ANTIOXIDANTS & REDOX SIGNALING
Volume 37, Numbers 10–12, 2022

DOI: 10.1089/ars.2021.0274

FORUM REVIEW ARTICLE

Circadian Control of Mitochondria in Reactive Oxygen Species Homeostasis

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Abstract

Significance: Mitochondria produce most of the cellular ATP through the process of oxidative phosphorylation. Energy metabolism in the mitochondria is associated with the production of reactive oxygen species (ROS). Excessive ROS production leads to oxidative stress and compromises cellular physiology. Energy metabolism in the mitochondria depends on nutrient flux and cellular metabolic needs, which are in turn connected with the feeding/fasting cycle. In animals, the feeding/fasting cycle is controlled by the circadian clock that generates 24-h rhythms in behavior, metabolism, and signaling.

Recent Advances: Here, we discuss the role of the circadian clock and rhythms in mitochondria on ROS homeostasis. The circadian clock is involved in mitochondrial ROS production and detoxification through the control of nutrient flux and oxidation, uncoupling, antioxidant defense, and mitochondrial dynamics.

Critical Issues: Little is known on the molecular mechanisms of circadian control of mitochondrial functions. The circadian clock regulates the expression and activity of mitochondrial metabolic and antioxidant enzymes. The regulation involves a direct transcriptional control by Circadian Locomotor Output Cycles Kaput/brain and muscle ARNT-like 1(CLOCK/BMAL1), nuclear factor erythroid-2-related factor 2 (NRF2) transcriptional network, and sirtuin-dependent posttranslational protein modifications.

Future Perspectives: We hypothesize that the circadian clock orchestrates mitochondrial physiology to synchronize it with the feeding/fasting cycle. Circadian coordination of mitochondrial function couples energy metabolism with diets and contributes to antioxidant defense to prevent metabolic diseases and delay aging. *Antioxid. Redox Signal.* 37, 647–663.

Keywords: metabolism, oxidative stress, antioxidant defense, circadian rhythms, gene expression, caloric restriction, fasting, longevity

Introduction

ENERGY METABOLISM IS central for cellular homeostasis. Cells generate energy by oxidizing nutrients: carbohydrates, amino acids, and lipids. Nutrient oxidation occurs within different cellular compartments, and the mitochondria play a primary role in the generation of energy-rich ATP molecules through the process of oxidative phosphorylation. Side products of oxidative phosphorylation are reactive oxygen species (ROS) such as superoxide anion, generated when high-energy electrons are transferred to molecular oxygen.

Peroxisome is another cellular organelle that oxidizes long-chain, branch-chain, and dicarboxylic fatty acids. Oxidation in the peroxisome is coupled with generation of hydrogen peroxide (H_2O_2), another major cellular ROS. Thus, energy production in cells is associated with the

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generation of ROS in different cellular compartments. ROS and reactive nitrogen species (RNS) are important signaling molecules (29). They regulate multiple cellular processes such as transcription, secretion, and proliferation. However, due to their high chemical reactivity, uncontrolled ROS production causes oxidative damage to DNA, lipids, and proteins. This oxidative stress compromises the functions of biological macromolecules, which in turn affects cellular physiology, leads to cellular damage, and ultimately induces cell death (29).

Mounting evidence connects oxidative stress with cardiovascular diseases, cancer, diabetes, neurodegeneration, and accelerated aging (12, 29, 42, 153). To prevent these unwanted events, cells developed a highly sophisticated network of proteins and cofactors, together known as the cellular antioxidant system (29, 39). ROS are detoxified through a chain of reactions catalyzed by different enzymes. The efficient detoxification of ROS would require coordinated expression and/or activity of these enzymes and their cofactors. It was proposed that one of the functions of the circadian system is the orchestration of cellular oxidative stress response (74, 98, 114).

The circadian system is a network of circadian clocks that are present in every tissue and cell in most organisms (21, 25, 67). Cellular molecular oscillators, formed as transcriptional/translational feedback loops, generate 24-h rhythms in gene expression and signaling (67, 101). Cellular rhythms integrate with organism rhythms in metabolism, physiology, and behavior. Circadian disruption in humans is associated with the development of cardiometabolic diseases, cancer, and neurodegeneration (45, 98, 124).

Interestingly, oxidative stress is a contributing factor for the same diseases. Several animal models of circadian clock deficiency display chronic oxidative stress and defective antioxidant defense (8, 72, 99, 117, 158). Reduced mitochondrial volume, diminished respiration rate, and increased oxidative damage were found in circadian clock mutants (4, 95, 99, 131, 162), which impacted their physiology. The circadian clock is implicated in coordinating the antioxidant defense through both transcriptional-dependent and transcriptional-independent mechanisms (48). The circadian clock regulates rhythms in the expression and activity of nuclear factor erythroid-2-related factor 2 (NRF2), which is a leucine zipper transcription factor and master regulator of antioxidant defense that drives the transcription of major antioxidant enzymes.

The circadian clock also regulates rhythmic production of melatonin, a recognized ROS scavenger (48). Transcription-independent circadian mechanisms of redox control also exist in red blood cells, and they are linked with rhythmic oxidation/reduction of peroxiredoxin (PRDX) proteins (30).

The role of the circadian clock in ROS detoxification is confirmed by multiple studies. Whether the circadian clock is involved in the control of ROS production is not fully resolved. Energy metabolism is linked with the feeding/fasting cycle and is under the control of the circadian clock (1, 49, 154, 155). Mitochondria explore several strategies to maintain ROS homeostasis. Here, we discuss the circadian clock as a master regulator of ROS and mitochondria physiology in the context of ROS.

Circadian Control of Mitochondrial ROS Production

ROS are generated in several cellular compartments: in peroxisomes during oxidation of fatty acids and degradation of xenobiotics, at cell plasmatic membrane by associated dehydrogenases, but the main site of cellular ROS production is the mitochondrion. Mitochondrial energy metabolism is tightly linked with ROS production (Fig. 1). Mitochondria are capable of oxidizing different classes of nutrients: pyruvate, amino acids, and fatty acids, which generate most of the cellular ATP. Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) molecules generated through glycolysis, β-oxidation, and TCA cycle will donate high-energy electrons to the mitochondrial electron transport chain (ETC).

The ETC is composed of four multisubunit transmembrane protein complexes (I–IV) and two diffusive carriers, ubiquinone and cytochrome C. Electron transport through the ETC and the free energy released from this process drives proton pumping from the mitochondrial matrix to the intermembrane space that creates an electrical gradient ($\Delta\Psi_m$) as well as a chemical gradient (ΔpH). The proton gradient is used to produce ATP molecules through a transmembrane ATP synthase. The oxidative phosphorylation mechanism is not 100% efficient, and naturally, some electrons leak out of the ETC system.

The major sites for electron escape are Complexes I and III. Complex II can generate ROS in the presence of $FADH_2$ (121). Complex IV is an endpoint of electron transfer, and whether complex IV is a direct source of ROS is unresolved. Escaped electrons interact with molecular oxygen and generate superoxide anion, from which other types of ROS such as H_2O_2 can be generated. High rates of electron transport create a high-proton motive force that causes the ETC to slow down because the protons are pumped against a stronger force. Slowing down the ETC results in electrons spending more time on complexes of the ETC, which increases their potential to escape. Mitochondria use different strategies to reduce electron escape from the ETC.

The transfer of electrons to ETC depends on NADH and $FADH_2$ supply (Fig. 1). NADH and $FADH_2$ are generated as a result of nutrient oxidation. The choice of the substrate for oxidation depends on the stage of the feeding/fasting cycle, with preferential carbohydrate oxidation during feeding and fatty acids during fasting (11). There are several recent reviews on circadian clock control of nutrient digestion and transport to the cell (45). The expression of enzymes involved in glycolysis, mitochondrial β-oxidation of fatty acids, and TCA cycle is highly rhythmic across the day and responds to the diets in a clock-dependent manner (88, 112, 115).

