Regulation of circadian clocks by redox homeostasis

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ABSTRACT

Living organisms possess biological clocks that resonate with environmental cycles in light, temperature and food availability. Recently, circadian oscillations in the redox state of peroxiredoxin have been described as an additional, non-transcriptional timekeeping mechanism. Of note, this redox cycle is conserved in both prokaryotes and eukaryotes. How the classical ‘transcription-translation feedback loop’ model and this redox oscillation are related is still poorly understood. In this review we describe the most recent evidence pointing to crosstalk between the circadian clock and the redox status of the cell.

Introduction

The integration of biological clocks into cellular physiology has represented an important evolutionary advantage for multicellular and unicellular organisms, allowing them to anticipate and adapt to cyclical changes in environmental cues such as light, temperature and food availability (1). The advantage conferred by resonating with environmental cycles has been technically challenging to demonstrate. Pioneering experiments, however, have shown that the coordination with the light/dark cycles can improve fitness in bacteria, flies and plants (2-5).

In mammals, the timing system is composed of a series of biological clocks organized in a hierarchical manner. The main clock, also known as the ‘master pacemaker’, resides in the paired suprachiasmatic nuclei (SCN)² of the hypothalamus, which receive and process light signals to achieve synchronization with the external environment. Through the release of hormones and neuropeptides, the SCN coordinate several other clocks distributed in different tissues and organs. These peripheral clocks in turn generate local, self-sustained circadian rhythms (from Latin circa diem, about a day) of physiological processes to control tissue-specific functions (6-8).

The first insights into the molecular mechanism of cellular rhythmicity came from relatively recent studies in Drosophila and Neurospora crassa. These studies showed that rhythmic oscillations in the expression of clock-controlled genes are generated by transcription-translation feedback loops (TTFLs) and that they were necessary to coordinate behavioral rhythmicity (9, 10). Similar timekeeping logic was later described in other organisms, although with different genes involved, and different levels of complexity in the transcriptional circuits (11, 12). In mammals, for example, two positive activators CLOCK and BMAL1 initiate the transcription of Period1/2 (Per1/2), Cryptochrome1/2 (Cry1/2), Rora, and Rev-erba/b genes. When the level of expression of PER and CRY proteins reaches a particular threshold, they translocate into the nucleus and inhibit the transcriptional activity of the CLOCK-BMAL1 heterodimer thereby
blocking their own transcription. An additional loop is created by REV-ERBα/β and RORα proteins, which instead respectively repress or activate the transcription of the Bmal1 gene (13) (Fig. 1). This classical model based on transcription has been slightly revisited in light of new data showing that proteosomal degradation, epigenetic modulation of gene expression and post-translational modifications of mRNA play key roles in generating molecular rhythmicity (11, 14-16). The turnover of PER and CRY is, for example, controlled by phosphorylation-mediated ubiquitination processes (17-21).

Although conserved in many organisms, the TTFL cannot be considered as a universal building block for circadian clocks (11). For instance, the yeast Saccharomyces cerevisiae and the worm Caenorhabditis elegans show circadian rhythms, but do not express the classical ‘clock genes’ (22-24). Also, the cyanobacterium Synechococcus elongatus and the filamentous fungus Neurospora crassa tend to favor protein phosphorylation as their basic timing mechanism (25, 26). Very recently, biochemical oscillations of the redox state of the protein peroxiredoxin (Prx) have been described as an additional timekeeping mechanism conserved in both eukaryote and prokaryote (12, 27, 28). These findings have thus revealed an intriguing link between the redox status of the cell and circadian clocks. We will discuss what we know about clock-relevant redox control systems and the reciprocal regulation between the redox state of the cells and circadian clocks.

Oxidative state and redox control systems

The redox cellular environment is determined by the balance between the generation of oxidants and free radicals, and the level of reducing agents. The most common oxidants are the Reactive Oxygen Species (ROS), which are generated by intracellular enzymes during metabolic reactions. Some examples include superoxide anion (O₂⁻), hydroxyl radical (HO⁻), and hydrogen peroxide (H₂O₂). In order to avoid oxidative damage, cells have adopted several detoxification strategies. Non-enzymatic mechanisms involve the synthesis of antioxidant molecules such as ascorbate, tocopherols (including vitamin E), and retinol (vitamin A). Enzymatic mechanisms include proteins such as superoxide dismutase (SOD), which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, and catalase (CAT), which mediates the decomposition of hydrogen peroxide to water and oxygen. Additional redox buffering systems are provided by oxidize/reduced glutathione (GSH) and oxidized/reduced thioredoxin (Trx) (Fig. 2).

