

FORUM REVIEW ARTICLE

Circadian Rhythm Connections to Oxidative Stress: Implications for Human Health

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Abstract

Significance: Oxygen and circadian rhythmicity are essential in a myriad of physiological processes to maintain homeostasis, from blood pressure and sleep/wake cycles, down to cellular signaling pathways that play critical roles in health and disease. If the human body or cells experience significant stress, their ability to regulate internal systems, including redox levels and circadian rhythms, may become impaired. At cellular as well as organismal levels, impairment in redox regulation and circadian rhythms may lead to a number of adverse effects, including the manifestation of a variety of diseases such as heart diseases, neurodegenerative conditions, and cancer. **Recent Advances:** Researchers have come to an understanding as to the basics of the circadian rhythm mechanism, as well as the importance of the numerous species of oxidative stress components. The effects of oxidative stress and dysregulated circadian rhythms have been a subject of intense investigations since they were first discovered, and recent investigations into the molecular mechanisms linking the two have started to elucidate the bases of their connection. **Critical Issues:** While much is known about the mechanics and importance of oxidative stress systems and circadian rhythms, the front where they interact has had very little research focused on it. This review discusses the idea that these two systems are together intricately involved in the healthy body, as well as in disease. **Future Directions:** We believe that for a more efficacious management of diseases that have both circadian rhythm and oxidative stress components in their pathogenesis, targeting both systems in tandem would be far more successful. *Antioxid. Redox Signal.* 19, 192–208.

Introduction

RHYTHMICITY AND OXYGEN are essential to life, from the beating of the heart for pumping oxygenated blood, to the rhythmic breathing of air by the lungs. Circadian rhythms are intimately involved in living systems at environmental, organismal, and cellular levels. Circadian rhythms, by definition, are physical, mental, and behavioral changes that follow an ~24-h cycle. This cycle is generally slightly longer than 24 h (an average of 24.2 h in sighted humans, and 24.5 h in blind humans), but can vary from person to person (48, 142). It is believed that the development of circadian rhythms is a response to the earth's rotation, both around its axis and around the sun, which dictates daily light and temperature changes (111). Similarly important are the role of oxygen in our universe and a myriad of biological processes. The balance of oxygen and its related compounds is one of the most

crucial parts of regulating life on the Earth, especially in humans. It is also an immensely intricate system involving numerous enzymes, biologically active compounds, and signaling molecules. Deregulation of normal circadian rhythms as well as the various biological activities of oxygen can compromise the quality or even the very existence of life, and there is much evidence that these two fundamental processes are intimately linked.

The toxic effects of oxygen were first appreciated in 1954 with Gershman's free-radical theory, suggesting that oxygen toxicity may happen due to partially reduced forms of oxygen (59). Commoner *et al.*, in the same year, suggested the presence of free radicals in a variety of biological materials (34). These new ideas triggered a surge of scientific research into the idea that although a necessary part of life, oxygen may not always be beneficial. The cell has evolved an intricate web of energy synthesis and signaling mechanisms that are

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dependent on oxygen and its more reactive forms, reactive oxygen species (ROS). Intense research has been done on ROS, their beneficial and detrimental effects on the organism, as well as the efforts mounted by the cell to counteract them. Interestingly, many of these efforts, including the production of antioxidants and protective enzymes, have been reported to be regulated or expressed in rhythmic fashions. Thus, oxidative stress seems to have a circadian rhythm connection. This gives rise to a myriad of questions. When an oxidative-stress-linked disorder manifests, is it because of a dysregulation of the protective mechanism, or just because of the increased ROS and their detrimental effects? Would targeting dysregulated circadian rhythm components help in the overall management of oxidative-stress-mediated diseases and conditions? Would restoring rhythmicity in the protective enzymes and antioxidants cure or mitigate the disease? It is important to consider, discuss, and investigate the connection between oxidative stress and circadian rhythms, especially in regard to human health and disease. In light of these questions, we offer support for the idea that oxidative stress and circadian rhythms are intertwined, both in the maintenance of homeostasis, as well as in certain diseases.

Oxidative Stress: The Disease Connection

ROS, such as superoxide radicals (O_2^-), peroxides (ROOR), and hydroxyl radicals (OH^-), are byproducts of normal cellular metabolism, mainly in the mitochondria. These molecules can benefit the cell by playing critical roles in cellular defense and other important cellular processes (19, 181). One such role that many of the ROS have, especially O_2^- and hydrogen peroxide (H_2O_2), is in cellular signaling controlling a variety of biological processes. The ROS have many features that make them excellent signaling molecules, and have been shown to be involved in many pathways, from kinase activation to insulin action [reviewed in Refs. (8, 52)]. However, a tight regulation of ROS is needed, as they may inflict serious detrimental effects if left unchecked. To place checks and balances on ROS, the cellular machinery has a sophisticated system to neutralize them before they become problematic. This includes producing protective enzymes (*e.g.*, catalases [CATs], superoxide dismutases [SODs], and glutathione peroxidases [GPxs]) and physiologically generated small-molecule antioxidants (*e.g.*, Vitamins C and E, glutathione [GSH], and uric acid) (70, 108). If these protective measures are not enough, due to exposure to environmental stresses (such as ultraviolet [UV] light, chemical pollutants, or heat) or due to other physiological reasons (poor or inappropriate diet, life style, *etc.*), the cell enters a state of oxidative stress. When this happens, it can be disastrous for the cells, causing DNA damage, lipid peroxidation, oxidation of amino acids, and ultimately death of the cell or disease in the host (181). Reactive nitrogen species (RNS), including nitric oxide (NO) and peroxynitrite ($ONOO^-$), also play an important role in oxidative stress. NO is produced in normal cells as an integral part of cellular signaling and is a mediator of cellular damage [reviewed in Ref. (175)]. NO can diffuse easily through cells, which makes it especially useful as a biological messenger, and although it is only mildly reactive, its chemical properties make it a very potent intermediate messenger for production of more reactive RNS [reviewed in Refs. (25, 124)], as well as other biological processes such as vasodilation (175). Excess

NO is normally inactivated by various cellular responses, including in the blood by a reaction with oxyhemoglobin to form nitrate (124). However, since NO is poorly reactive, it has been suggested that much of the cellular damage that occurs during oxidative stress comes from the oxidation products of NO, especially peroxynitrite (12, 181). This compound is formed from the reaction of O_2^- and NO, is a particularly active oxidative molecule, and has been implicated in several diseases and disorders, including hypertension, arthritis, and cancer [reviewed in Refs. (124, 181)].