One of the potential mechanistic connections is the peroxisome proliferator-activated receptor (PPAR) network, which is a master regulator of fat oxidation and energy production. PPARs make up a family of transcription factors that include three isoforms, α, β/δ, and γ (17). These transcriptional factors belong to a larger superfamily of nuclear receptors that are activated upon binding their endogenous fatty acid ligands along with various industrial, pharmaceutical, and phytological

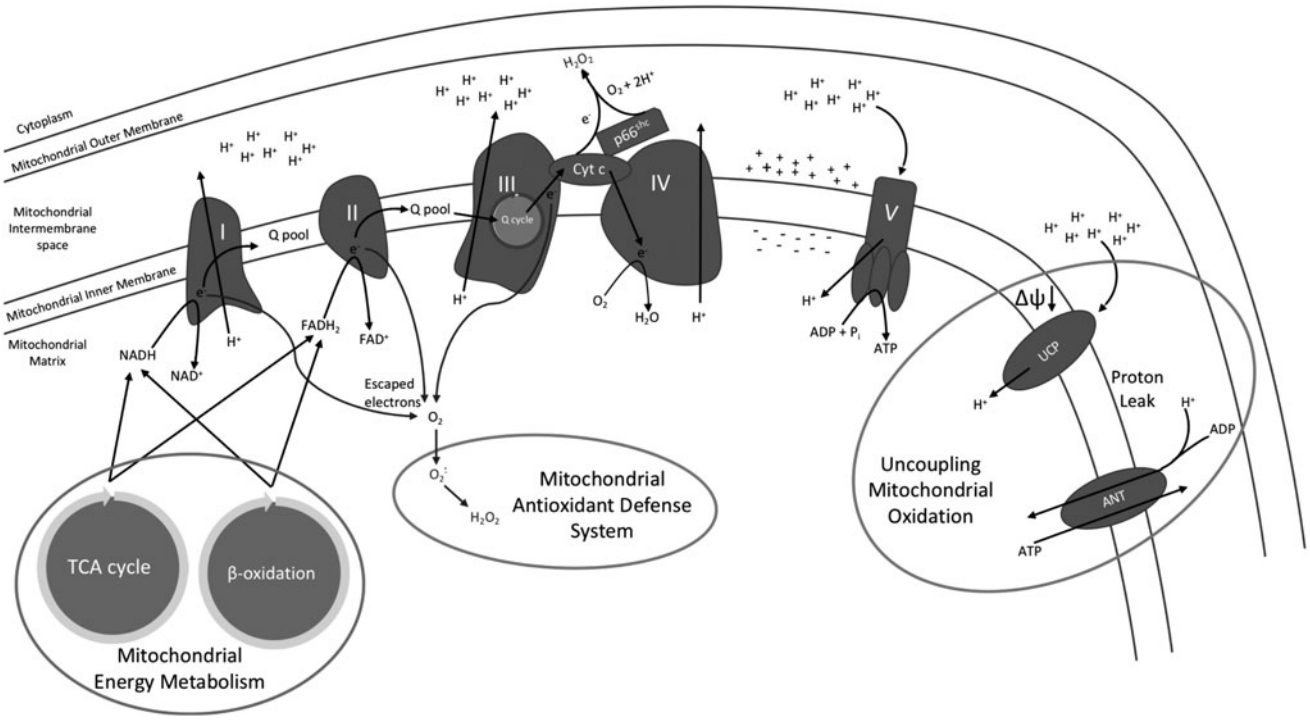

FIG. 1. Mitochondrial energy production and management linked with ROS. In the mitochondrial matrix, high-energy electron carrier molecules, including NAD^+ and FAD^+, are reduced during processes of energy metabolism, including the TCA cycle and β-oxidation. These high-energy molecules travel to complex I and II to be oxidized, which results in the reduction of ubiquinone (Q). Reduced Q, QH_2, will diffuse through the mitochondrial inner membrane to complex III for participation in the Q cycle, which oxidizes QH_2 and reduces Cyt C. Reduced cytochrome C will transfer electrons to molecular oxygen in complex IV to produce water. Some of the reduced cytochrome C will be oxidized by p66 to produce H_2O_2. During the process of electron transfer from NADH and $FADH_2$ through the electron transport chain to molecular oxygen, some electrons may be lost from the electron transport chain, which interact with molecular oxygen and produce superoxide anion, which is processed into H_2O_2 and then managed by the mitochondrial antioxidant defense system. Throughout the electron transfer chain, complexes I, III, and IV move protons from the matrix into the mitochondrial intermembrane space, which induces a chemical and electrical gradient that drives ATP synthase, complex V, and leads to the production of ATP while transferring protons down the gradient. In times of excess electrons being processed through the electron transfer chain, uncoupling of mitochondrial oxidation is necessary to alleviate production of ROS. This uncoupling process takes place through UCP along with ANT. Both proteins allow protons to leak back into the mitochondrial matrix, which reduces the magnitude of the chemical and electrical gradient and allows for electrons to move through the complexes with a lower chance of ROS production. ANT, adenine nucleotide translocator; Cyt C, cytochrome C; $FADH_2$, flavin adenine dinucleotide; H_2O_2, hydrogen peroxide; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; UCP, uncoupling proteins.

chemicals (17). Activated PPARs heterodimerize with retinoid X receptor and bind PPAR responsive elements to regulate transcription of target genes (17).

The three highly homologous isoforms are differentially expressed among tissues and elicit various cellular functions. All PPAR isoforms across tissues have demonstrated circadian rhythmicity in their expression, while the alpha and gamma isoforms display direct interaction with core clock genes (13, 58, 166). It is hypothesized that PPARα and PPARγ bind a PPAR response element (PPRE) in the promoter of *Bmal1* and *Nr1d2* genes to regulate their transcription (17). Plus, the expression of components of the ETC might also be under clock control (107).

In agreement with this, diurnal regulation of mitochondrial respiration was blunted in mice lacking PER1/2 (106). Thus, the circadian clock tightly controls the rate of nutrient oxidation to guarantee that it is in balance with ATP production to minimize ROS generation.

Transfer of electrons through the ETC and the movement of protons are coupled with ATP synthesis, and most protons are transferred back to the mitochondrial matrix through ATP synthase (Fig. 1). Some protons can leak through the inner mitochondrial membrane without ATP production, which results in uncoupling and energy dissipated as heat. Mild uncoupling lowers the electrochemical potential, minimizes electron leak, and prevents ROS production (12, 82). Basal proton leakage occurs by diffusion of protons through the inner membrane (62a) and through adenine nucleotide translocase.

Facilitated process of uncoupling is mostly supported by uncoupling proteins (UCP). The mammalian UCP family

consists of five proteins (UCP1–5). Members of the family have similar activities but different tissue distribution. UCPs are localized in the inner mitochondrial membrane and they catalyze the transport of protons across the membrane. UCP1 is expressed in brown adipose tissues, UCP2 is ubiquitously expressed in many tissues, and UCP3 is predominantly expressed in skeletal muscle. UCP4 and UCP5 are expressed predominantly in nervous tissues.

In addition, UCPs are linked with many diseases such as cancer, neurodegeneration, and chronic inflammation. UCP1 plays an important role in thermogenesis, but the data on UCP1 in ROS homeostasis are conflicting (27, 108, 132). The role of UCP2 in the regulation of mitochondrial ROS level is well documented. Increased expression of UCP2 is associated with reduced ROS production (78, 105, 145). UCP3 plays a similar role by inhibiting ROS production and oxidative stress in skeletal muscle (10) and in isolated mitochondria (147). UCP4 and UCP5 reduce oxidative stress in the neural system and might play some other roles.

The circadian clock regulates the expression and activity of UCPs. UCP1 is rhythmically expressed in brown adipose tissue and is directly regulated by the circadian transcriptional repressor Nr1D1 (also known as Rev-erb-α) (36). PER2 acts as a coactivator of PPARα transcriptional factor in FABP3/fatty acid-dependent activation of UCP1 (15). Brain and muscle ARNT-like 1 (BMAL1) regulates UCP2 expression and uncoupling in β cells (76) and the heart (69). UCP3 expression oscillates in the skeletal muscle in a circadian manner (87).

