This Review is in an ongoing thematic series on Mitochondria in Health and Disease, which includes the following articles:

Mitochondrial Control of Cellular Life, Stress and Death [Circ Res. 2012;111:1198–1207]
Mitochondria as a Drug Target in Ischemic Heart Disease and Cardiomyopathy [Circ Res. 2012;111:1222–1236]
Mitochondria and Endothelial Function [Circ Res. 2013;112:1171–1188]

Guido Kroemer & Lorenzo Galluzzi, Guest Editors

Cardiac Mitochondria and Reactive Oxygen Species Generation

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Abstract: Mitochondrial reactive oxygen species (ROS) have emerged as an important mechanism of disease and redox signaling in the cardiovascular system. Under basal or pathological conditions, electron leakage for ROS production is primarily mediated by the electron transport chain and the proton motive force consisting of a membrane potential (ΔΨ) and a proton gradient (ΔpH). Several factors controlling ROS production in the mitochondria include flavin mononucleotide and flavin mononucleotide–binding domain of complex I, ubisemiquinone and quinone-binding domain of complex I, flavin adenine nucleotide–binding moiety and quinone–binding pocket of complex II, and unstable semiquinone mediated by the Q cycle of complex III. In mitochondrial complex I, specific cysteinyl redox domains modulate ROS production from the flavin mononucleotide moiety and iron–sulfur clusters. In the cardiovascular system, mitochondrial ROS have been linked to mediating the physiological effects of metabolic dilations and preconditioning-like mitochondrial ATP-sensitive potassium channel activation. Furthermore, oxidative post-translational modification by glutathione in complex I and complex II has been shown to affect enzymatic catalysis, protein–protein interactions, and enzyme-mediated ROS production. Conditions associated with oxidative or nitrosative stress, such as myocardial ischemia and reperfusion, increase mitochondrial ROS production via oxidative injury of complexes I and II and superoxide anion radical–induced hydroxyl radical production by aconitase. Further insight into cellular mechanisms by which specific redox post-translational modifications regulate ROS production in the mitochondria will enrich our understanding of redox signal transduction and identify new therapeutic targets for cardiovascular diseases in which oxidative stress perturbs normal redox signaling. (Circ Res. 2014;114:524-537.)

Key Words: electron transport chain complex proteins ■ myocardial infarction ■ mitochondria ■ reactive oxygen species

Mitochondria are the powerhouses of the living cell, producing most of the cell’s energy by oxidative phosphorylation. The process of energy transduction requires the coordinated action of 4 major respiratory enzyme complexes and ATP synthase (F1F0ATPase). High-resolution structures are now available for the bacterial complex I, complete structures of mammalian complexes II to IV, and F1F0ATPase (peripheral or headpiece domain of F1F0ATPase). In addition, the mitochondria play a central role in the regulation of programmed cell death. The mitochondria trigger apoptosis by impairing electron transport and energy metabolism, by releasing cytochrome c and activating caspases that mediate apoptosis, and...
Oxidative Phosphorylation Is an Endogenous Source of ROS

Chance and Williams\(^3,4\) have proposed a convention following the typical order of addition of agents during an experiment using the Clark oxygen electrode, which allows definition of the so-called respiratory states and determination of the respiratory control index. In this experiment, mitochondria were added to an oxygen electrode chamber, followed by a mixture of glutamate and malate (NADH-linked; explained in Online Figure I) or succinate (FADH\(_2\)-linked) as substrate. Respiration is slow (state 2 respiration) because of a low amount of ADP and that the proton circuit is not completed by H\(^+\) re-entry through F\(_{0}\)F\(_{1}\)ATPase. A limited amount of ADP is added, allowing F\(_{0}\)F\(_{1}\)ATPase to synthesize ATP coupled to proton re-entry across the membrane, which is defined as state 3 respiration. Oxygen uptake is accelerated during state 3 respiration, and the total oxygen uptake is effectively used for ATP synthesis. When ADP is exhausted, respiration slows and finally anoxia is attained, which is state 4 respiration. State 4 respiration is not ADP-dependent and technically can be attained by oligomycin, an F\(_{0}\)F\(_{1}\)ATPase inhibitor.

In myocytes, the mitochondria comprise 30% to 40% of the volume and generate \(~90\%\) of ATP.\(^5\) The mitochondria are also the major source of ROS in the cardiovascular system.\(^5\)

Under most physiological conditions, electron transport to O\(_2\) is tightly coupled to oxidative phosphorylation for ATP synthesis. However, oxidative phosphorylation is the major endogenous source of ROS, such as \(\cdot O_2^-\) (superoxide anion radical), H\(_2\)O\(_2\), and \(\cdot OH\) (hydroxyl radical), which are toxic byproducts of respiration.\(^1,2\) \(\cdot O_2^-\) generation by mitochondria is primarily by electron leakage from the ETC (Figure 1). Under the physiological conditions of state 4 respiration, oxygen tension in the mitochondria is low; O\(_2\) consumption by the ETC does not meet the needs of oxidative phosphorylation. A decrease in the rate of mitochondrial phosphorylation increases electron leakage from the ETC and subsequent production of \(\cdot O_2^-\). \(\cdot O_2^-\) is converted to H\(_2\)O\(_2\) by mitochondrial MnSOD (SOD2) as in Equation 1, and H\(_2\)O\(_2\) is further converted to H\(_2\)O by glutathione peroxidase in the presence of glutathione (GSH in Equation 2). It was estimated that the mitochondria produce \(~0.5\) nmol H\(_2\)O\(_2\)/min per milligram of protein, accounting for \(~2\%\) of oxygen uptake, under the conditions of state 4 respiration.\(^5\)

Metabolic H\(_2\)O\(_2\) under state 3 and 4 respiration may modestly induce mitochondrial oxidative stress or diffuse to the cytosol, acting as a signaling molecule to trigger important physiological responses, such as endothelium-derived hyperpolarizing factor that mediates shear or acetylcholine-induced smooth muscle relaxation in small arteries.\(^7,8\) Therefore, H\(_2\)O\(_2\), generated at low to moderate concentrations under normal physiological conditions has been recognized as a nontoxic byproduct of cellular metabolism that can mediate physiological signaling:\(^6\)

\[
\begin{align*}
\cdot O_2^- + \cdot O_2^- + 2H^+ & \rightarrow H_2O_2 + O_2 \quad (1) \\
H_2O_2 + 2GSH & \rightarrow 2H_2O + GSSG \quad (2) \\
H_2O_2 + Fe^{2+} & \rightarrow Fe^{3+} + HO^- + HO^* \quad (3) \\
H_2O_2 + \cdot O_2^- & \rightarrow O_2 + HO^* + HO^* \quad (4)
\end{align*}
\]

