An oxidative fluctuation hypothesis of aging generated by imaging H_2O_2 levels in live *Caenorhabditis elegans* with altered lifespans

Xinmiao Fu [a], [1], Yan Tang [a], [1], Bryan C. Dickinson [b], [1], Christopher J. Chang [c], [*], Zengyi Chang [a], [*]

[a] *State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing 100871, China*
[b] *Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138, USA*
[c] *Department of Chemistry and the Howard Hughes Medical Institute, University of California, Berkeley, CA 94720, USA*

ABSTRACT

Reactive oxygen species (ROS) are important factors mediating aging according to the free radical theory of aging. Few studies have systematically measured ROS levels in relationship to aging, partly due to the lack of tools for detection of specific ROS in live animals. By using the H_2O_2-specific fluorescence probe Peroxy Orange 1, we assayed the H_2O_2 levels of live *Caenorhabditis elegans* with 41 aging-related genes being individually knocked down by RNAi. Knockdown of 14 genes extends the lifespan but increases H_2O_2 level or shortens the lifespan but decreases H_2O_2 level, contradicting the free radical theory of aging. Strikingly, a significant inverse correlation between lifespan and the normalized standard deviation of H_2O_2 levels was observed (p < 0.0001). Such inverse correlation was also observed in worms cultured under heat shock conditions. An oxidative fluctuation hypothesis of aging is thus proposed and suggests that the ability of animals to homeostatically maintain the ROS levels within a narrow range is more important for lifespan extension than just minimizing the ROS levels though the latter still being crucial.

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1. Introduction

Reactive oxygen species (ROS), including H_2O_2, O_2^-, and $\cdot$OH, and the related reactive nitrogen species such as [NO]$\cdot$ and peroxynitrite ($ONOO^-$), are commonly produced by the normal metabolic processes of organisms. The free radical theory of aging proposes that ROS will generate cumulative damage on biomolecules, and thus is one of the most predominant internal factors causing aging [1–3]. Consistent with this theory, the levels of oxidative damage to DNA and proteins in animals or humans have been reported to increase with age [4–6] and some longer-lived animals show reduced oxidative damage, decreased ROS level, and/or increased resistance to oxidative stress, or vice versa [3,7–13]. Nevertheless, an increasing number of observations apparently contradict this theory and suggest a more complex

relationship between ROS and aging (as extensively reviewed in Refs. [14–17]). For example, the disruption of superoxide dismutases did not shorten or even extended the lifespan of *Caenorhabditis elegans* [18,19]. Extended lifespan of *Drosophila* as achieved by mutation of an insulin receptor substrate protein [20] was even associated with an increase in H_2O_2 levels of the midgut enterocytes. The overexpression of antioxidant enzymes did not extend the lifespan [21] while the knockout of antioxidant enzymes did not shorten the lifespan in mice [22,23].

H_2O_2 is produced from O_2^- either spontaneously or catalyzed by superoxide dismutases and is considered a common mediator of, or at least considered correlated with, aging [14,24–26]. Nevertheless, directly measuring the H_2O_2 levels in living organisms remains extremely challenging [25,27,28]. Here we report that the H_2O_2-selective fluorescence probe Peroxy Orange 1 (PO1), previously proven by us to be able to image H_2O_2 largely in the cytoplasm of cultured mammalian cells [29], is also able to enter the cells of living *C. elegans* for correlative H_2O_2 imaging. Using this chemical fluorescence probe, we explored the relationship between the levels of H_2O_2 and resultant lifespan by assaying the *in vivo* H_2O_2 levels of a variety of epigenetically manipulated species of

C. elegans. Interestingly, our data suggest that the lifespan of *C. elegans* is inversely correlated better with the fluctuation (the normalized standard deviation) than with the absolute value of H_2O_2 levels.