The expression of UCPs is impaired in circadian clock mutants, which further supports the role of the clock in their regulation. Clock-dependent rhythmic expression of UCPs contributes to the circadian control of thermogenesis and other metabolic functions. Whether rhythms in UCP expression contribute to circadian ROS production in mitochondria is unknown. Such regulatory mechanisms are possible, but the effect might be tissue specific and needs to be investigated.

ETC-associated ROS are side products of oxidative phosphorylation. Mitochondria also have active and highly controlled mechanisms of ROS generation, probably for regulatory purposes. Recent findings have shown that activated $p66^{shc}$ is responsible for $\sim$30% of ROS produced in the mitochondria (39). Activated $p66^{shc}$ directs H_2O_2 production by oxidizing reduced cytochrome C and catalyzing the reduction of O_2 to H_2O_2 with electrons from the electron transfer chain (38, 39). ETC-generated ROS are in the mitochondrial matrix; in contrast to that, p66 generates ROS in the intermembrane space, which allows ROS to leak into the cytoplasm (39). Increased $p66^{shc}$ activity leads to mitochondrial H_2O_2 leakage to the cytoplasm, which induces apoptosis and may contribute to mitochondrial fusion or mitophagy (38, 39, 116).

Deficiency in $p66^{shc}$ reduces mitochondrial H_2O_2 production, stabilizes mitochondrial dynamics, and increases longevity (39, 90, 116) Furthermore, mice lacking $p66^{shc}$ display alterations in the hepatic circadian transcriptome, along with reduced levels of NAD^+ and ratio of oxidized to reduced NAD, which may be correlated to decreased expression of nicotinamide phosphoribosyltransferase (NAMPT) (116). $p66^{shc}$ gene contains an E-box element in its promoter, indicating potential transcriptional control by the core clock machinery (116).

$p66^{shc}$ expression is rhythmically expressed in the suprachiasmatic nucleus and liver and is critical for maintaining redox control of Circadian Locomotor Output Cycles Kaput (CLOCK) cysteinyl thiols (116). It has also been shown to be a critical component for maintaining normal circadian rhythms, which implicates it as a significant enzyme for managing mitochondrial H_2O_2 in regulating gene expression, metabolic homeostasis, and behavior.

Circadian Control of Mitochondrial Antioxidant Network

Oxidative stress occurs when the antioxidant system is not sufficient to match ROS production (118, 134). Cells keep their oxidative stress status in check by controlling the rate of ROS production and accumulation, along with the scavenging activity of antioxidants. The mitochondrion being a major site for ROS production also has its antioxidant system, which comprises superoxide dismutase (SOD), glutathione (GSH), and the PRDX-thioredoxin system (Fig. 2). The SOD proteins are the first line of defense against superoxide radicals (127).

There are three different SODs, SOD1, SOD2, and SOD3, located in different cellular compartments (33, 169). SOD2 is also referred to as the manganese-dependent SOD, and it is predominantly localized in the mitochondria. The mitochondrial pool of superoxide is mostly generated during the process of electron transfer during oxidative phosphorylation. This radical is very reactive and high concentrations have deleterious effects on the cell. SODs catalyze the dismutation of superoxide (O_2^-) to H_2O_2 (33, 114, 127, 169). H_2O_2 produced is further detoxified either *via* the glutathione or PRDX system.

Glutathione is synthesized in the cytosol and can be transported across the mitochondrial inner membrane. It is a tripeptide that is highly studied for its antioxidant attributes. Various cellular compartments maintain varying levels of glutathione, and the mitochondria comprise about 10%–15% of cellular glutathione content (84, 127). Most of the mitochondrial glutathione is maintained in its reduced form as GSH, which is needed for the detoxification of H_2O_2 (84, 97).

The glutathione system consists of two enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR). GPx is a selenium containing enzyme that catalyzes the reduction of H_2O_2 to water (H_2O), and this process utilizes GSH. Following the reaction, GSH is oxidized to GSSG. In turn, GSSG can be reduced back to GSH *via* the GR enzyme. The reduction of GSSG by GR requires NADPH (84, 97, 127) This is important for recycling glutathione back to its reduced state, making it available for another round of H_2O_2 reduction.

While this is out of the scope of the current review, we want to mention that glutathione-*S*-transferase (GST), the enzyme that conjugates glutathione to various electrophilic compounds, shows significant circadian oscillation in mammals (24, 59, 150), plants (34, 35), and flies (75). Rhythms in GST activity contribute to circadian variability in detoxification of xenobiotics, toxins, and pharmacological drugs.

PRDX is a thiol-dependent peroxidase, which confers another route for the detoxification of H_2O_2 to H_2O. In this process, PRDX becomes oxidized and needs to be recycled back to its reduced state for another cycle of H_2O_2 reduction. To do this, the system is coupled to thioredoxin. Thioredoxin

