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Research Article

Adaptive Response to Hydrogen Peroxide in Yeast: Induction, Time Course, and Relationship to Dose–Response Models

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The assay for *trp5* gene conversion and *ilv1-92* reversion in *Saccharomyces cerevisiae* strain D7 was used to characterize the induction of an adaptive response by hydrogen peroxide (H_2O_2). Effects of a small priming dose on the genotoxic effects of a larger challenge dose were measured in exponential cultures and in early stationary phase. An adaptive response, indicated by smaller convertant and revertant frequencies after the priming dose, occurred at lower priming and challenge doses in young, well-aerated cultures. Closely spaced priming doses from 0.000975 to 2 mM, followed by a 1 mM challenge, showed that the induction of the adaptive response is biphasic. In exponential cultures it was maximal with a priming dose of 0.125–0.25 mM. Very small priming doses were insufficient to induce the adaptive response, whereas higher doses contributed to damage. A significant adaptive response was detected when the challenge dose was administered 10–20 min after the priming exposure. It was fully expressed within 45 min, and the yeast began to return to the nonadapted state after 4–6 hr. Because of the similarity of the biphasic induction to hormetic curves and the proposal that adaptive responses are a manifestation of hormesis, we evaluated whether the low doses of H_2O_2 that induce the adaptive response show a clear hormetic response without a subsequent challenge dose. Hormesis was not evident, but there was an apparent threshold for genotoxicity at or slightly below 0.125 mM. The results are discussed with respect to linear, threshold, and hormesis dose–response models. Environ. Mol. Mutagen. 54:384–396, 2013. © 2013 Wiley Periodicals, Inc.

Key words: oxidative stress; genotoxicity threshold; hormesis; biphasic

INTRODUCTION

An adaptive response is a phenomenon whereby cells that are exposed to a low dose of a toxicant or radiation become less susceptible to damage caused by a subsequent larger dose. The first report of an adaptive response was that of Samson and Cairns [1977] who showed that a low dose of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) induces resistance to mutagenic effects of a larger dose of MNNG in bacteria. An adaptive response was later found in eukaryotes, where pretreating human lymphocytes with ionizing radiation conferred resistance to the induction of chromosome aberrations by larger doses [Olivieri et al., 1984]. Since then, various agents have been found to provoke adaptive responses, also called stress responses. They include oxidants, hypoxia, heat, salt, ionizing radiation and various toxicants and metabolic products [Calabrese et al., 2007; Hoffmann, 2009; Guan et al., 2012].

Oxidative stress describes the situation in which cellular antioxidant responses cannot cope adequately with the level of reactive oxygen species (ROS) present or the damage that they cause [Morano et al., 2012]. Hydrogen peroxide (H_2O_2) has been widely used in studies of oxidative stress responses, even though no single oxidant is truly representative of the totality of oxidative stress, which also occurs with organic peroxides, products of lipid peroxidation, and other oxidants [Morano et al., 2012]. Adaptive responses in which small priming doses of H_2O_2 cause resistance to a later challenging dose have been described in yeast [Collinson and Dawes, 1992; Jamieson, 1992; Davies et al., 1995; Temple et al., 2005; Morano et al., 2012] and in mammalian cell cultures

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[Laval, 1988] using cell viability as the measured endpoint. Genetic effects were not measured, but the evidence of an adaptive response to H_2O_2 was clear from the differences in toxicity. In reporting an adaptive response in yeast strain RZ53-6, Davies et al. [1995] presented a dose–response relationship that also fit the description of hormesis, in that viability increased to roughly 125% of the control value at 0.4 mM before declining at higher concentrations.

The hormesis dose–response model proposes that low doses of toxic substances or radiation elicit responses that are opposite to those at high doses of the same agent. The hormesis model differs from the threshold and linear-nonthreshold (LNT) dose-response models that are widely used in toxicology because hormetic curves are biphasic [Calabrese and Baldwin, 2001; Calabrese 2008; Hoffmann, 2009]. Evidence for the occurrence of hormesis is based largely on surveys of published literature [Calabrese and Baldwin, 2001] and analyses of databases from high-throughput screening of chemicals [Calabrese et al., 2006, 2010]. There is controversy about the prevalence of hormesis and its possible applications to toxicological risk assessment [Axelrod et al., 2004; Thayer et al., 2005, 2006; Cook and Calabrese, 2006a,b; Mushak, 2007, 2009; Calabrese, 2009; Elliott, 2011]. In our view, objections to possible applications of hormesis may be well founded, but they should not impede a dispassionate evaluation of the biological phenomenon.