2. Materials and methods

2.1. C. elegans culture and RNAi manipulation

Standard nematode growth medium plates seeded with *E. coli* OP50-1 bacteria were used for *C. elegans* culture at 20 °C. N2 Bristol was used for H_2O_2 treatment experiments and the RNAi-enhanced strain NL2099 *rrf-3*(pk1426) for RNAi experiments. Plasmids for RNAi were purchased from Geneservice (USA) and transformed into the *E. coli* strain HT115. Each RNAi bacteria (the bacteria carrying the empty pL4440 vector was used as a control) was grown overnight in LB with 100 μg/mL of ampicillin, seeded onto dishes containing 5 mM isoprophylthiogalactoside and incubated overnight for expressing double strand RNA. Eggs of NL2099 *rrf-3*(pk1426) were collected and cultured overnight on LB dishes in the absence of bacteria. Afterward, L1 larvae were collected, transferred onto *E. coli*-containing LB dishes and cultured for 51 h before the worms at the end of L4 larvae stage were collected and transferred to the RNAi *E. coli*-containing LB dishes in the presence of 0.1 mg/mL 5-fluorodexoyuridine. For lifespan analysis, worms were transferred to newly prepared dishes containing RNAi bacteria every three other days. Live worms in each dish were counted every day. In each batch of experiment, the worms with RNAi of a few target genes (usually not more than 5) and the control worm were all subjected to lifespan at the same time. The experiment in its entirety was repeated twice in our study.

2.2. H₂O₂ imaging and quantification in C. elegans

PO1 was synthesized as described earlier [29] and dissolved in DMSO at a stock concentration of 5 mM, which was freshly dissolved in M9 medium to prepare PO1 working solution (50 μM). For H_2O_2 treatment experiments (Fig. 1A), adult worms (three days old) were subjected to H_2O_2 treatment (0, 2, 6, or 18 mM) for 30 min, washed with M9 medium twice, and bathed in a 100 μL PO1 working solution for 8 h at 20 °C with mild shaking in the dark. For RNAi experiments, both experimental and control worms (after RNAi treatment for three days as described above) were collected, and washed with M9 medium twice before incubation with PO1 for 8 h. Afterward, the worms were washed twice with M9 medium, anesthetized with 10 mM levamisole and stationed with glass slides and coverslips, and then subjected to fluorescence imaging (excitation at 540 nm, emissions collected between 560 nm and 590 nm, imaging being completed within 10 min after anesthetization). The images were collected using the software AxioVision 4.5 at 16-bit with the raw pixel values within the linear range. Segmentation and fluorescence intensity quantification were performed using the software Image Pro Plus.

3. Results

3.1. H₂O₂ levels in live C. elegans can be correlatively imaged with the H₂O₂-specific fluorescence probe PO1

The data presented in Fig. 1A and B clearly demonstrate that the apparent fluorescence of PO1 will increase when the *C. elegans* were pre-incubated with increasing concentrations of H_2O_2, indicating that PO1 is a suitable probe for imaging H_2O_2 in living worms. In further support of this, worms treated with RNAi against *ctl-3* encoding the H_2O_2-decomposing gene catalase exhibit higher PO1 fluorescence than the control worms (Fig. 1C). In addition, we also found that the fluorescence intensity of PO1 is linearly proportional to the H_2O_2 concentration (within the range of 88–440 μM) in solution ($R^2 = 0.91$; Fig. S1B). Therefore, the relative levels of *in vivo* H_2O_2 in worms can be semi-quantitatively measured by PO1 fluorescence imaging.

3.2. A simple inverse correlation between lifespan and H₂O₂ levels does not hold true for many of the RNAi manipulated worms

To systematically study the relationship between the levels of *in vivo* H_2O_2 and longevity, we treated the RNAi-enhanced worm strain NL2099 *rrf-3* (pk1426) with RNAi against 41 different genes that impact lifespan. We choose these 41 genes in order to have a global characterization of aging process without biasing on a certain aspect of aging, and they represent main pathways or mechanisms known to affect aging process, including insulin/IGF signaling pathway [30], the TOR signaling pathway [30,31], the genome integrity maintenance [32], ROS related pathway, etc (for details, see Table S1). Apparently, these 41 genes are not exhausted for aging studies but only representative ones. For comparison, we also examined another 8 genes unrelated to aging (Table S1). The relative *in vivo* H_2O_2 levels as measured using PO1 imaging, as well as their mean lifespan for all the 49 RNAi manipulated worms are presented in Table S1.

