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Light-Activated Regulation of Cofilin Dynamics Using a Photocaged Hydrogen Peroxide Generator

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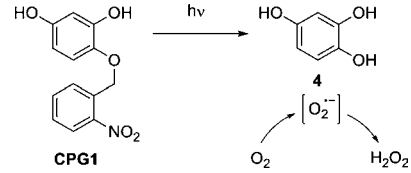
Abstract: Hydrogen peroxide (H_2O_2) can exert diverse signaling and stress responses within living systems depending on its spatial and temporal dynamics. Here we report a new small-molecule probe for producing H_2O_2 on demand upon photoactivation and its application for optical regulation of cofilin-actin rod formation in living cells. This chemical method offers many potential opportunities for dissecting biological roles for H_2O_2 as well as remote control of cell behavior via H_2O_2-mediated pathways.

Hydrogen peroxide (H_2O_2) is a potent small-molecule oxidant that can influence the growth, development, and fitness of living organisms in a wide variety of ways.[1] Aberrant production of H_2O_2 leads to oxidative stress and damage cascades connected to aging[2] and diseases ranging from cancer[2] to neurodegeneration,[3] whereas regulated H_2O_2 fluxes are used in phagocytic killing of invading pathogens.[4] In addition, newer studies link H_2O_2 signaling[5−9] to cell growth, proliferation, and migration events that form the basis for beneficial processes like wound healing[10] and neurotransmission.[11]

Because the localized dynamics of H_2O_2 are intimately linked to disparate physiological and/or pathological consequences, a major challenge in dissecting roles for H_2O_2 and its downstream effects is a dearth of methods for directly probing this reactive oxygen metabolite in complex biological settings. In this context, the vast majority of chemical tools developed to study H_2O_2 in living environments are fluorescent probes,[12−21] but another potentially powerful chemical approach for interrogating H_2O_2 biology is through the use of photocaged compounds,[22−24] which have been employed to release bioactive molecules such as ATP,[25] neurotransmitters,[26−28] metal cations,[29−31] peptides,[32] and proteins[33,34] in living cells with spatial and temporal fidelity by unmasking a photolabile protecting group. Such synthetic systems offer the opportunity to turn on a specific type of chemical reactivity within live biological specimens with precise spatial and temporal control. Here we report the synthesis and properties of Caged Peroxide Generator 1 (CPG1), a new type of synthetic small-molecule probe that can produce H_2O_2 upon photoactivation. Molecular imaging with H_2O_2-sensitive fluorescent reporters establishes that CPG1 can deliver H_2O_2 on demand to living cells by photochemical manipulation. Moreover, we have applied the photocaged H_2O_2 reactivity of CPG1 for optical control of cytoskeleton dynamics through redox-regulated cofilin-actin rod formation, presaging the utility of this new chemical method for elucidating roles for H_2O_2 in cell signaling and stress cascades as well as real-time, remote control of cell behavior via H_2O_2-mediated pathways.

Scheme 1. Photolysis of CPG1 Releases 1,2,4-Trihydroxybenzene (**4**), which Sequentially Reduces Molecular Oxygen to Give H_2O_2 via a Superoxide Intermediate



The design and action of CPG1 is shown in Scheme 1. We reasoned that electron-rich *ortho*- or *para*-hydroquinones, which are known to produce H_2O_2 by reduction of molecular oxygen with concomitant oxidation to the corresponding quinone species via semiquinone radical and superoxide (O_2^{-}) intermediates,[35] would provide a platform amenable to delivery of caged H_2O_2 in a fashion similar to that for endogenous H_2O_2-producing NAD(P)H oxidases. Whereas in both systems O_2^{-} is the initial ROS generated upon reduction of O_2, the relative cellular stability of H_2O_2 compared to O_2^{-} support the latter as the primary signaling agent.[1] In initial experiments we screened several hydroquinones for their ability to produce H_2O_2 in water as measured using the H_2O_2-specific fluorescent probe Peroxyfluor 1 (PF1).[12] The electron-rich polyphenol 1,2,4-trihydroxybenzene **4** produces more H_2O_2 than its structural isomer pyrogallol (Figures S1 and S2). H_2O_2 production by **4** is completely abrogated by the addition of 25 U/mL catalase, whereas addition of 50 U/mL superoxide dismutase (SOD) reduces H_2O_2 levels by 50%, establishing the role of superoxide as an intermediate in the CPG1-mediated production of H_2O_2 and suggesting that oxidation of **4** or its semiquinone radical competes with the spontaneous dismutation of superoxide in the absence of enzymes (Figure S3). When loaded into HEK 293 cells, the peracetylated form of **4** is membrane-permeable and, after *in situ* ester hydrolysis by intracellular esterases, generates levels of H_2O_2 that are detectable by PF1 (Figure S4).