As mentioned above, these reactive species can be produced in the mitochondria as a byproduct of metabolism or by environmental stressors such as exposure to UV light or exposure to chemical pollutants. Since it is difficult to directly measure the very short-lived ROS, much research has focused on studying the presence of the antioxidants and other enzymes produced by the body that work against the oxidant radicals (70). Interestingly, the cellular concentrations or activity levels of many of these antioxidants and protective small molecules (such as SOD, GPx, melatonin, and several others discussed in the next section) have been found to have circadian rhythmicity (37, 107, 134). This suggests an importance of both oxidative stress and the circadian rhythm in human diseases.

Circadian Rhythms and Zeitgebers: The Tempo of Life

Circadian rhythms are the natural patterns of physical, mental, and behavioral changes in living organisms. For a pattern to be considered circadian, it must have five features, including (i) the ability to become entrained (synchronized), (ii) the persistence of the cycle after the removal of external stimuli, (iii) the ability to shift the phase of the cycle, (iv) a period of around 24 h, and (v) the ability to maintain its period independent of temperature (13, 50). To determine if the expression pattern is circadian, data are traditionally fit to a cosine curve (the cosinor method) and displayed as a graph such as that shown in Figure 1. If the levels do not fit the regularly recurring wave, the pattern is most likely not circadian.

The cyclic pattern in the expression of circadian proteins and other rhythmic elements is dependent on a number of external cues or zeitgebers, including light exposure, feeding patterns, exercise, and temperature change (62, 183). The strongest zeitgeber, light, initiates synchronization of rhythms when the retinorecipient cells within the suprachiasmatic nucleus (SCN) in the hypothalamus region of the brain receive the light impulses from the retina (62). The SCN has been found to be the main regulator of circadian rhythms, which then sends signals to peripheral cells throughout the body, causing that rhythm to be passed on to the proper cells and tissues (53). However, the SCN can be stimulated by zeitgebers other than light, especially by serotonin and melatonin, although these are thought to be internal feedback regulators as opposed to primary circadian rhythm initiators (112, 113, 128). Melatonin has also been found to be one of the signaling molecules used by the body to synchronize certain peripheral cells. However, it is believed that many more molecules and hormones, including insulin and glucocorticoids, may be involved in this process (145, 154). These concepts are outlined in Figure 2.

It has been estimated that the body uses these cues to synchronize the oscillatory expression of up to 10% of genes,

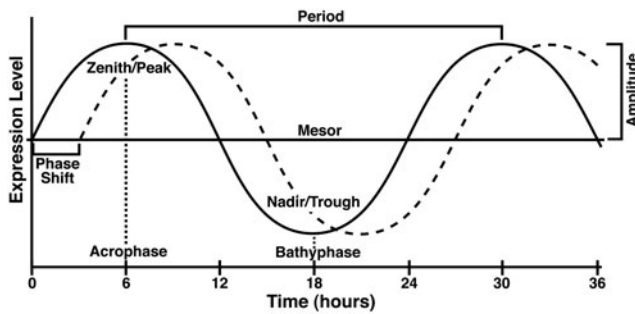


FIG. 1. Circadian curve and terminology. A model curve representing a circadian rhythm with a period of 24 h. Zenith or Peak=the maximum level of gene expression over the course of the oscillation. Acrophase=the time at which the curve reaches the Zenith/Peak. Nadir or Trough=the minimum level of gene expression over the course of the oscillation. Bathy phase=the time at which the curve reaches the Nadir/Trough. Period=the time required to complete one cycle, that is, the distance from one peak to the next. Midline estimating statistic of rhythm (Mesor)=the value halfway between the highest and lowest values of the cosine curve of best fit to the data. Amplitude=the distance from the mesor value to the Zenith/Peak. Phase shift=displacement of a curve along the time axis.

which thereby regulate many different physiological and biochemical processes (146). Many of these circadian genes are important in basic cellular processes, such as regulation of cellular division, proliferation, and DNA damage repair (17, 146). Physiologically, many processes have been found to

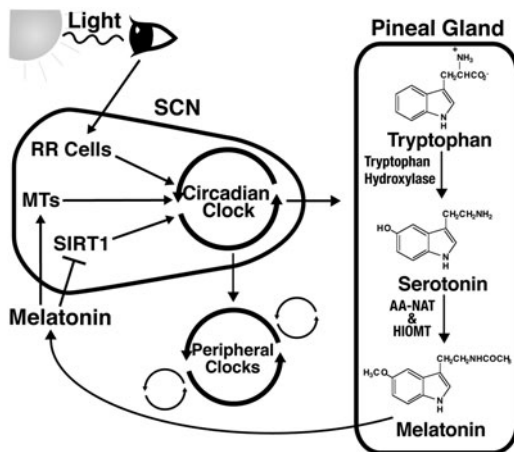


FIG. 2. Circadian rhythms are regulated by more than one zeitgeber. Light is presumably the strongest zeitgeber that acts directly on the suprachiasmatic nucleus (SCN). It is sensed by the retina, which signals to retinorecipient cells (RR Cells) in the SCN region of the hypothalamus. This entrains the circadian clock within the SCN, which in turn signals to peripheral clocks throughout the body. However, a number of other endogenous and exogenous cues are capable of entraining circadian rhythms. For example, the circadian clock also stimulates production of melatonin in the pineal gland, which can also act as a zeitgeber, entraining peripheral clocks. Melatonin can also bind to melatonin receptors (MTs) in the SCN or inhibit SIRT1 expression and activity, both of which can also affect circadian rhythms, thereby acting as a zeitgeber itself.

have circadian rhythmicity, such as sleep/wake cycles, body temperatures, and T-cell immune responses (94, 138, 144). As expected, a dysregulation of these rhythms may trigger a variety of adverse effects in the cell and living system. Below, we have described the roles and interaction of circadian rhythms and oxidative stress in biological systems and as well as discussed the consequences of their dysregulation.

