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Original Contribution

Exploring real-time in vivo redox biology of developing and aging *Caenorhabditis elegans*

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ABSTRACT

Reactive oxygen species (ROS) are no longer considered merely toxic by-products of the oxidative metabolism. Tightly controlled concentrations of ROS and fluctuations in redox potential may be important mediators of signaling processes. Understanding the role of ROS and redox status in physiology, stress response, development, and aging requires their nondisruptive, spatiotemporal, real-time quantification in a living organism. We established *Caenorhabditis elegans* strains bearing the genetically encoded fluorescent biosensors HyPer and Grx1-roGFP2 for the detection of hydrogen peroxide (H_2O_2) and the glutathione redox potential, respectively. Although, given its transparency and genetic tractability, *C. elegans* is perfectly suitable as a model organism for such approaches, they have never been tried before in this nematode. We found that H_2O_2 treatment clearly induces a dose-dependent, reversible response of both biosensors in the living worms. The ratio of oxidized to reduced glutathione decreases during postembryonic development. H_2O_2 levels increase with age and this effect is delayed when life span is extended by dietary restriction. In young adults, we detected several regions with distinct redox properties that may be linked to their biological function. Our findings demonstrate that genetically encoded biosensors can reveal previously unknown details of in vivo redox biology in multicellular organisms.

Reactive oxygen species (ROS) were long considered as merely unwanted toxic by-products produced during oxidative metabolism, as they are tightly associated with aging and age-related diseases [1]. More recently, ROS were also found to be involved in proliferation, differentiation, immune response, and apoptosis [1–3]. In particular, hydrogen peroxide (H_2O_2) seems to be a key second messenger operating through reversible thiol oxidation in redox-sensitive proteins [4]. This is consistent with its stability and membrane permeativity, key features for intercellular signaling. ROS production influences redox status, which in turn affects development, senescence, and death. The glutathione redox potential provides an accurate proxy of the total redox state, because of the very high ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) and the high intracellular GSH abundance (1–11 mM) [5].

Because redox signaling acts through small and local changes, there is a need for sensitive, selective spatiotemporal measurements of H_2O_2 and GSH/GSSG in vivo. Most conventional redox-sensitive fluorogenic probes are nonspecific, irreversible, and disruptive [6]. Genetically encoded fluorescent sensors can overcome these limitations and have several advantages [7]. Whereas the suitability of externally added probes largely depends on how well they are taken up at the site of interest, any type of expression of genetically encoded sensors can be achieved using the appropriate promoter or signaling peptide. Over the past few years, much effort has been put into constructing redox-sensitive genetically encoded sensors [7]. The hydrogen peroxide-specific sensor HyPer is composed of a circularly permutated yellow fluorescent protein inserted into the H_2O_2-sensitive regulatory domain of the bacterial transcription factor OxyR (OxyR-RD) [8]. Selective and sensitive oxidation of HyPer by H_2O_2 generates a disulfide bridge between the separated parts of OxyR-RD, subsequently inducing changes in the fluorescent properties of the protein. These changes are reversible and quantified ratiometrically and are therefore independent of the HyPer expression level or photobleaching. HyPer is reduced in vivo by glutaredoxin-1 (Grx1) and GSH [9,10] and under normal physiological conditions this reduction is not limited by the GSH concentration [10]. Grx1-roGFP2, another ratiometric biosensor, specifically detects glutathione redox potential. The fusion of the human Grx1 to the redox-sensitive roGFP2 (redox-sensitive green fluorescent protein 2) greatly enhances the response to glutathione redox changes [11]. To conclude,

Abbreviations: DR, dietary restriction; Grx1-roGFP2, human glutaredoxin-1–redox-sensitive green fluorescent protein 2; GSH, reduced glutathione; GSSG, oxidized glutathione; INR, intensity-normalized ratio; LM, linear model; LMM, linear mixed model; ROS, reactive oxygen species; SOD, superoxide dismutase.

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oxidized-to-reduced ratios of HyPer and Grx1-roGFP2 can be used as proxies for H_2O_2 levels and GSSG/2GSH ratios, respectively.

In this study, we explored the redox biology of development and aging in the nematode *Caenorhabditis elegans* with these fluorescent probes. The use of this model in this particular research area has several advantages. *C. elegans* has an entirely transparent body, allowing the observation of spatiotemporal redox processes in vivo. Isogenic populations can be cultured in large synchronic populations and they develop and age in a short period of time [12].

Earlier studies in *C. elegans* suggest a role for ROS during development, oxidative stress response, and aging [13–15]. In *C. elegans* it was found that GSH concentration strongly declines during the adult life span [16] and the H_2O_2 production capacity of isolated mitochondria decreases with age [17]. However, all redox-related studies mentioned above have one major limitation: the redox-related measurements were not performed in vivo and therefore could not take into account the influence of the cellular and subcellular environment on ROS generation and detoxification. Neither could ROS be localized at the anatomical level. Using the genetically encoded sensors described above, we gained unprecedented insight in the topology and timing of redox signaling and oxidative stress in this model organism.

Materials and methods

Construction of HyPer and Grx1-roGFP2 transgenic strains

To obtain a constitutive and ubiquitous expression of the biosensors we performed a screen of the expression profiles of several annotated promoters (http://elegans.bcgsc.ca/perl/eprofile/index) and selected the promoter of a large ribosomal subunit L17 protein, *rpl-17*. HyPer from the mammalian pHyPer-Cyto vector (Evrogen) and Grx1-roGFP2 from the bacterial expression vector pQE-60 (a generous gift from T.P. Dick) were integrated into an expression vector with the ribosomal *rpl-17* promoter by MultiSite Gateway cloning [18] using pDONR 221 (Invitrogen), pDONR P4-P1R (Invitrogen), and pDEST-MB14 [19], devoid of GFP. *unc-119(ed3)* mutants were transformed with the HyPer or Grx1-roGFP2 expression vector containing *unc-119(+)* by microparticle bombardment using a Biolistic PDS-1000/He particle delivery system (Bio-Rad) [20]. All homozygous transgenic worms were backcrossed at least twice into N2 Bristol wild type (*jrIs1[Prpl-17::HyPer]*, *jrIs2[Prpl-17::Grx1-roGFP2]*).