We used the well characterized assay in *Saccharomyces cerevisiae* strain D7 developed by F. K. Zimmermann [Zimmermann et al., 1975, 1984; Zimmermann, 1975, 1992] to explore an adaptive response to the induction of mitotic gene conversion and point mutations by H_2O_2. H_2O_2 gives rise to hydroxyl radicals through the Fenton reaction [Shackelford et al., 2000; Temple et al., 2005; Morano et al., 2012], and these highly reactive oxidant radicals account for most H_2O_2-induced DNA damage and toxicity [Shackelford et al., 2000]. Mitotic recombination may occur by several mechanisms [Prado et al., 2003], and double-strand breaks are the principal initiating lesions [Kupiec, 2000]. The induction of point mutations may involve imperfect repair of breaks [Chen and Stubbe, 2005]. Recombinagenic effects in strain D7 correlate with mutagenicity and serve as a general indicator of genotoxicity [Zimmermann et al., 1984]. We measured the dose-dependence of the adaptive response in stationary-phase and exponential cultures, and we analyzed its time course. In light of the suggestion that adaptive responses are a manifestation of hormesis [Calabrese et al., 2007], we also evaluated whether conditions of H_2O_2 exposure that unequivocally induce an adaptive response to a later challenge dose also clearly exhibit hormesis in the original sense of the term, using low-dose exposures without separate priming and challenge doses.

MATERIALS AND METHODS

Chemicals

Hydrogen peroxide (H_2O_2; Chemical Abstracts Service (CAS) Registry Number 7722-84-1) was purchased from Sigma–Aldrich Chemical Company, St. Louis, MO, as a 30% aqueous solution. It was stored at 4°C in the dark.

Media

The rich liquid medium for growing D7 cultures and treating growing cells was YEPD. It contained 1% Difco yeast extract, 2% Difco peptone, and 2% D-glucose [Zimmermann, 1975]. YEPD plates also contained 2% Bacto-agar. The yeast minimal medium (YM) used to quantify cells, measure toxicity, and select for convertants and revertants contained 2% glucose, 0.67% Difco Yeast Nitrogen Base without amino acids, and 2% Bacto-agar [Zimmermann, 1975]. To quantify cells and measure toxicity in strain D7, YM was supplemented with 5 mg adenine sulfate, 60 mg isoleucine, and 10 mg tryptophan per liter (YMAIT). Trp+ convertants and Ilv+ revertants were selected on the same medium lacking tryptophan (YMAI) or isoleucine (YMAT), respectively.

Microbiological Methods

Strain D7 of *Saccharomyces cerevisiae* (*a ade2-40 trp5-12 ilv1-92 /α ade2-119 trp5-27 ilv1-92*) was obtained from F.K. Zimmermann (Technische Hochschule, Darmstadt, Germany). Single colonies, isolated from YEPD plates, were grown to stationary phase in liquid YEPD. Trp^+ convertants and Ilv^+ revertants were counted by plating on YMAI and YMAT, respectively, and their spontaneous frequencies were determined using cell densities measured by plating dilutions on YMAIT. Colonies were counted after 3-days incubation at 28°C. Such cultures, derived from single-colony isolates and having characterized spontaneous convertant and revertant frequencies, were stored at 4°C and used for up to 6 weeks. They were subcultured by inoculating 5 ml of YEPD with 20 μl of yeast and grown for 18 hr at 28°C to generate actively growing cultures for use in experiments. These procedures tend to minimize variation between cultures and between experiments. In all experiments, the spontaneous frequencies of convertants and revertants were consistent with our historical controls and values in published literature [Zimmermann, 1975, 1992; Hoffmann et al., 1999, 2011].