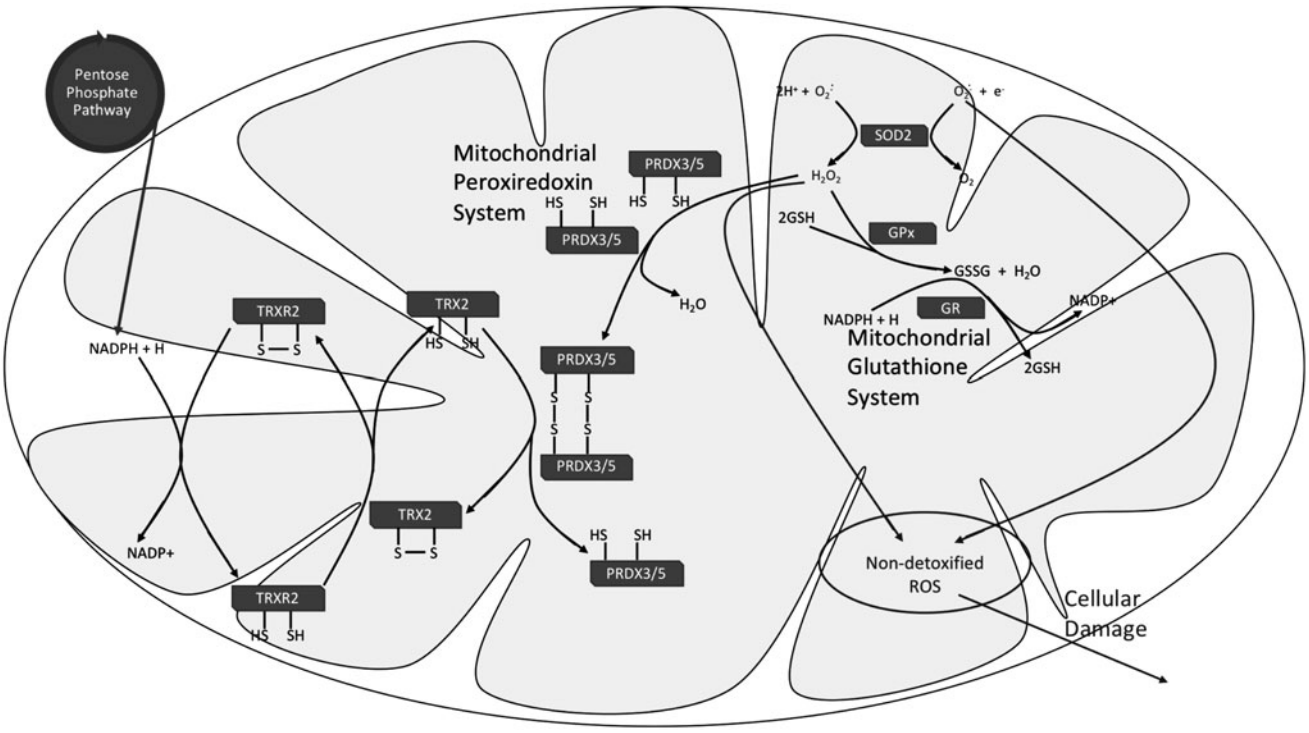

FIG. 2. Mitochondrial antioxidant system. The mitochondrial antioxidant defense comprises SOD, glutathione, and the PRDX system. SOD2 functions as the first line of defense against mitochondrial ROS by catalyzing the dismutation of superoxide (O_2^-) to H_2O_2 or molecular oxygen. The reduced form of GSH is used for the reduction of H_2O_2 to H_2O, and in turn glutathione is oxidized to GSSG and needs to be converted back to its reduced state to maintain the cycle. GPx catalyzes the GSH-dependent reduction of H_2O_2, while GR catalyzes the reduction of GSSG to GSH in a reaction that requires NADPH. Another route for H_2O_2 reduction is the PRDX system. PRDX3/5 in their reduced state is used for the reduction of H_2O_2 and this is followed by the subsequent oxidation of PRDX3/5. Oxidized PRDX3/5 can be recycled back to its reduced state by accepting hydrogen from reduced thioredoxin TRX2. Mitochondrial TRXR2 is needed for maintaining reduced levels of TRX2 and this reaction requires NADPH. GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; PRDX, peroxiredoxin; SOD, superoxide dismutase; TRX2, thioredoxin-2; TRXR2, thioredoxin reductase 2.

in its reduced state serves as a hydrogen donor used for the reduction of oxidized PRDX, and this is followed by the subsequent oxidation of thioredoxin (127). Conversion of thioredoxin back to its reduced state is catalyzed by the NADPH-dependent thioredoxin reductase, and this process is important for the continuation of the PRDX scavenging cycle (127).

Excessive ROS production may have detrimental effects on cell physiology by damaging biological macromolecules, which compromises cellular functions and could lead to cellular death.

The cycling of fuel substrates and the level of oxidative phosphorylation occur across the day. Therefore, it is surprising that the antioxidant defense system is highly rhythmic. Daily oscillations of antioxidant enzymes and the redox ratio of scavenging molecules occur in both the cytoplasm and mitochondria (117, 119). These rhythms are disrupted in circadian clock mutants, and clock deficiency is associated with oxidative stress.

Circadian transcriptional factor BMAL1 is central for circadian antioxidant defense, and mice deficient for BMAL1 represent the most striking example of oxidative stress upon circadian disruption. Total *Bmal1*$^{-/-}$ mice have increased ROS level in the liver (72). Total and neuron-specific *Bmal1*$^{-/-}$ mice demonstrate synaptic degeneration and neuropathology most likely through increased oxidative stress (99). *Bmal1*$^{-/-}$ fibroblasts also have an increased ROS level, which is associated with senescence (65).

β cell-specific BMAL1 deficiency leads to accumulation of ROS and mitochondrial uncoupling (76). Circadian regulation of redox control is conserved in Drosophila (74), but not every knockout of the circadian clock is associated with increased ROS. *Per* and *Tim* deficiency in Drosophila males extends the life span, increases uncoupling of respiration, and lowers intestinal ROS level (152).

The expression of many antioxidant enzymes is regulated by the clock directly through clock responsive elements or indirectly through antioxidant response elements (ARE) and retinoid response elements in their promoters (Fig. 3). NRF2 plays one of the central roles in the circadian antioxidant defense. NRF2 regulates the transcription of genes that manage mitochondrial oxidative stress and redox status (128, 159) by binding to ARE in their promoters (23, 159).

There are over 250 genes with ARE in their promoters (23), including antioxidant enzymes and enzymes involved in the homeostasis of ROS scavengers such as glutathione, NADH, and NADPH (28, 44, 54, 94, 117, 119, 126, 140, 163). NRF2 activity, localization, and expression are directly impacted by the oxidative state of the cell through interactions with Kelch-like ECH-associated protein 1 (KEAP1)

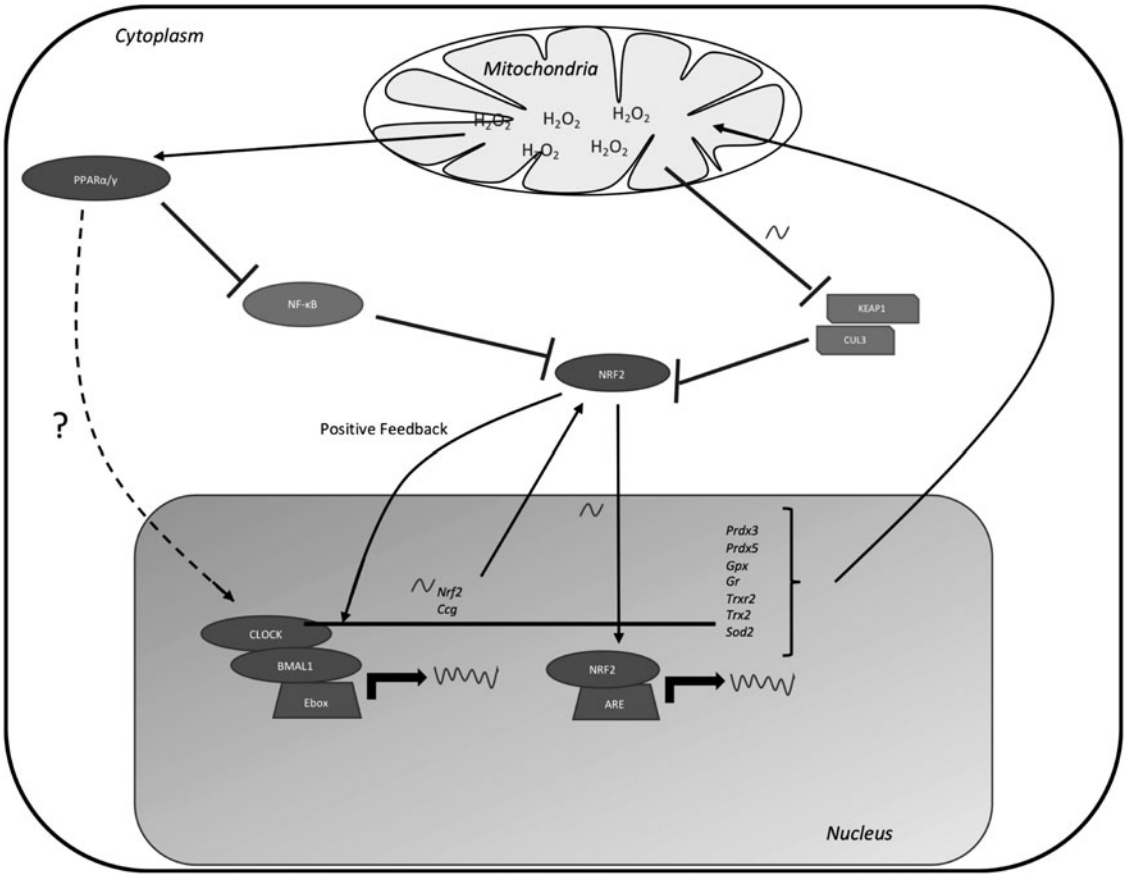

FIG. 3. Circadian interaction with transcriptional regulation of antioxidant defense system. Core clock components drive the rhythmic expression of *Nrf2*. Mitochondrial H_2O_2 that escapes the mitochondria rhythmically inhibits KEAP1, which allows NRF2 to translocate into the nucleus and regulate expression of mitochondrial antioxidant defense enzymes and pentose phosphate pathway enzymes. NRF2 interacts with the core clock through positive feedback to regulate its own expression. Mitochondrial H_2O_2 drives PPAR nuclear receptors to inhibit activity of NF-κB and is speculated to promote activity of core clock transcriptional regulation for management of mitochondrial oxidative stress enzymes. KEAP1, Kelch-like ECH-associated protein 1; NF-κB, nuclear factor-κB; NRF2, nuclear factor erythroid-2-related factor 2; PPAR, peroxisome proliferator-activated receptor.