Culture and sampling

Synchronous populations of the transgene and control strains were initiated from eggs prepared by alkaline hypochlorite treatment of gravid adults [21]. Worms were grown on cholesterol-supplemented nutrient agar (OXOID) plates containing a lawn of freshly grown *Escherichia coli* K12 cells. The nematode cultures used for external H_2O_2 stress experiments and confocal scanning were grown at 24 °C and sampled on the first day of egg laying. To examine the H_2O_2 levels and GSSG/2GSH ratios during postembryonic development, worms were cultured at 16 °C on agar plates seeded with K12. Larvae were sampled daily until they reached adulthood. Larval stage was determined with bright-field microscopy. Because large populations of nematodes were required for our fluorometric assays, we chose to culture synchronically aging cohorts in monoxenic liquid medium at 24 °C. This setup also enables the rigorous control of food supply needed to adequately induce dietary restriction (DR). The culture conditions we used in this study have been described previously and were clearly shown to induce a characteristic physiological aging phenotype [12,22]. By using an *hsp-16.2::GFP* reporter strain, we confirmed that 24 °C does not induce thermal stress (Supplementary Fig. S1). Synchronized L4 larvae, grown at 24 °C, were suspended in monoxenic liquid medium at densities not exceeding 2000 worms/ml. This medium contained S-buffer (43.55 mM KH_2PO_4, 6.45 mM K_2HPO_4, 100 mM NaCl, pH 6.0), 100 μM FUdR (to suppress reproduction), and frozen *E. coli* K12 cells (~3×10^9 cells/ml for fully fed conditions and ~8×10^8 cells/ml for dietary restriction). The dietary restriction bacterial concentration induced a maximal life-span extension. These liquid cultures were transferred to Fernbach flasks, incubated at 24 °C, and shaken at 120 rpm in a gyratory shaker incubator (New Brunswick Scientific, NJ, USA). Bacterial concentration in the culture was kept constant by measuring turbidity at 550 nm daily and K12 was added as needed. Samples were taken at adult day 1 and at ~50 and <25% survival. Juvenile stages were cultured different from adult stages for practical reasons: juveniles were grown at 16 °C on plate culture, because they develop more synchronously on plates. Culture temperature at 16 °C allowed sampling of one juvenile stage per day (light green bars in Fig. 2). Adults, on the other hand were grown in liquid at 24 °C (dark green bars in Fig. 2), thus allowing a better control of food supply and speeding up the aging process without stressing the worms. The samples harvested for fluorescence measurements were freed from dead worms, bacteria, and debris through Percoll and dense sucrose washing [23]. To remove the cuticular bacterial biofilm, the nematodes were washed with S-buffer containing 2.5 mM EDTA [24,25]. The resulting suspension of clean worms was used for spectrofluorometry and confocal microscopy.

Spectrofluorometry

Dense pellets of at least 1000 worms were loaded in a black, flat-bottom 96-well microtiter plate (Greiner, Frickenhausen, Germany). Fluorescence was measured for ~15 min at 25 °C with a Victor^2 1420 multilabel counter (PerkinElmer, Boston, MA, USA) with 490-nm (oxidized HyPer and reduced Grx1-roGFP2) and 405-nm (reduced HyPer and oxidized Grx1-roGFP2) excitation filters and a 535-nm emission filter. Unless otherwise noted, all data shown are averages of at least three biological repeats. For each biological repeat, at least three technical repeats were averaged over the time measured. The fluorescence for each technical repeat was normalized to protein content using the bicinchoninic acid (Pierce, Rockford, IL, USA) method. Corrections for autofluorescence in the transgenic strains were made by subtracting the 535-nm emission autofluorescence of matched wild types per protein content after 405- and 490-nm excitation, respectively, from that of HyPer or Grx1-roGFP2-expressing lines:

$$Oxidized/Reduced\ \ HyPer = \frac{\left[\left(\frac{HyPer\ \ Ex490Em535}{protein(mg)}\right)-\left(\frac{N2\ \ Ex490Em535}{protein(mg)}\right)\right]}{\left[\left(\frac{HyPer\ \ Ex405Em535}{protein(mg)}\right)-\left(\frac{N2\ \ Ex405Em535}{protein(mg)}\right)\right]}$$

$$Auto\,fluorescence = \frac{\left(\frac{HyPer\ \ Ex490Em535}{protein(mg)}\right)}{\left(\frac{HyPer\ \ Ex405Em535}{protein(mg)}\right)} - Oxidized/Reduced\ \ HyPer$$

For the quantification of oxidized/reduced Grx1-roGFP2 and fraction of autofluorescence, similar calculations were made with Ex405Em535 as the numerator and Ex490Em535 as denominator.

Confocal microscopy

For the anatomical localization of H_2O_2 levels and GSSG/2GSH ratios, we used a Nikon A1r confocal laser scanning microscopy system mounted on a Nikon Ti-E inverted epifluorescence microscope, equipped with a 405-nm diode laser and 488-nm multiline Ar laser. After treatment with 10 mM H_2O_2, HyPer transgenic worms were immobilized by being mounted on 2.5% agarose pads and scanned with a Plan Fluor 10×/0.5 objective, sequentially excited with both