J|A|C|S COMMUNICATIONS

Published on Web 11/15/2010

A Highly Selective Fluorescent Probe for Visualization of Organic Hydroperoxides in Living Cells

Boxuan Simen Zhao,[†,‡] Yujie Liang,[†,‡] Yanqun Song,[†,‡] Chunhong Zheng,[§] Ziyang Hao,[†,‡] and Peng R. Chen*[,†,‡]

Department of Chemical Biology, College of Chemistry and Molecular Engineering, Beijing National Laboratory for Molecular Sciences, and Department of Advanced Materials and Nanotechnology, College of Engineering, Peking University, Beijing 100871, China

Received August 11, 2010; E-mail: pengchen@pku.edu.cn

Abstract: The transcriptional regulatory protein OhrR is converted into a fluorescent bioprobe capable of detecting organic hydroperoxides in living cells with high sensitivity and selectivity.

Cellular damage generated by reactive oxidative species (ROS) is widely recognized as one of the major causes of human chronic diseases including cancer, diabetes, and aging diseases.[1−3] Discrete cellular impacts have been observed among many interconvertible forms of ROS.[1] For example, hydrogen peroxide often induces short-term cellular stress, while the stimulation evoked by organic hydroperoxides (OHPs) is constant.[4] A variety of fluorescent probes have been developed recently for the selective detection of these highly reactive molecules in living organisms.[3,5−7] Such strategies have dramatically facilitated our study of the physiological roles of specific types of ROS including H_2O_2, one of the most frequently encountered ROS. The genetic-encoded H_2O_2 fluorescent sensor, Hyper, works as a reversible indicator for real time imaging of H_2O_2 *in situ*.[6] Another elegant approach based on a H_2O_2 mediated boronate-deprotection reaction has yielded a broad spectrum of fluorescent indicators with high selectivity and sensitivity toward H_2O_2.[7] However, there is currently no report on similar fluorescent probes for selective visualization of OHPs, a highly reactive form of ROS that can cause fast lipid peroxidation and cardiovascular disease.[8] Excessive OHP generation has also been implicated in the pathogenesis of a panel of chronic diseases, with the suggested mechanism being different from that of H_2O_2.[9] The lack of a selective intracellular probe for OHPs has prevented us from fully elucidating their functional roles at the molecular level. Finally, as the beneficial effects of certain types of ROS are recognized,[10] it is important to develop species-specific indicators for probing ROS signaling dynamics in living cells.

We present here the design and characterization of a novel genetically encoded fluorescent probe for selective visualization of OHPs in living systems. Our design strategy is inspired by the unique feature of OhrR, a transcriptional regulator in control of the OHP detoxification apparatus that is widely conserved in bacteria.[11] OhrR senses OHP stress via the oxidation of a strictly conserved cysteine residue near its N terminus. The subsequent conformational change leads to derepression of corresponding genes that defend against OHPs through reduction to less harmful alcohols. OHPs are a much better inducer of these genes than the smaller and less nonpolar H_2O_2, as is exemplified by Xc-OhrR from the soil bacterium *Xanthomonas campestris* (Xc).[12] Xc-OhrR uses an intersubunit disulfide bond formation mechanism to control transcription upon OHP oxidation.[13] The recently solved crystal structures of the reduced and OHP-oxidized forms of Xc-OhrR demonstrate that OHP oxidation generates significant protein conformational changes that result in the formation of a disulfide bond between Cys 127 near its C terminus and the N terminal cysteine (Cys 22′) from the opposing subunit[14] (Figure 1A, B). Because our previous work on this family of proteins[15] intrigued us of their superselectivity of OHPs over H_2O_2, we decided to convert the OhrR protein family into selective OHP biosensors, using Xc-OhrR as a proof-of-concept.