Based on these results, we synthesized CPG1 as shown in Scheme S1. Installation of a photolabile *ortho*-nitrobenzyl ether at the 1-hydroxy position effectively blocks quinone formation by imposing a *meta* arrangement between the two remaining hydroxyl groups, rendering CPG1 oxidatively inert. In turn, removal of this protecting group by photolysis furnishes the reactive hydroquinone H_2O_2 generator. Alkylation of bis-MEM protected phenol **1** with 2-nitrobenzyl bromide in acetone affords nitrobenzyl ether **2** in 86% yield. Removal of the MEM groups using *para*-toluenesulfonic acid monohydrate in ethanol delivers nitrobenzyl-caged CPG1 **3** in 74% yield. In DPBS at pH 7, CPG1 features a moderate-intensity UV absorbance band centered at 280 nm (ϵ = 6400 M^{-1} cm^{-1}).

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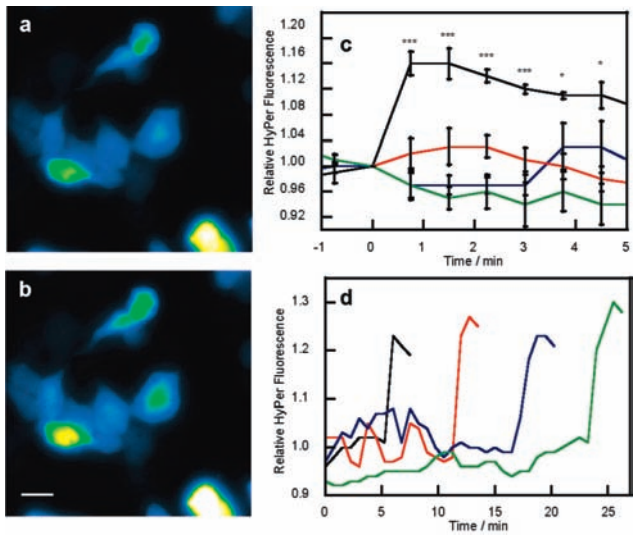

Figure 1. Controlled generation of intracellular H_2O_2 by photolysis of CPG1. (a) Live-cell imaging of HyPer-expressing HEK 293 cells loaded with 100 μM Ac-CPG1. (b) Live-cell imaging of the same cells after photodecaging of CPG1 with UV light to initiate H_2O_2 production from 1,2,4-trihydroxybenzene. (c) Real-time fluorescence intensity readouts of HyPer-expressing HEK cells upon UV irradiation (40 s) in the presence or absence of CPG1, showing cells loaded with Ac-CPG1 and activated by UV light (black line), cells loaded with Ac-CPG1 but not exposed to UV irradiation (blue line), cells without CPG1 under UV irradiation (red line), and cells without CPG1 that are also not exposed to UV activation (green line). HyPer fluorescence is calculated from 10−15 cells per experiment. Error bars are ±SEM for at least three separate experiments. * = $p < 0.05$. *** = $p < 0.01$ (Student's t test). (d) Temporal control of phototriggered release of H_2O_2 in living HEK cells by CPG1, as monitored by HyPer fluorescence. HyPer-expressing HEK cells were loaded with Ac-CPG1 as in panel (a) and then washed and imaged on stage. UV light (40 s) was delivered at varying times, and plots show data where UV excitation to elicit CPG1-mediated generation of H_2O_2 occurs after 5 min (black line), 11 min (red line), 17 min (blue line), or 23 min (green line) in separate experiments.

