Mitochondrial Physiology of Cellular Redox Regulations

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Received October 31, 2023
Accepted February 24, 2024

Summary
Mitochondria (mt) represent the vital hub of the molecular physiology of the cell, being decision-makers in cell life/death and information signaling, including major redox regulations and redox signaling. Now we review recent advances in understanding mitochondrial redox homeostasis, including superoxide sources and H₂O₂ consumers, i.e., antioxidant mechanisms, as well as exemplar situations of physiological redox signaling, including the intramitochondrial one and mt-to-cytosol redox signals, which may be classified as acute and long-term signals. This review exemplifies the acute redox signals in hypoxic cell adaptation and upon insulin secretion in pancreatic β-cells. We also show how metabolic changes under these circumstances are linked to mitochondrial cristae narrowing at higher intensity of ATP synthesis. Also, we will discuss major redox buffers, namely the peroxiredoxin system, which may also promote redox signaling. We will point out that pathological thresholds exist, specific for each cell type, above which the superoxide sources exceed regular antioxidant capacity and the concomitant harmful processes of oxidative stress subsequently initiate etiology of numerous diseases. The redox signaling may be impaired when sunk in such excessive pro-oxidative state.

Key words
Mitochondrial superoxide formation • Redox regulations • Redox signaling • Pancreatic β-cells • β-oxidation • Peroxiredoxins

Mitochondrial reactive oxygen species (ROS) sources

Primary sources of mitochondrial superoxide
According to the classification of M. Brand [1-4], flavin (F) and ubiquinone (Q) containing binding sites, typically within structures of respiratory chain (RC) complexes, belong to the most critical superoxide formation sites in mitochondria [5-7], besides particular loci of dehydrogenases [1,2] (Fig. 1). Recent progress in understanding mechanisms involved in proton-coupled electron transfer via the RC and in resolving supercomplexes formation and single cristae architecture [7-9] then calls for reconsiderations of these mechanisms and more precise determination of superoxide formation sites within the given and already resolved protein structures.

A general requirement for superoxide (O₂•−; and its conjugated acid – hydroperoxyl radical, HO₂•, pKa 4.9) to be formed is a local retardation of the electron transfer or an enzyme reaction process so that intermediate radicals have enough lifetime to react with oxygen. These intermediates are typically semiquinone anion radical (Q•−; or semiquinol QH•) for Q sites and flavosemiquinone radical FMNH• for F sites. Thus, the flavin site on Complex I (termed Ip) can produce superoxide at a higher NADH/NAD⁺ ratio after the direct H⁻ transfer between NADH and FMN [10]. When an excessive electron cannot pass through the existing FeS chain within the Complex I matrix arm, the NAD⁺ binding is interrupted, and the pairing of FMN⁻ and NADH form FMNH⁺. At lower NADH, indeed, NAD⁺ can pair with FMNH⁺, and superoxide cannot be formed [11].
Fig. 1. Sites of superoxide formation in mitochondria. Schema depicts locations for the identified sites of superoxide formation, acting at the ~280 mV redox potential of the NADH/NAD+ iso-potential pool (index F, flavin; dark blue capitalized fonts) and sites acting at the ~20 mV redox potential of the ubiquinol/ubiquinone (QH2/Q) iso-potential pool (index Q; red capitalized fonts), according to the Brand’s nomenclature introduced by Martin Brand [1]. Thus the major sites I F, IQ and IIIQo exists for Complex I and III, respectively; at certain circumstances also Complex II/succinate dehydrogenase forms superoxide at site II F; and sites for probable superoxide formation by dehydrogenases (DH) are shown (G Q for α-glycerolphosphate dehydrogenase, GPDH; D Q for dihydroorotate DH; AF for 2-oxoadipated DH; B F for branched-chain ketoacid DH, BCKDH; O F for 2-oxoglutarate DH, 2OGDH; P F for pyruvate DH. In case of acylCoA dehydrogenases acting in fatty acid β-oxidation, two electron transfer flavoproteins (ETF) are required to transfer electrons to the membrane-attached ETF:ubiquionone oxidoraductase (ETF:QOR, depicted by its structure) containing a site E F (though a site E Q also potentially exists). The scheme also depicts a situation when retardation fo cytochrome c shuttling induces superoxide formation on site IIIQo; as well as attenuation of superoxide formation by uncoupling proteins (UCP) based on the fatty acid -cycling mechanism [119]. Finally, formed superoxide is converted to H2O2 by MnSOD in the matrix or by CuZnSOD within the intermembrane space. H2O2 may readily penetrate to the cell cytosol (for special relations of such diffusion, see Reference [7].

Complex I, as an H+-pumping NADH:quinone oxidoreductase, possesses a Q-tunnel structure, where an ongoing inhibition by a product (ubiquinol, QH2) can form superoxide at the phenomenologically defined site IQ [1] (Fig. 1). This typically occurs when the whole Complex I runs backward during so-called reversed electron transfer (RET). Disputes still exist whether under conditions of e.g. reperfusion after ischemic accumulation of succinate, superoxide is formed at I0 or I1 site [12,13]. Due to the suppression of the electron leak to oxygen at site I0 by a specific antioxidant S1QEL, the site I0 is more plausible to act in RET-derived superoxide formation [1].

We have also revealed that the maximum superoxide was formed only when electron transport and H+ pumping were retarded [14,15]. H+ pumping may be attenuated by a high electrochemical gradient of protons established at the inner mitochondrial membrane (IMM), termed protonmotive force, Δp (when expressed in mV units) [16-18]. In pathologies this can be induced by mutations of the ND5 subunit (or other mitochondrion-encoded subunits) of the Complex I membrane arm.

