{-9} M) [87] 0.175 ms (10^{-9} M + SOD 10^{-6} M) [87]	28.4 pM (normal condition)/mitochondria [88] Formation rate (to 6 μM/s) [88] MnSOD-catalyzed dismutation ($k = 2 \times 10^{9}$ M^{-1} s^{-1}) [88] 9.15×10^{-8} pmol production/s/mitochondria * 690 nM production/s/mitochondria * 5.5×10^{4} superoxide molecules /s/mitochondria *	—	2.1×10^{-6} (SBPC) [59] 7.6×10^{-8} (EYPC) [59]	$+0.94$ [72] $+0.94$ [73]	—	1×10^{5} (pH 7.4) [61] 2.7×10^{5} (pH 7.4) [61]	~10 to 10^{3} [72] 2×10^{2} [81] $1.1 \pm 0.04 \times 10^{3}$ [74] 6.7×10^{5} (reduced GSH) (pH 7.8) [89]

Table 2. *Cont.*

	ROS or RNS	Half-Life Time	Amount/Cell	Diffusion Distance (μm)	Permeability Coefficients (P_m) (cm s^{-1})	$E^{0\prime}$; One-Electron Reduction Potential (V) at pH 7	pK_a	k (AscH$^-$)/M^{-1} s^{-1}	k (GSH)/M^{-1} s^{-1}
HO$_2\bullet$	Hydroperoxyl radical	51~422 s (pH 2~10) [90] HO$_2\bullet$ radicals in organic or lipophilic media could have a longer half-life. The half-life of superoxide cannot be calculated unless the concentrations of SOD and all reactive substrates are known [67]	9.15×10^{-8} pmol production/s/mitochondria * 690 nM production/s/mitochondria * 5.5×10^4 superoxide molecules/s/mitochondria *	—	4.9×10^{-4} (EYPC) [59]	+1.05 (pH 7) [60] +1.06 [72] +1.05 (pH 7, 25 °C) [86]	4 [62] 4.8 [89] 4.8 [90] 4.8 [91]	1×10^5 (pH 7.4) [61] 2.7×10^5 (pH 7.4) [61]	—
$\bullet$NO	Nitric oxide	ms to s depending on the available concentration of O$_2$, otherwise stable [66] Second [67] 1~10 s [69] 445 s [92] $\bullet$NO:1200 nM in saline: binding with Hb: 2×10^5 M^{-1} s^{-1} [92] Seconds [93]	pM~μM [93] pM~μM in physiological milieu [94] cGMP-mediated processes; <1~30 nM [95] Akt phosphorylation; = 30~100 nM stabilization of HIF-1α; = 100~300 nM [95] phosphorylation of p53; > 400 nM [95] nitrosative stress; 1 μM [95]	—	73 (EYPC) [59] 66 (EYPC: 30% Chol) [59] 18 (RBC human) [59]	−0.52 (pH 7) [60] −0.35 [63] −0.80 [72] −0.80 [73]	—	—	Nondetectable [72] 1.0×10^1 [75]
$\bullet$NO$_2^-$	Nitrogen dioxide	Second [67] <10 μs [96]	Typically 0.2~0.3 μM [96]	0.4 (GSH+) [78] 0.2 in the cytoplasm [96] <0.8 in blood plasma [96]	~5 (EYPC) [59]	+1.04 (pH 7) [60] +1.04 [63] +1.04 [72] +1.04 [73]	—	1.8×10^7 [96] 3.5×10^8 [96] 3.54×10^6 (pH 5.4~6.5, 55 °C) [97]	3.0×10^7 [72] 2.2×10^7 [75] 3×10^7 [78] 2×10^7 [81] ~2×10^7 [96]
ONOO$^-$	Peroxynitrite	0.8 s (pH 7.4) [64] 10^{-3} s [67] 0.05~1 s [69] 0.8 s (pH 7.4) [98] 0.9 s [98] Stable [98] Relatively stable [99] Less than 1 s (pH 7.4, 37 °C) [99] 0.8 s (pH 7.4) [100]	A total peroxynitrite and peroxynitrous acid concentration that exceeds 0.1 mM [101]	60 (GSH+) [78] 0.42 [101]	—	—	—	7×10^2 [78] $2.35 \pm 0.04 \times 10^2$, 25 °C [91]	6.6×10^2 (pH 7.4, 25 °C) [71] 7.0×10^2 [73] 6.6×10^2 [75] 1.36×10^3 (pH 7.4, 37 °C) [78] 2.81×10^2 (pH 5.75, 37 °C) [100]
ONOOH	Peroxynitrous acid	Fairly stable [67] 0.90 s, 25 °C [98] Less than 1 s at physiological pH and 37 °C [99] 0.6 s; 1.13 s−1 in phosphate buffer (pH 7.4, 37 °C) [102]	A total peroxynitrite and peroxynitrous acid concentration that exceeds 0.1 mM [101]	—	8×10^{-4} (DMPC) [59] 1.3×10^{-3} (EYPC) [59] 6.3×10^{-4} (DMPC) [59] 4×10^{-4} (DPPC) [59]	+1.40 [72]	6.8 [86] 6.8 [90] 6.8 [98] 6.8 [103]	—	6.6×10^2 [72] 1.35×10^3 [81]
RO$\bullet$	Alkoxyl radicals	10^{-6} s [67] 10^{-6} s (100 mM) [68] 10^{-6} s [69]		—	—	+1.60 [61] +1.60 [72] ~+1.60 [73]	—	1.6×10^9 (pH 7.4) [61]	$2.76 \pm 0.15 \times 10^6$ [74]
ROO$\bullet$	Peroxyl radicals	Seconds to hours depending on conditions [66] 17 s [67] 7 s (100 mM, 37 °C) [68] 7 s [69]		—	—	+1.00 [61] +0.77~1.44 [73] +1.00 [72]	—	1-2 $\times 10^6$ (pH 7.4) [61]	—