Circadian Rhythms: Molecular Clockwork

While the central pacemaker is located in the SCN region of the hypothalamus, each peripheral cell in the body contains its own clock that adjusts its rhythm in response to entrainment signals (116, 190, 200, 205). A molecular mechanism consisting of a transcriptional–translational feedback loop is responsible for this time-keeping capability. The positive branch of this loop consists of the proteins aryl hydrocarbon receptor nuclear translocator-like (ARNTL), also known as BMAL1, and circadian locomotor output cycles kaput protein (CLOCK) (4, 58, 93). The negative branch of the loop consists of period homologs 1–3 (Periods [PER]1–3), and cryptochrome 1 and 2 (CRY1 and 2) (98, 205). As shown in Figure 3, the heterodimerization of BMAL1 and CLOCK or its homolog NPAS2 starts the circadian cycle. Once dimerized, BMAL1 and CLOCK bind to E-box elements in the promoters of their target genes, initiating transcription of various rhythmic proteins (58). Two groups of these transcriptional targets are the *PERS* and the *CRYS*. The PER and CRY proteins then form a second heterodimer that translocates into the nucleus and interferes with the activity of BMAL1 and CLOCK at the promoter sites, completing the cycle (156).

The regulation of *BMAL1*, *CLOCK*, and *NPAS2* transcription adds a second regulatory loop to the core circadian mechanism. The orphan nuclear receptors REV-ERB α and β repress *BMAL1* transcription through binding to retinoic acid-related orphan receptor-response elements (ROREs) in the *BMAL1* promoter (66, 130). Recent studies have shown that REV-ERB α also represses both *CLOCK* and *NPAS2* transcription (41, 69). Competing with the REV-ERBs for RORE-binding sites in the *BMAL1* promoter are RAR-related orphan receptors (RORs) α , β , and γ (also known as ROR α , β , and γ), all of which activate *BMAL1* transcription (2, 66, 150). ROR α has also been shown to activate *NPAS2* transcription, but not the transcription of *CLOCK* (41). Like the PERs and the CRYs, the REV-ERBs and the RORs are clock-controlled (2, 3, 66, 150, 177), thereby forming a second, stabilizing feedback loop. However, the status of each individual ROR as a clock-controlled element depends on whether circadian regulation is being studied in the master regulator in the SCN, or in peripheral tissues, as ROR α expression oscillates only in the SCN; ROR γ expression oscillates only in peripheral tissue; and the circadian status of ROR β expression is currently unknown (66, 150).

Interestingly, this regulation of *BMAL1*, *CLOCK*, and *NPAS2* expression is affected by the redox state of REV-ERB β . REV-ERB β activity is facilitated by heme binding that allows for the recruitment and binding of a histone deacetylase (HDAC), which when complexed with REV-ERB β yields its active form. The reduced dithiol state of REV-ERB β binds heme more tightly than the oxidized form of REV-ERB β (69). Since ROS oxidize thiol groups, oxidative stress is predicted to reduce REV-ERB β activity, thereby allowing for increased *BMAL1*, *CLOCK*, and *NPAS2* transcription and

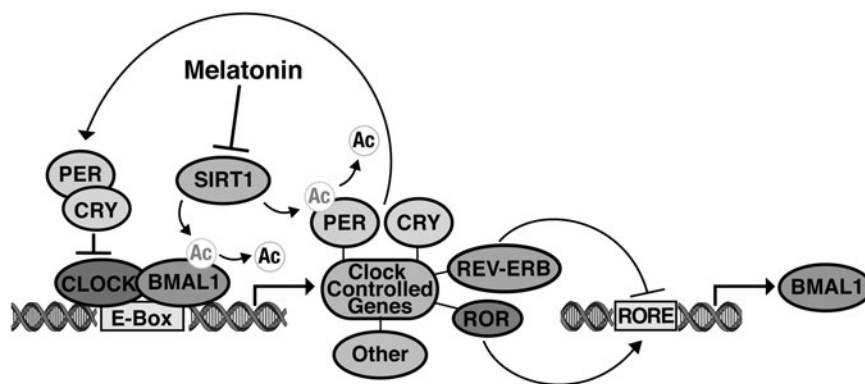


FIG. 3. The roles of SIRT1 and melatonin in the molecular mechanism of the circadian clock. The basic circadian mechanism consists of a transcriptional-translational feedback loop. Circadian locomotor output cycles kaput protein (CLOCK) and BMAL1 form a heterodimer in the nucleus and bind to E-box elements in the promoters of their target genes. Known target genes include the Periods (PER) and cryptochromes (CRY), which form a heterodimer, enter the nucleus, and interfere with CLOCK-BMAL1 activity. BMAL1 acetylation is required for the recruitment of CRY, and SIRT1 disrupts this recruitment through deacetylation of BMAL1. SIRT1 also deacetylates PER2, targeting it for degradation by the proteasome. Melatonin is capable of inhibiting SIRT1 expression and activity, thereby preventing BMAL1 and PER2 deacetylation. An auxiliary loop-regulating BMAL1 expression is controlled by two groups of clock-controlled proteins, the REV-ERBs, and the RAR-related orphan receptors (RORs). The RORs promote BMAL1 expression by binding to the retinoic acid-related orphan receptor-response elements (RORE) site in the *BMAL1* promoter, while the REV-ERBs inhibit BMAL1 transcription through competitive binding to the same site. Note that while both REV-ERB α and β are clock-controlled proteins, ROR α expression oscillates only in the SCN; ROR γ expression oscillates only in peripheral tissues; and it is currently unknown if ROR β expression is clock controlled.

disrupting circadian rhythms. However, this has not yet been directly tested.

A number of post-translational modifications are also known to regulate the core mechanism. Briefly, phosphorylation by CKI ϵ facilitates cytoplasmic sequestration of the PER1 and 2 proteins. Nuclear export of PER1 and PER2 is facilitated by a nuclear export signal, which is activated upon phosphorylation (186). PER1 also contains a nuclear localization signal that is masked upon phosphorylation at the adjacent CKI ϵ -binding site (185). CLOCK's nuclear localization is dependent on its binding to BMAL1 and subsequent phosphorylation, and this localization oscillates with circadian rhythmicity (96). The acetylation status of at least two of the core circadian proteins is important in regulation of this system. During a normal circadian cycle, CLOCK acetylates its heterodimerization partner, BMAL1, at its Lys537 residue, allowing for the recruitment of CRY1 in the negative limb of the cycle (73). The class III HDAC SIRT1 then deacetylates BMAL1, allowing for the system to reset for a new cycle (118).