Complex III, a ubiquinol-cytochrome c reductase, contributes to O2•− generation by autoxidation of the
semiquinone anion radical ($Q^*$) within the so-called Q cycle [1,5,7,19,20], while it releases $O_2^{-*}$ about equally to both sides of IMM [20,21]. Typically, when cytochrome c turnover is delayed for some reason, then a feedback inhibition of the Q-cycle within CIII is induced, causing the superoxide formation at the Complex III site, termed $\text{III}_{QO}$ (o for outer, which is located in proximity to the intracristal space, [1]) (Fig. 1). This is because of the increased lifetime of $QH^+$ and oxygen diffusion into this site [22]. In vivo, a physiological delay of the Q-cycle occurs at hypoxia or pathological one with specific mutations in Complex IV [23]. Retardation of the cytochrome c cycling automatically exists at the escape of cytochrome c from the cristae lumen during initiation of mitochondria-related apoptosis.

Also, Complex II (succinate dehydrogenase, SDH) may form superoxide under specific conditions but not at high succinate concentrations [24,25]. But superoxide is formed when the flavin site II$_F$ within the SDHA subunit is less occupied, such as when succinate concentrations approach to $K_m$ of 100-500 µM [26-28]. Pathologically, with the blocked SDHD subunit and hence interrupted electron transfer to Q, Complex II/SDH produces $H_2O_2$ (with a 70 % capacity) directly due to the ability of existing three FeS clusters to provide two-electron transfer to oxygen [29]. The 3Fe-4S cluster may also theoretically provide superoxide [30].

Evidence was also reported for superoxide formation within the dehydrogenase (DH) complexes in isolated mitochondria when excessive particular substrates for given DHs were used. Hence with excessive 2-oxoglutarate (2OG, 2-ketoglutarate) for OGDH, pyruvate for pyruvate dehydrogenase (PDH) and substrates of branched-chain 2-ketoacid (2-oxoacid) dehydrogenase (BCKDH) superoxide/$H_2O_2$ formation in skeletal muscle mitochondria was eightfold, fourfold, and twofold higher, respectively, than that one ascribed to the site II$_F$ [31]. Phenomenological sites were termed as site $O_F$, $P_F$, and $B_F$, respectively, but mechanisms and occurrence in vivo must be further investigated.

Also, isolated mitochondria respiring with glycerol-3-phosphate partly produced superoxide at site $G_0$ of the glycerol-3-phosphate dehydrogenase [4,26,32-34]. Analogously, dihydroorotate dehydrogenase was reported to form superoxide at side $D_0$ [1,4,33,35]. Moreover, ongoing fatty acid (FA) β-oxidation also produces superoxide. Its portion may originate from the site $E_F$ of the electron-transferring flavoprotein – ubiquinone oxido reductase (ETFQOR) [27] (Fig. 1).

### Superoxide dismutation into $H_2O_2$

Manganese superoxide dismutase (MnSOD or SOD2) is localized in the mitochondrial matrix, whereas CuZnSOD (SOD1) localizes to the mitochondrial intermembrane space, besides residing in the cell cytosol. MnSOD dismutates the majority of superoxide released to the matrix into $H_2O_2$. It is not known whether CuZnSOD is exclusively located between the outer mitochondrial membrane (OMM) and the inner boundary membrane (IBM, the unfolded part of IMM) in the so-called intermembrane space peripheral (IMSp), or whether in also resides in the intracristal space (ICS). In the latter case, it could more effectively convert superoxide therein [36], namely the part released from the site $\text{III}_{QO}$.

MnSOD activity was found to be regulated. Rather fast posttranslational modifications (PTMs) were reported for NAD+-dependent sirtuin-3- (SIRT3-) mediated deacetylation of MnSOD, activating the enzyme [37-40]. These results and derived conclusions need to be validated since not always a large population of MnSOD molecules in the matrix is acetylated/deacetylated. Only a fraction is affected, therefore, one should expect that this particular MnSOD molecule fraction is activated and, for example, only the resulting fraction of $H_2O_2$ may thus participate in intramitochondrial redox signaling or a weak redox signal directed to the cytosol. MnSOD regulations rather proceeded within an hour-time frame and could be regarded as chronic regulations [41-45]. Thus a subtle change in $H_2O_2$ release, accumulated during sufficient time, can be effective.

#### Ultrastructure of mitochondrion vs. $H_2O_2$ diffusion to the cell cytosol

We have published several reviews on how mitochondrial morphology and ultrastructure affect the diffusion of $H_2O_2$ into the cell cytosol and/or other organelles and up to the plasma membrane or even diffusion into the extracellular space [5-9,46-48]. Hence, let’s briefly summarize 3D architecture of mitochondria (Fig. 2). Actually the plural is adequate for isolated fragments of the original mitochondrial network, the mitochondrion [6-8,49-51]. Note that such a network also exists in skeletal muscle and the heart [52,53]. Small fragments are constantly separated from the main mitochondrial network by fission machinery (Fig. 2E), while at the same time, fragments join the main network by fusion (Fig. 2D), which is aided by the pro-fusion proteins [54,55]. This process is important, since
Fig. 2. Cristae in mitochondrial network of pancreatic islet β-cells. (A) An exemplar 3D image of crista lamellae within a 4-µm segment of the mitochondrial tubule, obtained by the focused ion beam/scanning electron microscopy (FIB/SEM); (B) detail of the single crista lamella from A). (C) Comparison with the 3D image of a single crista with resolved ATP-synthase dimers at the lamella edge, adapted from Ref. [67]. Also, structures of respiratory chain supercomplexes are visible on a lamella flank, which represents the crista membrane lipid bilayer leaflet oriented toward the matrix. The distances are marked for a minimum path of proton diffusion (mild blue arrows), providing a substantial coupling between the respiratory chain proton pumping and the ATP-synthase. The purple arrow indicates a shuttling of cytochrome c at the supercomplex surface. The distances are also marked for a short ubiquinol QH2 (or ubiquionone Q) diffusion between Complex I (C1) and Complex III (CIII) around supercomplexes (red arrow) and a much longer diffusion path from Complex II (red arrow) to C11 or from oxidoreductases and dehydrogenases to C11 (dashed red arrows). Inside the broken portion of crista lamella at the inner (intracristal space) surface, a QH2-diffusion path is indicated by orange arrows. This path must be followed by the flip across the membrane to C11. (D, E) Mitochondrial reticular network in pancreatic islet β-cells of Wistar (D) and diabetic Goto-Kakizaki rats (E) in 3D images adopted from Ref. [49]. Note the nearly continuous mitochondrial network in intact β-cells (D), but the fragmented network in diabetic β-cells (E).