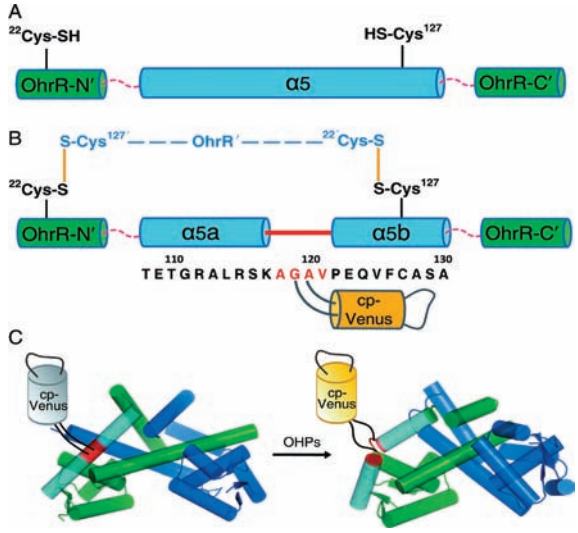

Figure 1. Design and construction of OHSer. (A) The reduced form of Xc-OhrR has an intact helix α5. Two cysteine residues involved in the formation of an intersubunit disulfide bond are shown. (B) Helix α5 of the OHP-oxidized Xc-OhrR is broken into two distinct helices (α5a and α5b) connected by a new loop (residues 118−121) where we introduced cpVenus. Two intersubunit disulfide bonds (colored in yellow) in an Xc-OhrR protein dimer are shown with the name and residues from the opposing subunit colored in blue. (C) OHSer contains a cpVenus inserted through two short, flexible linkers between residues 119 and 120 in each monomer. OHSer exhibits an enhanced fluorescent signal in the presence of OHPs.

A striking interesting feature observed on Xc-OhrR upon OHP oxidation is the breakage of helix α5 into two shorter helices, α5a and α5b, which are now connected by a new loop (residues 118−121). This disruption allows a dramatic rotation (135°) of helix α5b upon which Cys 127 resides. This rotation and a further 8.2 Å movement of Cys 127 toward Cys 22′ on the opposing monomer bring the two Cys residues into close proximity for disulfide bond formation. After close inspection of helix α5 before and after Xc-OhrR oxidation, we speculated that the conformational switch of

[†] Department of Chemical Biology.
[‡] Beijing National Laboratory for Molecular Sciences.
[§] Department of Advanced Materials and Nanotechnology.

residues 118−121 between the rigid helix and the flexible loop is key to the subsequent structural reorientation and disulfide bond formation upon OHP treatment (Figure 1B, C). We envisioned that insertion of an environmentally sensitive fluorescent reporter into this region could yield a fluorescent indicator that is sensitive and selective to OHPs.

Circular permutation was performed on a yellow version of green fluorescent protein Venus by connecting the original N and C termini with a pentapeptide linker GGSGG, rendering D174 and E173 the new N and C termini, respectively. This version of cpVenus (cpVenus173/174) has been shown to be highly conformation sensitive.[16] We created two chimeras by introducing cpVenus between residues in the above-mentioned loop region of Xc-OhrR (Supporting Information). The first version had a cpVenus inserted between residues 119 and 120 (119-cpVenus-120, Figure 1B), while the entire loop was replaced by cpVenus to yield a second version (118-cpVenus-122) (the numbering refers to residue position on Xc-OhrR). Short amino acid linkers, Ser-Ala-Gly and Gly-Thr, were introduced between Xc-OhrR and cpVenus in a similar fashion as the previously reported cpYFP-based biosensors.[6,17] Both chimeric proteins showed an emission peak at 526 nm upon excitation at 519 nm. The first version 119-cpVenus-120 exhibited a better performance and gave a higher increase of fluorescent signal with the addition of 20 μM TBHP. We decided to focus on this chimeric indicator, named OHSer (Organic Hydroperoxide Sensor), for further characterization.

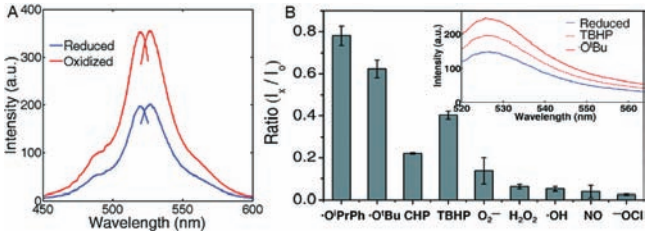

Figure 2. Characterization of OHSer. (A) Excitation and emission spectra for reduced (blue lines) and oxidized (red lines) OHSer. (B) Fluorescence response of OHSer to various ROS. Values are normalized to the fluorescence ratio of fully oxidized/fully reduced OHSer. The inset graph presents the fluorescence spectra of the probe in the presence of TBHP and ·O^{t}Bu. All data were acquired in 20 mM HEPES buffer, pH 7.4, after incubation with the appropriate ROS at 25 °C for 30 min.