GSH is a low molecular weight antioxidant involved in the reduction of disulphide bonds and in the reduction of hydroperoxides by glutathione peroxidases (GPx). Oxidized GSH, the disulphide GSSG, is potentially dangerous for the cell (29, 30), but it is normally reduced to GSH by glutathione reductases (GR) via an NADPH-dependent reaction. Disulphide bridges in proteins are also reduced by glutaredoxins (Grx), which rely on GSH for their non-enzymatic regeneration. GSH can also be conjugated to cysteine (Cys) residues on proteins by Glutathione-S-transferase in a process called glutathionylation, which protects proteins from oxidation (31, 32). Similarly to Grx, Trx proteins facilitate the reduction of several proteins by cysteine thiol-disulphide exchange. Oxidized Trx are eventually reduced by Trx reductases via NADPH-dependent reactions. Among these antioxidant systems, peroxiredoxins (Prx) have recently emerged has key players in the control of circadian rhythms.

Prx cyclical oxidation as the prototype for redox regulated cytosolic clocks

Prxs are a highly conserved family of antioxidant proteins classified in class 1-Cys and class 2-Cys, depending on the number of Cys involved in catalysis. In their catalytic site, Prxs contain a ‘peroxidatic’ Cys residue that can be oxidized to a sulphenic acid (Cys-SOH) by an incoming peroxide (Fig. 2). In class 2-Cys Prxs after oxidation, this residue reacts with a ‘resolving’ Cys to form an intermolecular (typical and atypical class 2-Cys) or intramolecular (atypical class 2-Cys) disulphide bond, which is eventually reduced by Trx. Typical class 2-Cys Prxs can undergo further oxidation (termed hyperoxidation) which generates sulphinic (Cys-SO₂H) and sulphonic acid forms (Cys-SO₃H) of the catalytic cysteine (33-35). The sulphonic acid form is catalytically inactive but can be
reactivated by sulphiredoxin through an ATP-dependent reduction reaction (36). On the contrary, the sulphinic acid form is irreversibly oxidized and its physiological occurrence is controversial (37, 38).

It has been recently demonstrated that Prx follow circadian cycles of oxidation. In a recent paper, in fact, the levels of dimeric Prx-\(SO_3H\) were shown to oscillate with a period of 24 hours with peaks of hyperoxidation at 12 hours (circadian time) (27). Strikingly, these oscillations were demonstrated to occur in red blood cells (RBCs), which do not possess DNA, showing that Prx oscillations occur even in the absence of gene transcription (27). Oscillations in Prx have also found in the small protist *Ostreococcus tauri*, which contrary to RBCs, possesses an endogenous clock driven by transcription and translation of recognized plant clock genes (39). Importantly, oscillations in Prx could be detected also when this organism was shifted into dark environment, a condition under which gene transcription of *O. tauri* is known to stop (28). In addition, when the organism was brought back to light, the clock did not reset, suggesting that a mechanism must be in place to keep track of time even in the absence of gene transcription. These studies therefore showed that Prx redox cycling events could be an important mechanism for time keeping. Of note, circadian oxidation of Prx have been found not only in eukaryotes (including algae, fungi, flies, worms and mammals), but also in archaea and bacteria, suggesting that these oscillations might have been integrated early in evolution and likely co-evolved with differing TTFLs in each organism (12, 24, 27, 28). An unanswered key question though, is what determines Prx oscillations. Srx, which reduces the inactive sulfinic acid form into the active sulfenic acid form might indeed account for these oscillations. However, some organisms that display oscillations in Prx do not express Srx homologues (e.g. *C. elegans* and *N. crassa*) suggesting that other mechanisms might be in place.