MITochondrial-specific autophagy, termed mitophagy, eliminates those fragments that do not possess a sufficient IMM membrane potential (or Δp) as a result of local predominance of the mutated mt-DNA-encoded RC and ATP synthase subunits.

The mitochondrial tubular network possesses a complex ultrastructural organization of mt cristae, i.e., rich invaginations of IMM from the IBM, which shrink or inflate according to the metabolic performance or other reasons [7] and might exhibit dynamics in a short
time scale [56]. To understand mitochondrial compartments, one must recognize their three-dimensional (3D) architecture. The cristae form rather lamellae with bottleneck connections to the IBM, where ICS meets IMSp (Fig. 2A, B). The mitochondrial cristae organization system (MICOS) complex is attached to the OMM SAM complex and thus forms crista junctions (CJs) [57-59] around the crista outlets [7,60]. Note that at the edges of single crista lamella, the ATP-synthase dimers form rows or arrays [61-67] (Fig. 2C), the dynamic of which may also affect the cristae morphology [68-70]. Small MICOS subunits may intercalate between ATP-synthase dimers, such as Mic10, bound to the ATP-synthase membrane subunit e [71]; or Mic27 [72]. 

RC supercomplexes (typically C1 CH1; CIV1 [73-75] then reside at flanks of crista lamellae [61,67] (Fig. 2C). Just below CJs (crista outlets) other cristae-shaping proteins reside within cristae membranes (CM) facing ICS (crista lumen), such as various oligomers of OPA1 [76,77] or even filaments of OPA1 ortholog MGM1 [78]; and scaffolding proteins prohibitins, forming hetero-oligomeric 20-27 nm rings [79]. Positive curvature of 90° bends of the crista outlets, when IBM meets CM, is provided by oligomers of MICOS subunit MIC10 [80,81], while the negative curvature of crista lamellae is established by FAM92A1 protein, which binds cardiolipin and phosphatidylinositol 4,5-bisphosphate [82]. 

The rich lamellar cristae organization affects H2O2 diffusion into the cytosol [6]. If even H2O2 released into ICS (crista lumen) diffuses across the crista membrane, it will still reach the mt matrix in ~99% of CM surface. Only at the proximity of CJs the ICS-located H2O2 might escape into the IMSp, across CM or IBM or through the crista outlet; and subsequently to the cytosol via the OMM. So, taking into account the mitochondrion ultrastructure, we see the limitations of H2O2 diffusion from ICS to the cytosol. On the contrary, pancreatic β-cells exhibit a less abundant glutathione/GRX system [6,18,86-88]. 

Glutathione is present in a reduced (GSH) or oxidized (GSSG, glutathione disulfide) form. Glutathione reductase (GRX; EC 1.8.1.7) catalyzes the NADPH-dependent reduction of GSSG to GSH [84], and hence oxidized glutathione is regenerated. Glutathione provides/GRX a major mt matrix redox buffer in numerous cells [85]. On the contrary, pancreatic β-cells exhibit a less abundant glutathione/GRX system [6,18,86-88]. 

Mitochondrial redox buffers and/or antioxidant systems

Mitochondrial glutathione reductase & glutathione peroxidase system

Redox buffers and antioxidant enzymes detoxify the produced ROS and may exert specific roles in redox signaling. Similarly to the cell cytosol, in addition to small antioxidant molecules such as vitamin E (α-tocopherol), ascorbate, and uric acid, enzyme systems of glutathione peroxidase (GPX) and peroxiredoxin represent the most critical intracellular antioxidants and a primary defense system. Catalase is absent in mitochondria except in the heart [5,9]. 

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Mitochondrial vs. cytosolic peroxiredoxin system

Peroxiredoxins (PRDX) are hydroperoxide reductases, either of the 2-Cys type (cytosolic peroxiredoxins PRDX1 and PRDX2 (Fig. 3); PRDX3 residing in the mt matrix; and PRDX4 of the endoplasmic reticulum) or 1-Cys type (PRDX6) [92-98]. The second mitochondrial PRDX, PRDX5, also contains two narrowing of cristae was observed after dimethyl-2-oxoglutarate addition to hypoxia-preadapted HEPG2 cells [63] or upon glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells, i.e., when high glucose was set [46].
Possible modes of peroxiredoxin participation in redox signaling. Possible ways of redox signal spreading from vicinity of the outer mitochondrial membrane (OMM) to the plasma membrane – from left to right: i) direct superoxide diffusion (range only in OMM proximity); ii) direct H$_2$O$_2$ diffusion; iii) peroxiredoxin-mediated redox signal transfer, including diffusion of peroxiredoxin decamers allowed by the flood-gate model mechanism; iv) hypothetical redox relay via an array of peroxiredoxins. Note, that according to the flood-gate model, H$_2$O$_2$ oxidizes PRDX to higher states than a sulfenic state, allowing distant decamers in a S-S state to migrate to the target and exchange the two target sulfhydryls for PRDX S-S bridge. In (iv) the target is the PRDX itself. Left inset: color coding of PRDX monomers: green – PRDX S-S bridge; the very light green – basic reduced state (sulfhydryl and thiolate anionic form); the light green – the first-degree of oxidation, i.e., sulfenic state; yellow – oxidation into the second degree, i.e., sulfinic state; orange – oxidation into the third degree, irreversible, sulfonic state. Note, thioredoxin (TRX) converts either disassembled S-S state dodecamers to the basic state; and, together with sulfiredoxin (SRX), TRX regenerates PRDX in sulfenic state into the sulfenyl state.