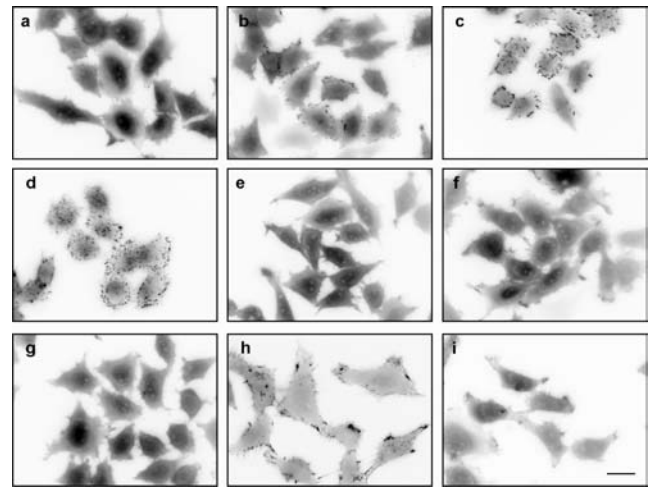

Figure 2. Live-cell imaging of H_2O_2-induced cofilin-actin rod formation in eGFP-cofilin expressing HeLa cells by exogenous H_2O_2 addition or light-induced generation of H_2O_2 with CPG1. (a) eGFP-cofilin HeLa cells treated with 10 μM H_2O_2 show no rod formation. (b) Cells stimulated with 50 μM H_2O_2 produce a regulated pattern of rod formation that localizes to the periphery of the cell membrane. (c) Samples treated with 100 μM H_2O_2 form numerous intracellular cofilin-actin rods. (d) Samples treated with 500 μM H_2O_2 also form numerous intracellular cofilin-actin rods. (e) Control eGFP-cofilin HeLa cells without CPG1 or UV irradiation show no rod formation. (f) Control eGFP-cofilin HeLa cells loaded with 200 μM Ac-CPG1 without UV irradiation do not show rod formation. (g) Control cells without CPG1 that were treated with an identical pulse of UV excitation show negligible rod formation, establishing that cofilin activation requires light-initiated decaging of CPG1 to elicit H_2O_2 production. (h) CPG1-loaded HeLa cells activated with UV light exhibit controlled rod formation in a global pattern similar to that observed upon stimulation with 50 μM H_2O_2. (i) Control cells from the same imaging dish as (h), which were not exposed to UV light and do not show rod formation. All images displayed were taken 26 min after treatment with either H_2O_2 or UV light, except for (i), which was taken immediately after (h). Cells treated with Ac-CPG1 were loaded for 10 min at 37 °C, washed, and then imaged. UV light was provided in 1 s pulses for the first 9 min of the experiment. Scale bar = 30 μm.

Photolysis of a 50 μM CPG1 solution with a hand-held UV lamp (304 nm, 8 W) results in almost complete cleavage of the nitrobenzyl cage after 30 min and the liberation of 1,2,4-trihydroxybenzene **4** as confirmed by LC-MS (Figure S5). The quantum yield for CPG1 photolysis was determined to be $\Phi = 0.19$ using caged P_i as a standard (Supporting Information). H_2O_2 production by photodeprotection of CPG1 can also be monitored using PF1. Photolysis of a 200 μM CPG1 solution for 30 min (304 nm, 8 W) produces ca. 20−30 μM H_2O_2 by fluorometric analysis with PF1, a level suitable for triggering cellular responses (Figure S6).

We next tested the ability of CPG1 to deliver H_2O_2 to living cells with spatial and temporal control using light as a trigger. To avoid potential issues of small-molecule fluorescent dye uptake and retention, we monitored the photoinitiated production of intracellular H_2O_2 by CPG1 using the genetically encodable protein sensor HyPer. HyPer-expressing HEK 293 cells loaded with 100 μM of the acetylated, cell-permeable form of CPG1 (Ac-CPG1) for 30 min exhibit basal levels of intracellular fluorescence as determined by epifluorescence microscopy (Figure 1a). Upon CPG1 photolysis, cells show a prompt 20% rise in H_2O_2-induced HyPer fluorescence within 2 min of UV irradiation as seen in Figure 1b−1c. Importantly, control experiments displayed in Figure 1c establish that no significant increases in HyPer fluorescence are observed under conditions where CPG1-loaded cells are not irradiated with UV light, cells without CPG1 are irradiated with UV light, or cells without CPG1 are not exposed to UV light, verifying that observed increases in intracellular H_2O_2 detected by HyPer depend upon both the presence of CPG1 and light activation. Moreover, CPG1 also allows for temporal control of H_2O_2 delivery to living cells by varying the time at which UV light is delivered to the sample as shown in Figure 1d.