Abbreviations: Chol, cholesterol; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; RBC, red blood cell. * Assuming a 70 kg man, O$_2$ consumption/day is estimated as 14.7 mol/day [14,104]. Assuming that 2% of electrons leak from the mitochondrial electron transport chain (ETC) and that these are trapped by oxygen and made into superoxide, the superoxide production from the ETC is thus estimated as 3402.8 nmol/s. The number of cells/body is estimated as 3.72×10^{13} [105]. Thus, superoxide production is calculated as 5.51×10^7 mol/s/mitochondria. Assuming that the volume of mitochondria is 1.32×10^7 m^3, then considering mitochondrial volume, 1.32×10^{-16} m^3, superoxide production is estimated as 6.90×10^2 μmol/s/m^3. It is noted that this number is the amount of superoxide produced and that superoxide is modified by other molecules and enzymes, and thus the amount of superoxide existing in cells is much less.

3. Results

The results for the calculated dipole moment (in D) and experimental permeability coefficient (in cm s^{-1}) are listed in Table 1.

Table 2 shows the predictive performance of the mitochondria-originating ROS. The intracellular amount (amount/cell); the half-life; the diffusion distance (μm); permeability coefficients (Pm; cm s^{-1}); E^o, the one-electron reduction potential (V vs. NHE) at pH 7.4; pK_a; and the rate constants for the reaction with ascorbate (AscH$^-$) (k (AscH$^-$)/M^{-1} s^{-1}) and glutathione (GSH) (k (GSH)/M^{-1} s^{-1}) were examined. For considerations of reactions of ROS in the mitochondria, we used AscH$^-$ and GSH. Finally, we detected the ONOOH and HO$_2$• for the responsible ROS, which crossed the mitochondrial membrane and initiated the intracellular signaling in cytosol (Figure 1).
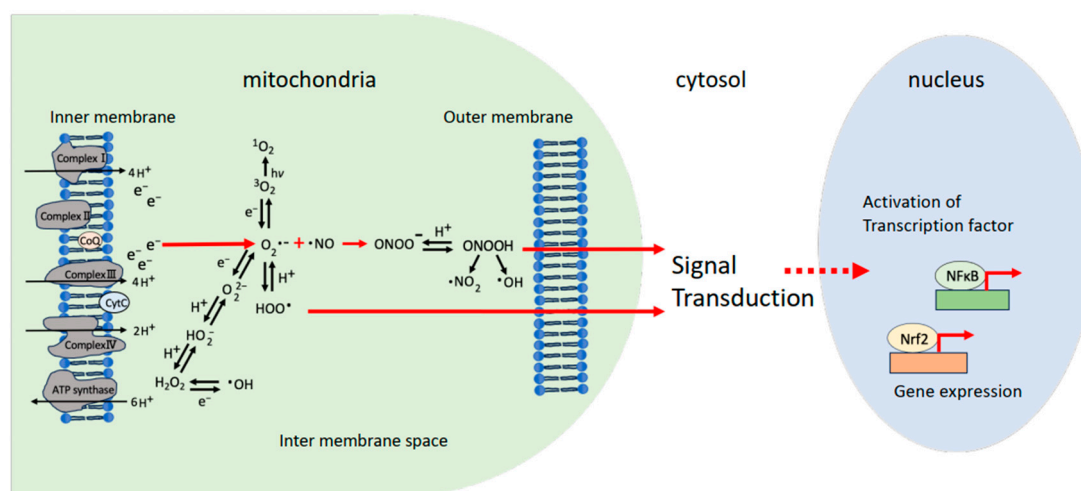
Figure 1. In the mitochondria, 2~3% of electrons leak from the electron transport chain (ETC), and then oxygen traps the electrons, turning them into superoxide anions (O$_2$•$^-$), and subsequently various ROS are produced: •OH, ^{1}O$_2$, H$_2$O$_2$, O$_2$•$^-$, HO$_2$•, •NO, •NO$_2$, ONOO$^-$, and ONOOH. In the intermembrane space, ten times higher amounts of H$^+$ (protons) exist compared to those in the matrix. Among the ROS, ONOO$^-$ and HOO• (HO$_2$•) can couple with H$^+$, and ONOOH and HOO• are produced and penetrate through the membrane, entering the cytosol to initiate intracellular signals, such as NF-κB and Nrf2.