Overall, the regulation of circadian rhythms is a very complex system with many details still to be explored. Despite the clear role of the transcriptional-translational feedback loops in the regulation of these patterns, a recent study indicates that there is a significant contribution to normal clock function outside of the core feedback loops (119). In this study, the authors have shown that human red blood cells display circadian oscillation in peroxiredoxin (Prx)-redox cycles in spite of the fact that they lack nuclei, and are therefore incapable of the transcription necessary to sustain the transcriptional-translational feedback loops of the core circadian mechanism (119). While much remains to be explored concerning the intricacies of circadian rhythm control by external cues, these findings suggest that they play a more significant role than previously understood.

Rhythmicity of the Cellular Antioxidant System: Patterns of Protection

A growing collection of data suggests that the circadian regulation of protein expression plays a significant role in the cellular response to oxidative stress. Several studies have shown evidence of differences in DNA damage, lipid peroxidation, and protein oxidation at different times of the day, thus indicating circadian oscillations of oxidative stress responses (51, 90, 97, 100, 176). These oscillations relate directly to the daily rhythm of antioxidant expression and protective enzyme activity levels (Fig. 4). This rhythmicity in antioxidant levels may be exploited for a more precise targeting of the ROS, thereby offering better protection for the cells. One of the biggest causes of oxidative damage in the cell is the O $_2^-$ molecule. As elucidated below and in Figure 5, many of the enzymes used to transform it into less-reactive species are rhythmically expressed.

One key group of antioxidant enzymes that oscillate with circadian rhythmicity is SODs. These enzymes protect against oxidative damage by catalyzing the dismutation of O $_2^-$ into O $_2$ and H $_2$ O $_2$. In eukaryotes, there are two main types of SODs: copper/zinc (Cu/Zn), found cytoplasmically and extracellularly, and manganese (Mn), found mitochondrially (127). Mn SOD is thought to be especially important in maintaining and influencing the redox status of the cell, since it is the form present in the mitochondria where most ROS are produced. Also, it can directly influence the flux of certain ROS in some instances [reviewed in Ref. (21)]. Daily rhythmicity in SOD activity was first reported by Diaz-Munoz *et al.* in 1985. They found that in the rat cerebral cortex, SOD activity peaks in the dark phase, coinciding with the peak level of malondialdehyde, which is a product of lipid peroxidation (43). Later studies showed that both Cu/Zn and Mn SOD

due to the low levels of protective enzymes at that point in the day.

Oxidative Stress and Circadian Rhythms: Possible Links and Applications

In addition to the oscillation of protective enzymes that directly connects circadian rhythms to oxidative stress defense mechanisms, there are several other ways in which the two are closely linked. In 2008, Krishnan *et al.* demonstrated that the rhythmicity of oxidative damage extends past the lipid peroxidation mentioned above to include cases of protein oxidation as well (97). The authors studied H₂O₂-induced oxidative stress in *Drosophila melanogaster* at different time points over a 24-h period (97). They found that there was a significantly higher mortality level in flies treated during the day than in those treated at night (97). This higher daytime mortality was accompanied by higher levels of protein carbonyls, which are a byproduct of protein oxidation. To demonstrate that these effects were directly related to the circadian clock, the authors demonstrated that flies lacking the protein PER did not show a difference in mortality rate between daytime and night-time exposure to H₂O₂ (97). Interestingly, there was also no difference in mortality rate between daytime and night-time in flies that were exposed to constant light, a condition that is shown to disrupt circadian rhythms (97, 115, 121, 172). Mammalian studies in the Syrian Hamster have supported this finding, showing that continuous light greatly diminishes or abolishes the circadian rhythms that are observed under normal light/dark cycles in SOD, GR, and CAT (176).

Circadian rhythms and oxidative stress are also linked through the activity of at least two known factors that play a significant role in both processes. The first and most well known is the pineal hormone melatonin. Its circadian oscillation was first discovered in 1964 (134), and it has been studied extensively as a marker of circadian rhythms ever since. Its antioxidant properties, however, were not discovered for almost another 30 years, when Ianas *et al.* first published an article discussing the involvement of melatonin in the oxidative process (78). Since then, melatonin's antioxidant properties have been explored and exploited by many researchers in various fields (28, 29, 135, 173). More recently, SIRT1 has been linked to both oxidative stress and circadian rhythms as well. SIRT1 plays a role in oxidative stress through the initiation of several downstream effectors, including p53 and FOXO transcription factors (187). In 2008, two independent studies linked SIRT1 to the core circadian mechanism through the deacetylation of the proteins BMAL1 and PER2. As outlined above and in Figure 3, SIRT1 is responsible for deacetylating BMAL1, thereby resetting the clock to its original state (118). In addition, SIRT1 deacetylates PER2, targeting it for degradation (5). This prevents the heterodimerization of PER2 with the CRYs in the negative feedback portion of the core circadian mechanism. Interestingly, melatonin and SIRT1 have also been shown to interact, as melatonin is an inhibitor of both SIRT1 expression and activity (87, 89). Thus, not only is melatonin expression controlled by the circadian clock, but circadian rhythms are controlled by melatonin feedback *via* SIRT1 (see Figs. 2 and 3). Further linking SIRT1 to both oxidative stress and circadian rhythms is the coenzyme nicotinamide adenine dinucleotide (NAD⁺).

The mechanism of protein deacetylation by SIRT1 involves the use of NAD⁺ as a cofactor, and thus SIRT1 activity is dependent upon the availability of NAD⁺ (74). Therefore, through its role as a coenzyme of SIRT1, NAD⁺ affects circadian rhythm regulation. NAD⁺ and its redox partner NADH are also intimately involved in redox reactions, primarily driving oxidation, while their phosphorylated forms NADP⁺/NADPH drive reduction reactions (74). These reactions include the reduction of GSSG to GSH as depicted in Figure 6, as well as the generation of O₂⁻ through the donation of electrons from NADPH in a reaction catalyzed by NADPH oxidase. Thus, the initiation of oxidative stress and the general redox state of the cell affects the availability of NAD⁺ and consequent activity of SIRT1. Finally, both NADH and NADPH have been shown to enhance DNA binding of the CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers to their transcriptional targets, whereas NAD⁺ and NADP⁺ inhibit this activity (141). Thus, there is a direct link between the redox state of NAD⁺/NADH and NADP⁺/NADPH and circadian rhythms.