Finally, we sought to employ this new caged H_2O_2 system to simultaneously control and observe downstream effects elicited by cellular H_2O_2 reactivity by releasing this molecular signal in a light-dependent manner. In this regard, recent studies establish that H_2O_2 produced by membrane-bound NAD(P)H oxidase (Nox) enzymes can direct and facilitate the leading edge progression of cell populations and their membrane dynamics.[36] A primary molecular target of H_2O_2 in these cascades is the actin depolymerization factor (ADF) cofilin, which results in the regulated assembly of cofilin-actin rods upon oxidative activation. To probe cofilin-actin rod formation mediated by H_2O_2 signaling, we utilized a HeLa cell line stably expressing eGFP-cofilin. In the absence of a redox signal, eGFP-cofilin exhibits a diffuse cytosolic staining pattern in these modified HeLa cells (Figure 2e and 2f). Treatment of these live specimens with H_2O_2 in a dose-dependent manner initiates formation of bright bands of rods to various extents as eGFP-cofilin is incorporated into cofilin-actin rod structures. Although a variety of stimuli,[37] including excessive glutamate or AMPA stimulation in cultured neurons or ATP depletion,[38] induce cofilin rod formation, treatment with H_2O_2 alone provides sufficient impetus for the formation of cofilin-actin rods.[36] Stimulation of the eGFP-cofilin HeLa cells with 50 μM H_2O_2 produces a regulated pattern of rod formation that localizes to the periphery of the cell (Figure 2b),

whereas addition of 10 μM H_2O_2 fails to generate a rod-forming response after extended treatment (Figure 2a). Higher doses of H_2O_2 (100 or 500 μM) result in intense intracellular rod formation (Figure 2c and 2d). To our delight, loading the eGFP-cofilin cells with 200 μM Ac-CPG1 followed by irradiation with UV light triggers a rod formation pattern that is strikingly similar in appearance to treatment with the 50 μM H_2O_2 dose (Figure 2h), suggesting that CPG1-derived H_2O_2 production in this context is on the order of tens of micromolar. More importantly, control cells in the same imaging dish that were not exposed to UV irradiation did not show H_2O_2-induced coflin-actin rod patterns, highlighting the utility of CPG1 for delivering H_2O_2 within precise, spatially defined regions in the same experiment (Figure 2i). In addition, control cells that are either loaded with CPG1 and not activated with UV light or not loaded with CPG1 and treated with UV excitation do not form rods (Figure 2f and 2g, respectively), establishing that the downstream phenotype is due to controlled photoactivation of CPG1 to release the molecular signal H_2O_2. As further evidence that photogeneration of H_2O_2 from CPG1 and not a miscellaneous factor induces rod formation, we treated cells loaded with a nitrobenzyl-protected resorcinol compound. UV-irradiation releases the nonredox active resorcinol along with the nitrosobenzaldehyde side product resulting from photodeprotection. No rod formation was observed under conditions identical to cells in Figure 2, confirming byproducts of photodecaging do not contribute to rod formation (Figure S7). Finally, analysis of the cellular morphology and corroboration by MTT viability assay following experimental treatments confirm that CPG1 is nontoxic to HeLa cells at concentrations up to 300 μM (Figure S8).

In summary, we have developed a new type of small-molecule probe for on-demand delivery of H_2O_2 to living cells by photoactivation. CPG1 is capable of generating physiologically relevant μM bursts of H_2O_2 upon photolysis, and molecular imaging verifies that this reagent can induce rises in intracellular H_2O_2 within live biological specimens with precise temporal control. Furthermore, CPG1 can be used to trigger and regulate downstream cellular phenotypes via H_2O_2 signaling pathways in spatially defined patterns of cells, as demonstrated by light-initiated cofilin-actin rod formation. The ability to rapidly and precisely deliver H_2O_2 to living systems by a chemically orthogonal light stimulant offers new opportunities for elucidating the functions of this transient small molecule in heterogeneous biological samples as well as real-time remote control of downstream cell behavior through H_2O_2-regulated pathways.

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Supporting Information Available: Synthetic and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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