The mitochondrial ETC proteins are rich in metal cofactors such as hemes (complexes II, III, and IV) and iron–sulfur clusters (complexes I, II, and III). Oxidative stress in the mitochondria can be greatly enhanced in the presence of reduced transition metals because H\(_2\)O\(_2\) can be converted to the highly reactive hydroxyl radical (HO\(^\cdot\)) via the Fe\(^{3+}\)-dependent Fenton reaction (Equation 3) or via the Fe\(^{3+}\)-catalyzed Haber–Weiss mechanism (Equation 4). Under pathophysiological conditions of cardiovascular disease, decreased or impaired functioning of the ETC and oxidative phosphorylation increases mitochondrial ROS production. Acute ROS exposure can inactivate the iron–sulfur centers of complexes I, II, and III, and Krebs cycle aconitase, resulting in the shutdown of mitochondrial energy production, and chronic ROS exposure can result in oxidative damage to mitochondrial and cellular proteins, lipids, and nucleic acids. Therefore, oxidative stress induced by ROS overproduction in the mitochondria is closely associated with disease pathogenesis.
proton pumping and represents the potential energy driving proton re-entry to the matrix for ATP synthesis. Δp consists of a membrane potential (ΔΨ) and a proton gradient (ΔpH), which partially contribute to the source of •O2− mediated by the mitochondria (Figure 1). In vitro studies have firmly established the effect of membrane potential on •O2− production by complex III.9–11 The production of •O2− mediated by isolated mitochondria under the conditions of state 2 respiration can be induced by glutamate plus malate and measured by electron paramagnetic resonance (EPR) spin trapping with 5′,5′-dimethyl pyrroline N-oxide. The addition of ADP dissipates the proton gradient, initiates the conditions of state 3 respiration, and diminishes mitochondria-mediated •O2− generation, indicating that the coupling of enhanced O2 consumption with oxidative phosphorylation decreased electron leakage, and a decrease in ΔpH is correlated with decreasing •O2− generation by the mitochondria. The addition of oligomycin A inhibits the F0F1ATPase activity and initiates the conditions of state 4 respiration, which gradually restores ΔpH and ΔΨ. Because of restored Δp, •O2− generation induced by glutamate plus malate at state 4 respiration is enhanced to the level of state 2 respiration. The addition of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone, a proton ionophore, to the mitochondria uncouples oxidative phosphorylation, which dissipates ΔΨ and ΔpH. EPR analysis indicated that proton ionophore reduces mitochondria-mediated •O2− production to the level of state 3 respiration. The analysis of H2O2 production by isolated mitochondria indicated that the rate of •O2− generation by the ETC is decreased by uncouplers or inhibitors of Δp.12,13 Therefore, increasing ΔΨ and ΔpH-supported proton backpressure induced electron leakage from the normal pathway for •O2− production in the mitochondria.

Factors Involved in •O2− Generation Mediated by the ETC

Under normal physiological conditions, a decrease in the rate of oxidative phosphorylation can increase oxygen free radical production, in the form of •O2−, from the early stages of the ETC. Two segments of the ETC have been widely hypothesized to be responsible for •O2− generation. One, on the NADH dehydrogenase (NDH; or flavin subcomplex) of complex I, operates via electron leakage from the reduced flavin mononucleotide.14,15 The other, on complex III, mediates •O2− production through a Q-cycle mechanism, in which electron leakage results from auto-oxidation of ubisemiquinone16 and reduced cytochrome b (low potential blow or blo).11 Furthermore, an increasing body of evidence links •O2− overproduction with complex II17,18 or a defect in complex II.19,20 Under ischemic conditions, phosphorylation of complex IV may also increase electron leakage and •O2− production.21 Therefore, mediation of •O2− production by complex II or complex IV is relevant in disease conditions.
Mediation of \( \text{O}_2^- \) Generation by Complex I
Mitochondrial complex I (EC 1.6.5.3—NADH:ubiquinone reductase [NQR]) is the first energy-conserving segment of the ETC. Purified bovine heart complex I contains 45 different polypeptides with a total molecular mass approaching 980 kDa. Complex I can be resolved into 3 subcomplexes using a chaotropic agent: a flavoprotein fraction, an iron–sulfur protein fraction, and a hydrophobic fraction. The flavoprotein fraction contains the catalytic activity of NDH or NADH ferricyanide reductase. The redox centers, apart from the flavin mononucleotide (FMN), are iron–sulfur centers, which cannot be studied by optical spectroscopy but instead require the more labor-intensive technique of low-temperature EPR. Complex I catalyzes the transfer of 2 electrons from NADH to ubiquinone in a reaction that is coupled with the translocation of 4 protons across the membrane, and it is inhibited by rotenone and piericidine A (Table). The redox centers of mammalian complex I that are involved in the mediation of 2-electron transfer include a noncovalent FMN, 8 iron–sulfur clusters, and ubiquinone. Current evidence suggests that proton translocation stoichiometry is 4H+/2e−. In addition to electron transfer and energy transduction, the catalysis of complex I provides the major source of ROS generation in the mitochondria with detectable \( \text{O}_2^- \) formed under the conditions of enzyme turnover. Studies using isolated mitochondria indicate that \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) production by complex I are mainly controlled by NADH/NAD⁺ redox coupling and succinate-induced reverse electron transfer. Two segments of complex I are widely recognized to be responsible for enzyme-mediated \( \text{O}_2^- \) generation. One is involved in the cofactor of FMN and FMN-binding moiety at the 51 kDa polypeptide (FMN-binding subunit), and the other is located on the ubiquinone-binding site that mediates ubiquinone reduction.

**ROS Generation by the FMN Moiety of Complex I**
The mechanism of \( \text{O}_2^- \) generation is likely derived from 1-electron transfer of reduced FMN (FMNH₂) to molecular oxygen. Based on investigations using models of intact complex I and its flavoprotein fraction subcomplex (NDH) along with EPR spin trapping with 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide, enzyme-mediated \( \text{O}_2^- \) generation by intact complex I or NDH isolated from bovine heart can be inhibited by the general flavoprotein inhibitor diphenyleneiodonium chloride. The addition of free FMN can increase enzyme-mediated \( \text{O}_2^- \) generation by intact complex I or NDH. However, FMN addition could not reverse the inhibition by either diphenyleneiodonium chloride treatment or heat denaturation of either intact complex I or NDH. These results support the involvement of FMN and the FMN-binding protein moiety in the mediation of \( \text{O}_2^- \) generation by the flavoprotein fraction of complex I. More evidence has been provided by redox titration of mitochondrial \( \text{O}_2^- \) generation using submitochondrial particles. The redox potential of \( \text{O}_2^- \) production at the site of complex I was determined to be \( n\approx 295 \text{ mV} \). The midpoint potential of the \( \text{O}_2^- \) producing site at complex I resembles the value of free FMN (\( n\approx 310 \text{ mV} \) for the redox couple of FMN/FMNH⁺), also supporting the FMN moiety as a potential site of \( \text{O}_2^- \) generation.