cysteines in the monomer [99] but allows an atypical mechanism, while forming the intra-subunit S-S bridge within the single monomeric subunit [92-96,98]. However, PRDX5 is located also in the cytosol and peroxisomes and prefers lipid peroxides and peroxynitrite over H$_2$O$_2$. Artificial PRDX5 expression in IMSp attenuated hypoxic transcriptome reprogramming [100] and cancerogenesis [101].

PRDX6 is a 1-cys-PRDX, which can also be recruited to mitochondria (probably to OMM) [102-104]. PRDX6 forms only homodimers, cannot form disulfide bonds, and is not reduced by sulfanyl reductase (SRX). PRDX6 reduces oxidized phospholipids. The sulfenic moiety of PRDX6 is subsequently reduced with GSH/GRX system but not with thioredoxins. PRDX6 also exerts Ca$^{2+}$-independent phospholipase A2 activity.

Cytosolic peroxiredoxins are decameric, containing five homodimers. Mitochondrial PRDX3 is dodecameric, consisting of six homodimers. Thus, PRDX1, PRDX2, and PRDX3 form a toroid (doughnut-like) structure of five (six) homodimers, which can split from the toroid in an unstable disulfide conformation (see below). Such mechanism allows disulfide regeneration.

**Peroxiredoxin catalytic cycle**

PRDX monomers of the 2-Cys type contain the peroxidatic cysteine, $C_P$ and the resolving cysteine, $C_R$. After reaction with H$_2$O$_2$, the peroxidatic cysteine $C_P$ of the first monomer within a homodimer forms an inter-subunit disulfide bond with the resolving cysteine $C_R$ of the second homodimer subunit [92-98]. As an
intermediate, sulfenic acid (R-SOH) is first formed by two-electron reversible oxidation of the CP. Subsequently, the disulfide (S-S bridge) between CP and CR is formed. Interestingly, the PRDX ring is destabilized when such disulfide bonds are formed [92,94], thus allowing homodimers (monomers) to interact with their regenerating enzyme systems, completing the cycle. Such regeneration is catalyzed either by a couple of thioredoxin (TRX) plus NADPH-dependent TRX reductase (TRXR) [105] or by glutathione (GSH)/glutaredoxin (GRX) [92-98]. The disulfide bonds of homodimers are thus converted back to two cysteines. PRDXs react with H$_2$O$_2$ faster than other peroxidases (catalases and GPX); hence they outcompete them and serve as the primary regulators of cytosolic H$_2$O$_2$ and in specific tissues also of the mt matrix H$_2$O$_2$. The latter is valid for pancreatic β-cells. Therefore PRDXs have been considered major players in cancerogenesis [93,97] and are promising targets for therapies of cardiovascular [106] or neurodegenerative diseases [107] and for defense against oxidative stress in pancreatic β-cells [108].

**Peroxiredoxins enable redox signaling**

There are two other unique PRDX properties, making them essential players in the redox homeostasis regulations and even redox signaling. The first such property is the formation of stacks of decamers/dodecamers, thus establishing high molecular weight complexes (HMW), which can even form filaments with chaperone function [109]. Since this formation happens only with PRDXs oxidized into higher oxidation state (sulfinyl and sulfonyl), the HMW formation effectively withdraws PRDX molecules from their entire population. This instantly leads to a higher local H$_2$O$_2$ concentration in the HMW loci.

The second property lies in the specific interaction of PRDXs with other proteins containing the two proximal cysteines, which enables their direct redox regulation (targeting the redox signal). The PRDX disulfides (S-S bridges) oxidize those proximal cysteines of the target protein into the S-S bridge between them, while PRDX homodimer become reduced back into two cysteines, CP and CR.

Indeed, when sulfinyls of PRDX1,2 and PRDX3 are oxidized into higher oxidized states, i.e., sulfinyls or sulfonyls, HMW complexes are formed [109]. The sulfinyls within HMW complexes or filaments can still be slowly reduced back to cysteines after their reduction by the SRX system. Mitochondrial SRXs were reported to act even in the transfer of circadian rhythms to the mt matrix. This is enabled by periodically enhanced SRX expression intermittent with enhanced SRX degradation by LON-protease under control of the clock genes in the adrenal gland, brown adipose tissue and heart [109,119,120]. It should be further investigated whether such elegant circadian regulation of the mitochondrial matrix redox homeostasis exists in pancreatic β-cells.
Mild uncoupling attenuates mitochondrial ROS generation at intact mtDNA

Oxidative phosphorylation (OXPHOS) represents an ATP synthesis by the mt ATP-synthase (Complex V), which is driven by the protonmotive force, $\Delta p$. $\Delta p$ is formed by the respiratory chain $H^+$ pumping at Complex I, III, and IV [7,16-18,119]. The IMM domain (membrane domain) of the ATP-synthase ($F_0ATPase$) consumes an adequate $\Delta p$ portion in a state, historically termed state-3, for isolated mitochondria with an ADP excess. In vivo, cellular respiration is governed by the metabolic state and/or availability of substrates. Hence, a finely tuned spectrum of various states-3 can be established, depending on the substrate load (e.g., increasing glucose). A state-4, is then given by zero ATP synthesis, when zero $H^+$ backflux via the $F_0ATPase$ exists, while respiration and $H^+$ pumping are given by so-called $H^+$ leak, mediated by mitochondrial carrier proteins, as their side-function and by the native $H^+$ permeability of IMM. Since $\Delta p$ exists predominantly in the form of $\Psi_m$ (IMM electrical potential), $\Psi_m$ is maximum at state-4 with the maximum substrate load.