Given the highly conserved redox component of circadian oscillations, it is an important goal to now understand the relationship between the classical TTFL and Prx oscillations (12). Interestingly, when the transcriptional machinery is disrupted, for example, in behavioral arrhythmic *Drosophila* mutants, or in *N. crassa* mutants exhibiting a lengthened period, Prx oscillations are perturbed in phase, suggesting that gene transcription is not necessary, but is related to cellular metabolic cycles. On the same lines, when the Prx clock system is abolishes, as occurs in mutants of *Synechococcus elongatus* and *Arabidopsis thaliana* deficient for well-annotated 2-Cys Prx genes, circadian rhythms of ‘clock genes’ persisted with the same period as in control organisms, but were perturbed in either phase or amplitude (12). Taken together, these studies showed that TTFL and Prx cycles are intertwined, but potentially autonomous components of the circadian system. These results also raise the possibility that redox status of the cell fluctuates, and that these oscillations have critical, and as yet incompletely understood, biological consequences.

**The reciprocal relationship between redox state and circadian system**

Initial hints that redox metabolism might be linked to the circadian clock was shown by Rutter and colleagues, in which the ratio between oxidized and reduced forms of NAD and NADPH were shown to regulate the DNA binding activity of CLOCK/NPAS2:BMAL heterodimer (40). However, these studies were purely biochemical, based solely on the use of purified recombinant proteins, and used concentrations of reactants much higher than is seen physiologically, making their wider interpretation difficult, especially in an *in vivo* context. More recently, *in vivo* oscillations in the redox state of FAD and NADPH have been described in organotypic slices of SCN (41). In this study, the authors demonstrated that the redox state of SCN oscillates in a self-sustained fashion and that these oscillations contribute to determining the excitability of SCN neurons via non-transcriptional regulation of potassium (K\(^+\)) channels. However, the connection between the transcriptional clock and redox oscillations in this tissue requires further investigation. Whether redox fluctuations are an output of circadian rhythms, or whether they can act as input, or indeed both, is still under intense investigation.
In favor of a mechanistic link between redox fluctuations and the regulation of gene expression, studies in *Zebrafish* demonstrate that changes in redox state actively control the expression of light-dependent genes. Light, which is the key entraining stimulus in this organism, generates H$_2$O$_2$, which in turn regulates the expression of the clock genes zCry1a and zPer2. Interestingly, oscillations in the mRNA levels of these genes are paralleled by antiphasic oscillations in mRNA and the activity of catalase, suggesting that this enzyme is involved in the control of H$_2$O$_2$-mediated circadian gene expression (42). Recently, LdpA (light-dependent period A), a component of the cyanobacterial circadian clock, was proposed to act as a redox sensor and to be used by the clock to adjust the period length (43). LdpA contains iron-sulfur centers and can sense the redox state of the cell, which correlates with the amount of light (high light correlates with reduced redox state, whereas low light is associated with an oxidized redox state). Interestingly, on the basis of the light conditions, LdpA modulates the levels of CikA and KaiA, the latter of which is a key component of the central oscillator (44), thereby affecting the period length. Furthermore, cyanobacteria exposed to high light conditions show short periods whereas cyanobacteria exposed to low light display long periods. Finally, the effects of altered ROS and the circadian clock have also been observed in *Neurospora crassa* (45, 46) and in the cyanobacterium *Microcystis aeruginosa* (47), in which H$_2$O$_2$ has been shown to impact on the daily expression pattern of ‘clock genes’ as well as clock-controlled genes, including those involved in coordinating photosynthesis. These results clearly show that fluctuations in the redox state of the cells have an impact on the expression of clock-related genes in multiple diverse systems.

This scenario is further complicated by the finding that clock genes can in turn regulate the expression of antioxidant enzymes, thus providing an important, and novel, feedback loop (Fig. 3). For instance, in *Arabidopsis thaliana*, the circadian clock coordinates ROS homeostasis and ROS-responsive genes, and H$_2$O$_2$ production and scavenging exhibit diurnal rhythms (48). Importantly, mutations in the core clock regulator CCA1, or other components of the TTFL, affect this time of the day specific pattern. In addition, the authors observed that ROS can feed back to affect the transcription of clock-regulated genes. The importance of this crosstalk has been underlined in *Drosophila melanogaster*, in which the period gene has been shown to be essential for maintaining anti-oxidant defence. Indeed, flies exposed to H$_2$O$_2$ show daily mortality rhythms and are more susceptible during the late light phase. Mutation in the period gene abolishes this time of the day sensitivity and renders flies more susceptible to oxidative stress in general (49). Bmal1 +/- mice show higher accumulation of ROS in several tissues as compared to wt animals. This impairment in ROS homeostasis correlates with early aging and age-dependent pathologies. These data again suggest a connection between the circadian clock and redox homeostasis (50).