Spectroscopic experiments with OHSer were performed under simulated physiological conditions (20 mM HEPES buffer, pH 7.4). The fluorescence emission (at 526 nm) of purified OHSer, when excited at 519 nm, increases almost 2-fold under oxidizing conditions (100 μM ·O^{t}Bu) over the reduced state (Figure 2A). The relative response of OHSer upon addition of various ROS is compared in Figure 2B, which indicates that OHSer is highly selective toward OHPs. It exhibits a much higher response to OHPs such as cumene hydroperoxide (CHP) and TBHP over similar ROS such as H_2O_2, $^-$OCl, O_2^-, or NO. Further experiments were conducted to rule out the possibility that the fluorescent increase is due to cpVenus responding to the addition of OHP or pH variation. The results showed that cpVenus alone exhibits no fluorescent change to OHPs in comparison with other ROS (Figure S1). We also found that OHSer has a very similar fluorescent response to pH variation as cpVenus (Figure S2), while no pH variation was detected after OHP treatment (Supporting Information). These results verify that the OHP selectivity of OHSer originates from Xc-OhrR. Since the OHP induced helix α5 breakage can be reversed upon reduction,[14] we surmise that OHSer might be a reversible probe depending on Xc-OhrR's disulfide bond formation and disruption. Detailed investigation on the reversibility of this probe is ongoing.

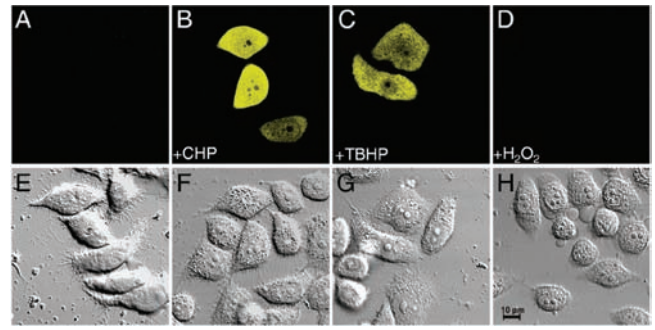

Figure 3. Live-cell imaging of oxidative stress by confocal microscopy. Fluorescence images of live HeLa cells expressing OHSer without stimulation (A), or with the addition of 100 μM CHP (B), 100 μM TBHP (C), or 100 μM H_2O_2 (D) at 37 °C for 30 min. (E−H) DIC images of live HeLa cells after the treatment of ROS to confirm viability. All figures are at the same scale as (H).

We then assessed the ability of OHSer to operate within living cells. After transfection with expression vectors encoding OHSer, HeLa cells showed negligible intracellular background fluorescence (Figure 3A), which is consistent with the reduction of protein by the cytosolic environment. Prompt increases in cytosolic fluorescence were observed upon addition of physiologically relevant concentrations of exogenous CHP and TBHP (100 μM, Figure 3B, C), as determined by scanning confocal microscopy on live samples. A control experiment performed with the same concentrations of H_2O_2 gives an extremely faint fluorescent response (Figure 3D). DIC transmission measurements after ROS addition (Figure 3E−H) confirmed that the cells are viable throughout the imaging experiments. The above experiments were also performed in live CHO cells and live HEK293T cells, yielding similar results (Figures S3 and S4). Finally, time-lapse microscopy was performed before and after the addition of TBHP (100 μM) to live HeLa cells expressing OHSer, which allowed the real-time visualization of a remarkable fluorescence increase triggered by OHP (Figure S5A). In contrast, only very faint fluorescence was observed following the treatment of an equal amount of H_2O_2 on live HeLa cells (Figure S5B). Similar time-lapse imaging results were obtained on live HEK293T cells (Figure S6). Taken together, these data show that OHSer is both selective and sensitive to micromolar changes in OHP concentrations within living cells.

In conclusion, we have presented the design, construction, and characterization of OHSer, a novel protein-based fluorescent indicator for optical imaging of intracellular OHPs. The unique oxidative response mechanism of the template protein Xc-OhrR enables OHSer to effectively discriminate OHPs against competing cellular ROS. Its long-wavelength excitation and emission help to minimize possible damage to living samples and avoid the interference of autofluorescence from native cellular species. Furthermore, we have demonstrated the value of this probe by monitoring intracellular OHPs within living mammalian cells. This genetically encoded probe might be well-suited for detection of OHPs in various cellular compartments. Current efforts are directed toward applying OHSer and analogous optical probes for studying the oxidation biology of living systems, especially physiological redox activities in mitochondria.