**Table. Summary of Inhibitors Used to Study the Components of Mitochondrial Electron Transport Chain (ETC) and Proton Motive Force (PMF) In Vitro and In Vivo**

<table>
<thead>
<tr>
<th>ETC/PMF Components</th>
<th>Inhibitors</th>
<th>Experimentation</th>
<th>Mechanism of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>Diphenyleneiodinium chloride</td>
<td>In vitro</td>
<td>Inhibiting electron leakage at flavin mononucleotide–binding subunit</td>
</tr>
<tr>
<td>Complex I</td>
<td>Rotenone, piericidine A</td>
<td>In vitro and in vivo</td>
<td>Blocking the oxidation of iron–sulfur clusters of complex I</td>
</tr>
<tr>
<td>Complex I</td>
<td>Amytal</td>
<td>In vitro and in vivo</td>
<td>Inhibiting flavoprotein subcomplex activity and subsequently block oxidation of iron–sulfur clusters</td>
</tr>
<tr>
<td>Complex II</td>
<td>TTFA</td>
<td>In vitro</td>
<td>Blocking ubiquinone reduction by occupying ubiquinone-binding site</td>
</tr>
<tr>
<td>Complex II</td>
<td>Malonate oxaloacetate</td>
<td>In vitro</td>
<td>Competitive inhibitor for succinate binding and blocking FADH₂-linked ROS production</td>
</tr>
<tr>
<td>Complex II</td>
<td>Diazoxide</td>
<td>In vitro and in vivo</td>
<td>Inhibiting complex II and weakening succinate cytochrome c reductase supercomplex assembly, increasing ROS mediated by complex III</td>
</tr>
<tr>
<td>Complex III</td>
<td>Antimycin A</td>
<td>In vitro</td>
<td>Blocking electron transfer between ( b_6 ) and semiquinone bound at the Q site, increasing ROS production</td>
</tr>
<tr>
<td>Complex III</td>
<td>Myxothiazol</td>
<td>In vitro</td>
<td>Blocking electron transfer between ubiquinol and Reiske iron–sulfur cluster, inhibiting ( \text{Q}_6^- ) and ROS production by complex III</td>
</tr>
<tr>
<td>Complex III</td>
<td>Stigmatellin</td>
<td>In vitro</td>
<td>Inhibiting electron transfer to cytochrome ( c_1 ), inhibiting ( \text{Q}_6^- ) and ROS production by complex III</td>
</tr>
<tr>
<td>Complex IV</td>
<td>Cyanide, azide, CO</td>
<td>In vitro</td>
<td>Inhibiting electron transfer to ( q_1 ) by binding tightly with iron coordinated in cytochrome ( a_6 )</td>
</tr>
<tr>
<td>F,F,ATPase</td>
<td>Oligomycin A</td>
<td>In vitro</td>
<td>Blocking the flow of protons through the channel by binding to the F₆ portion of F,F,ATPase</td>
</tr>
<tr>
<td>ΔpH</td>
<td>Nigericin</td>
<td>In vitro and in vivo</td>
<td>( \text{Na}^+/\text{K}^+ ) antiporter, collapsing proton gradient and increasing ( \Delta \Psi )</td>
</tr>
<tr>
<td>ΔΨ</td>
<td>Valinomycin</td>
<td>In vitro and in vivo</td>
<td>( \text{K}^+ ) mobile carrier ionophore, collapsing mitochondrial ( \Delta \Psi )</td>
</tr>
<tr>
<td>Δp</td>
<td>DNP, FCCP</td>
<td>In vitro and in vivo</td>
<td>Protonophore, mediating the net electric uniport of protons and uncoupling the process of ( \Delta p )</td>
</tr>
</tbody>
</table>

\( \Delta \Psi \) indicates membrane potential; \( \Delta p \), proton motive force; \( \Delta \text{pH} \), proton gradient; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; ROS, reactive oxygen species; and TTFA, 2-thenoyltrifluoroacetone.
ROS Generation by the Ubiquinone-Binding Domain of Complex I

The second site associated with complex I-mediated ‘O\textsubscript{2}’ generation has been proposed to be the site of ubiquinone reduction because of the formation of an unstable ubisemiquinone radical (SQ) that is the source of ‘O\textsubscript{2}’\textsuperscript{−}.\textsuperscript{13,27,32,33} The ubisemiquinone radical is formed via incomplete reduction of ubiquinone under the conditions of enzyme turnover. Evidence of SQ involvement has been investigated using EPR detection of enzymatic systems in the presence or absence of spin trap.\textsuperscript{25} SQ can be categorized as stable ubisemiquinone radical that is EPR-detectable and unstable ubisemiquinone radical that is not detected by EPR because of a short half-life at room temperature. Unstable ubisemiquinone radical is thus a source of ‘O\textsubscript{2}’\textsuperscript{−} under physiological conditions. Studies using isolated complex I and EPR spin trapping indicated that enzyme-mediated ‘O\textsubscript{2}’ generation driven by NADH was enhanced 2-fold in the presence of ubiquinone-1 (Q\textsubscript{1}) compared with that in the absence of Q\textsubscript{1}. More than 80% of the enhanced ‘O\textsubscript{2}’ generation can be inhibited by rotenone, thus supporting a ‘O\textsubscript{2}’ generation mechanism involving the reduction of ubiquinone to an unstable semiquinone radical.\textsuperscript{25} The formation of an unstable semiquinone radical under enzyme turnover conditions is likely mediated through the ubiquinone-binding site of the hydrophobic fraction subcomplex in complex I. Ohnishi et al\textsuperscript{19} have reported the presence of 2 distinct semiquinone species with different spin relaxation times in complex I in situ based on a study using the submitochondrial particle from rat heart\textsuperscript{44} and proteoliposomes of isolated complex I. The unstable semiquinone as a source of ‘O\textsubscript{2}’ is likely to be SQ\textsubscript{af} (semiquinone with fast relaxing time), which is highly sensitive to rotenone or piericidin A.\textsuperscript{25}

Studies from Lambert and Brand\textsuperscript{27} using isolated mitochondria suggest that ‘O\textsubscript{2}’\textsuperscript{−} production from the ubiquinone moiety of complex I is large under conditions of reverse electron transport mediated by respiring to succinate\textsuperscript{13} or conditions of forward electron transport in the presence of ATP and piericidin A (or rotenone). ‘O\textsubscript{2}’\textsuperscript{−} generation by complex I during reverse/forward electron transport can be inhibited by uncoupler and nigericin, suggesting that ‘O\textsubscript{2}’\textsuperscript{−} production by complex I is more dependent on ΔpH than ΔΨ.\textsuperscript{13,22} Note that nigericin is an ionophore functioning as a specific carrier of protons and catalyzing a K\textsuperscript{+}/H\textsuperscript{+} exchange that abolishes ΔpH, thus allowing ΔΨ to increase to the full magnitude of the proton motive force. Whereas, an uncoupler [eg, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone] functions as a carrier of protons and charge that collapses both ΔpH and ΔΨ.