Among these 41 types of RNAi-manipulated worms, 27 matched the predicted trends for the free radical theory of aging in that an increase of lifespan was inversely correlated to a decrease of H_2O_2 levels or vice versa (Fig. 2A). This somehow reflects the marginally significant inverse correlation between the lifespan and the mean level of H_2O_2, which is also indicated by the P value of 0.051 (Fig. 2A) (Note: $P < 0.05$ is considered as statistically significant with a confidence of 95%). In particular, a low level of H_2O_2 increase led to an increase of lifespan (the six red triangles (in the web version) in Fig. 2A) while a high level of H_2O_2 increase exclusively led to a decrease in the lifespan (the five brown squares (in the web version) above the solid line in Fig. 2A).

Nevertheless, 7 RNAi-manipulated worms (the red triangle symbol (in the web version) in Fig. 2A) exhibited an increase in lifespan correlating with an increase in H_2O_2 levels, while another 7 (the green circle symbol) exhibited a decrease in lifespan correlating with a decrease in H_2O_2 levels, both of which are inconsistent with the free radical theory of aging. Additional inconsistent cases include two of the aging-unrelated control genes (*rpl-36* and *unc-86*), the RNAi knockdown of which was found to greatly decrease the mean H_2O_2 levels though not increase the lifespan of the worms (Table S1).

3.3. The lifespan of RNAi manipulated worms is inversely correlated with H₂O₂ fluctuation under both normal and heat shock conditions

The lack of a simple correlation between the lifespan and H_2O_2 levels motivated us to explore whether these two parameters are related in other ways. To this end, we fortuitously noted that the standard deviation values of the H_2O_2 levels of worms that exhibited an increased lifespan tend to be significantly smaller in comparison with those that exhibited a decreased lifespan (Table S2). We thus decided to introduce the parameter of "H_2O_2 fluctuation", to be defined as σ^2/μ^2, where σ represents the standard deviation value and μ the mean H_2O_2 level, and tried to determine whether this parameter is correlated to the lifespan with any statistical significance (for detail, see the legend of Table 1). A similar parameter has been used to evaluate the fluctuation (i.e., noise) of gene expression levels measured under different experimental conditions [33,34].
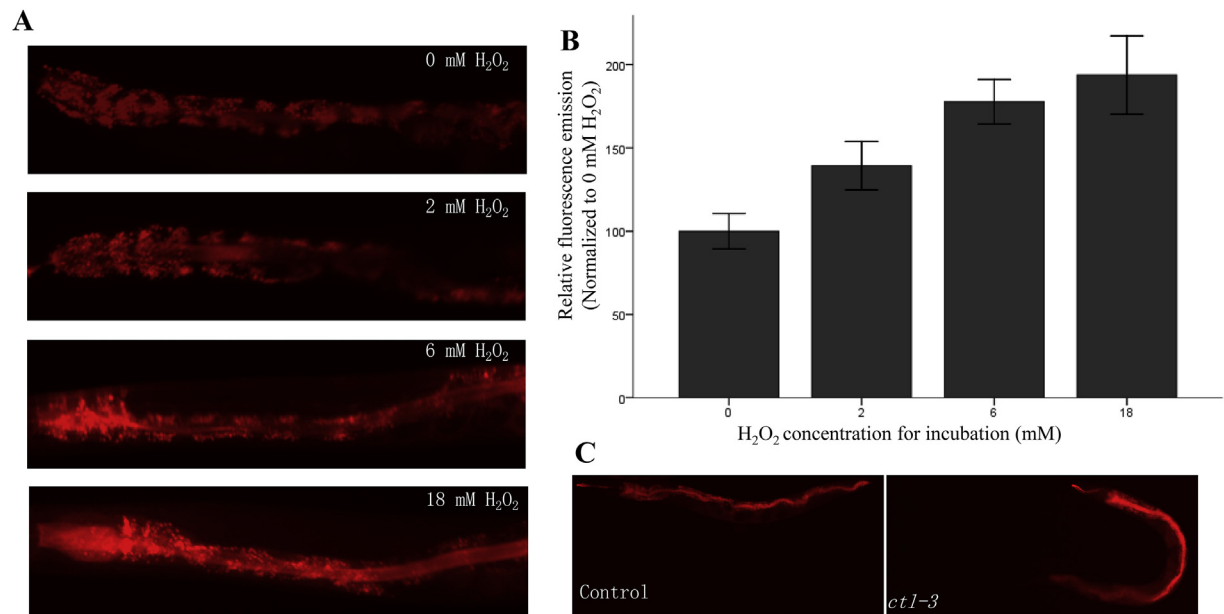
Fig. 1. PO1 fluorescence intensity correlates with H_2O_2 levels *in vivo*. (**A**) PO1 fluorescence image results for adult worms (N2 strain) after being treated with increasing concentrations of H_2O_2. One representative result from three repeating experiments is shown here. (**B**) Semi-quantification of the PO1 fluorescence intensity of the worms displayed in Panel A. Statistical significance was determined by ANOVA. Error bars represent the s.e.m. of fluorescence measurement from five worms. (**C**) PO1 fluorescence image results for the adult worm with the catalase gene (*ctl-3*) being knocked down by RNAi and the control worm NL2099 *rrf-3*(pk1426) strain.