Although some overlap has been found between the two systems, there is still an incomplete understanding of the molecular mechanisms linking circadian rhythms and oxidative stress. However, the fact that the two are related has been exploited by researchers and physicians in the form of chronotherapy. As defined by Merriam-Webster, chronotherapy is the "administration of medication in coordination with the body's circadian rhythms to maximize effectiveness and minimize side effects." Research suggests that applying our current knowledge of circadian rhythms to timed medical treatment yields better outcomes in a number of diseases. For example, a study of diabetic patients who took hypertensive medications at bedtime rather than upon waking resulted in significantly reduced cardiovascular morbidity and mortality rates, as well as improved ambulatory blood pressure control (72). This benefit in the timed dosing of cardiovascular medications extends to nondiabetic patients as well, and has been shown to significantly improve patient health for a variety of blood pressure medications (166). Rheumatoid arthritis, a systemic inflammatory disease with links to oxidative stress and circadian rhythms, is another disease in which a benefit has been shown when timing treatments with biological rhythms. A slow-release form of prednisone has been developed, so that the rise of proinflammatory cytokines in the morning could be counteracted (23). In cancer, chronotherapeutic treatments have been studied more extensively [reviewed in Ref. (80)]. Overall, these studies have shown that cancer treatments also show greater benefit when administered at specific times of day. The identification of circadian disruptions in a number of other diseases suggests that this timed treatment could also be useful in diabetes, asthma, arthritis, and Parkinson's disease (PD), to name a few (20, 22, 39, 91). The following section provides a brief review of several of these diseases and their relationship to oxidative stress and circadian rhythms.

Oxidative Stress and Circadian Rhythms: Partnership of the Processes

Oxidative stress and circadian rhythms have each been studied extensively in their own right, as they relate to different conditions and disorders. However, because of the

links that have been found between many of their individual components at a cellular level, we feel that an evaluation of the intersections of the systems in certain diseases is merited. Here, we discuss several diseases that have been found to have strong oxidative stress and circadian rhythm links, including aging, heart disease, cancer, diabetes, and neurodegenerative disorders.

Aging

Aging is a complex process characterized by the slowing or alteration of cellular and bodily processes over time, resulting in reduced functionality of cells, increased susceptibility to disease, and ultimately the death of the organism. This is a process that happens naturally to all organisms, and can ultimately not be avoided. All of the factors involved in aging are not known, and intense research is currently ongoing in this direction. In the cell, indicators of aging can be seen in changes/damage to DNA, proteins, and other cellular components. One of the key factors that have been found to be involved in the aging process is oxidative stress. Among environmental stressors, UV radiation causes significant oxidative stress, primarily in skin cells. UV exposure from the sun or other artificial sources can lead to a buildup of DNA damage and destruction of skin cells, leading to photoaging [reviewed in Ref. (114)]. There are several ways the DNA can be repaired, depending on the exact form of damage, but only one has been found to be linked to circadian rhythms (146, 147). When UV light reaches the DNA, it can cause cyclobutane pyrimidine dimers. The system that the cell uses to repair this damage is called nucleotide excision repair (NER, Fig. 7) (147). NER involves several critical proteins, including the

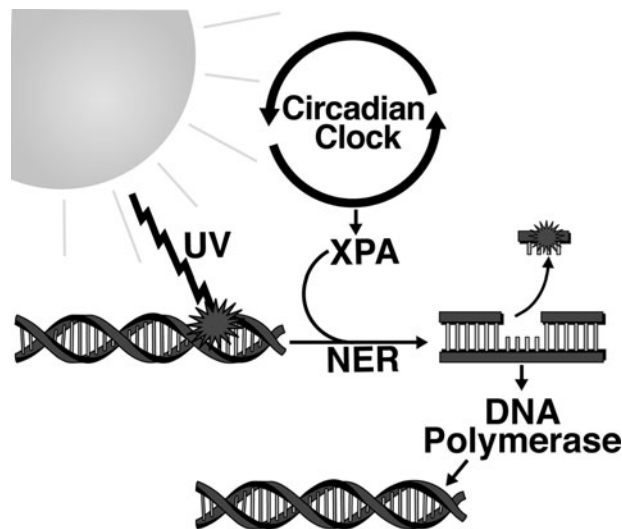


FIG. 7. Nucleotide-excision repair (NER) and circadian rhythms. NER is initiated upon DNA damage by ultraviolet light. Xeroderma pigmentosum A (XPA) is the rate-limiting protein in the process of NER initiation. XPA is also clock-controlled, indicating that circadian rhythms have a significant influence on the NER process. After initiation, the damaged piece of DNA is excised, and the repair process is completed when DNA polymerase fills the gap left by the excised DNA using the undamaged single strand of DNA as a template.

protein xeroderma pigmentosum A (XPA), which shows circadian rhythmicity due to its transcription and ubiquitination by other rhythmic elements in the cell, and is also a deacetylation target of SIRT1 (57, 169). This is especially important when we realize that SIRT1 activity has been found to be proportional to lifespan in several model organisms [reviewed in Refs. (35, 120, 162)].

Calorie restriction-related longevity increases have been shown in various species, from yeast to mammals (92, 162). It is believed that the reasons for this are that a reduced amount of ROS is produced by calorie restriction, effectively lowering overall oxidative stress in the cell, and that calorie restriction increases the ability of sirtuins to moderate the stress response in the cell, thereby countering aging two ways (35, 136). In addition to the effects of restrictive diets on the oxidative stress system, a decreased calorie diet has also been shown to entrain the SCN, consequently changing the circadian rhythm of an organism (54). This may affect aging, as a direct disruption of many of the core circadian rhythm genes has been shown to reduce lifespan in transgenic mice (47, 201).

As described in the earlier section, many oxidative stress enzymes have been found to oscillate rhythmically. This rhythmicity has been found in some instances to change as an organism ages, making it less reliably circadian. Goncharova *et al.* have found this to be true in a group of 10 rhesus monkeys (5 older and 5 younger) (64). The monkeys were subjected to a stressor either in the morning or in the evening and found that a circadian rhythm in corticosteroids and GR was present in the erythrocytes of younger animals. However, this rhythm was mitigated in the older animals (64). This group had earlier demonstrated a similar result in erythrocyte SOD levels with the same type of monkeys (63). This phenomenon has also been observed in a study in humans where Stritesky Larssen *et al.* found that in a group of 66 individuals, the variations of ROS production in leukocytes oscillated with circadian rhythmicity, and had a higher antioxidant status in newborns, as opposed to older volunteers (168). This leads us to believe that both circadian rhythms and the redox state of cells are both very important in aging in all species.