Role of Iron–Sulfur Clusters in ROS Generation by Complex I

Mammalian complex I hosts as many as 8 iron–sulfur clusters, which are essential redox centers controlling electron transfer during enzyme turnover. Iron–sulfur clusters identified with the use of EPR include the binuclear clusters N1a and N1b and the tetranuclear clusters N2, N3, N4, and N5. Two more tetranuclear clusters, N6a and N6b, were identified in the subunit TYKY. Based on the x-ray structure of complex I from Thermus thermophilus, the organization of iron–sulfur clusters in mammalian complex I has been inferred. The main route for electron transfer within the enzyme is likely to be NADH→FMN→N3→N1b→N4→N5→N6a→N6b→N2→ubiquinone.\textsuperscript{36} The iron–sulfur–clusters in complex I should play a secondary role in the generation of ‘O\textsubscript{2}’ by complex I. Studies using intact mitochondria from rat brain indicate that rotenone-insensitive ‘O\textsubscript{2}’\textsuperscript{−} generation by complex I is far less sensitive to p-chloromercuribenzoate (an iron–sulfur–cluster blocker) treatment than to diphenylenediamine chloride treatment.\textsuperscript{29} However, several research groups have proposed that in intact mitochondria and submitochondrial particles, most of the ‘O\textsubscript{2}’\textsuperscript{−} may be generated around the N2/ubiquinone site, suggesting that electron leakage at the N2 center is coupled to incomplete reduction of ubiquinone.\textsuperscript{32}

The 75 kDa subunit hosts 3 iron–sulfur clusters (N1b, N4, N5) of mammalian complex I. Residues 100 to 120 of the protein precursor of mammalian complex I. Residues 100 to 120 of the protein data bank accession code 3I9V, as a template.\textsuperscript{36} (peptide of p75), exhibit a β-sheet-α-turn-helix conformation (blue region in the homolog model of 75 kDa subunit in Figure 2B). The peptide of p75 was designed as a B-cell epitope to make an antibody that has been used to probe the structure and function of complex I.\textsuperscript{25,37} The polyclonal antibody...
generated against p75 is named Ab75. The binding of Ab75 antibody to mammalian complex I resulted in the inhibition of \( \text{O}_2^- \) generation by 35% as measured by EPR spin trapping. Therefore, (1) the binding of Ab75 is likely to stabilize the conformation of protein matrix surrounding the iron–sulfur clusters, thus enhancing electron transfer efficiency; and (2) Ab75 binding may protect the protein matrix from molecular oxygen accessing the iron–sulfur clusters, including N1b, N4, and N5 in bovine complex I (Figure 2B), minimizing electron leakage from iron–sulfur clusters.

**Role of Cysteinyl Redox Domains**

The mitochondrial redox pool contains a high physiological concentration of glutathione. The overproduction of \( \text{O}_2^- \) and \( \text{O}_2^- \)-derived oxidants increases the ratio of oxidized glutathione to glutathione. Moreover, the proteins of mitochondrial ETC are rich in protein thiols. Mammalian complex I is a major component of the ETC to host the reactive/regulatory thiols have been implicated in the regulation of respiratory function, NO utilization, and redox status of the mitochondria. It is documented that the 51 kDa (FMN-binding protein) and 75 kDa (iron–sulfur protein) subunits of complex I are the 2 major polypeptides hosting redox thiols.4-24,26,38-40

The C206 (Cys206) moiety of 51 kDa subunit plays a unique role as a redox thiol in oxidative damage to complex I. The C206 of 51 kDa subunit is also involved in site-specific S-glutathionylation (via binding of glutathione).4,24,26 The peptide identified to form and bind glutathione is \text{GAGAYIC}_{206}\text{GEETALIESIEGK}_{219}, which is highly conserved in bacterial, fungal, and mammalian enzymes.23 An x-ray crystal structure of the hydrophilic domain of complex I from *T.thermophilus* reveals that this conserved cysteine (Cys182 in *T.thermophilus*) is only 6 Å from the FMN, which is consistent with the role of Cys206 as a redox-sensitive thiol and FMN serving as a source of \( \text{O}_2^- \).26

The polypeptide of 75 kDa is the other subunit of complex I to be involved in redox modification via S-glutathionylation or protein thiol radical formation.24,26,40 Based on LC/MS/MS analysis, S-glutathionylation of C367 can be induced by oxidized glutathione through protein thiol disulfide exchange. C354 and C727 were S-glutathionylated when complex I of rat heart mitochondria was oxidatively stressed by diamide.20 The glutathione–binding peptides identified include

\[
\text{VDSDDLTC}\text{EEVEPTAAGTDLR_{544}MLFLLAGDGC{ITR_{170}and_{717}AVTEGAHAVEEPSIC}_{727}}.
\]

Based on EPR spin trapping studies, oxidized glutathione–induced S-glutathionylation of complex I at the 51 kDa and 75 kDa subunits affects the \( \text{O}_2^- \) generation activity of complex I by marginally decreasing electron leakage and increasing electron transfer efficiency.26 High dosage of oxidized glutathione or diamide-induced S-glutathionylation tends to decrease the catalytic function of complex I and increase enzyme-mediated \( \text{O}_2^- \) generation.39,40 The binding of antibodies against the peptide of \text{GAGAYIC}_{206}\text{GEETALIESIEGK}_{219} (pGSCA206 in Figure 2A) decreases complex I–mediated \( \text{O}_2^- \) generation by 37%.25 Because FMN serves as a source of \( \text{O}_2^- \), the binding of antibodies may prevent molecular oxygen from accessing FMN, resulting in a subsequent decrease in \( \text{O}_2^- \) production. In addition, the binding of antibodies against the peptide of \text{K-LDLLNKVDSDLTC}^{206}\text{EEVEPTAAGTDLR}_{544} (pGSCB367 in Figure 2B) inhibits \( \text{O}_2^- \) production by complex I by 57%. The distance of pGSCB367 to iron–sulfur clusters is relatively long; thus, the binding of antibodies to the 75 kDa subunit of complex I likely triggers long-range conformational changes in the 75 kDa polypeptide to reduce \( \text{O}_2^- \) interactions. The binding of either antibody does not affect the electron transfer activity of complex I. Therefore, the redox domains involved in S-glutathionylation are responsible for regulating electron leakage for \( \text{O}_2^- \) production by complex I.

**Mediation of \( \text{O}_2^- \) Generation by Complex II**

Mitochondrial complex II (EC 1.3.5.1—succinate:ubiquinone reductase [SQR]) is a key membrane complex in the Krebs cycle that catalyzes the oxidation of succinate to fumarate in the mitochondrial matrix. Succinate oxidation is coupled to the reduction of ubiquinone at the mitochondrial inner membrane as one part of the ETC. Complex II mediates electron transfer from succinate to ubiquinone through the prosthetic groups of flavin adenine nucleotide (FAD)—[2Fe-2S] (S1), [4Fe-4S] (S2), and [3Fe-4S] (S3)—and heme b. The enzyme is composed of 2 parts: a soluble succinate dehydrogenase (SDH) and a membrane-anchoring protein fraction. SDH contains 2 subunits, a 70 kDa protein with a covalently bound FAD, and a 30 kDa iron–sulfur protein containing S1, S2, and S3 iron–sulfur clusters. The membrane-anchoring protein fraction contains 2 hydrophobic polypeptides (14 and 9 kDa) with heme b binding.