(60, 68, 142). KEAP1 is a negative regulator of NRF2 nuclear translocation by promoting its ubiquitination through CULLIN E3 ligase, which leads to the subsequent proteasomal degradation of NRF2 (60, 142).

Daily oscillations in ROS, RNS, and electrophiles create oscillatory interactions with reactive cysteine residues of KEAP1, allowing for circadian timekeeping of NRF2 activity and degradation (116, 159, 165). Plus, the positive arm of the circadian system holds transcriptional control over NRF2 through an E-box element in its promoter (141, 143). NRF2 expression and activity are regulated by the clock in several mouse models such as lung fibrosis, neurodegeneration, and human lens epithelial cells, which suggest that the NRF2/clock interaction exists in different tissues and warrants further study (19, 99, 117).

Above in the Circadian Control of Mitochondrial ROS Production section, we have already discussed the PPAR-clock cross talk. PPAR transcriptional factor family is also implicated in antioxidant defense. Several antioxidant enzymes such are SODs, catalase, and Gpx-3 are under PPAR transcriptional control through PPREs in their promoters (57, 149). Treatment of cells with the PPARγ agonist increases the expression some antioxidant genes such as *Sod2* and *Gpx-3*, while the PPARα agonist had an antioxidant and antifibrotic effect in mice (26, 40). PPARγ exerts antioxidant effects through suppressing nuclear factor-κB and allowing ROS to be depleted and antioxidant enzymes to be promoted (3, 113).

Interestingly, several reports have associated PPAR with NRF2 in the management of mitochondrial oxidative stress through the context of metabolic disorders and drug-induced injury (81).

Circadian Clock and Mitochondrial Sirtuins

Sirtuins (SIRTs) are members of class III histone deacetylases (100). They play an important role in the regulation of protein function by regulating their posttranslational modifications such as acetylation, malonylation, succinylation, and glutarylation. There are currently seven known SIRTs (SIRT1–7) that have been identified in mammals, and they are distributed across various subcellular compartments (89). Localized in the nucleus are SIRT1, SIRT6, and SIRT7. SIRT2 is situated mostly in the cytoplasm, but can also be found in the nucleus. While SIRT3, SIRT4, and SIRT5 are in the mitochondria (100).

Activity of SIRTs relies on the availability of NAD^+ for their function. NAD^+ is synthesized *via* different routes (Fig. 4); *de novo* biosynthesis from tryptophan, the deamidated pathway from nicotinic acid, and through the amidated route (96, 138). The amidated route occurs either through nicotinamide riboside kinase 1 (NRK1), which synthesizes NAD^+ from dietary supplies of nicotinamide ribose, or through NAMPT, which synthesizes NAD^+ by recycling nicotinamide (86). Most of the mammalian NAD^+ is synthesized *via* the amidated route (96). BMAL1 binds to the *Nmrk1* gene (which codes for NRK1 protein) and promotes its expression and rhythmicity. The rhythms in the expression of *Nmrk1* are absent in *Bmal1*$^{-/-}$ and *Cry1,2*$^{-/-}$ mice (73, 86). CLOCK and BMAL1 dimerize and bind to E-box elements in the *Nampt* gene and promote its transcription (Fig. 4).

Both the mRNA and protein expressions of the NAD^+ salvage enzyme NAMPT are rhythmic and the rhythms in the expression are compromised in circadian clock mutants (103, 122). In agreement with changes in NAMPT expression in circadian clock mutant mice, total and mitochondrial levels of NAD^+ are affected (86, 115, 122). There is feedback interaction between SIRTs and the circadian clock. CLOCK acts as a histone acetyltransferase and directly acetylates BMAL1, and this is important for promoting the transcriptional activity (51). SIRT1, which is mainly localized in the nucleus, regulates this process by deacetylating BMAL1 and PER2 (5, 102).

SIRT3 is the major mitochondrial protein deacetylase (Fig. 4). Acetylation is receiving more attention as a key posttranslational modification that can regulate various

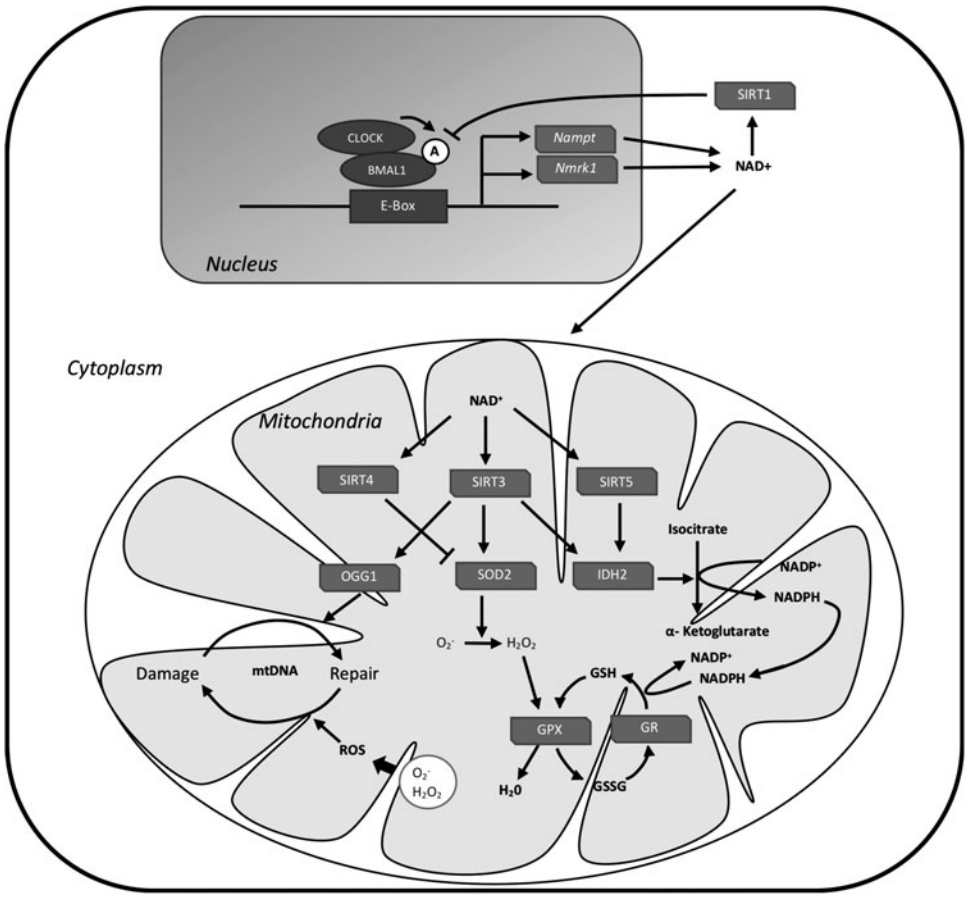

FIG. 4. Regulation of mitochondrial SIRTs by the circadian clock. CLOCK and BMAL1 heterodimerize and bind to E-box elements to promote the transcription of *Nampt* gene. This transcriptional process is favored by CLOCK-dependent acetylation of BMAL1. NAMPT is an important enzyme in the salvage route in NAD^+ biosynthesis. Another route for NAD^+ biosynthesis is *via* NRK1 enzyme, which produces NAD^+ from dietary supplies of nicotinamide ribose. This process is also under circadian control. BMAL1 binds to *Nmrk1* gene and promotes its expression, but it is not clear if it has an E-box element. Circadian rhythms in NAD^+ are responsible for circadian rhythms in the activity of SIRTs. SIRT3, SIRT4, and SIRT5 are localized in mitochondria and they contribute to mitochondrial antioxidant defense. SIRT3 deacetylates SOD2, IDH2, and OGG1. IDH2 produces NADPH used to reduce oxidized glutathione, and OGG1 is a DNA repair enzyme that protects mitochondrial DNA from ROS damage. SIRT5 contributes to antioxidant defense *via* IDH2 desuccinylation. SIRT4 may have an opposing effect on antioxidant defense; it prevents the deacetylation of SOD2 by SIRT3. Feedback regulation occurs between SIRTs and the circadian clock *via* SIRT1-dependent deacetylation of BMAL1. BMAL1, brain and muscle ARNT-like 1; CLOCK, Circadian Locomotor Output Cycles Kaput; IDH2, isocitrate dehydrogenase 2; NAMPT, nicotinamide phosphoribosyltransferase; NRK1, nicotinamide riboside kinase 1; OGG1, 8-oxoguaninine-DNA glycosylase 1; SIRT, sirtuin.