Besides other proteins, such as the ADP/ATP carrier, $\Delta p$ dissipation by a protonophoric short-circuit, termed uncoupling, can be physiologically provided by mitochondrial uncoupling proteins (UCPs) [119], frequently in synergy with mitochondrial phospholipases cleaving nascent fatty acids [120-123]. A mild uncoupling exists when carrier-mediated protonophore activity plus the native IMM $H^+$ leak do not overwhelm the $F_0ATPase$ protonophoric activity, and hence, ATP synthesis still takes place. This contrasts to a complete uncoupling when $\Delta p$ approaches zero, such as established by agents termed uncouplers. The mild uncoupling is able to decrease mitochondrial $O_2^••$ formation at Complex I [62,63] and Complex III [124]. In cell types where such mitochondrial ROS source predominates, even redox homeostasis in the cytosol may be more pro-oxidant. However, oxidative stress originating from irreversible changes, such as stress due to mutated subunits encoded by mitochondrial DNA (mtDNA), cannot be counteracted by mild uncoupling [62].

Previously, an antioxidant role for UCP2 has been demonstrated in vivo [123,125,126]. For example, Duval et al. [127] have shown that UCP2-mediated uncoupling in endothelial cells is able to decrease extracellular ROS in co-incubated low-density-lipoproteins (LDL). Mice with deleted LDL receptors exhibited extensive diet-induced atherosclerotic plaques when they received bone marrow transplants from UCP2 (+/+) mice [128]. We have also demonstrated that UCP2 function suppresses mitochondrial superoxide production in vitro [121,123,129,130].

Physiological redox signaling vs. oxidative stress

Oxidative stress

In principle, there exists no net oxidative stress without the other consequences, such as proteinaceous stress due to the disrupted turnover of intact proteins, concomitantly impaired autophagy and/or mitochondria-specific autophagy, i.e., mitophagy, or without the endoplasmic reticulum stress. Oxidative stress cannot be separated from the possible initiation of apoptosis, ferroptosis, or other forms of the cell death, as well as from impaired mitochondrial biogenesis. Moreover, all these phenomena are projected to an abnormal mt network morphology, frequently also to an abnormal cristae morphology (e.g., apoptosis). Redox-sensitive transcriptomic reprogramming sets altered metabolism and changes in epigenetics, which may further accelerate pathogenesis. In addition, the internal causes should be distinguished from the external ones, such as macrophage attacks and other immune system stimuli. That is why oxidative-stress-related pathologies must always be analyzed in a complex way, and frequently it is difficult to establish the primary cause. The reason is that under oxidative stress, cellular constituents are oxidized, i.e., covalently modified, deteriorating function and/or quality with serious consequences. To avoid the encyclopedic description, next, we will only briefly describe the oxidative stress of mitochondrial origin and how we can distinguish it from the redox signaling.

Moreover, recently, mitochondria are regarded as signaling organelles when signals of different origins are produced, not only the redox signals [55,130,131]. The evoked signals affects not only cells and tissues but also the systemic levels of the organism. The latter exists with the metabokin/mitokine signaling [133-135], mt-nuclear crosstalk [136-139], and mt-initiated epigenome remodeling [140-143].

Oxidative stress of mitochondrial origin

Theoretically, when an overly excessive superoxide/H$_2$O$_2$ formation exceeds the mitochondrial redox buffering, i.e., antioxidant capacity in the
mitochondrial redox signaling at hypoxia

One would not expect increasing ROS with a lowering oxygen. This paradox has been investigated, and mitochondrial contribution to oxygen sensing and hypoxic transcriptome reprogramming is still debated [159,160]. The central cytosolic mechanism of oxygen sensing is based on prolyl hydroxylases (PHD1 to 3, or Egl nine homolog 1 proteins, GLNs)[159,161-163], which catalyze hydroxylation of hypoxia-induced factor HIF-1α, -2α or -3α in a ferrous iron- (FeII) plus 2OG- plus O2-dependent manner. The resulting hydroxylation promotes its constant proteasome degradation after ubiquitination by pVHL ubiquitin ligase (Von Hippel-Lindau tumor suppressor protein) [164-166]. Therefore, by decreasing O2, by lowering 2OG and by oxidation of FeII to FeIII due to increasing cytosolic ROS, PHDs are inhibited, and HIF-1α stabilization occurs. Also, another O2- and 2OG-dependent dioxygenase, termed factor inhibiting HIF (FIH), hydroxylates HIFα, but at asparagines. This blocks the binding of the coactivators CBP (CREB-binding protein) and p300, and thereby disables HIF-1-mediated transcription. Upon hypoxia, both PHD and FIH are inhibited; hence, HIF-α is stabilized and binds HIF-β plus coactivators VBP and p300, which allows activation of transcription of >400 genes [167-172]. In this way, the HIF system is activated, resulting in transcriptome reprogramming important in cancerogenesis and numerous physiological and pathological situations.