The catalysis of complex II is thought to contribute to ROS generation in mitochondria. Two regions of the enzyme complex are responsible for generating \( \text{O}_2^- \). One is located on the FAD cofactor, whereas the other is located on the ubiquinone-binding site, which acts in the mediation of ubiquinone reduction.27,41

**ROS Generation by the FAD Moiety of Mammalian Complex II**

The generation of \( \text{O}_2^- \) by the FAD moiety of complex II may arise from FADH \_ auto-oxidation or FADH \_ semiquinone auto-oxidation. Evidence has been provided from the mammalian enzyme via the use of inhibitor 2-thienyltrifluoroacetonate (TTFA).42,43 TTFA is a classical inhibitor for the ubiquinone reduction of complex II by occupying its ubiquinone-binding sites.26 Therefore, the binding of TTFA to the enzyme induces electron accumulation at the early stage of complex II. Based on EPR spin trapping studies using isolated complex II and a supercomplex (succinate cytochrome c reductase) hosting complex II and complex III, the inhibitory effect of TTFA on \( \text{O}_2^- \) generation by complex II or succinate cytochrome c reductase indicated that FADH \_ auto-oxidation mediated by FAD-binding moiety partially contributes to \( \text{O}_2^- \) production.43 The production of \( \text{O}_2^- \) by complex II or succinate cytochrome c reductase is minimized under the conditions of enzyme turnover.42,43

**ROS Generation by the Ubiquinone-Binding Site of Complex II**

Mammalian complex II contains \( \geq 2 \) ubiquinone-binding sites (namely, Qp and Qd).26 Qp is near the matrix side,
and Qd is near the intermembrane space site. Because the ubiquinone-binding site in complex II is close to the TTFA-binding site, the EPR signal derived from ubisemiquinone of complex II is sensitive to TTFA. The location of Qp site and its quinone-binding pocket has been revealed by the x-ray structure of porcine heart complex II (protein data bank, 1ZOY). The amino acid residues involved in the ubiquinone-binding site are determined by the x-ray structure to be Trp B173 and Tyr D91. UQ denotes ubiquinone.

**Figure 3. Hydrophobic residues and polar interactions (dashed lines) in the ubiquinone-binding (Qp) site of mammalian complex II.** The quinone-binding pocket is revealed by the x-ray structure (protein data bank, 1ZOY) and involves the residues Trp B172, Trp B173, His B216, Arg C46, Asp D80, and Tyr D91. The residues of the ubiquinone-binding site are determined by the x-ray structure to be Trp B173 and Tyr D91. UQ denotes ubiquinone.

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**Mediation of 'O2' Generation by Complex III**

**Q-Cycle Mediated by Complex III**

Mitochondrial complex III catalyzes electron transfer from ubiquinol (QH2) to ferricytochrome c, which is coupled to proton translocation for ATP synthesis. The redox centers of complex III consist of QH2; hemes b1, bH (high potential b or bH2), and c1; and the Rieske iron–sulfur protein. The electron transfer from QH2 to cytochrome c follows the Q-cycle mechanism.

In the Q-cycle mechanism, there are 2 semiquinones formed in different parts of the cycle (Figure 4A). An unstable semiquinone (Q−) is formed near the cytoplasmic side (outer site or positive site). The other semiquinone (Q−), formed near the matrix side (inner site or negative site), is a stable semiquinone and EPR-detectable. During the enzyme turnover of complex III, 1 electron from QH2 is sequentially transferred to Rieske iron–sulfur protein, then cytochrome c1, and then ferricytochrome c. QH2 contains 2 electrons, but cytochrome c only accepts 1 electron. This leaves an unstable semiquinone (QH−) formed at the cytoplasmic side. One electron from the unstable semiquinone is transferred to bL, and then transferred to bH. One electron from bH is then transferred to ubiquinone (Q) to form a stable semiquinone at the matrix side. The stable semiquinone can accept 1 electron from a second single turnover to complete the cycle, forming QH2.

**'O2' Generation by the Q-Cycle**

The Q-cycle mechanism provides an important theoretical basis to explain why mammalian complex III is an endogenous source of ROS in mitochondria.9,43,45,46 'O2' formed near the cytoplasmic space is a source of 'O2' because of its poor stability. Antimycin A, myxothiazol, and stigmatellin are inhibitors of complex III (Figure 4A). The mechanism of inhibition by these 3 inhibitors strongly supports Qo site as the source of 'O2' generation. Antimycin A inhibits electron transfer from bH to bH2, preventing the formation of the relatively stable 'O2', increasing 'O2' formation and subsequent 'O2' production. Myxothiazol inhibits electron transfer to the Rieske iron–sulfur protein and blocks the formation of 'O2', whereas stigmatellin inhibits electron transfer to cytochrome c1, raising the midpoint potential of the Rieske iron–sulfur cluster from +290 to +540 mV, thus decreasing 'O2' formation and 'O2' production.

**Role of Cytochrome bL and Membrane Potential in 'O2' Generation by Complex III**

As indicated in Figure 4A of the Q-cycle mechanism, bH accepts 1 electron from the unstable semiquinone. The midpoint potential of bH is ~50 mV, which tends to donate an electron. In vitro evidence supports the notion of ferrocytochrome bH to be a source of 'O2'. Experimental evidence has shown the involvement of 'O2' generation during intramolecular electron transfer from ferrocytochrome bL to ferricytochrome c1.43

In vitro studies using vesicles of phospholipid-reconstituted complex III indicated that the rate of 'O2' generation can be controlled by the modulation of ΔΨ.10 Experimental evidence has revealed that the rate of 'O2' generation by phospholipid-reconstituted complex III is enhanced exponentially by increased ΔΨ and nigericin treatment,10 suggesting that 'O2' generation by complex III is more dependent on ΔΨ than on ΔpH. Opposing ΔΨ inhibits electron transfer from heme bL to heme bH2, thereby promoting 'Qo' formation and stimulating 'O2' generation from reduced bL or 'Qo'. (Figure 4A).

**Bidirectionality of Superoxide Release as Mediated by Complex III**

Because the anionic form of 'O2' is too strongly negatively charged to readily pass the inner membrane, 'O2' production mediated by complex III can exhibit a distinct membrane sidedness or topology. The Qo site, one of the sources of 'O2', is located near the intermembrane space (Figure 4B). The close proximity of the Qo site to the intermembrane space can result in invariable release of a fraction of complex III–derived 'O2' to the cytoplasmic side of the inner membrane.48,49 Experimental evidence has supported that ~50% of total electron leakage in the mitochondria lacking CuZn-SOD accounts for extramitochondrial 'O2' release.50 The remaining ~50% of electron leakage is because of 'O2' released to the matrix. H2O2 derived from dismutation of 'O2' in the mitochondrial matrix can induce aconitase inhibition. The inhibition of aconitase by H2O2 in the matrix can be relieved by the
Q$_i$ site inhibitor stigmatellin. These results indicate that 'O$_2^-$' is released to both sides of the inner mitochondrial membrane by semiquinone at the Q$_o$ site.49,50

Protein–Protein Interaction With Complex I Minimizes 'O$_2^-$ Generation by Complex III

NADH cytochrome $c$ reductase is the supercomplex hosting complex I and complex III, mediating electron transfer from NADH to ferricytochrome $c$. Direct evidence from EPR measurements shows that NADH cytochrome $c$ reductase–mediated 'O$_2^-$' generation is controlled by the enzyme turnover of complex I, whereas 'O$_2^-$' generation mediated by the enzyme turnover of complex III is minimized in the intact supercomplex of NADH cytochrome $c$ reductase, indicating that the protein–protein interaction with complex I modulates 'O$_2^-$' production by complex III. Impairment of protein–protein interaction via oxidative injury of complex I may amplify complex III–mediated 'O$_2^-$' generation.51