mitochondrial processes, including fatty acid oxidation and the TCA cycle (50, 52, 123). Studies on SIRT3 knockout mice reveal increased global liver mitochondrial protein acetylation, decreased β-oxidation, decreased ketogenesis, and increased oxidative stress (50, 118, 133). In addition, SIRT3 has been shown to confer cell-protective properties against ROS toxicity by boosting the antioxidant defense and by protecting against mitochondrial damage (80).

A notable mitochondrial protein that is regulated by SIRT3 through deacetylation is SOD2. Decreased activity of SIRT3 leads to increased acetylation and decreased activity of SOD2 (120). Some other key acetylated mitochondrial proteins are ornithine-transcarbamoylase (OTC) and isocitrate dehydrogenase 2 (IDH2) (47). IDH2 contributes to the production of NADPH by catalyzing the oxidation of isocitrate to α-ketoglutarate. The production of NADPH is required for the reduction of oxidized glutathione and is thereby needed for antioxidant defense (171). SIRT3 deacetylates and promotes IDH2 activity (136, 168). In addition, SIRT3 also deacetylates 8-oxoguaninine-DNA glycosylase 1 (OGG1), which catalyzes the base excision repair of mitochondrial DNA and protects it from ROS toxicity (18, 66).

The circadian clock regulates SIRT3 activity *via* the availability of NAD^+ (115) and is in agreement with rhythmic acetylation of SOD2 (86). SIRT3 activity is decreased in *Bmal1*$^{-/-}$ mice, and this leads to an increase in mitochondrial protein acetylation, including SOD2, OTC, and IDH2 (115). SIRT4 and SIRT5 are two other members of the SIRT family that are localized in the mitochondria and play an important role in mitochondrial physiology. While it is not clear if the circadian clock interacts with SIRT5 or SIRT4, it remains plausible considering that the activities of SIRT deacetylases are regulated by NAD^+ availability, which is under the control of the circadian clock.

Circadian Regulation of Mitochondrial Dynamics

Mitochondria are dynamic organelles that respond to various challenges by changing their shape through fusion/fission processes and their numbers through mitophagy and mitogenesis. Balance in these processes has a dramatic effect on cellular homeostasis (Fig. 5). Under stress, fusion of mitochondria occurs, which increases their oxidative capacity. Fusion of damaged and undamaged mitochondria compensates for loss of function (167) and induces a prosurvival response to stress (148). Mitochondrial fission is essential for cell growth as well as elimination and replacement of damaged organelles and components. Fragmentation of mitochondria networks is associated with apoptosis (53, 139).

Nutrient challenges can affect mitochondrial dynamics: nutrient excess causes mitochondria fission and creates fragmented mitochondria. Organismal fasting or nutrient depletion in cell culture induces mitochondria fusion, which is associated with increased capacity for ATP synthesis (79, 157). Mitochondrial fission is controlled by dynamin-related protein 1 (DRP1), a cytosolic GTPase that is recruited to the mitochondria by several proteins, including fission 1 (FIS1) (93). Mff1, Mief1, and MiD49/51 also were proposed to regulate DRP1 recruitment and function (110, 111, 170).

The key players in fusion machinery are mitofusins 1 and 2, located on the outer mitochondrial membrane, and

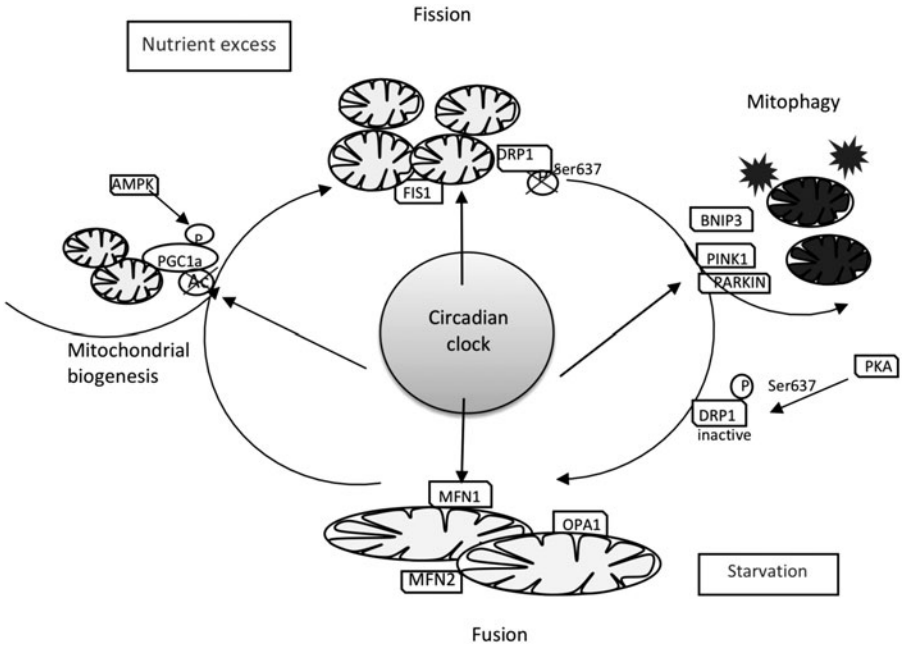

FIG. 5. Circadian clock control of mitochondrial dynamics. Feeding/fasting cycle creates temporal oscillation of nutrient flux to mitochondria. Both nutrient excess and nutrient starvation are a challenge, and to respond to it, mitochondria engage mitogenesis/mitophagy and fission/fusion processes. The circadian clock contributes to these processes to maintain healthy mitochondria. The circadian clock regulates the expression of several key proteins in mitochondrial fission/fusion such as DRP1, FIS1, and MFN1. Circadian control of mitophagy occurs through regulation of *PINK*1 and BNIP3 expression. Cross talk between the circadian clock and PGC1a pathways is involved in mitochondrial biogenesis. BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; DRP1, dynamin-related protein 1; FIS1, fission 1; MFN1, mitofusin 1; PINK1, PTEN-induced kinase 1.

optic atrophy 1 (OPA1), which resides in the inner membrane or intermembrane space (137). OPA1 was also proposed to regulate mitochondria cristae structure (2, 31).

Circadian oscillations of mitochondrial morphology were noticed about 40 years ago (151). It was hypothesized that the circadian clock regulates mitochondrial dynamics and helps prepare cells for day/night feeding/fasting cycles, while clock disruption is associated with an inability to adapt to different nutrient conditions (Fig. 5).

The expression of several fission/mitophagy genes in the liver is regulated in response to feeding, and this response was disrupted in the liver of liver-specific *Bmal1*$^{-/-}$ mice. In agreement with that, the mitochondrial dynamics was significantly impaired in this circadian mutant, which led to dysfunctional mitochondria (61). Schmitt *et al.* demonstrated that clock regulates mitochondrial morphology through DRP1 (130). DRP1 is a key mediator of mitochondria fission, and its activity is regulated by phosphorylation. DRP1 phosphorylation by PKA inhibits its activity and leads to the formation of an elongated mitochondria (43, 109). Calcineurin dephosphorylates DRP1 and activates mitochondrial fission (14). The activity of calcineurin is under circadian control (55).