Mutagenic Treatments

Adaptive responses were explored using sequential treatments with a priming dose and a later challenge dose. Priming-dose treatments of fresh subcultures in 1 ml YEPD were timed so that challenge doses could be given at different stages of culture growth. To challenge stationary-phase cells, which tend to be hypoxic, the priming dose was given to a 14-hr culture (i.e., 14 hr after subculture) at 28°C in YEPD in a shaker; a 1-hr challenge dose was given 4 hr later, and cultures were terminated at 19 hr. To give both priming and challenge doses to exponential cultures, the challenging treatment was added to 10-hr cultures that had received an earlier priming treatment. The interval between priming and challenge depended on the purpose of the experiment. For time-course experiments, the priming dose was given at culture times between 4 hr and 9 hr, 57 min, and the time of the challenging dose was held constant at 10 hr. After a total of 18-hr growth, cultures were terminated by 1:10 dilution in cold saline (0.9% NaCl), centrifugation in a Beckman TJ-6 centrifuge for 15 min at 2000 RPM, decanting, and resuspension in 1 ml of cold saline at an approximate cell density of 2×10^8 cells ml^{-1}.

Quantification of Toxicity, Convertants, and Revertants

Unless otherwise noted, yeast suspensions were diluted in saline and spread on plates at cell densities of $\sim 2 \times 10^7$ cells on YMAT, 2×10^6

TABLE I. Genotoxic Effects of H_2O_2 in Stationary-Phase Cells of *Saccharomyces cerevisiae* Strain D7 With and Without a Prior Exposure to a Lower Dose of H_2O_2

H_2O_2 priming (mM)[a]	H_2O_2 challenge (mM)	Relative cell density	Ilv^+ revertants per plate[a]	Trp+ convertants per plate[a]	Revertants per 10^6 cells[b]	Convertants per 10^5 cells[b]
0	0	1.00	2.0	7.3	0.15 ± 0.04	0.56 ± 0.17
0.375	0	0.93	2.3	17.3	0.19 ± 0.05^{NS}	1.43 ± 0.17^{NS}
0	1	0.89	5.7	21.0	0.48 ± 0.10	1.80 ± 0.13
0.375	1	1.03	4.7	19.3	0.34 ± 0.05^{NS}	1.43 ± 0.11^{NS}
0	2	0.93	4.7	44.7	0.38 ± 0.17	3.68 ± 0.44
0.375	2	0.93	5.3	26.3	0.44 ± 0.10^{NS}	2.16 ± 0.05^{NS}
0	4	1.02	13.3	123.7	0.99 ± 0.07	9.21 ± 0.78
0.375	4	0.97	7.3	34.0	0.58 ± 0.07^{NS}	2.67 ± 0.16***
0	8	0.88	34.3	189.7	2.98 ± 0.51	16.48 ± 1.28
0.375	8	0.88	11.5	102.0	1.00 ± 1.04***	8.86 ± 0.17***
0	16	1.02	42.7	265.0	3.19 ± 0.14	19.81 ± 0.71
0.375	16	0.82	24.3	150.0	2.25 ± 0.22^{NS}	13.89 ± 0.39***
0	32	0.96	35.3	239.7	2.80 ± 0.23	18.99 ± 0.45
0.375	32	0.90	27.0	164.3	2.28 ± 0.37^{NS}	13.90 ± 0.56***

[a]Treatment of a 14-hr culture with a priming dose of H_2O_2 in YEPD at 28°C in a shaker. After 4-hr, D7 was given a 1-hr challenging dose of H_2O_2 in YEPD. The cell density in the untreated control was 1.3×10^8 cells per ml.

[b]Frequencies are means ± SEM. The significance of differences from the same challenge dose with no priming dose was determined by ANOVA with a Bonferroni multiple comparisons test (NS: nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). In the absence of a priming dose, the increases in convertant frequency were significant at 2 mM ($P < 0.05$*) and higher doses ($P < 0.001$***); the increases in revertant frequencies were significant between 8 and 32 mM ($P < 0.001$***).

on YMAI, and 150 on YMAIT. Plating was in triplicate except where larger numbers of replicates are so indicated. Plates were incubated for 72 hr at 28°C. The relative cell density, defined as the number of colony-forming cells on YMAIT relative to that of the untreated control, served as an indicator of toxicity. For the evaluation of dose–response relationships at low doses, 8-hr cultures were pooled and then divided into a sufficient number of 1 ml cultures to allow multiple replicates. H_2O_2 was added, and incubation at 28°C was continued for 10 hr before culture termination for plating. Plating cell densities were adjusted to 8×10^6 cells on YMAI and 160 cells on YMAIT, with plating in triplicate from each replicate culture.