Hyperphosphorylation of Complex IV Increases Enzyme-Mediated ROS Generation

Mitochondrial complex IV is cytochrome $c$ oxidase that catalyzes the sequential transfer of 4 electrons from ferrocytochrome $c$ to O$_2$, forming 2H$_2$O. The reduction of O$_2$ to H$_2$O provides the driving force for proton translocation and ATP synthesis. The catalytic function of complex IV is mainly controlled by subunit I and subunit II with both subunits encoded by mitochondrial DNA.52,53 The redox centers of complex IV include 2 CuA (in a cluster with sulfur atoms in subunit II), 2 a-type hemes (heme a and heme a$_3$ are located in subunit I), and CuB (in subunit I). Subunit II is the location of 2 CuA sites that undergo a 1-electron oxidation–reduction reaction. The binuclear Cu$_A$ center receives electrons, 1 at a time, from ferrocytochrome $c$. One axial coordination position to the iron of heme a$_3$ is not occupied by an amino acid ligand. This is the position where oxygen binds before its reduction to water. Heme a$_3$ is also the site of binding of several inhibitors, including cyanide, azide, NO, and CO. Cu$_B$, the third copper, is immediately adjacent to heme a$_3$; it has 3 histidine ligands, suggesting that a fourth coordination position may be occupied by a reaction product during a specific stage of the oxygen–reduction reaction. One of 3 ligands, histidine-240 (H$_{240}$ in bovine heart complex IV), is crosslinked to a nearby specific tyrosine residue (Y$_{244}$ in bovine heart complex IV) through a covalent bond. This specific tyrosine has been proved to participate in the formation of a protein–tyrosyl radical intermediate.
during enzyme turnover or under oxidant stress induced by \( \text{H}_2\text{O}_2 \).\textsuperscript{54-56} \( \text{Cu} \) and nearby heme \( \alpha \) form the bimetallic center, which tightly controls oxygen binding, formation of intermediates (oxy, compound P, and compound F), and reduction of oxygen to \( \text{H}_2\text{O} \). Electron leakage for \( \text{"O}_2^- \) generation is not likely occurring during the enzyme turnover of complex IV or under normal physiological conditions. However, \( \text{"O}_2^- \) production can be mediated by hyperphosphorylated complex IV under ischemic conditions.\textsuperscript{21}

**Aconitase Is a Source of Hydroxyl Radical**

Mitochondrial aconitase hosts a \([4\text{Fe}-4\text{S}] \) cluster in its active site, which catalyzes the conversion of citrate to isocitrate (a reaction of stereospecific hydration and rehydration) in the Krebs cycle. Aconitase activity in the mitochondria was reported to be a redox sensor of ROS.\textsuperscript{57-59} The reaction of aconitase with superoxide (\( k=10^7 \text{M}^{-1} \text{second}^{-1} \)) rapidly inactivates its enzymatic activity by producing an inactive \([3\text{Fe}-4\text{S}] \) cluster (\( g=2.018 \)), free iron (II), and \( \text{H}_2\text{O}_2 \). Consequently, the inactivation of mitochondrial aconitase by superoxide facilitates the formation of hydroxyl radical via a Fenton-type mechanism (Figure 1).\textsuperscript{60}

**Effects of Mitochondrial ROS in the Heart and Role in Disease**

**Effects of Mitochondrial ROS on Myocytes**

The heart requires a constant supply of energy to support contractile activity. This obligation is met by the daily synthesis of ATP via oxidative phosphorylation. In normal heart, \( \approx 70\% \) of ATP requirement is met by the catabolism of free fatty acids via \( \beta \)-oxidation, giving rise to a greater ATP yield than with glucose. Oxidative phosphorylation is the endogenous source of mitochondrial ROS production, and the overproduction of ROS can establish a vicious cycle of oxidative stress in the mitochondria and mitochondrial permeability transition induction with ROS-induced ROS release,\textsuperscript{61} which has important pathophysiological effects. Excess ROS production under pathophysiological conditions executes the detrimental effects on myocytes via mitochondrial dysfunction and bioenergetic decline. The chronic exposure of myocytes to ROS leads to the impairment of excitation–contraction coupling, causing arrhythmias and contributing to cardiac remodeling by inducing cardiac hypertrophy, apoptosis, necrosis, and fibrosis.\textsuperscript{62-64}

**Effects of Mitochondrial ROS on Endothelial Cells**

In the vasculature, excess production of ROS from the mitochondria is responsible for the inflammatory vascular reaction that has been implicated in the pathogenesis of atherosclerosis, hypertension, diabetes mellitus,\textsuperscript{65} and corruption of coronary collateral growth.\textsuperscript{66} However, basal low levels of mitochondrial ROS released under normal metabolic activity may exert beneficial effects on cardiovascular physiology and function.\textsuperscript{7} Myocardial or cardiac work is tightly controlled by the coronary blood flow and heart rate. In the endothelium of coronary arterioles, the generation of \( \text{H}_2\text{O}_2 \) in mitochondria is induced by shear stress. Mitochondrial \( \text{H}_2\text{O}_2 \) subsequently mediates flow-induced dilation of vessel via the activation of large-conductance \( \text{Ca}^{2+} \)-activated potassium channel coupled with intracellular signaling of protein kinase A-1 (activation in the smooth muscle cells).\textsuperscript{67,68} \( \text{H}_2\text{O}_2 \) produced in response to flow was demonstrated to be a byproduct of complex I and complex III from the endothelial ETC.\textsuperscript{8} Therefore, \( \text{H}_2\text{O}_2 \) originating from mitochondrial \( \text{"O}_2^- \) in the vasculature has been described as an endothelium-derived hyperpolarizing factor that acts as an intracellular signaling molecule to mediate shear- or acetylcholine-induced smooth muscle relaxation (Figure 5).

In the cardiac myocytes, \( \text{H}_2\text{O}_2 \) derived from mitochondrial \( \text{"O}_2^- \) has also been proposed to regulate collateral blood flow via underlying diffusion to the microvascular system of the heart.\textsuperscript{69} This has important physiological implications providing a link between mitochondrial \( \text{H}_2\text{O}_2 \) and metabolic flow regulation and couples the production of \( \text{H}_2\text{O}_2 \) as an endothelium-derived hyperpolarizing factor to myocardial metabolism. Evidence has indicated that mitochondrial \( \text{H}_2\text{O}_2 \) is the vasoactive product of metabolically active cardiac myocytes, functioning as a coronary metabolic dilator coupling myocardial oxygen consumption to coronary blood flow in a redox-dependent manner. The above coronary metabolic dilation is mediated by the activation of voltage-dependent potassium channels and thiol redox–dependent signaling.\textsuperscript{69,70} Therefore, it is expected that metabolic \( \text{H}_2\text{O}_2 \) produced by increased perfusion during intense exercise likely contributes to endothelium-dependent hyperpolarization.