Heart disease

Both circadian rhythms and oxidative stress have been studied for a long time in various diseases and disorders of the heart. Oxidative stress has been shown to play a primary role in the development of heart disease, while various cardiovascular factors have been found to have circadian rhythms, including basic blood pressure (191). At lower levels, numerous ROS have been implicated as signaling factors in angiogenesis, but higher levels are associated with cardiovascular disease (180). The intricate balance of ROS and overall reduction of oxidative stress is extraordinarily important in heart tissue, as the cells that make up the heart have been estimated to have a renewal rate of 1% or less, depending on age (15). This is considerably less than most other organs and tissues, especially the skin, which has shown to have a turnover rate of 39 days for the entire tissue (189).

Angiotensin is one hormone that has been implicated in several necessary processes, including angiogenesis, regulation of blood pressure, induction of apoptosis, and ROS production. Angiotensin enhances ROS production *via* increasing cellular production of NADPH oxidase, which is one of the

key enzymes that produces O₂⁻ inside the cell (125). This increase in O₂⁻ appears to be an integral part of the signaling mechanism used by angiotensin to affect the vascular tissue in the fashions listed above (125). A study done in 2002 in hypertensive rats found rhythmicity in the precursor to angiotensin, angiotensinogen, suggesting that this cascade could be important in regulating oxidative stress in cells in the heart, and that the changes are made in a rhythmic fashion (117). It is well documented that sudden cardiac death and heart attacks are more common in the morning, when blood pressure starts rising after leaving its low point overnight (129). Interestingly, it has recently been found that a dysregulated blood pressure rhythm could be corrected by blocking the angiotensin II type 1 receptor, showing that this rhythmic body process is intimately involved with oxidative stress regulation, which ultimately can lead to heart disease if incorrectly regulated (56). As mentioned earlier, the knowledge that blood pressure and other cardiovascular events follow a circadian rhythm has also been exploited in therapeutics for best response, when giving certain medications, especially angiotensin receptor blockers and other antihypertensive drugs (72, 104, 192).

Interestingly, several of the players that interact in the regulation of circadian rhythms and oxidative stress also play a role in heart disease. Ota *et al.* demonstrated that statin treatment of human umbilical vein endothelial cells after H₂O₂-induced oxidative stress induction was able to increase the levels of SIRT1 and CAT (123). SIRT1 has been found to be important in revascularization, especially in tissues that have had a restricted blood supply (196). In addition, one of the forms of GST, GST-pi, was found by investigators to be critical in restoring balance to the ischemic heart, due to its nonclassical ability to bind NO and NO-containing compounds (137, 184). The rhythmicity of both the pi form and mu form of GST was found in a study done in the mouse livers in 2012 (195). As discussed previously, melatonin is an especially important hormone in circadian rhythm regulation, as well as a potent antioxidant. It has been found that melatonin has a beneficial effect of lowering blood pressure in both men and women (158). Several studies have also shown that there is a significant reduction in circulating melatonin levels in patients with heart disease as compared to control patients [reviewed in Ref. (45)]. These findings suggest that using agents to correct arrhythmic components of diseased patients, including blood pressure, can play key roles in survival after heart injury and in preventing injury and/or death.

Cancer

The expression of the core circadian rhythm genes is dysregulated in many types of cancer, as is the expression of proteins involved in oxidative stress. Table 1 provides information regarding the reported expression pattern of circadian rhythm and oxidative stress components in different cancers in humans. Interestingly, in many cancers, the antioxidants and protective enzymes involved in preventing oxidative stress still oscillate with circadian rhythmicity, but have been found to have altered levels of expression. For example, Kolanjiappan and Manoharan found that in rats with mammary tumors, there was a delay in the acrophase of SOD, CAT, GPx, and lipid peroxidation levels in blood as compared to controls (95). In addition, the mesor value (roughly, the average expression level) for lipid peroxidation was found to be increased while values for SOD, CAT, and GPx activities decreased, indicating a correlation between lowered antioxidant levels and increased lipid peroxidation in rats during mammary carcinogenesis (95). This trend of altered levels of circadian rhythmicity in oxidative stress components was also found in human gynecological malignancies and in oral cancer (106, 159).

While these studies show a significant difference in the circadian oscillation of antioxidants, protective enzymes, and consequently a difference in oxidative damage, these studies do not clearly show whether the disrupted circadian rhythm is a cause or consequence of cancer. However, a recent article by Gaddameedhi *et al.* shows that UVB radiation, which is a significant source of oxidative stress in the skin, causes five-fold higher frequency and a faster growth rate of skin cancer in mice when applied in the early morning rather than the early evening (57). In addition, XPA, which is a critical rate-limiting protein in the DNA damage response (shown in Fig. 7), was shown to oscillate with circadian rhythmicity in mouse skin, peaking in the early evening (57). This suggests that even when they are not disrupted, circadian rhythms in the body's defense system can play a significant role in cancer development, simply due to their impact on expression levels of protective proteins.

Recent studies in hepatocellular carcinomas suggest that melatonin treatment can reverse abnormal changes in daily oxidative stress rhythms, as well as tumor development. In the first study, rats displayed altered circadian rhythm characteristics (acrophase, amplitude, and mesor values) of

TABLE 1. CIRCADIAN RHYTHMS AND OXIDATIVE STRESS COMPONENTS IN CANCER

	<i>BMAL1</i>	<i>CLOCK</i>	<i>PER1</i>	<i>PER2</i>	<i>CAT</i>	<i>SOD</i>	<i>GSH</i>	<i>Lipid peroxidation</i>	<i>References</i>
Ovarian	↑	↓	↓	↓	↓	↑/↓	↑	↑	(76, 85, 148, 155, 174)
Prostate	↑	↓	↓	↓	↓	↑	↓	↑	(10, 27, 87)
Colorectal	=	↑	↓	↓	↓	↑	↓	↑	(122, 163, 164, 188)
Liver	=	=	↓	↓	↓	↓	↓	↑	(14, 31, 36, 102, 139)
Squamous cell	↓		↓	↓	↓	↓	↓	↑	(61, 67, 75)
Gliomas			↓	↓	↑	↓	↓	↑	(131, 194, 202)
Breast	↓		↓	↓	↓	↑	↑	↑	(30, 99, 132, 193, 197)
NSCL			↓	↓	↓	↑	↑	↑	(33, 60, 65, 79, 199)
Endometrial			↓	↓	↓	↓	↓	↑	(126, 198)