Pharmacological inhibition of calcineurin in cells blocks circadian oscillations in DRP1 phosphorylation and mitochondrial rhythms (130). CLOCK can bind to *Drp1* mRNA, which affects its stability and regulates mitochondrial dynamics and function (164). Circadian control of mitochondrial dynamics through expression of fission protein FIS1 was abolished in Bmal1$^{-/-}$ mice, and liver overexpression of *Fis1* normalized mitochondria function and reduced oxidative damage (61).

PGC1α and PGC1β are master regulators of mitochondrial biogenesis and energy production through activation of several transcriptional factors, such as NRF1 and 2, PPARs, and ERRs (64, 156), while PINK1, PARKIN, and BNIP3 are involved in removing damaged mitochondria through mitophagy (41). PGC1α activity is regulated by many proteins that are connected to the energy status of the cell such as AMPK, SIRT1, MAPK, CaMKIV, and PKC.

In agreement with that, PGC1α phosphorylation and acetylation are affected by the feeding/fasting cycle (37, 41, 62). PGC1α expression is also regulated by diet such as caloric restriction (CR) (20). Mitochondrial pathology observed in circadian clock mutants was linked with disrupted expression of PGC1α. Interestingly, PGC1α regulates circadian clock gene expression, thus providing a feedback to the clock from mitochondria. Several pieces of evidence connect the circadian clock and mitophagy. BMAL1 is involved in quality control of mitochondria through mitophagy regulation. Expression of PINK1 is rhythmic, and it is the direct target of BMAL1 (71, 125). In addition, BMAL1 promotes the expression of *Bnip3* gene by binding to an E-box element in its promoter (77).

ROS Signals to Clock in Feedback Regulation

ROS, RNS, and electrophilic molecules are recognized as signaling molecules that lead to changes in cellular redox status (29). Mitochondria-produced ROS directly oxidize numerous cellular proteins such as receptors, kinases, and phosphatases, which impacts their biological functions, and,

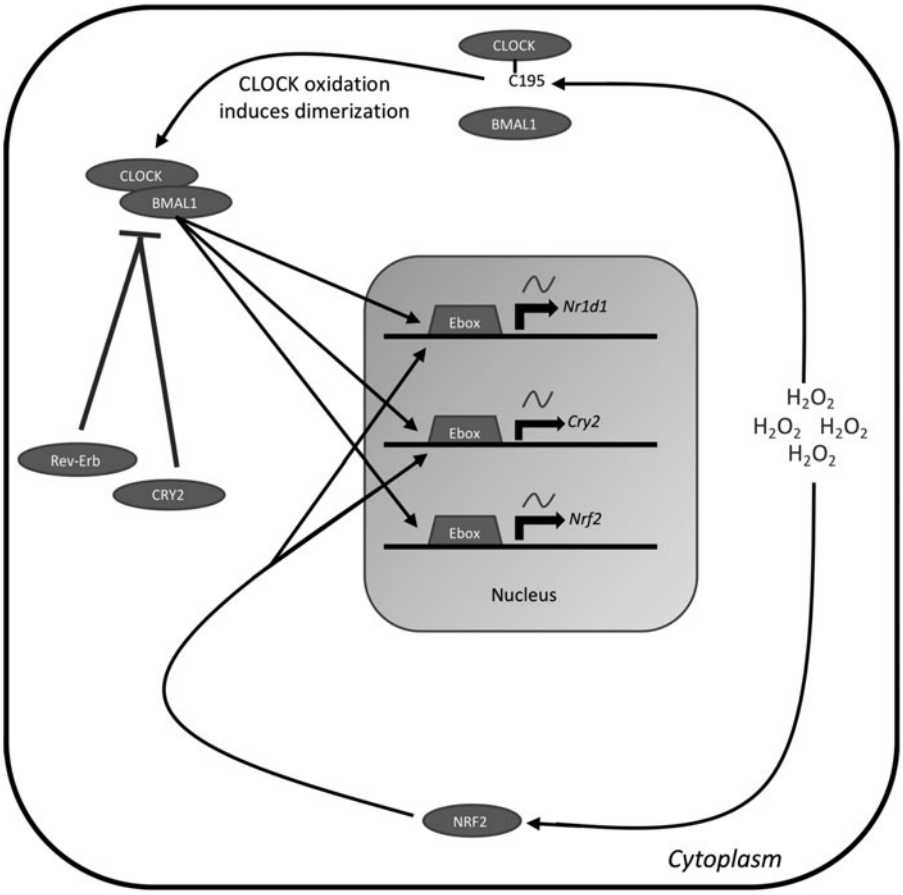

FIG. 6. ROS feedback to the clock. Cytoplasmic ROS act as a posttranslational modifier and signaling molecule, directing the cell to increase antioxidant defense. H_2O_2 in the cytoplasm will interact directly with CLOCK and oxidize cysteine195. This oxidation event promotes dimerization between CLOCK and BMAL1 and their subsequent nuclear translocation. Cytoplasmic H_2O_2 will also stimulate NRF2 nuclear translocation and activity. BMAL1 targets NRF2, which will promote further antioxidant defense. NRF2 targets *Cry2* and *Nr1d1*, which will act as a negative feedback mechanism to inhibit BMAL1 transcriptional activity and reduce the antioxidant response.

ultimately, modulates signal transduction pathways, gene expression, and metabolism (94, 160, 161). Cellular circadian oscillators are also a target for ROS (Fig. 6). Cellular redox status provides a rhythmic signal for maintaining circadian regulation of metabolic homeostasis (56, 91, 104, 116). Increased ROS lead to a decrease in the ratio of $NADP^+$/NADPH to manage the oxidative environment, and in turn provides a regulatory component for the circadian clock (126, 163).

Overwhelming the mitochondrial antioxidant system with increased oxidation causes the clock to be shifted and reset, and may even be attenuated depending on how long the oxidative stress persists (143, 144). This indicates a threshold for which oxidative stress uncouples the circadian system from redox homeostasis and coordinates prosurvival signals and gene expression through stress-resistant and stress-responsive pathways, including heat shock factors and proteins for maintaining protein homeostasis (143, 144). Organismal oxidative stress is critical for maintaining circadian function and timing, and CLOCK and BMAL1 are heavily impacted by the levels of ROS and electrophiles (116).

Protein interaction with H_2O_2 is an important rhythmic modification that regulates signal transduction and enzymatic activity. Specifically, oxidation of cysteine195 by H_2O_2 in the PAS domain of CLOCK promotes CLOCK interaction with BMAL1, DNA binding, and transcriptional control of downstream targets. H_2O_2 targeting of CLOCK provides a direct coupling between redox signaling and the circadian clock (116). The production of H_2O_2 regulates core clock genes, including retinoic acid-related orphan receptor (ROR) and NR1D1/2, through a PRDX/STAT3 pathway (63). Interestingly, it is well documented that a robust circadian clock increases longevity, however, it may be speculated that increased longevity can be attained with a disrupted circadian clock if oxidative stress is reduced (9, 116).

Transcriptional factor NRF2 mediates the clock effects on antioxidant defense as discussed above in the Circadian Control of Mitochondrial Antioxidant Network section. Recent data suggest that ROS can signal to the clock through the NRF2-dependent pathway. Indeed, NRF2 interacts with the clock by binding the promoters of *Cry2* and *Nr1d1* genes, which induces their expression (159).

Increased expression of *Cry2* drives negative feedback that inhibits its own gene expression along with the expression of *Nrf2* and other core clock genes (159). *Nr1d1* induction by NRF2 will interact with ROR elements in the *Bmal1* promoter to inhibit the production of *Bmal1* and halt the transcription of *Nrf2* and its target genes. This interaction between the circadian clock and NRF2 creates a direct link between regulation of the circadian system and redox homeostasis (7).

