Diphenyleneiodonium Chloride, an Inhibitor of Reduced Nicotinamide Adenine Dinucleotide Phosphate Oxidase, Suppresses Light-Dependent Induction of Clock and DNA Repair Genes in Zebrafish

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In most species, solar light is both a DNA-damaging agent and the key entraining stimulus for the endogenous circadian clock. The zebrafish is an attractive vertebrate system in which to study the influence of light on gene expression because the DNA repair proteins and circadian oscillators in this species are light-responsive. At the molecular level, light treatment of zebrafish cells induces the production of reactive oxygen species (ROS). ROS both alters the reduction–oxidation (redox) state of these cells and stimulates intracellular extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) cascades that transduce photic signals activating the transcription of particular light-responsive genes, including some clock genes and some DNA repair genes involved in photoreactivation. To date, however, the phototransducing molecules responsible for light-dependent ROS production have not been identified. Flavin-containing oxidases, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, are versatile flavoenzymes that catalyze molecular oxidation in numerous metabolic pathways. Importantly, light induces the photoreduction of the flavin adenine dinucleotide (FAD) moiety in these oxidases, leading to ROS production. Here, we show in cultured zebrafish cells that diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase, both suppresses ERK/MAPK activation and efficiently reduces light-dependent expression of clock and photoreactivation genes. Our results suggest that flavin-containing oxidases may be responsible for light-dependent ROS production and thus light-dependent gene expression in zebrafish. Our findings also support the existence of a regulatory link between photoreactivation and the circadian clock in this species.

Key words circadian clock; flavin-containing oxidase; reduction–oxidation; reactive oxygen species; zebrafish

Solar light has both beneficial and harmful effects on most species. Beneficial uses include photosynthesis and the entrainment of circadian clocks. However, the UV component of solar light produces cytotoxic, mutagenic and carcinogenic lesions in DNA that can transform or kill cells. Photoreactivation is a mechanism by which visible light can reverse some of the lethal and mutagenic effects of UV irradiation.

Circadian clocks are endogenous oscillators that drive the daily rhythms of organisms ranging from bacteria to humans. These clocks regulate various biochemical, physiological, and behavioral processes with a periodicity of approximately 24 h. Under natural conditions, circadian rhythms are entrained to this 24 h day by environmental time cues, with light level being the most important. The core of the clock mechanism in almost all organisms studied to date is a transcription/translation-based negative feedback loop that relies on positive and negative oscillators. In vertebrates, three basic helix–loop–helix PAS (PER-ARNT-SIM) domain-containing transcription factors, called CLOCK, NPAS2 and BMAL, constitute the positive elements. CLOCK or NPAS2 heterodimerizes with BMAL to form an transcriptionally active complex that binds to E-box elements (CACGTG) present in the promoters of members of the Period (Per) and Cryptochrome (Cry) gene families. Once the PER and CRY proteins have been translated, they form heterodimers that can then translocate to the nucleus to repress CLOCK (NPAS2):BMAL-mediated transcription through direct protein–protein interaction. These interactions then set up the rhythmic oscillations of gene expression that drive the circadian clock.

Zebrafish have been established as an attractive vertebrate model suitable for the examination of the light signaling pathway and its impact on the circadian clock. Not only are the molecular components of the mammalian and zebrafish circadian oscillators highly similar, but zebrafish clocks have the unusual attribute of being directly light-responsive.

In zebrafish organs, tissues and cultured cells, exposure to an acute light pulse transactivates two clock genes, zCry1a and zPer2, and entrains oscillations of clock gene expression to a new light-dark cycle. Zebrafish have also been used to study the light-dependent triggering of DNA repair via photoreactivation. It has recently reported that the light-dependent induction of 64 photolase (z64Phr), a DNA repair enzyme, is essential for successful photoreactivation in zebrafish.