Remarkably, analysis of the 41 RNAi manipulated worms reveals a statistically significant inverse correlation between the relative H_2O_2 fluctuation and the relative lifespan (Fig. 2B), as reflected by a Pearson's correlation coefficient of −0.60 and a P value lower than 0.0001. Among them, as many as 34 worms were found to exhibit an inverse correlation between the lifespan and the H_2O_2 fluctuation (as indicated by 13 dark blue symbols and 21 brown symbol (in the web version) in Fig. 2B). The remaining 7 worms did not exhibit such inverse correlation (the red and green symbols (in the web version) in Fig. 2B).

Furthermore, we examined worms that were subjected to RNAi knockdown of three representative pro-aging genes, *akt-1*, *age-1* or *glp-1* [35–37] and three anti-aging genes *daf-16*, *jnk-1* or *old-1* [37–39] under heat shock conditions (as diagrammed in Fig. S2A).

We found that the RNAi manipulated worms for each of the three pro-aging genes exhibited a significantly lower level of H_2O_2 fluctuation while that for each of the three anti-aging genes exhibited a significantly higher level of H_2O_2 fluctuation (Fig. S2B). In contrast, the mean H_2O_2 level was found to be significantly decreased in the short-lived worms treated with RNAi against *daf-16* or *jnk-1* (indicated by asterisks in Fig. S2B), apparently inconsistent with the traditional free radical theory of aging.

4. Discussion

We propose an expanded model of the free radical theory aging — the oxidative fluctuation hypothesis of aging. Whereas the traditional one emphasizes the absolute levels of ROS, our
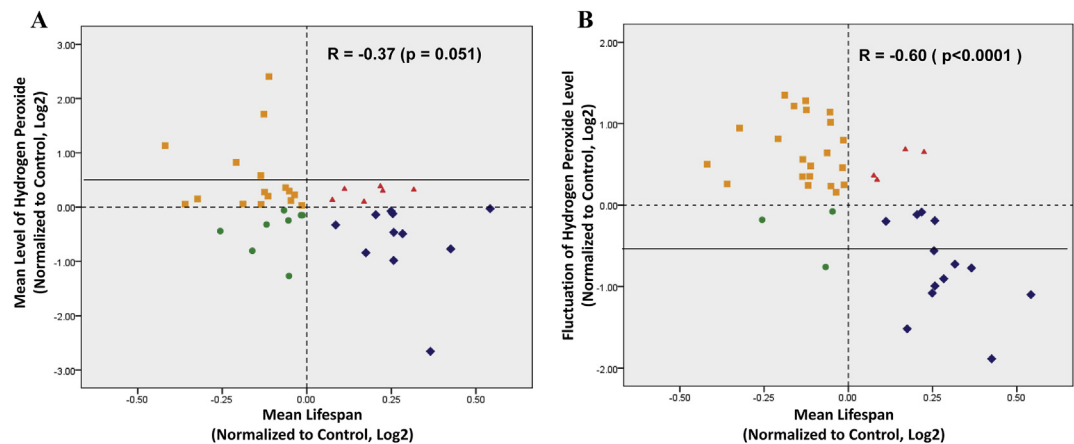
Fig. 2. The H_2O_2 fluctuation amplitude, rather than the mean level, is inversely correlated with lifespan. (**A**) Plot of the mean H_2O_2 level against the mean lifespan of worms. The data points with a small increase in H_2O_2 levels and those with a large increase are separated by the solid line. (**B**) Plot of the H_2O_2 fluctuation amplitude against the mean lifespan of worms. The data points with a small decrease in H_2O_2 fluctuation and those with large decrease are separated by the solid line. The mean H_2O_2 level of worms for each type of RNAi manipulation was calculated based on around 15 adult worms and the mean lifespan on around 100 worms. The normalized data (as shown in Table 1) were plotted here all in the scale of logarithm to base 2. The experiment in its entirety was repeated twice.