**Role of Mitochondrial ROS in Myocardial Infarction**

Mitochondrial dysfunction is a critical consequence of myocardial infarction. Impairment of mitochondrial function during myocardial ischemia/reperfusion injury is caused by oxidative stress.\textsuperscript{71-75} In the ischemic heart, oxygen delivery to the myocardium is not sufficient to meet the need for substrate oxidation in mitochondria during the physiological conditions of hypoxia, leaving mitochondrial ETC in a highly reduced state. This results in increased electron leakage from the ETC that in turn reacts with residual \( \text{O}_2 \) to give \( \text{"O}_2^- \). Ischemia converts most ATP to ADP and AMP. On reperfusion, most ADP is converted back to ATP and AMP as catalyzed by adenylate kinase. Because of the significant depletion of ADP in the early stage of reperfusion,\textsuperscript{8} the reintroduction of \( \text{O}_2 \) with reperfusion will greatly increase electron leakage along with a decrease in scavenging capacity, leading to a marked overproduction of \( \text{"O}_2^- \) and \( \text{O}_2^- \)-derived ROS in the mitochondria. Specifically, increased hyperoxygenation induced by reperfusion in the postischemic heart has been detected in vivo EPR oximetry.\textsuperscript{76} The overproduction of mitochondrial ROS also initiates oxidative inactivation of ETC proteins, antioxidant enzymes, lipid peroxidation, and mtDNA damage. Mitochondrial dysfunction caused by this injury to the ETC was marked in the postischemic rat heart. Pretreatment of the heart with the radical trap, \( 5,5' \)-dimethyl pyrroline \( \text{N-oxide} \), protected the ETC from postischemic injury, preventing oxidative damage to the ETC that occurred after myocardial ischemia and reperfusion.\textsuperscript{77} The activity of mitochondrial aconitase is highly susceptible to oxidative stress, which further predisposes the enzyme as a source of hydroxyl radical. The physiological conditions
of myocardial ischemia have been reported to impair aconitase activity in the mitochondria.\textsuperscript{79} Postischemic reperfusion increased mitochondrial \( \cdot O_2^- \) production and further enhanced oxidative protein damage of aconitase.\textsuperscript{79} Therefore, oxidative inactivation of mitochondrial aconitase activity may serve as an additional disease marker of myocardial infarction (Figure \textsuperscript{5}).

Myocardial ischemia and reperfusion provide a stimulus to alter NO metabolism, including the involvement of endothelial NO synthase,\textsuperscript{77,80} increased nitrite disproportionation,\textsuperscript{81–83} and increased inducible NO synthase expression in chronic reperfusion injury.\textsuperscript{80,84} Therefore, myocardial ischemia and reperfusion indirectly change the balance between NO and \( \cdot O_2^- \) in the mitochondria. NO production, subsequent peroxynitrite (ONOO\(^-\)) formation, and related protein tyrosine nitration were greatly increased in the mitochondria of postischemic heart.\textsuperscript{85,85}

\textbf{Mitochondrial Complex I in Postischemic Heart}

Complex I is viewed as a major contributor of mitochondrial ROS metabolism in cells.\textsuperscript{86} ROS generated by complex I in the cardiovascular system are thus proposed to mediate the physiological effects related to vascular signaling.

However, pathophysiological production of excess ROS by a leak at mitochondrial complex I has been implicated in myocardial infarction (Figure \textsuperscript{5}). Oxidative impairment of mitochondrial complex I is detected in isolated rat hearts subjected to global ischemia and reperfusion\textsuperscript{51,87} and in animal disease models of myocardial ischemia and reperfusion.\textsuperscript{25,77} In the mitochondria of postischemic hearts, more severe impairment of ETC was observed in complex I, which correlated with mitochondrial dysfunction, diminished NADH-linked state 3 oxygen consumption, and enhanced NADH-linked \( \cdot O_2^- \) generation and the capacity of mitochondria to produce \( H_2O_2 \).\textsuperscript{51,87} Furthermore, the activity of intact complex I was impaired with no significant loss of NADH ferricyanide reductase activity (the enzymatic activity of NADH dehydrogenase or flavoprotein fraction subcomplex) due to the restoration of flavoprotein fraction function during reperfusion. The functional recovery of flavoprotein fraction likely plays a role in \( \cdot O_2^- \) generation during reperfusion, because a functional flavoprotein fraction is one of the major sources of myocardial ischemia.

**Figure 5.** Schematic of the proposed physiological (highlighted with light green/yellow boxes) and pathophysiological (highlighted with light red boxes) roles of \( \cdot O_2^- \) generation by each respiratory electron transport complex, the proton motive force (\( \Delta p \)), and aconitase of Krebs cycle. \( H_2O_2 \) derived from \( \cdot O_2^- \) by complex I and complex III can function as endothelium-derived hyperpolarizing factor (EDHF) to mediate the intracellular signaling of metabolic dilation via smooth cell (SMC) relaxation. FMN indicates flavin mononucleotide. Intramitochondrial \( H_2O_2 \) formed at the matrix site and extramitochondrial \( H_2O_2 \) formed at the Qo site can diffuse to cytosol to trigger the physiological responses. Unlike complex I, complex II–mediated \( \cdot O_2^- \) may modulate preconditioning via mitochondrial ATP-sensitive potassium channel (mK\textsubscript{ATP}) activation. However, the physiological role of \( \cdot O_2^- \) mediated by the Qp site of complex II remains unclear. In addition to the physiological role of EDHF, complex III-mediated \( \cdot O_2^- \) also has been linked to diazoxide-induced transient opening of mK\textsubscript{ATP}. Under the pathophysiological conditions of myocardial ischemia and reperfusion (I/R), \( \cdot O_2^- \) generation by complexes I, III, and flavoprotein of complex II all mediate I/R injury and increase the pro-oxidant activity of aconitase, thus further augmenting I/R injury. Protein kinase A (PKA)-mediated phosphorylation of complex IV under ischemic conditions predisposes complex IV to generate \( \cdot O_2^- \) and augment I/R injury. Proton motive force is proposed as a source of \( \cdot O_2^- \) for I/R injury because reperfusion partially restores the membrane potential. The overproduction of \( \cdot O_2^- \) from the Qp site of complex II is associated with the disease of head-to-neck paraganglioma; however, its pathological role in I/R injury remains unexplored. (Illustration credit: Ben Smith.)
for complex I–mediated \( \text{O}_2^- \) production.\(^{51}\) In the postischemic heart, ROS-induced damage of mitochondrial cardiolipin and respiratory supercomplex assemblies has been attributed to a defect in complex I activity, increasing electron leakage for complex I–mediated \( \text{O}_2^- \) generation, perpetuating a cycle of oxidative damage, and ultimately leading to mitochondrial dysfunction.\(^{85,86}\) Furthermore, protein tyrosine nitration of complex I at the flavoprotein fraction and iron–sulfur protein fraction subcomplexes was marked in the postischemic heart, implicating that peroxynitrite-mediated complex I inactivation occurs during myocardial infarction.\(^{85}\)

**Mitochondrial Complex II in Postischemic Heart**

The role of complex II in mitochondrial ROS production in vivo remains uncertain, and further investigation is required to determine its role in the cardiovascular system. To date, there is no evidence indicating that complex II–mediated ROS metabolism is involved in vascular signaling (unlike complex I). However, specific complex II inhibitors diazoxide or atenolol at sub-toxic concentrations have been used as pharmacological agents to induce preconditioning-like cardioprotective effects by activating mitochondrial ATP-sensitive potassium (mKATP) channels.\(^{89–92}\) The basal levels of ROS generated by complex II are likely physiologically relevant in enabling the mitochondria to modulate cardiac function and exert cardioprotection via mKATP channels (Figure 5).

