Circadian control by the reduction/oxidation pathway: Catalase represses light-dependent clock gene expression in the zebrafish

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Light is the key entraining stimulus for the circadian clock, but several features of the signaling pathways that convert the photic signal to clock entrainment remain to be deciphered. Here, we show that light induces the production of hydrogen peroxide (H_2O_2) that acts as the second messenger coupling photoreception to the zebrafish circadian clock. Treatment of light-responsive Z3 cells with H_2O_2 triggers the induction of *zCry1a* and *zPer2* genes and the subsequent circadian oscillation of *zPer1*. Remarkably, the induction kinetics and oscillation profile in response to H_2O_2 are identical to those initiated by light. Catalase (*Cat*), an antioxidant enzyme degrading H_2O_2 , shows an oscillating pattern of expression and activity, antiphasic to *zCry1a* and *zPer2*. Interestingly, overexpression of zCAT results in a reduced light-dependent *zCry1a* and *zPer2* gene induction. In contrast, inhibition of zCAT function enhances light-mediated inducibility of these clock genes. These findings implicate the enzymatic function of zCAT enzyme in the negative regulation of light-dependent clock gene transcriptional activation. Our findings provide an attractive link between the regulation of the cellular reduction/oxidation (redox) state and the photic signaling pathways implicated in circadian control.

light signaling | hydrogen peroxide | transcription | circadian rhythms

Organisms ranging from bacteria to humans have daily rhythms driven by endogenous oscillators called circadian clocks that regulate various biochemical, physiological, and behavioral processes with a periodicity of ≈ 24 h (1–3). Under natural conditions, rhythms are entrained to a 24-h cycle by environmental time cues, light being the most important. These circadian clock mechanisms have been investigated and specific “clock genes” that affect the daily rhythm have been identified. The core of the clock mechanism in *Drosophila*, *Neurospora*, mammals, and cyanobacteria is commonly represented by a transcription/translation-based negative-feedback loop that relies on positive and negative oscillator elements. Although the organization of the negative feedback loop in *Drosophila*, *Neurospora*, mammals, and cyanobacteria is conceptually similar, its components differ among species (1). In mammals, two basic helix–loop–helix PAS(PER-ARNT-SIM) domain-containing transcription factors, CLOCK and BMAL1, constitute the positive elements (4, 5). Upon heterodimerization, CLOCK:BMAL1 drives the transcription of the negative components of the clock machinery, three *Period* genes (*Per1*, *Per2*, and *Per3*) and two *Cryptochrome* genes (*Cry1* and *Cry2*). The PERs and CRYs negatively regulate their own expression, setting up the rhythmic oscillations of gene expression that drive the circadian clock (6–8).

The zebrafish constitutes an attractive alternative to the mammalian system to study the complexity of the circadian clock machinery and the influence that light has on it (9, 10). Characterization of the molecular components of the zebrafish circadian oscillator has revealed that the zebrafish negative-feedback loop consists of components similar to those of mam-

mals (10). Organ and tissue culture explant experiments have demonstrated that peripheral circadian oscillators are present throughout the tissues and organs of the zebrafish and that they display the remarkable feature of being light-responsive (11–13). We have established a cell line derived from zebrafish embryos, designated Z3, in which circadian clock components display distinct and differential light-dependent activation and expression profiles under various light conditions (14). Z3 cells, therefore, nicely recapitulate the zebrafish clock system and constitute an invaluable tool for investigating the link between light-dependent gene activation and the signaling pathways responsible for the generation of vertebrate circadian rhythms.

Hydrogen peroxide (H_2O_2) is one of the reactive oxygen species (ROS) which is generated from various sources, including the mitochondria and the peroxisome, as a by-product of normal metabolism (15, 16). H_2O_2 can be generated by specific enzymes, such as NADPH oxidase, Acyl-CoA oxidase (AOX), and amine oxidase (17–19). A variety of extracellular stimuli, like visible light, low and high temperature, and wounding, enhance cellular H_2O_2 generation through the activation of these enzymes (20, 21). Excess production of H_2O_2 has deleterious effects because H_2O_2 can react with various cellular targets such as DNA, proteins, and lipids, causing cellular damage (22). As a protective system against increased intracellular levels of H_2O_2 , cells possess several antioxidant enzymes that degrade H_2O_2 . One prominent antioxidant enzyme in the cell is catalase, which converts H_2O_2 to O_2 and H_2O (23). Thus, a critical balance between the production and the metabolism of H_2O_2 is required to determine the cell fate.

Although H_2O_2 can act as a toxic metabolite, it is ideally suited to be a signaling molecule because of its small size, so it can diffuse short distances within the cell, and because several mechanisms for its production and its rapid removal are present in the cell. Indeed, various studies have established that H_2O_2 has an intracellular signaling role in many biological systems. For example, H_2O_2 has been implicated in a wide range of plant responses, which include the closure of stomata and the regulation of cell expansion and development (24, 25). In addition, it has been demonstrated that H_2O_2 controls the expression of many genes, such as *c-fos*, *c-jun*, and *c-myc* (26), and that it activates cell signaling cascades, such as those involving mitogen-activated protein kinases (MAPKs), to mediate responses to a variety of extracellular stimuli (27). It is thereby established that H_2O_2 can act as a signaling molecule to induce a range of molecular, biochemical, and physiological responses in cells, and

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Fig. 1. Light produces H₂O₂ in Z3 cells. Quantitative analysis of DCF fluorescence. Z3 cells were loaded with CM-H₂DCFDA and then were exposed to light. CM-H₂DCFDA fluorescence was detected by spectrofluorimetry. Values are means $\pm$ SE of three independent experiments. In each experiment, the CM-H₂DCFDA fluorescence of the nontransduced cells kept in the dark was set as 1. Where indicated, the cells were transduced with *zCat* or *luciferase* genes by retroviral vector (shown by zCAT and LUC, respectively).

it is therefore conceivable that it could mediate signaling pathways for additional biological events. Here, we show that H₂O₂ is a key signaling mediator for light-dependent circadian gene expression in the zebrafish. In addition, we provide several lines of evidence that the antioxidant enzyme catalase is involved in circadian gene control by governing intracellular levels of H₂O₂. Our data strongly suggest that the alteration of the reduction-oxidation (redox) status within a cell is used as a signaling mechanism to transduce photic signals to circadian transcriptional regulation.