4. Discussion

Majima et al. were the first to report that reactive oxygen species (ROS) generated from the mitochondria promote apoptosis [106], while Itoh et al. described the function of the Nrf2-Keap1 intercellular signal for the first time [107,108]. A recent study described that ROS generated from the mitochondria initiates cellular transduction in the cytosol [34,35]. The further roles of ROS and the subsequent intracellular signals, proteins, and molecule transport change need to be clarified. The establishment of cellular signaling and metabolism change based on mitochondrial ROS augmentation is in demand. Thus, studies on the physiological and pathological functions of mitochondrial ROS will be necessary.

This paper aims to consider the roles of mitochondrial ROS in the activation of intracellular signals. The dipole potential (represented by Ψd) is shown as the potential difference that arises due to the nonrandom orientation of dipolar residues of the lipids and associated water molecules within the membrane [109,110]. ROS with a positive or negative charge cannot escape mitochondria by passive diffusion through phospholipid bilayer due to their large number of dipoles. The results of the dipole moments (Table 1) show that H$_2$O$_2$ is permeable (the dipole moment is 0.00 D). The dipole moment of •NO$_2$ was 0.35 D, indicating permeability. Although the dipole moment of O$_2$•$^-$ is 0.00 D, the negative charge in O$_2$•$^-$ precludes its penetration into the membrane. ONOO$^-$ is non-permeable.

H_2O (with a dipole moment of 1.89 D), $\bullet OH$ (with a dipole moment of 1.67 D), $ONOOH$ (with a dipole moment of 1.77 D), and $HO_2\bullet$ (with a dipole moment of 2.23 D) might be permeable. The candidates that can escape from the mitochondria include ROS with small dipole moments, i.e., H_2O_2, $\bullet NO$, $\bullet NO_2$, $HO_2\bullet$, $ONOOH$, $\bullet OH$, and H_2O. It is well known that $\bullet NO_2$ reacts with urate, ascorbate, and GSH at 10^7 M^{-1} s^{-1} [96]. Therefore, the reaction of $\bullet NO_2$ with specific targets in the cytoplasm, where GSH is present at μM~mM levels [111,112], likely occurs with very low frequency [113]. The candidates that can escape from the mitochondria thus include ROS with small dipole moments, i.e., H_2O_2, $HO_2\bullet$, $ONOOH$, $\bullet OH$, and $\bullet NO$.

The reactivity of ROS/RNS should be essential. However, if the molecules disappear in a short period, there is less chance of the reaction occurring. A greater amount, a long half-life, a greater diffusion distance, a greater Pm, a greater I, a greater one-electron reduction potential, a smaller pK_a, and greater rate constants for the reaction with ascorbate and GSH would be preferable for the studied ROS/RNS. Molecules with electrical charges cannot pass the phospholipid bilayers of mitochondrial membranes [36]. Short-lived molecules, such as $\bullet OH$, are difficult to contribute to intracellular signaling due to the characteristics of the short-lived molecule (Table 2). For signal activation inside the cytosol, again, H_2O_2, $HO_2\bullet$, $ONOOH$, $\bullet OH$, and $\bullet NO$ can be selected as candidates (Table 2).