Since both PHD and FIH are affected by ROS, both can be targets of redox signaling. Various ROS may oxidize FeII of PHD to FeIII [173]. Still, also reactive cysteines were recognized in PHD2, which, after oxidation, inactivate the enzyme (probably inactive PHD homodimers are formed due to S-S bridges) and initiate HIF-response upon oxidation [174-176]. Cytosolic peroxiredoxins may also be involved. In any case, PHDs sense oxygen independently of mitochondria, however, mitochondrial metabolism and mt redox signaling may also independently participate. Since PHDs are also inhibited by the lack of 2OG-related substrates, such as fumarate, succinate, malate isocitrate, and lactate [177,178], suppression of mitochondrial metabolism may stimulate HIF system.

Moreover, participation of mt redox signaling linked to HIF-1α stabilization was suggested by the ΔΨm
restoration, which returned a higher mt superoxide formation in cells with deleted mtDNA polymerase when respiration and hence Krebs cycle turnover was largely abolished [179]. After an instant hypoxia switch-on, a hypoxic burst of mt matrix superoxide release was observed [180] but delayed by several hours [181, 182]. Other reports described an instant hypoxic ROS burst in endothelial, HeLa, and HK2 cells [183]. Originally, the Complex III site III\textsubscript{OX} was considered as the superoxide source for the mt hypoxic ROS burst [180, 184-188]. A similar hypoxic mt ROS burst was also detected for normoxic HIF activation [189]. The emanated mt H\textsubscript{2}O\textsubscript{2} to the cytosol was suggested to oxidize Fe\textsuperscript{II} in PHDs. When certain Complex III subunits, such as Rieske iron-sulfur protein [190] or others were ablated, HIF-1\textalpha was stabilized [180], unlike in anoxia [187]. Moreover, suppressors of site III\textsubscript{OX} electron leak (S3QELs) prevented the HIF response [34]. The key evidence for mt redox signal participation in HIF-system signaling was provided by PRDX5 overexpression in the mt intermembrane space, which abolished HIF-1\textalpha stabilization [100].

**HIF strikes back on mitochondria**

The chicken-and-egg problem of the steady-state established upon HIF-signaling can be solved by precisely time-resolved events. This is because the execution of HIF-mediated transcriptome reprogramming affects redox homeostasis, which is then different than that one allowing HIF-system initiation. Indeed, HIF activates transcription for the expression of proteins, decreasing ROS formation or scavenging ROS [171].

**Mitochondrial cristae inflate with dormant ATP synthesis in hypoxic cells and shrink with its restoration**

We have also encountered that mitochondrial cristae inflate after adaptation of HepG2 cells to hypoxia [60] (Fig. 4). It is recognized as cristae widening in transmission electron microscopic (TEM) images (Fig. 4A) and as inflation (widening in 2D projections) of 3D super-resolution images of mitochondrial cristae stained with Eos-Lactamase-\beta (Fig. 4B, D). Due to the HIF transcriptome reprogramming, hypoxic HepG2 cells exhibited a low-intensity (dormant) ATP synthesis and respiration [60, 182]. Partial degradation of mitofilin/MIC60 protein led to the decrease of cristae junctions and the widening of cristae outlets from the inflated cristae to the intermembrane space [60, 63].

In contrast, after addition of respiratory substrate to the hypoxia-adapted HepG2 cells, a sudden narrowing of cristae in 2D projections (shrinkage of cristae lamellae in a space) resulted from the restored respiration and ATP-synthesis [63, 188] (Fig. 4B). We have observed similar changes in rat pancreatic \beta-cells, INS-1E, after the addition of a substrate, i.e. glucose (which stimulates secretion of insulin) [46]. This observation led us to a hypothesis assuming that strengthening and ordering the ATP-synthase dimers at the crista lamellar edges leads to sharpening of these edges and that the two lamellae flanks mechanistically come close together [46, 63]. When metabolic conditions and signaling allow disordering of the ATP-synthase dimers at the crista lamellar edges, this allows a more flat edge, which mechanistically puts apart the two lamellae flanks of the crista, resulting in cristae inflation [7, 46, 63].

However, considering that individual mechanistic tension within the single crista is responsible for the lamella inflation and shrinkage seems insufficient. Hence, recently, we came up with a novel hypothesis [7], which may be valid simultaneously, that the osmotic forces are the real engines of the crista lamellae inflation and shrinkage (Fig. 4E). The hypothesis expects that at low ATP-synthesis and low \Delta\text{p}, ion fluxes allow the salt to be extruded from the matrix (cations such as K\textsuperscript{+} and Na\textsuperscript{+} and anions such as phosphate and Krebs cycle intermediates). After a switch-on of the ATP synthesis and before the built-up of the high ATP concentration, i.e., at the beginning of cristae morphology changes, the open mitochondrial ATP-sensitive K\textsuperscript{+} channel (mtK\textsubscript{ATP}) allows an influx of K\textsuperscript{+} from the intracristal space to the matrix. Since simultaneous phosphate uptake to the matrix diminishes a salt content in the ICS and enriches it in the matrix, water uptake to the matrix occurs concomitantly to the salt influx. As a result, this osmotic force shrinks the ICS. However, to prevent an infinite cristae shrinkage, a built-up of ATP closes the mtK\textsubscript{ATP}, stopping salt leakage from the ICS and water transport to the matrix. Also, mt K\textsuperscript{+}/H\textsuperscript{+} and Na\textsuperscript{+}/H\textsuperscript{+} antiporters, being driven by \Delta\text{p}, prevent the infinite shrinkage of cristae.