Arrows indicate direction of expression or activity change of the component; = indicates no change from control. CAT, catalase; CLOCK, circadian locomotor output cycles kaput protein; GSH, glutathione; NSCL, non-small cell lung; PER, periods.

lipid peroxidation, SOD, CAT, GPx, and GSH after *N*-nitrosodiethylamine-induced hepatocarcinogenesis (40). Treatment with melatonin decreased many of these differences. This indicates that melatonin significantly improves lipid peroxidation levels during the development of hepatocarcinomas through an increase in the levels of antioxidants and circadian phase shifts. A second study showed that melatonin treatment within this system also leads to a significantly lower tumor burden relative to untreated controls (171). Thus, melatonin is an effective tumor suppressor, possibly through its role as an antioxidant or its effects on circadian rhythm regulation, or both. It is therefore not surprising that low levels of melatonin have been associated with several types of cancer (9, 110, 151–153).

Interestingly, a number of studies in the past decade have suggested an involvement of SIRT1 in cancer (32, 77, 83, 88, 170, 203). Since SIRT1 impacts both circadian rhythms and oxidative stress, its aberrant expression could lead to disruptions in both systems. In the case of circadian rhythms, since SIRT1 deacetylation targets PER2 for degradation, this disruption could contribute to tumor development as illustrated in the case of PER2 knockout in mice (55). Mice deficient in the circadian gene *PER2* develop more tumors with reduced apoptosis after exposure to gamma-radiation. This effect is accompanied by an absence of the core circadian gene induction observed in wild-type mice and deregulation of cell cycle, cellular proliferation, and apoptosis (55). Studies from our laboratory have shown that in prostate cancer, inhibition of endogenously overexpressed SIRT1 results in decreased cell growth and viability, and an increase in FOXO1 activity (88). Interestingly, when p53 is present in this system, SIRT1 inhibition yields an increase in apoptosis, *versus* increased senescence in p53 knockout lines (86). Thus, p53 and FOXO1 are involved in the mechanism by which SIRT1 promotes tumor growth (86, 88, 187). Further, we have demonstrated that melatonin treatment inhibits SIRT1 expression and activity, also leading to decreased cell growth and viability, which suggests a second mechanism by which melatonin can impact both oxidative stress and circadian rhythms (89). Thus, as an upstream regulator of circadian rhythms and tumor suppressors that also impact oxidative stress, SIRT1 could play a large part in the possible role of both systems in tumor development.

Neurodegenerative diseases

Many neurodegenerative conditions show the presence of plaques of different proteins in the brain, neuronal cell death, and an onset later in life. In addition, many have been linked to both increased oxidative stress and the disruption of circadian rhythms. Two of these disorders are Alzheimer's disease (AD) and PD. As both of these diseases are age related, it is no surprise that sirtuins and antioxidants are potential targets for preventing their progression (46, 178). As discussed earlier, SIRT1 plays a major role in increased longevity due to calorie restriction and is important in both oxidative stress and circadian rhythm regulation. Qin *et al.* determined the relative levels of SIRT1 and amyloid- β proteins in the brains of Squirrel monkeys (133). They found that monkeys kept on a 30% calorie-restricted diet had increased levels of SIRT1 protein in the temporal cortex of the brain, and that those same areas had reduced levels of two amyloid- β pep-

tides (133). This suggests that SIRT1 may play a part in preventing the accumulation of the peptides. In addition to the role played by SIRT1 in the oxidative stress effects on AD, mitochondrial malfunction driven by increased oxidative stress has also been implicated in increasing amyloid- β plaques (157), leading to further evidence of the importance of oxidative stress and its regulation in the development of AD. This can be further confirmed by considering evidence that both the physiology and genetic expression of important circadian rhythm regulators are impacted in patients suffering from AD. In a review by Slats *et al.*, it was pointed out that AD patients were shown to have a severely atrophied SCN, as well as a marked decrease in melatonin levels and CLOCK expression, suggesting the importance of circadian rhythms in this disease (165). Conversely, this may also suggest that some component of AD acts to decrease the effectiveness of circadian rhythm regulation.

In PD, many of these same components have been found to be important. PD is a disease centered on the degeneration of the central nervous system, with most patients displaying a significant amount of cell death in dopaminergic neurons (41). It is thought that these neurons are especially susceptible to increases in oxidative stress, as they already have high levels of free radicals due to the cellular processes used to break down dopamine. Melatonin has been shown to offer some protection to dopaminergic neurons in a mouse model of two types of the disease, as well as attenuation of increases in lipid peroxidation and messenger RNA (mRNA) expression of cytochrome P-450 2E1, which has been shown to increase ROS (161). However, it is not known whether this observed response was due to melatonin's antioxidant property or its circadian rhythm regulation abilities. To illustrate the importance of circadian regulation in PD, several studies have looked at central circadian pathway members. For example, a study done by Cai *et al.* looked at expression levels of key components of the circadian rhythm machinery in leukocytes over a 12-h period in patients with and without PD (24). They found decreased levels of BMAL1, but not PER1, and further described a correlation between PD severity and BMAL1 expression levels (24). Another study found that BMAL2 also had significantly lower levels of expression in patients with PD than controls, suggesting a dysregulation of molecular clock components in patients with PD (44). Thus, both of these diseases, and many neurodegenerative diseases in general, seem to have strong oxidative stress and circadian rhythm components.

Interestingly, Prxs have been suggested to be protective agents for neurological disorders, because they are key regulators in inflammatory pathways, and as we discussed earlier, they have also been shown to be rhythmic (204). Inflammation has been shown to play a role in many neurodegenerative diseases, as well as many others, including heart disease (16, 140). Intriguingly, in addition to oxidative stress regulators being important in regulating the inflammatory response, it has been suggested that circadian rhythm regulation may also be involved in the regulation of inflammation. Radogna *et al.* have recently discussed a role of melatonin in regulating the inflammatory response in humans, both in a circadian fashion and through antioxidant activity, which also ties in with our premise that circadian rhythms and oxidative stress are intimately linked in many human pathologies (135).