More recently, the circadian system has been shown to also modulate the pathways involved in production and utilization of GSH (51). Wild type Canton S (CS) flies show daily rhythms in the mRNA levels of glutamate cysteine ligase (GCL), the rate-limiting enzyme in glutathione biosynthesis, and glutathione S-transferase D1, which utilizes GSH in cellular detoxification. Importantly, mutants lacking the clock genes per and cyc showed no rhythms in the expression of these proteins underlying the link between GSH metabolism and the circadian system.

**Compartmentalization of oxidative state and redox signaling: future perspectives**

An emerging feature of redox signaling is its spatial and temporal compartmentalization. Recent developments highlight that different ROS signaling and redox buffering systems are spatially segregated, and can have unique, compartmentalized functions (52-55) (Fig. 4). For example, pools of mitochondrial, cytosolic and nuclear GSH are separated within cells and the trafficking of GSH, from the cytosol to the mitochondrial intra-membrane space, is tightly regulated by porins in their membranes (56). Importantly, the maintenance of localized redox states is critical for cell function. Mitochondria-specific depletion of GSH make them more sensitive to oxidative damage (57), whereas overexpression of the mitochondrial Grx, Grx2 protects against oxidative stress to prevent apoptosis (58). The nuclear redox state is similarly pivotal for the activation of several...
redox-regulated transcription factors such as CLOCK and NPAS2 (40), NFkB (59), the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (60) and Reverbβ (61).

Although evidence suggests that ROS are bona fide signaling molecules, some skepticism has been raised because of their high reactivity and low substrate specificity. However, there is evidence of tight coupling of ROS generators to the activity of antioxidant buffering systems and to specific targets, which would explain how the specificity of ROS signaling is brought about (62-64). In the adrenal gland for example, H$_2$O$_2$ is involved in a feedback control loop to regulate corticosteroid synthesis (65). In the last phase of adrenocorticotropic hormone-induced steroidogenesis, cholesterol is imported in mitochondria where cytochromes P450 enzymes catalyse the oxidative cleavage of its side chain. As a by-product of their activity, cytochromes generate H$_2$O$_2$ which is eliminated by PRX3. During the catalytic cycle, PRX3 can be inactivated by hyperoxidation. Its activity is normally reverted by SRX. However, when corticosteroid synthesis increases, so does H$_2$O$_2$, and sulfiredoxin activity is no longer sufficient to reduce and reactivate PRX3. This causes a further increase in H$_2$O$_2$ levels and overflow of H$_2$O$_2$ into the cytosol. This last event triggers a signaling cascade involving p38 MAPK kinase which eventually inhibits corticosteroid synthesis. Of note, levels of inactivated PRX3, activated p38 MAP kinase and sulfiredoxin exhibit circadian oscillations. In addition, tissue specific ablation of sulfiredoxin results in suppression of the adrenal circadian rhythms of corticosterone production, suggesting that Prx hyperoxidation, corticosteroids synthesis and the circadian clock are interconnected.

Interestingly, oxidative signals can cause selective oxidation of specific redox couples. For example, endothelial growth factor (EGF)-mediated ROS signaling selectively oxidizes the cytosolic pool of Trx1, but not the mitochondrial pool of Trx2, suggesting that these pools are independently regulated (66, 67) (Fig. 4). Furthermore, one of the major transcription factors activated by oxidative stress, Nrf2 can be differentially activated by redox signals: its translocation is promoted by a redox switch of Keap1, which is controlled by GSH, whereas its nuclear activity is under control of Trx1 (60) (Fig. 4).

It is tempting to speculate that different redox systems are strategically located within the cell not only to protect substrates from excessive oxidation but also to regulate specific signaling pathways. In addition, different redox couples might act in concert to specifically modulate the response to ROS signals in proximity of key redox-sensitive proteins. Determining how this compartmentalized nature of cellular redox systems links to the clockwork will be critical to fully understand how the cell en masse keeps daily time. We believe this will be an exciting area of investigation in the next few years.

Conclusions

Substantial evidence highlights the capability of living organisms to resonate with environmental cycles, which confers an evolutionary advantage, since perturbing the clockwork reduces fitness. However, the biological mechanisms underlying the regulation of circadian rhythms are still elusive in the light of new insights coming from redox biology. In the post-genomic era, the dominance of gene regulation at the heart of circadian rhythms needs to be reconciled with mounting evidence demonstrating the importance of redox cycles and post-transcriptional/post-translational modifications (68).