We have already obtained the first evidence supporting the relevance of the osmotic hypothesis of cristae morphology changes. In hypoxia-adapted HepG2 cells where the addition of dimethyl-2-oxoglutarate initiated respiration and cristae shrinkage, glibenclamide, an inhibitor of the mtK\textsubscript{ATP}, blocked such shrinkage [60] (Fig. 4C). Further investigations are required to reveal whether the mechanistic or osmotic hypothesis is relevant, or whether both are relevant; as well as to describe possible regulations which transfer metabolic changes to the activation of relevant proteins which initiate cristae morphology changes.
Fig. 4. Hypoxic cristae inflation, its reversal at restored respiration and ATP synthesis and possible osmotic mechanism involving mitochondrial ATP-sensitive K⁺ channel. (A) Illustration of mitochondrial cristae widening after adaptation of HepG2 cells to hypoxia in transmission electron microscopic (TEM) sections (adapted from Ref. [60]). (B-D) Mitochondrial cristae widening indicated by the Eos-Lactamase-β 3D-superresolution fluorescence microscopy (adapted from Ref. [60]). Since Lactamase-β stains the intracristal space, an apparent width of its fluorescence contour on 2D sections of 3D images (panels Da for normoxia, Db, Dc for hypoxia) semi-quantifies the volume of crista lamellae. Thus, panel (B) illustrates on histograms of such width the existence of bulky cristae after hypoxic adaptation and their shrinkage after the addition of respiratory substrate, dimethyl-2-oxoglutarate. In contrast, panel (C) shows inhibition of the cristae lamellae shrinkage by glibenclamide, a known inhibitor of ATP-sensitive K⁺ channel, including its mitochondrial form (mtKATP). (E) Osmotic hypothesis for participation of mtKATP in cristae shrinkage. For an explanation, see text (Chapter 3.6).
Pancreatic β-cells as an exemplar mitochondrial redox system

Oxidative phosphorylation and NADPH-oxidase-4-mediated redox signaling as essential determinant of glucose-stimulated insulin secretion

Previously, an effect of antioxidants upon exhausted glutathione in pancreatic β-cells has been reported as an unspecified link between glucose-stimulated insulin secretion (GSIS) and external H$_2$O$_2$ [191]. Recently, it has been established that the essential conditions for GSIS involve the elevated OXPHOS and consequent ATP/ADP elevation in the peri-plasma membrane space [87,192,193] plus essential redox signaling, mediated by the NADPH-oxidase 4 (NOX4) [18,88,194] (Fig. 5). Together with ATP, the cytosolic redox (H$_2$O$_2$) signal closes the plasma membrane ATP-sensitive K$^+$ channels (K$_{ATP}$), together with the elevated ATP [18,88,194]. For a closing of the entire K$_{ATP}$ population and setting a threshold membrane potential to -50 mV, opening of other non-specific calcium channels (NSCCs, such as TRMP2 channels, [195]) or Cl$^-$ channels is required [87]. At -50 mV an intermittent opening of voltage-dependent Ca$^{2+}$ channels (Ca$_V$) is initiated, being instantly counteracted by voltage-dependent K$^+$ channels (K$_V$). This leads to a pulsatile Ca$^{2+}$ entry into the cytosol and in-phase exocytosis of insulin granule vesicles (IGV) [18,87].

Upon GSIS, an NADPH supply to the constitutively expressed NOX4 originates from the two enzymes of the pentose phosphate pathway (PPP), producing NADPH, i.e., glucose-6-phosphate dehydrogenase (G6PDH) 6-phosphogluconate dehydrogenase (6PGDH) [196]; plus from so-called (pyruvate redox) shuttles [18,88,193,197]. Interestingly, these shuttles do not allow synthesis of one NADH molecule in the mt matrix, but instead, NADPH is formed in the cytosol after a few transport steps and enzyme reactions [197]. As a result, production of superoxide released to the mt matrix is slowed down, mostly probably due to the decreased NADH/NAD$^+$ ratio affecting the Complex I superoxide formation site I$_F$ [7,197]. We have linked two redox shuttles with the decreasing superoxide formation upon GSIS, the pyruvate-malate shuttle and the pyruvate-isocitrate shuttle [197]. Other shuttles were also reported [193,198]. The pyruvate-malate shuttle is allowed by the pyruvate carboxylase (PC). Such a bypass of pyruvate dehydrogenase makes possible a reverse reaction of malate dehydrogenase (MDH2), consuming NADH. A concomitant malate export from the mt matrix enables the cytosolic malic enzyme (ME1) to convert malate into pyruvate while yielding NADPH. The pyruvate-isocitrate shuttle stems from a truncated Krebs cycle after citrate synthase so that isocitrate dehydrogenase 3 (IDH3) does not form NADH. Instead, matrix NADPH is converted by IDH2 together with 2-oxoglutarate (2OG) into isocitrate, allowing its export from the mt matrix and subsequent reaction of cytosolic IDH1, transforming cytosolic isocitrate back to 2OG and synthesizing NADPH. $^{13}$C-glutamine-assisted isotope tracing enabled to verify the existence of this redox shuttle [197,199].

Relative easy spread of redox changes in pancreatic β-cells is possible due to a rather weak antioxidant defense system and low capacity of redox buffers [200,201]. Such a delicate redox homeostasis is then disturbed by a rather weak insult. Expression and activity of antioxidant enzymes is low in rodent β-cells as compared to other organs [202].

Oxidative phosphorylation and mitochondrial redox signaling as essential determinant of branched-chain-ketoacid- and fatty-acid-stimulated insulin secretion

Insulin is also stimulated by other metabolites, collectively termed secretagogues [7,88] (Fig. 5). Thus, a leucine metabolite, keto-isocaproate (KIC) was demonstrated to stimulate insulin secretion, while its oxidation (termed commonly as β-like oxidation) provides both elevated ATP and redox (H$_2$O$_2$) signal [194]. The mitochondrial origin of this redox signal was suggested by the blockage of KIC-stimulated insulin secretion with mt-matrix-targeted antioxidant SkQ1 [194]. This excludes the previous hypothesis that leucine itself stimulate IGV exocytosis.