Diets, Feeding/Fasting Cycle, and Circadian Mitochondria

Diets have a strong impact on metabolism and physiology. Some diets such as high-glucose or high-fat diets disrupt metabolism and provoke the development of pathologies such as cardiovascular diseases, metabolic syndrome, and diabetes (7). Other diets such as CR or diets that explore periodic fasting might improve metabolism, reduce the rate of diseases, and increase longevity (22, 92). Oxidative stress contributes to development of the above diseases, and diets are known to impact ROS homeostasis, for example, a high-fat diet is associated with increased oxidative stress, while CR is associated with reduced oxidative stress. Diets also significantly impact circadian clock and rhythms in the liver and other tissues.

Indeed, there is a large body of evidence that circadian rhythms are significantly reprogrammed in response to various diets (46, 83, 129, 135, 146). Some genes become rhythmic, while others lose rhythmicity. Interestingly, about the same fraction of genes, 10%–20%, oscillates on different diets; the overlap of rhythmic transcripts between different diets is less than 25%. The expression of clock genes is also affected by diet. Diets that have a negative impact on health dampen the rhythms (70), and diets that improve metabolism enhance the rhythms (6, 16). This leads to the hypothesis that positive or negative metabolic effects of diets are linked with the effect of diet on circadian clock and rhythms. Diets frequently impact the pattern of food consumption, and therefore, feeding/fasting rhythms, which are linked with rhythms in energy metabolism (7, 32).

As discussed above, mitochondria adjust their physiology responding to nutrient flux and metabolic needs to balance ATP and ROS production. We hypothesize that reprogramming of circadian rhythms in response to various diets plays an important role in mitochondrial antioxidant defense

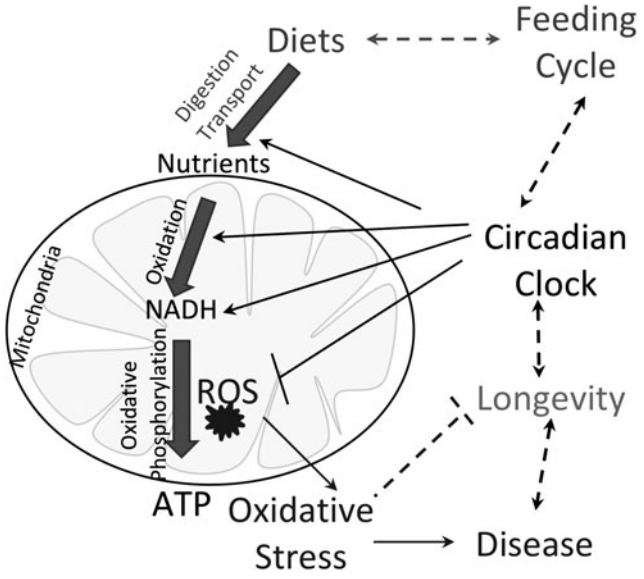

FIG. 7. Circadian coupling of mitochondria and ROS homeostasis. Daily feeding/fasting cycle dictates nutrient flux to mitochondria, where the nutrient oxidation is coupled with production of energy-rich ATP molecules in the process of oxidative phosphorylation. Due to electron leakage, mitochondrial energy production is associated with ROS generation. Excessive ROS lead to oxidative damage and contribute to diseases, and impact longevity. Almost every stage of the nutrient oxidation/energy production chain and mitochondrial antioxidant defense is under circadian control. The circadian clock is intertwined with daily rhythms, including the feeding cycle. Due to that, the circadian clock is capable of coordinating ROS homeostasis with organism daily activity and optimizes metabolism to reduce the risk of disease and delay aging.

by orchestrating the expression of various proteins in mitochondria to synchronize their activities with rhythms in ROS generation (Fig. 7). Periodicity of the feeding/fasting cycle will entrain rhythms in antioxidant defense in a manner similar with other circadian metabolic entrainments. Some of the metabolic benefits of periodic fasting based diets might be due to this entrainment.

Thus, a robust circadian clock helps to prevent oxidative stress and damage to cellular structures and ultimately contributes to health and longevity. Feeding during the wrong time, for example, during the time of rest or expected fasting will result in ROS production when clock-controlled antioxidant defense is not ready to manage it. Consequently, the antioxidant defense will be compromised resulting in oxidative damage. Circadian disruption is associated with disrupted feeding rhythms, and increased oxidative stress in circadian clock mutants is in line with the model. However, it is important to keep in mind that some of the effects of the circadian mutations might be rhythm independent.

Conclusions

Mitochondria have a broad range of functions that include energy production, lipid metabolism, calcium homeostasis, generation and detoxification of ROS, and the initiation of apoptosis. Maintenance of proper mitochondrial physiology is essential for cellular metabolism and organismal survival. ROS are produced as side products of mitochondrial energy metabolism, mostly in the electron transfer chain. Excessive ROS production might have detrimental effects on cell physiology by damaging biological macromolecules, which compromise cellular function and may result in cell death. Therefore, the mitochondrial antioxidant defense system is critically important for cell life cycle and survival. The circadian clock is integrated with mitochondrial physiology and contributes to ROS homeostasis by orchestrating mitochondrial ROS production and antioxidant defense.

Authors' Contributions

All authors conceived and designed the study. All authors wrote the article. All authors contributed to reading, revision, and approval of the article.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This work was supported by the U.S. National Institutes of Health/National Institute on Aging Grant R01AG039547 and funds from the Center for Gene Regulation in Health and Disease (Cleveland State University) to Roman V. Kondratov.

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Date of first submission to ARS Central, December 22, 2021; date of acceptance, January 6, 2022.

Abbreviations Used

AMPK = AMP-activated protein kinase
ANT = adenine nucleotide translocator
ARE = antioxidant response elements
BMAL1 = brain and muscle ARNT-like 1
BNIP3 = BCL2/adenovirus E1B 19kDa protein-interacting protein 3
CaMKIV = calcium/calmodulin-dependent protein kinase IV
CLOCK = Circadian Locomotor Output Cycles Kaput
CR = caloric restriction
CRY1 = cryptochrome circadian regulator 1
CRY2 = cryptochrome circadian regulator 2
Cyt C = cytochrome C
DRP1 = dynamin-related protein 1
ETC = electron transport chain
FABP3 = fatty acid binding protein 3
$FADH_2$ = flavin adenine dinucleotide
FIS1 = mitochondrial fission 1 protein
GPx = glutathione peroxidase
GR = glutathione reductase
GSH = reduced glutathione
GSSG = oxidized glutathione
GST = glutathione-*S*-transferase
H_2O_2 = hydrogen peroxide
IDH2 = isocitrate dehydrogenase 2
KEAP1 = Kelch-like ECH-associated protein 1
MAPK = mitogen-activated protein kinase
MFF1 = mitochondrial fission factor 1
MFN = mitofusin
NADH = nicotinamide adenine dinucleotide
NAMPT = nicotinamide phosphoribosyltransferase
NF-κB = nuclear factor-κB
Nr1d1 = nuclear receptor subfamily 1 group D member 1
Nr1d2 = nuclear receptor subfamily 1 group D member 2
NRF1 = nuclear factor erythroid 2-related factor 1
NRF2 = nuclear factor erythroid-2-related factor 2
NRK1 = nicotinamide riboside kinase 1
OGG1 = 8-oxoguaninine-DNA glycosylase 1
OPA1 = optic atrophy 1; OPA1 mitochondrial dynamin-like GTPase
OTC = ornithine-transcarbamylase
Per1 = period circadian regulator 1
Per2 = period circadian regulator 2
PGC1a = peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PINK1 = PTEN-induced kinase 1
PKA = protein kinase A
PKC = protein kinase C
PPAR = peroxisome proliferator-activated receptor
PPRE = PPAR response element
PRDX = peroxiredoxin
RNS = reactive nitrogen species
ROR = retinoic acid-related orphan receptor
ROS = reactive oxygen species
SIRT = sirtuin
SOD = superoxide dismutase
TRX2 = thioredoxin-2
TRXR = thioredoxin reductase
UCP = uncoupling proteins