Table 1
Relative lifespan and H_2O_2 level for the 41 types of RNAi-manipulated worms.

| Gene	Mean lifespan[a]	Mean H_2O_2 level[a]	H_2O_2 fluctuation (15 worms)[a]	Gene	Mean lifespan[a]	Mean H_2O_2 level[a]	H_2O_2 fluctuation (15 worms)[a]
aak-2	0.91	1.50	1.27	iftb-1	1.20	0.72	0.50
age-1	1.34	0.59	0.27	jnk-1	0.88	1.04	2.55
akt-1	1.13	0.56	0.35	K04A8.5	0.97	1.09	0.95
bec-1	0.96	1.28	1.56	let-363	1.46	0.98	0.47
C01F6.1	1.12	1.07	1.60	lin-35	0.96	0.41	2.02
C50F7.10	0.91	1.03	1.48	mev-1	0.92	1.21	2.25
cco-1	1.05	1.10	1.29	mtl-2	0.92	0.80	1.18
cep-1	1.06	0.80	1.24	nhr-49	0.87	1.77	1.76
clk-1	1.25	1.25	0.61	old-1	0.89	0.57	2.32
ctl-3	0.96	1.23	1.18	pes-2	1.15	0.91	0.92
cyc-1	1.19	0.92	0.68	rps-23	1.22	0.71	0.53
daf-12	0.92	1.15	1.28	sel-12	0.99	0.90	1.19
daf-16	0.92	3.27	2.43	sgk-1	1.08	1.26	0.87
daf-18	0.99	1.02	1.74	sir-2.1	0.99	0.90	1.38
daf-2	1.16	1.30	0.94	skn-1	0.78	1.04	1.20
daf-21	0.96	0.84	2.21	smk-1	0.80	1.11	1.92
E02H9.5	0.98	1.17	1.12	sod-1	0.84	0.74	0.88
eat-2	1.17	1.23	1.57	tag-237	1.19	0.51	0.88
glp-1	1.29	0.16	0.59	unc-83	0.95	0.96	0.59
hsf-1	0.75	2.19	1.42	wrn-1	0.93	5.30	1.40
ife-2	1.19	0.95	0.47				

[a] The mean lifespan and mean H_2O_2 level of the control worms were normalized to 1. The normalized H_2O_2 fluctuation amplitude was calculated as $(\sigma^2/\mu^2)/(\sigma_0^2/\mu_0^2)$, in which σ is the standard deviation and μ is the mean value of H_2O_2 level of around 15 subject worms, and σ_0 is the standard deviation and μ_0 is the mean value of H_2O_2 level of around 15 control worms. Original data are presented in Table S1.

hypothesis adds to this model by underscoring the homeostatic control capacity of ROS pools (as schematically illustrated in Fig. 3A and B). Conceivably, a lower ROS fluctuation for a given animal means that ROS levels are kept within a more narrow range, which might allow the animals to more efficiently detoxifying ROS as well as repairing the ROS-mediated damages and eventually result in longevity (Fig. 3B). As an excellent example in support of this, the long-lived daf-2 mutant was found to exhibit much lower levels of oxidative damages than the wild type worm as revealed both by F3-isoprostane measurement [40] and by Oxyblot-based protein damage assay [18], and meanwhile the RNAi knockdown of daf-2 in our study led to a higher level of H_2O_2 but a lower level of H_2O_2 fluctuation (Table 1). In further support of this, we did observe that very low degree of H_2O_2 fluctuation was associated with shorter lifespan in only one type of RNAi-manipulated worm but with

extended lifespan in 9 types of RNAi-manipulated worms (the green circle versus the dark blue squares (in the web version) below the solid line in Fig. 2B).