Also, free fatty acids (FAs) have been regarded to augment GSIS [203], meaning that insulin secretion in the presence of FAs required certain higher glucose concentrations [204-206]. Nevertheless, we and others have demonstrated that the net FA-stimulated insulin secretion (FASIS) exists [18,88,123,207-210], i.e., insulin secretion stimulated by FAs at low glucose concentration, subsequently spread up to the plasma membrane [9]. Previously, mitochondrial ROS resulting from the addition of monooleoyl-glycerol [211] have been suggested to modulate insulin secretion, and mt-derived
ROS were regarded as obligatory signals for insulin secretion [212].

FASIS is more complex than the KIC-stimulated insulin secretion (Fig. 5), since also metabotropic GPR40 receptors, residing presumably on the plasma membrane, sense FAs and initiate a complex downstream signaling. When this proceeds via Gaq11 heterotrimeric G-proteins, followed by the Ca^{2+}-dependent phospholipase-C-(PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol-3-phosphate (IP3), a non-metabolizable GPR40 agonist can stimulate insulin secretion even at low glucose and ATP (Jezek et al., unpublished). Indeed, PIP2 was known to stabilize the open K_{ATP}, hence its degradation by PLC-hydrolysis facilitates the K_{ATP} closure [213]. The reaction product DAG stimulates protein kinase-C (PKC) iso-enzymes, some of which phosphorylate TRPM4 and TRPM5 [214], which opens these channels, enabling them to activate Ca^{2+} channels, similarly to the TRPM2 action upon GSIS.

Moderately elevated cytosolic [Ca^{2+}] should be required for PLC activity, however, DAG could also originate from the so-called glycerol/FA cycle [203], if it exists at “fasting” glucose. Interestingly, novel PKCs (nPKCs) are activated by DAG alone, but not by Ca^{2+} [215]. Hence, GPR40-signaling to final targets via nPKCs, promoting IGV-exocytosis, could exist even at low glucose.

The other product IP3 acts in the Ca^{2+}-induced
Ca^{2+}\)-release from endoplasmic reticulum, enabled by the IP3-receptor (IP3R), functioning as a Ca^{2+} channel. Note also that in vivo, FASIS is not separated but acts in parallel with the signaling by monoacyl-glycerols (MAG) via the GPR119-Gs-PKA(EPAC2) pathway. The PKA and EPAC2 pathways also serve as a biased pathways for certain GPR40 agonists. The protein kinase A (PKA) phosphorylates glucose transporter GLUT2 to facilitate glucose entry. PKA also phosphorylates K<sub>ATP</sub> and C<sub>A</sub> to ease action potential triggering. The EPAC pathway acts similarly by phosphorylating TRPM2, releasing PIP2 from K<sub>ATP</sub> and affecting Rim2a interaction with SNARE proteins, thus facilitating IGV exocytosis [18,87,88].

Yet another phenomenon is concomitant to FASIS. Interestingly not the extracellular FAs, but FAs cleaved from the mt phospholipids by the redox-activated mt phospholipase A2, isoform γ (iPLA2γ) stimulate the GPR40 receptors [123]. Silencing of iPLA2γ led to a profound decrease of FASIS [123] despite the redox signaling up to the plasma membrane was not attenuated (Jabůrek et al., unpublished). There was a paradox encountered which has to be resolved. Due to the antioxidant synergy provided by a couple of iPLA2γ and uncoupling protein 2 (UCP2), the FA addition to pancreatic β-cells first attenuates mitochondrial superoxide formation released to the matrix. This mechanism exists since the redox-activated iPLA2γ provides nascent free FAs for UCP2 to initiate a mild uncoupling and thus reduce the mt superoxide formation [123]. However, when H<sub>2</sub>O<sub>2</sub> is monitored in the cell cytosol or extracellularly at the same time, it is elevated (MJ, unpublished data). This paradox could be speculatively explained by MnSOD activation or by the involvement of the peroxidrein system [9]. Indeed PRDX3 silencing in INS-1E cells partly inhibited FASIS (MJ, unpublished data).

**Future perspectives**

It is an experimental challenge to track or monitor acute redox signaling by observing changes in particular reactive oxygen species in a given compartment [9]. Any event has to be studied to reliably identify the source, the path, and the target of redox signaling. Tracking changes in the surrounding proteins, e.g., in cysteine oxidation, cannot frequently distinguish the real target from those collateral ones. Correlations must be found for the initiation of the signal with the initiation of the effect and particular molecular changes in the effector proteins. It has to be investigated, for example, whether the changes in mitochondrial cristae morphology are governed by certain redox signals. With already identified redox signals, such as NADPH-oxidase 4 essential participation in glucose-stimulated insulin secretion (GSIS), it must be further studied, whether a cytosol-targeted antioxidant therapy would not rather promote a certain harm. Strong artificial suppression of redox signals could inevitably suppress GSIS and even redox signals, which otherwise contribute to the fitness of pancreatic β-cells [194]. Thus, instead of healing, this would amplify symptoms of prediabetes. In contrast, mitochondria-targeted antioxidants would not harm physiological redox signaling (except that of oxoacids and fatty acids) and might avoid the premature oxidative stress in the matrix of β-cells at the prediabetes stage. In conclusion, future studies of redox signaling should answer not only the basic questions of molecular physiology, but will also lead to novel translational aspects.

**Conflict of Interest**

There is no conflict of interest.

**Acknowledgements**

This work has been supported by Grant Agency of the Czech Republic, grant No. 21-01205S and No. 24-10132S to PJ, 23-05798S to HE, 22-02203S to AD, 22-17173S to MJ and by the project National Institute for Research of Metabolic and Cardiovascular Diseases (Programme EXCELES, ID Project No. LX22NPO5104) – Funded by the European Union – Next Generation EU.

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