Importantly, studies in zebrafish have revealed critical roles for the cellular redox state and mitogen activated protein kinase (MAPK) signaling pathways in the regulation of light-dependent gene expression. In a variety of organisms, light induces ROS production that alters the reduction–oxidation (redox) state within cells. In zebrafish cells, a light-induced change in redox state stimulates intracellular extracellular signal-regulated kinase (ERK)/MAPK signaling that transduces photic signals. When zCry1a and zPer2 expression is induced by such signals, zCry1a and zPER2 proteins can initiate the light-dependent entrainment process in zebrafish cells cultured permanently in the dark. Similarly, in small fishes such as zebrafish and medaka, the expression of phr genes essential for photoreactivation has been associated with light-induced reactive oxygen species.
(ROS) production and ERK/MAPK signaling.6,14)

The phototransducing molecules responsible for light-dependent ROS production and its associated effects on gene expression have yet to be identified. Flavin-containing oxidases are versatile flavoenzymes that catalyze molecular oxidation in numerous metabolic pathways, generating ROS as a by-product.15) Importantly, it has been shown that flavin-containing oxidases can produce ROS in a light-dependent manner.16) In this study, we provide evidence suggesting that photoreduction of flavin-containing oxidases may underlie the light-dependent ROS production that regulates both the circadian clock and photoreactivation in zebrafish.

MATERIALS AND METHODS

Reagents and Antibodies Diphenyleneiodonium chloride (DPI; NADPH oxidase inhibitor) and l-NG-nitroarginine methyl ester (l-NAME; nitric oxide synthase inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Anti-ERK and anti-phospho-ERK primary antibodies were purchased from Cell Signaling and New England Biolab, respectively.

Cell Culture, Enzyme Inhibition and Light Induction Cultured zebrafish cells were prepared as described previously8,17) and maintained at 28 °C in L-15 medium (Sigma-Aldrich) containing 10% fetal bovine serum. For inhibitor experiments, cells were cultured in the above medium for 2 d in constant darkness prior to treatment (in the dark) for 60 min with dimethyl sulfoxide (DMSO) (vehicle control), 1 µM or 10 µM diphenyleneiodonium chloride (DPI), or 10 µM or 100 µM l-NAME. Treated cells were then exposed to light for 120 min before extracts were prepared for mRNA expression analysis (see below), or for 0—120 min before extracts were prepared for immunoblotting (see below).

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA extraction was carried out using TRIzol (Invitrogen) according to the manufacturer’s instructions. Total RNA was reverse-transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen) and random oligohexamers. Each quantitative real-time RT-PCR reaction was performed using the Chromo4 real-time detection system (Bio-Rad). For a 20 µl PCR reaction, 10 µl containing cDNA template mixed with the appropriate primers to a final concentration of 200 nM was combined with 10 µl iQ SYBR Green Supermix (Bio-Rad). The reaction was incubated at 95 °C for 3 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. PCR primers for the zCry1a, zPer2, z64Phr and zActin genes have been described previously.5)

Immunoblotting Cells were homogenized in binding buffer (150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet P-40, 1 mM ethylene glycol bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 5% glycerol, and 20 mM Tris–HCl pH 7.4) containing protease inhibitor mixture tablets. Lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electroblotting onto polyvinylidene difluoride membranes. Membranes were blocked with 2% or 5% nonfat milk and incubated for 10 h at 4 °C with primary anti-total ERK or anti-phospho-ERK antibody. Blots were then incubated with the appropriate secondary anti-
tibody and developed with the ECL Western blotting detection system (Amersham Biosciences).

Measurement of Intracellular ROS Levels Zebrafish cultured cells were pretreated with DPI or with the vehicle (DMSO, control). Cells were then loaded with 5 µM 2,7-dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich) and exposed to light. The resultant oxidized DCF was monitored from fluorescent intensity using a microplate fluorometer (ARVO MX, Perkin-Elmer) with excitation and emission at 485 and 530 nm, respectively. Cells were cultured in hanks’ balanced salt solution (Sigma-Aldrich) during the assay.

RESULTS

The NADPH Oxidase Inhibitor DPI Blocks Light-Induced Gene Expression In zebrafish, redox signaling couples photoreception to the induction of the zCry1a and zPer2 genes, an event that initiates the light-dependent entrainment of circadian clock.9—11) Redox signaling has also been linked to the induction of zPhr genes during photoreactivation.3,14,18) Nitric oxide (NO) is a well-established second messenger involved in redox signaling.19) We therefore investigated whether NO might be involved in light-dependent zCry1a, zPer2 and/or z64Phr expression. Cultured zebrafish cells were kept in constant darkness for 2 d prior to treatment for 60 min in the dark with DMSO (control) or l-NAME (10 or 100 µM), a specific NO synthesis inhibitor.20) The treated cells were then exposed to light for 120 min and gene expression was measured by RT-PCR. Consistent with previous reports,10,11) light strongly induced expression of zCry1a and zPer2 in the presence of vehicle alone (Figs. 1A, B). Notably, neither concentration of l-NAME had any effect on this induction. Similarly, l-NAME treatment had no inhibitory effect on the light-dependent induction of z64Phr expression (Fig. 1C). Thus, NO plays no role in the light-dependent expression of genes associated with either the circadian clock or photoreactivation.