Statistics

The significance of differences from controls or between effects of challenging doses with and without a priming dose was determined by ANOVA with Dunnett or Bonferroni multiple comparisons tests as indicated. Calculations were done with Prism 5.0 (GraphPad Software). To compare a sequential treatment with the sum of the separate priming and challenging treatments, means and standard errors were generated by a bootstrap method using the program Resampling Stats 4.1; data were resampled 15,000 times using per-plate convertant and revertant frequencies, and the SEM values were scaled to three replicate plates. A two-tailed t test was used to compare the mean and standard error for the sum of treatments with the sequential treatment. These results were expressed as an Interaction Ratio [Hoffmann et al., 2011], defined as the induced frequency of convertants or revertants in a combined (i.e., sequential) treatment divided by the sum of the induced frequencies of convertants or revertants in the separate treatments. Thus, for convertants, the calculation is as follows:

Interaction Ratio = $(CF_{combined} - CF_o) \div [(CF_{priming} - CF_o) + (CF_{challenge} - CF_o)]$,

where $CF_{combined}$ is the convertant frequency from the combined priming-and-challenge treatment; CF_o is the spontaneous convertant frequency; $CF_{priming}$ is the frequency from treatment with the priming dose alone; and $CF_{challenge}$ is the frequency from the challenge dose alone. Interactions were similarly measured for revertants. An interaction ratio significantly <1.0 indicates an adaptive response.

RESULTS

Table I shows that 1-hr H_2O_2 treatments of stationary-phase cells after 18 hr of culture induce gene conversion at the *trp5* locus and point mutations at *ilv1*. In the absence of the priming dose, the genotoxic effects were dose-dependent between 1 and 8 mM, followed by a plateau between 16 and 32 mM. The elevation in convertant frequency was statistically significant at 2 mM ($P < 0.05$*) and all higher doses ($P < 0.001$***), and the increases in revertant frequencies were significant at all doses between 8 and 32 mM ($P < 0.001$***). Yeast that had been given a priming dose of 0.375 mM 4 hr earlier showed less susceptibility to the genotoxicity of H_2O_2. The reduction in response affected both genetic endpoints but was most evident for convertants after a 4–8 mM challenge dose.

The differences in convertant and revertant frequencies with and without a priming dose actually underestimate the adaptive response, in that induced convertant or revertant frequencies for the combined (i.e., sequential) treatments are more properly compared with the sum of the induced frequencies for the separate treatments. These comparisons are shown in Table II. An adaptive response is indicated when the induced convertant and revertant frequency from a combined treatment is smaller than the sum of the induced frequencies from the separate treatments. This comparison can be expressed as an

TABLE II. Interaction Between Priming and Challenging Doses of H_2O_2 in an Assay for Gene Conversion and Mutations in Stationary-Phase Cells of *Saccharomyces cerevisiae* Strain D7