Acknowledgment. This work was supported by the National Key Basic Research Foundation of China (2010CB912300),

National Natural Science Foundation of China (20932006). B.S.Z. is supported by the President's Undergraduate Research Fellowship of Peking University. We thank Dr. Yanyi Huang and Dr. Xing Chen for helpful discussions and Mr. Tao Chen for confocal imaging.

Supporting Information Available: Experimental details, supplemental data, and complete ref 3. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Winterbourn, C. C. *Nat. Chem. Biol.* **2008**, *4*, 278.
(2) Giorgio, M.; Trinei, M.; Migliaccio, E.; Pelicci, P. G. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 722A.
(3) Wang, W. *Cell* **2008**, *134*, 279.
(4) Oh, S. Y.; Shin, J. H.; Roe, J. H. *J. Bacteriol.* **2007**, *189*, 6284.
(5) (a) Kojima, H.; Nakatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. *Anal. Chem.* **1998**, *70*, 2446. (b) Sun, Z. N.; Liu, F. Q.; Chen, Y.; Tam, P. K. H.; Yang, D. *Org. Lett.* **2008**, *10*, 2171. (c) Lim, M. H.; Xu, D.; Lippard, S. J. *Nat. Chem. Biol.* **2006**, *2*, 375.
(6) Belousov, V. V.; Fradkov, A. F.; Lukyanov, K. A.; Staroverov, D. B.; Shakhbazov, K. S.; Terskikh, A. V.; Lukyanov, S. *Nat. Methods* **2006**, *3*, 281.
(7) (a) Miller, E. W.; Albers, A. E.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2005**, *127*, 16652. (b) Miller, E. W.; Tulyanthan, O.; Isacoff, E. Y.; Chang, C. J. *Nat. Chem. Biol.* **2007**, *3*, 263.
(8) (a) Garcia-Cohen, E. C.; Marin, J.; Diez-Picazo, L. D.; Baena, A. B.; Salaices, M.; Rodriguez-Martinez, M. A. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 75. (b) Aherne, S. A.; O'Brien, N. M. *Free Radical Biol. Med.* **2000**, *29*, 507.
(9) Sestili, P.; Guidarelli, A.; Dacha, M.; Cantoni, O. *Free Radical Biol. Med.* **1998**, *25*, 196.
(10) Veal, E. A.; Day, A. M.; Morgan, B. A. *Mol. Cell* **2007**, *26*, 1.
(11) Fuangthong, M.; Atichartpongkul, S.; Mongkolsuk, S.; Helmann, J. D. *J. Bacteriol.* **2001**, *183*, 4134.
(12) Sukchawalit, R.; Loprasert, S.; Atichartpongkul, S.; Mongkolsuk, S. *J. Bacteriol.* **2001**, *183*, 4405.
(13) Panmanee, W.; Vattanaviboon, P.; Poole, L. B.; Mongkolsuk, S. *J. Bacteriol.* **2006**, *188*, 1389.
(14) Newberry, K. J.; Fuangthong, M.; Panmanee, W.; Mongkolsuk, S.; Brennan, R. G. *Mol. Cell* **2007**, *28*, 652.
(15) (a) Chen, P. R.; Bae, T.; Williams, W. A.; Duguid, E. M.; Rice, P. A.; Schneewind, O.; He, C. *Nat. Chem. Biol.* **2006**, *2*, 591. (b) Poor, C. B.; Chen, P. R.; Duguid, E.; Rice, P. A.; He, C. *J. Biol. Chem.* **2009**, *284*, 23517.
(16) Chiang, J. J. H.; Li, I.; Truong, K. *Biotechnol. Lett.* **2006**, *28*, 471.
(17) (a) Miyawaki, A.; Nagai, T.; Shimozono, S.; Fukano, T.; Mizuno, H. *RIKEN Rev.* **2002**, 55. (b) Frommer, W. B.; Davidson, M. W.; Campbell, R. E. *Chem. Soc. Rev.* **2009**, *38*, 2833.

JA1071114