Redox signaling is also influenced by ROS, and ROS has been identified as a key signaling mediator in zebrafish circadian transcription.5,10,13,18) However, the mechanism(s) underlying the ROS production responsible for light-dependent clock gene expression have not been identified. Flavin-containing oxidases have been shown to produce ROS in a light-dependent manner.16) We therefore speculated that these enzymes might be involved in light-induced gene expression in zebrafish. To test this possibility, we examined the effects of DPI, an inhibitor of the flavin-containing enzyme NADPH oxidase, on light-dependent gene expression in zebrafish cells. Cultured zebrafish cells were treated with DMSO or DPI (1 or 10 µM) and assayed for mRNA levels as described for l-NAME above. Strikingly, DPI significantly suppressed the light-induced upregulation of the zCry1a and zPer2 genes (Figs. 1D, E). The light-induced expression of the z64Phr gene was also blocked by DPI (Fig. 1F). Although DPI has been reported to inhibit NO synthesis,21) it was clear from our earlier results (Figs. 1A—C) that the specific NO synthesis inhibitor l-NAME did not affect light-dependent gene expression in zebrafish cells. Our results thus strongly implicate flavin-containing oxidases as playing a key role in the light-dependent expression of zebrafish circadian clock and photoreactivation genes.
The NADPH Oxidase Inhibitor DPI Suppresses Light-Induced ROS Production

Next we evaluated ROS production in cultured zebrafish cells after light exposure by a fluorometric assay. Zebrafish cultured cells were loaded with a nonfluorescent, membrane-permeable derivative of DCFH-DA that fluoresces when oxidized by ROS and then were exposed to light. As shown in Fig. 2, light treatment led to a significant increase in DCFH fluorescence in cells. Notably, DPI efficiently decreased the fluorescent signal. Because the flavin-containing oxidases have been shown to produce ROS in a light-dependent manner,16) these results provide evidence that DPI acts on these oxidases to suppress the light-induced intracellular ROS production.

The NADPH Oxidase Inhibitor DPI Impairs Light-Induced ERK/MAPK Activation

We have previously reported that light can directly activate ERK/MAPK signaling in zebrafish cells, and that this activation is critical for light-dependent expression of both circadian (zCry1a and zPer2) and DNA repair (z64Phr) genes.5) We therefore examined the effects of DPI and l-NAME on light-induced ERK/MAPK activation. Cultured zebrafish cells were treated with 10 \mu M DPI or 100 \mu M l-NAME for 60 min in the dark prior to exposure to light. Cells were harvested at various time points from 0—120 min after light stimulation, and extracts were immunoblotted to detect total and phospho-ERKs. Consistent with our previous report,5) phospho-ERK levels increased significantly at 5—15 min after light exposure in the absence of DPI or l-NAME (Figs. 3A, B, left). However, no phospho-ERK was detectable in DPI-treated cells at any time point (Fig. 3A, right). In contrast, l-NAME did not produce the drastic effect on light-induced ERK/MAPK activation as DPI did (Fig. 3B, right), indicating that DPI’s effect on ERK/MAPK activation is not due to suppression of NO synthesis. Taken together, our results suggest that DPI negatively regulates the light-dependent expression of circadian and DNA repair genes by inhibiting the activation of ERK/MAPK signaling. These findings indicate that the role of flavin-containing oxidases in light-dependent gene expression involves effects on ERK/MAPK signaling.

DISCUSSION

Visible light induces photo-oxidative stress in living organisms,24) and it has been proposed that cellular responses
to such photo-oxidative stress are the evolutionary origin of circadian rhythms.\textsuperscript{3,25} The fact that these processes are regulated by alteration of a cell's redox state strongly suggests that oxidative stress may act as the triggering signal. The toxic effects of oxidative stress have been linked to cellular stress responses, DNA repair, and cell cycle regulation.\textsuperscript{5,13,18,29} Our results suggest that the photo-oxidative stress that regulates the expression of these genes is exerted by ROS produced by light-activated flavin-containing oxidases. These enzymes would thus be newly-identified core components of the cellular response to photo-oxidative stress.

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