Additional evidence supporting our hypothesis comes come from an earlier report, in which the mean H_2O_2 levels of shorter lifespan flies due to excessive diet intake was hardly altered but the standard deviation was significantly larger (being 2–3 fold of the control animals; for details, refer to Figs. 6C and D in Ref. [41]). On the other hand, our hypothesis may explain the general lack of efficacy of antioxidants in modulating the lifespan of animals [42–45], assuming that these antioxidants cannot enhance the capacity of the animals in maintaining ROS homeostasis.

Several important questions remain to be explored. First, the H_2O_2 fluctuation as determined in our study was apparently due to the difference in the levels of H_2O_2 among individuals.
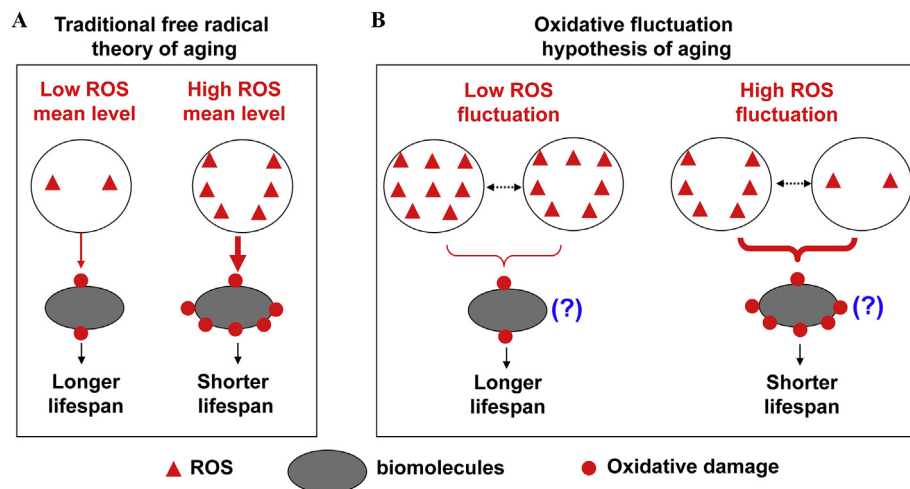
Fig. 3. Comparison between the traditional free radical theory of aging and the oxidative fluctuation hypothesis of aging. (**A**) The traditional free radical theory of aging emphasizes that a low level of ROS leads to a low level of oxidative damage to the animal and thus longer lifespan, and vice versa. (**B**) In the context of the oxidative fluctuation hypothesis of aging, low ROS fluctuation for an animal means that ROS levels are kept within a narrow range, which likely (as indicated by the blue question mark) generates a relatively low level of oxidative damage and thus longer lifespan. In contrast, an animal possessing a high ROS fluctuation result in a shorter lifespan. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Determination of the H_2O_2 fluctuation for each individual worm by measuring H_2O_2 at different time points, for example via reversible ROS imaging (using a probe other than PO1), may provide direct evidence for our hypothesis. However, new methodologies would have to be developed for such multiple time-point measurements of H_2O_2 in a single worm, since our current method requires the worms to be anesthetized to keep them motionless and could only be used for one round of imaging. Second, quantitative measurements on the steady level of the oxidative damages of cellular components (including proteins, DNA, RNA, lipids and others) for all the RNAi worms merit further explorations for verifying our hypothesis. In addition, whether the observed correlation between oxidative fluctuation and aging can be applied to ROS in general and whether the observed phenomenon of C. elegans can be applied to other animal species is also worth further exploration.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.055.

Transparency document

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