It now appears that control of ROS signaling is deeply intertwined in the circadian clock system. Disruption of circadian rhythms in humans has been linked to several diseases such as breast cancer, obesity, diabetes, sleep disorders, and neurodegenerative diseases (69). Given the role of ROS in human pathophysiology, it is tempting to speculate that some of the pathologies associated with the deregulation of clock signaling are partially caused by alteration in redox signaling and possibly their compartmentalized nature. Thus, we propose that the understanding of how localized ROS production affects the activity of oscillators within cells will have important consequences for the development of dedicated therapies aimed at restoring aberrant signaling.
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FOOTNOTES
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2The abbreviations used are: SCN, suprachiasmatic nuclei; TTFL, transcription-translation feedback loop; CLOCK, Circadian locomotor output cycles kaput; BMAL1, Brain and muscle ARNt like protein 1; Per, period; Cry, Cryptochrome; Ror, Retinoic acid receptor-related orphan receptor; ROS, reactive oxygen species; O$_2^-$, superoxide anion; HO-, hydroxyl radical; H$_2$O$_2$, hydrogen peroxide; SOD, Superoxide Dismutase; CAT, Catalase; GSH, Glutathione; Trx, Thioredoxin; GPx, Gluthathione Peroxidases; GST, Glutathion S-transferase; Grx, Glutaredoxin; Prx, peroxiredoxin; RBC, red blood cell; NPAS, Neuronal PAS domain protein 2; LdpA, light-dependent period A; CCA1, core clock regulator 1; EGF, endothelial growth factor; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; Keap1, Kelch-like ECH-associated protein 1.

FIGURE LEGENDS
Figure 1. Mammalian Transcription-Translation Feedback Loop (TTFL)
The mammalian clock is sustained by a series of feedback loops that involve several genes and the proteins that they encode. The two positive activators, CLOCK and BMAL1, initiate the transcription of the ‘clock genes’ Per1/2, Cry1/2, Rora, and Rev-erbα/β. PER1/2 and CRY1/2 proteins accumulate, dimerize and translocate into the nucleus where they bind to the CLOCK-BMAL1 dimer thereby inhibiting its transcriptional activity. Eventually, proteosomal degradation of PER1/2 and CRY1/2 relieves the transcriptional repression on the CLOCK/BMAL1 complex and the cycle can restart again. An additional loop involves the nuclear receptors RORα and REV-ERBa/β which respectively activate and repress the transcription of Bmal1.

Figure 2. Redox systems
Schematic representation of the cellular redox systems and main antioxidant enzymes. (A) Glutathione-Glutaredoxin system; (B) Thioredoxin system; (C) Peroxiredoxin system.

Figure 3. Crosstalk between the circadian clock and redox homeostasis
The circadian clock and the redox state of the cell are interconnected. The expression level and activity of antioxidant enzymes determine the levels of intracellular ROS, which have been shown to impinge on the expression pattern of clock genes. In addition, some antioxidant enzymes have been shown to follow circadian pattern of expression, suggesting that the clock system can regulate redox homeostasis.

ROS, reactive oxygen species.
Figure 4. Compartmentalization of redox systems

Redox systems are compartmentalized and pools of antioxidant enzymes are distributed differently in the cell. Pools of GSH and Trx have been described in the cytosolic, mitochondrial and nuclear compartments. The cytosolic pool of Trx1 has been shown to limit ROS generated upon EGFR activation. Nrf2 nuclear translocation is regulated by a redox switch controlled by GSH and Keap1 oxidation, whereas its DNA binding activity is regulated by a nuclear pool of Trx1.

EGF, endothelial growth factor; EGFR, EGF receptor; GSH, glutathione; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; Kelch-like ECH-associated protein 1, ox, oxidized; red, reduced.
Figure 1

- **Cytoplasm**
  - CRY1/2
  - PER1/2
  - Clock-controlled genes
  - REV-ERBa/b
  - RORa

- **Nucleus**
  - CLOCK
  - BMAL1
  - DNA
  - RORE

- Proteasomal degradation
Figure 3

Antioxidant enzymes

Clock genes

ROS

Expression levels vs. time (h)

Intracellular levels vs. time (h)
Regulation of circadian clocks by redox homeostasis
Alessandra Stangherlin and Akhilesh B. Reddy

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