The defects in mammalian complex II leading to \( \text{O}_2^- \) overproduction have been linked with the disease pathogenesis of pheochromocytoma and head-to-neck paraganglioma as well as myocardial infarction.\(^{42,44,45,93–97}\) The disease phenotype attributed to a point mutation of complex II is associated with pheochromocytoma and head-to-neck paraganglioma, causing electron leakage from the pro-apoptotic factors or the Qp site in complex II, producing excess ROS and leading to tumor formation (Figures 3 and 5).

In animal models of myocardial ischemia and reperfusion injury, oxidative damage of the electron transfer activity of complex II is marked in regions of myocardial infarction.\(^{42,44,98}\) A damage of complex II is associated with loss of FADH\(_2\)-linked oxygen consumption in the postischemic heart. Further evaluation of the redox biochemistry of complex II indicated that alterations of oxidative post-translational modification are present in postischemic myocardium, including modified thiols of the 70 kDa FAD-binding subunit (loss of glutathione binding) and an increase in the level of protein tyrosine nitration of the 70 kDa subunit. Because S-glutathionylation of complex II at the FAD-binding subunit decreases electron leakage that leads to \( \text{O}_2^- \) generation in vitro, it is thus expected that complex II deglutathionylation that occurs during myocardial ischemia and reperfusion enhances complex II–mediated \( \text{O}_2^- \) generation.\(^{42}\) The overproduction of \( \text{O}_2^- \) and NO results in subsequent oxidative inactivation and protein nitration of complex II, which can serve as a disease marker of myocardial infarction.\(^{84}\)

**Mitochondrial Complex III in Postischemic Heart**

Uninhibited complex III (versus complex I) is considered a more minor source of mitochondrial ROS with a physiological role in the regulation of metabolic dilation.\(^{7}\) Despite this minor role, the extramitochondrial role and effect of \( \text{O}_2^- \) produced at the Q site are expected to be relevant. Furthermore, electron leakage at complex III has been linked to pharmacological preconditioning by diazoxide via mKATP channel opening through the inhibition of complex II and promoting transient generation of ROS for signaling at complex III\(^{10}\) (Figure 5).

Complex III is proposed as one of the major sources for mitochondrial \( \text{O}_2^- \) production in postischemic heart (Figure 5). Increased unstable semiquinone radical, \( \text{Q}^- \), is attributed to be a source of \( \text{O}_2^- \).\(^{99,100}\) Evidence includes increased lipid peroxidation of cardiolipin required for complex III activity\(^{101}\) and increased protein tyrosine nitration at the polypeptide of Rieske iron–sulfur protein,\(^{85}\) which may increase \( \text{Q}^- \) formation. Oxidative injury or protein nitration of complex I and complex II weakens the supercomplex interaction with complex III, which can subsequently enhance \( \text{O}_2^- \) generation by complex III in the mitochondria of postischemic heart.\(^{51}\)

**Mitochondrial Complex IV in Ischemic Heart**

\( \text{O}_2^- \) production can be mediated by complex IV under ischemic conditions. The activity of complex IV is modulated in response to \( \text{O}_2^- \) tension. A marked decrease in complex IV activity has been measured in the mitochondria of ischemic hearts\(^{21,51}\) and submitochondrial particles exposed to hypoxic conditions in vitro.\(^{102}\) Hypoxic exposure of murine monocyte macrophages results in cAMP-mediated inhibition of complex IV activity.\(^{21}\) The inhibition of complex IV is accompanied by phosphorylation mediated by protein kinase A (PKA) and markedly increased \( \text{O}_2^- \) generation by phosphorylated complex IV. The conditions of myocardial ischemia also activate mitochondrial PKA, enhancing hyperphosphorylation of complex IV, vastly increasing ROS production by complex IV, and augmenting ischemic and subsequent reperfusion injuries\(^{21}\) (Figure 5). Therefore, specific inhibitors of PKA have been proposed to render cardiac protection against ischemia/reperfusion injury via suppression of the pro-oxidant activity of complex IV. Furthermore, blocking \( \beta_2 \)-adrenoceptor activation during ischemia/reperfusion reversed PKA-mediated depression of complex IV activity and reduced myocardium at risk for postischemic injury,\(^{79}\) implying that \( \beta_2 \)-adrenergic stimulation contributes to myocardial ischemic and reperfusion damage via the mechanism of PKA/cAMP-mediated complex IV inactivation.\(^{79}\)

**Conclusions and Perspective**

Mitochondrial ROS play critical roles in disease pathogenesis and redox signal transduction. Figure 5 summarizes the proposed physiological role and ischemia/reperfusion-related pathophysiological role of mitochondrial ROS generated by complexes I to IV, \( \Delta \psi \), and aconitase. Complexes I and III are the most extensively characterized enzyme complexes mediating ROS generation in the mitochondria and seem to be responsible for the majority of mitochondrial ROS in cardiovascular physiology and disease. The primary physiological role of mitochondrial ROS is to mediate intracellular signaling of vascular metabolic dilation in the cardiovascular system. In the disease setting of myocardial ischemia/reperfusion, oxidative injury of complex I with increased tyrosine nitration or protein thiol radical–dependent S-glutathionylation can...
weaken supercomplex interaction and enhance ROS production by complex III. Recently, deglutathionylated complex II and phosphorylated complex IV have also been demonstrated to be involved in mitochondrial ROS generation in the disease progression of myocardial infarction. Increased peroxynitrite-mediated complex II nitration in postischemic heart further supports the role of complex II in ROS generation and disease. ROS mediated by ETC may also synergistically enhance oxidative stress in the mitochondria via induction of the pro-oxidant activity of aconitase. The redox domains of S-glutathionylation in complex I modulate electron leakage that leads to ROS production from FMN and iron–sulfur clusters. The ongoing evaluation of the role of the aforementioned redox domains in normal and postischemic hearts will enable increased understanding of how mitochondrial ROS mediate cellular signaling in cardiovascular physiology and disease.

Further identification of specific redox domains that regulate mitochondrial ROS generation under physiological or pathological conditions is a major frontier in the redox regulation of the ETC. Currently, specific S-glutathionylated domains of complex I and complex II have been identified. In addition to S-glutathionylation, specific redox modifications such as cysteine nitrosylation and tyrosine nitration have been detected in complexes I to III, but it is currently unknown whether any of these modifications regulates mitochondrial ROS production. Future studies are likely to reveal new layers of complexity, including more specific oxidative post-translational modifications and more diverse mechanisms of mitochondrial ROS production and regulation, including how oxidative post-translational modifications regulate ROS generation by ETC in vivo, topology of O$_2^-$ production by ETC, and how O$_2^-$ production by ETC provokes the pro-oxidant activity of Krebs cycle enzymes such as aconitase. As this frontier of knowledge advances, continued attention to the fundamental processes of mitochondrial ROS production will be needed to understand cardiovascular disease pathogenesis with the goal of developing improved treatments to ameliorate disease.

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Disclosures

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