Diabetes

Diabetes represents another excellent example of a disease that has deregulations in both oxidative stress and circadian rhythm machinery. Type I diabetes has been found to result from the loss of beta-cells in the pancreas, thereby destroying the body's ability to produce insulin and regulate blood sugar on its own (7). Type II diabetes is characterized by a reduction in the body's ability to respond to insulin or by a reduced secretion of insulin, often due to a poor diet, and generally shows up later in life (68). Among other possible causes, it has been suggested that one reason for the loss of beta-cells in Type I diabetes could be the buildup of an excessive amount of NO and other reactive species due to an external stressor (7, 49). Following this idea, a study by Van Dyke *et al.* found that an NO oxidizer may prevent diabetes formation in a rodent model of streptozotocin-induced diabetes (182). Thus, oxidative stress in the form of NO plays a role in diabetes.

The connection between oxidative stress and circadian rhythms in diabetes has been illustrated in several different ways. A study by Kanabrocki *et al.* found that many by-products of an increase in oxidative stress are regulated rhythmically in diabetics, stressing the importance of ROS levels. They found a marked circadian rhythm in DNA oxidation (in urine), uric acid (in serum), and nitric oxide (in serum) levels in nondiabetic men, but not in diabetic subjects (90). This study found that the peak levels of serum concentrations of the markers occurred in the early evening, suggesting a correlation between oxidative damage and increased metabolism. The significance of rhythmic factors in diabetes was also shown in a study that looked at mRNA expression of the core clock genes in cells isolated from patients with and without type 2 diabetes, in addition to insulin secretion (167). The authors found that PER2, PER3, and CRY2 mRNA levels in human islets were significantly lower in patients with type II diabetes than in normal controls. These levels correlate positively with insulin content, suggesting that dysregulation of the circadian clock may contribute to the pathology of Type 2 diabetes. It is therefore most likely that both increased oxidative stress (in the form of ROS and RNS) as well as a dysregulation of key circadian clock genes are instrumental in the onset and exacerbation of diabetes.

In addition to these diseases, other critical body processes and diseases are affected by both circadian rhythms and oxidative stress working in concert. Although we have discussed several here, there are still many more that have links to either circadian rhythms or oxidative stress, with no research done on the other. This could be an interesting direction for new research in diseases that show promise in using a cross-disciplinary approach. However, the diseases we have discussed appear to represent some of the most promising diseases to benefit from a concerted effort to target both oxidative stress and circadian rhythm dysregulation.

Conclusion

Oxidative stress has been a hot research topic in a number of scientific disciplines for more than five decades. Substantial research has also been done on circadian rhythms and its effects at cellular and organismal levels. Because these two important processes are so integral to the proper functioning of cellular activity, it is not a surprise that both have been implicated in many different diseases and disorders (Fig. 8).

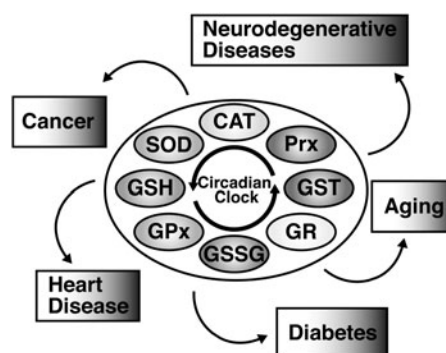


FIG. 8. Circadian rhythms, oxidative stress, and disease. Circadian rhythms have a significant impact on the oxidative stress defense system. Several key enzymes and antioxidants involved in protection from free radicals oscillate with circadian rhythmicity in expression or activity level. These include SOD, CAT, Prx, GSH, GPx, GSSG, GR, and GST. Circadian rhythms and oxidative stress are further connected by the role that they both play in several different diseases. Whether their disruption is a cause or an effect of these diseases remains to be determined, but further investigation into the connections between circadian rhythms, oxidative stress, and disease could lead to valuable new treatments in the future.

However, in-depth studies have not been done to determine the cause-and-effect association between circadian rhythm and oxidative stress-signaling components. There is a need to intensify our efforts to conduct such studies in appropriate model systems. One caveat of the current status of circadian rhythm research is that the rodent models, which are most widely used animal models, may or may not be directly relevant to humans, especially when developing human therapies. This is because mice, rats, and hamsters are nocturnal animals. However, it is conceivable that most of the findings could be phase-adjusted (101). Another important consideration is that many of the antioxidant enzymes have several isoforms or family members, many of which were discovered after the cited research was done. This makes it difficult to determine which isoform is responsible for rhythmicity, as it is likely that not all of these members are rhythmically regulated. However, the evidence suggests that at least one form of each enzyme is regulated in a circadian fashion, and it appears to be enough to affect disease pathologies. We believe that the diseases that have both circadian rhythm and oxidative stress components in their pathogenesis may benefit from a two-pronged approach of targeting both components. For example, a combinatorial approach comprised of antioxidants and circadian rhythm-resynchronizing approaches (*i.e.*, melatonin) could be beneficial to patients with these conditions. Intense concerted and collaborative efforts of scientists from diverse discipline are needed to explore these possibilities.

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Abbreviations Used

AD = Alzheimer's disease
ARNTL = aryl hydrocarbon receptor nuclear
translocator-like
BMAL1 = aryl hydrocarbon receptor nuclear
translocator-like (also known as ARNTL)
CAT = catalase
CLOCK = circadian locomotor output cycles kaput protein
CRY = cryptochrome
GPx = glutathione peroxidase
GR = glutathione reductase
Cu/Zn = copper/zinc
GSH = glutathione
GSSG = glutathione disulfide
GST = glutathione S-transferase
H₂O₂ = hydrogen peroxide

HDAC = histone deacetylase
NAD⁺ = nicotinamide adenine dinucleotide
NER = nucleotide excision repair
NSCL = non-small cell lung
PD = Parkinson's disease
PER = period
Prx = peroxiredoxin
RNS = reactive nitrogen species
ROR = RAR-related orphan receptors
RORE = retinoic acid-related orphan receptor-response
elements
ROS = reactive oxygen species
SCN = suprachiasmatic nucleus
SOD = superoxide dismutase
UV = ultraviolet
XPA = xeroderma pigmentosum A