It is also essential to consider the conditions that ROS/RNS must overcome to pass through the mitochondrial membrane to become signaling molecules in the cytosol. The plasma membrane consists of both lipids and proteins. The fundamental structure of the membrane is the phospholipid bilayer, which forms a stable barrier between two aqueous compartments. [36]. Most biologically important solutes require protein carriers to cross cell membranes via a process of either passive or active transport. Active transport requires the cell to expend energy to move the materials, while passive transport can be achieved without using cellular energy [37]. Certain substances easily pass through the membrane via passive diffusion, such as O_2 and CO_2, along with small relatively hydrophobic molecules, fatty acids, and alcohols [37]. In this study, in the mitochondria, we study which ROS can pass through the mitochondrial membrane.

The ROS produced in the mitochondrial matrix can pass through the two membranes in the mitochondria and enter into the cytosol in order to initiate intracellular signals. Lynch and Fridovich (1978) addressed the question of whether superoxide permeates membranes [114]. The pH of the intermembrane space is lower than that in the matrix due to proton pumping into the intermembrane space; in the intermembrane space (IMS), the concentration of protons is about ten times higher than in the matrix [115]. The pH values obtained were 6.88 ± 0.09 in the IMS, 7.78 ± 0.17 in the matrix, and 7.59 ± 0.01 in the cytosol using a human endothelial cell line, ECV304. [103]. $HO_2\bullet$ and $O_2^{\bullet-}$ are of considerable importance in oxidation processes, and the pK_a of $HO_2\bullet/O_2^{\bullet-}$ is 4.8 [62,90]. Therefore, at the physiological pH, $HO_2\bullet$ hardly exists. In addition to covalent, there is also ionic bonding. There are almost 10 times more protons in the IMS compared to in the matrix. Thus, it may be possible for H^+ to bind anion molecules, leading to protonation [116]. ROS produced in the mitochondria, HOON- and $O_2^{\bullet-}$, can be easily protonated in the IMS through ionic bonding. Whereas $O_2^{\bullet-}$ generated in the mitochondrial matrix may be easily and completely detoxified by mitochondrial SOD, any $O_2^{\bullet-}$ generated on the outside of the inner membrane will have a longer lifetime and, due to the more acidic environment there than in the matrix, it is likely that $O_2^{\bullet-}$ will be protonated to $HO_2\bullet$ and react with a phospholipid in the membrane [117]. Which radicals can penetrate through the mitochondrial membrane? Gus'kova et al. (1984) determined the permeability of the liposomal membrane for $O_2^{\bullet-}$ and $HO_2\bullet$, being $P'O_2^{\bullet-} = (7.6 + 0.3) \times 10^{-8}$ cm s^{-1} and $P'HO_2\bullet = 4.9 \times 10^{-4}$ cm s^{-1}, respectively [51]. Cordeiro (2014) described simulations that showed that molecular oxygen (O_2) accumulated at the interior membrane. Superoxide ($O_2^{\bullet-}$) radicals and hydrogen peroxide (H_2O_2) remained in the aqueous phase and could not enter the membrane. Both hydroxyl ($\bullet OH$) and hydroperoxyl ($HO_2\bullet$) radicals were able to penetrate deep into the lipid headgroup region in the membrane [118]. ROS are

produced in the mitochondria, and to establish which ROS can pass through the membrane, we needed to establish the interactions between ROS and the lipid membrane. Cordeiro evaluated HO_2, $O_2^{\bullet-}$, $\bullet OH$, and H_2O_2 in terms of the residence times in the phospholipid headgroup region, reported in units of ns [118]. The results show that HO_2 and $O_2^{\bullet-}$ have residence times of 17.3 and 12.4 ns, respectively, while $\bullet OH$ and H_2O_2 have residence times of 3.8 and 1.5 ns, respectively. A longer residence time suggests a higher affinity for the ROS and phospholipids, and a shorter residence time suggests a lower affinity for the ROS and phospholipids. $O_2^{\bullet-}$ in the mitochondrial intermembrane space can penetrate the outer membrane mitochondrial membranes through voltage-dependent anion channels (VDACs) [114,119]. However, how much $O_2^{\bullet-}$ can penetrate through VDACs is unknown. It may be possible for $HO_2\bullet$ to pass through the membrane without difficulty.

5. Conclusions

As a result, $HO_2\bullet$ and ONOOH were found to be the top candidates to initiate intracellular signaling among the mitochondrial ROS from Tables 1 and 2. Figure 1 shows the possible ROS that can initiate signal transduction in cells, which are $HO_2\bullet$ and ONOOH. Further experiments to prove that $HO_2\bullet$ and ONOOH go out of mitochondria and initiate signals inside cells will be necessary.

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