Results

Light Induces H₂O₂ Production in Z3 Cells. H₂O₂ is recognized to function as a ROS signaling molecule that mediates responses to various stimuli in plants and animals (15, 16). Because light has been reported to induce production of H₂O₂ in cultured cells (19, 28), we hypothesized that it could be the signaling molecule that would convey the photic signal to the circadian transcriptional machinery in Z3 cells. We therefore evaluated H₂O₂ production in Z3 cells after light exposure. Z3 cells were loaded with a nonfluorescent, membrane-permeable derivative of CM-H₂DCFDA that fluoresces when oxidized by ROS and then were exposed to light. Light treatment leads to a robust increase in CM-H₂DCFDA fluorescence in Z3 cells (Fig. 1). To assess the selectivity of the fluorescent response for H₂O₂, we infected Z3 cells with retroviral vectors expressing either the *catalase* or the *luciferase* genes. Catalase converts H₂O₂ to O₂ and H₂O (23). Expression of transduced genes was confirmed by catalase or luciferase activity assay (data not shown). The response of CM-H₂DCFDA intensity was shown to be coupled to H₂O₂, because catalase-mediated transduction markedly decreased the fluorescent signal, whereas luciferase had no effect (Fig. 1). Taken together, these results demonstrate that light induces H₂O₂ production in Z3 cells.

H₂O₂ Induces *zCry1a* and *zPer2* Expression in Z3 Cells. We have previously established that light induces *zPer2* gene expression, an event that initiates the light-dependent entrainment process of Z3 cells permanently cultured in the dark (14, 29). To obtain evidence that H₂O₂ functions as signaling mediator for light-dependent circadian transcription, we examined the effect of H₂O₂ on circadian clock gene expression. Z3 cells were kept in constant darkness for 3 days and then stimulated with 100 μ M H₂O₂. *zCry1a* and *zPer2*

Fig. 2. H₂O₂ induces *zCry1a* and *zPer2* expression in Z3 cells and this induction is mediated by MAPK pathway. (A) A time course of circadian gene induction upon H₂O₂ treatment of confluent Z3 cells was examined by RNA protection assay. Z3 cells permanently cultured in the dark were treated with H₂O₂ (100 μ M). At indicated time points after the treatment, cells were harvested for RNA preparation. tRNA serves as our negative control reaction (t). Relative amounts of total RNA used for each sample are displayed (RNA). (B) Circadian gene induction by different concentrations of H₂O₂. Z3 cells kept in the dark and stimulated with H₂O₂ at the concentrations of 10⁻³, 10⁻², 10⁻¹, 1, 10, 50, and 100 μ M. After 100 min, cells were harvested for RNA preparation. As the negative control (0 μ M H₂O₂ concentration), we added water to the culture media. (C) Z3 cells kept in dark and stimulated with light (L), H₂O₂ (H) (100 μ M), UV (U) (10 mJ/m², 30 seconds), or MNU (M) (2 mM). After 100 min, cells were harvested for RNA preparation. (D) Confluent Z3 cells were treated with the indicated inhibitors or with the vehicle (DMSO, 0 M). The concentrations of U0126 and SB203580 inhibitors used were 40 μ M and 10 μ M, respectively. After 1 h, cells were stimulated with light (L) or H₂O₂ (100 μ M) (H), or were left in the dark (D). After 100 min, cells were harvested, and RNA was prepared. (E) Confluent Z3 cells were treated with the indicated inhibitors or with the vehicle (DMSO, 0 M). The concentrations of U0126 and SB203580 inhibitors used were 40 μ M and 10 μ M, respectively. After 1 h, cells were stimulated with light (L) or were left in the dark (D). After 7 h, cells were harvested, and RNA was prepared.

expression was strongly induced by H₂O₂, reaching maximal levels 2 h after treatment (Fig. 2A). Importantly, the profile of H₂O₂-induced gene expression is analogous to that of light-dependent activation of *zCry1a* and *zPer2*, peaking 2 h after light onset (compare Figs. 2A and 6A). On the other hand, H₂O₂ induced a slight reduction in *zPer1* expression and had no apparent effect on *zCat* and *zAox* (Fig. 2A).

To better characterize the response of cells to H₂O₂, Z3 cells were stimulated for 100 min with H₂O₂ at various concentrations. Interestingly, a concentration as low as 1 nM caused a significant increase in *zCry1a* and *zPer2* expression (Fig. 2B).

Because H₂O₂ induces DNA damage in cells, we reasoned that DNA damage could represent a stimulus for clock gene expression. To test this possibility we treated Z3 cells with other genotoxic reagents, such as UV light or *N*-methyl-*N*-nitrosourea (MNU). Importantly, none of these DNA damage-inducing

Catalase Activity. Z3 cells were washed twice with PBS, homogenized in medium consisting of 50 mM phosphate buffer (pH 7.0), and then clarified by centrifugation for 10 min at 10,000 ×

g. The supernatant was used for the assay. Total catalase activity was measured by monitoring the decomposition of 10 mM H₂O₂ (47).

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