H_2O_2 priming (mM)[a]	H_2O_2 challenge (mM)[a]	Sum of separate treatments	Combined treatment	Interaction ratio (combined/Σ)	Significance of interaction (P)[b]
Induced Trp^+ convertants/10^5 cells					
0.375	1	2.11 ± 0.21	0.87 ± 0.11	0.41	0.006**
0.375	2	3.99 ± 0.48	1.60 ± 0.05	0.40	0.008**
0.375	4	9.52 ± 0.80	2.11 ± 0.16	0.22	0.0008***
0.375	8	16.78 ± 1.29	8.30 ± 0.17	0.49	0.003**
0.375	16	20.11 ± 0.73	13.32 ± 0.39	0.66	0.0012**
0.375	32	19.30 ± 0.48	13.35 ± 0.56	0.69	0.0013**
0.375	64	14.84 ± 0.88	18.26 ± 0.88	1.23	0.051^{NS}
0.375	128	10.87 ± 1.49	12.90 ± 0.64	1.19	0.28^{NS}
0.375	256	8.50 ± 0.90	10.37 ± 0.62	1.22	0.16^{NS}
Induced Ilv^+ revertants/10^6 cells					
0.375	1	0.37 ± 0.12	0.19 ± 0.05	0.51	0.23^{NS}
0.375	2	0.27 ± 0.18	0.29 ± 0.10	1.07	0.96^{NS}
0.375	4	0.88 ± 0.09	0.42 ± 0.07	0.48	0.014*
0.375	8	2.86 ± 0.51	0.85 ± 0.04	0.30	0.055^{NS}
0.375	16	3.08 ± 0.15	2.10 ± 0.22	0.68	0.021*
0.375	32	2.68 ± 0.23	2.13 ± 0.37	0.79	0.27^{NS}
0.375	64	1.99 ± 0.56	2.05 ± 0.08	1.03	0.92^{NS}
0.375	128	2.04 ± 0.28	1.80 ± 0.18	0.88	0.51^{NS}
0.375	256	0.63 ± 0.18	0.89 ± 0.40	1.41	0.58^{NS}

[a]Treatment as described in Table I.

[b]Frequencies are means ± SEM. All values are induced frequencies (spontaneous subtracted). The test for interaction was a two-tailed t test comparing the sequential treatment with the sum of the separate priming-dose and challenging-dose treatments, using means and error terms generated by resampling statistics (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

interaction ratio, as defined in the Materials and Methods. Ratios significantly <1 indicate an adaptive response. The adaptive response for convertants was statistically significant at all challenge doses up to 32 mM. For revertants, there was a significant adaptive response at two doses; three others also had an interaction ratio <1 but the reduction did not rise to the level of statistical significance. As a whole, the data indicate a modest adaptive response for genotoxic effects in stationary-phase cells induced by the priming dose of H_2O_2.

The dose-dependence of the induction of the adaptive response was explored using the same procedure with priming doses ranging from 0.03125 to 2 mM given 4 hr before a challenge dose of 8 mM H_2O_2 in early stationary phase. Figure 1 shows the reduction in susceptibility to the induction of gene conversion conferred by the priming dose of H_2O_2. The response is biphasic, and the frequencies reflect a maximal adaptive response occurring after a priming dose of roughly 0.25 mM H_2O_2.

Adaptive responses in exponential-phase yeast were studied by giving a priming dose of 0.25 mM H_2O_2 to an actively growing culture 4 hr after subculturing. A challenge dose of 1 mM was given at 10 hr, and plating was at 18 hr. Table III shows that the challenge dose induced gene conversion and point mutations, while the priming dose alone did not have a significant genotoxic effect. The genotoxic effect of the challenge dose was significantly smaller if it had been preceded by a priming dose, indicating an adaptive response. Three replicates conducted under identical conditions were consistent. The interaction ratio was <1.0 for both genetic endpoints in all three replicates, and resampling statistics showed the difference from 1.0 to be statistically significant in five of six cases.

Exponential cultures were more susceptible to H_2O_2 than stationary-phase cultures, as indicated by the strong genotoxicity of a 1 mM challenge in the former (Table III) but not the latter (Table I). In stationary phase, doses several fold higher did not have genotoxic effects as great as those of 1 mM H_2O_2 in exponential phase. The adaptive response, however, was evident under both growth conditions, as reflected in the overlapping and generally similar interaction ratios under conditions of effective adaptation in stationary (Table II) and exponential (Table III) cells.

The dose dependence of the induction of the adaptive response was studied by giving a 1 mM challenge to an exponential culture 2 hr after exposure to a broad range of priming doses, extending from slightly less than 1 μM through 2 mM. Table IV shows that in the absence of a challenge dose, the expected dose-dependent induction of *trp5* gene conversion and *ilv1* reversion was observed with priming doses from 0.5 to 2 mM, while lower doses did not differ significantly from the untreated control. The 1 mM challenge dose alone also showed the expected genotoxicity. In the combined treatments, priming doses

of 0.0625 to 0.5 mM H_2O_2 caused a reduction in susceptibility to the genotoxicity of the later challenge dose. There was an optimal priming dose at roughly 0.25 mM, above and below which the adaptive response was less effective.

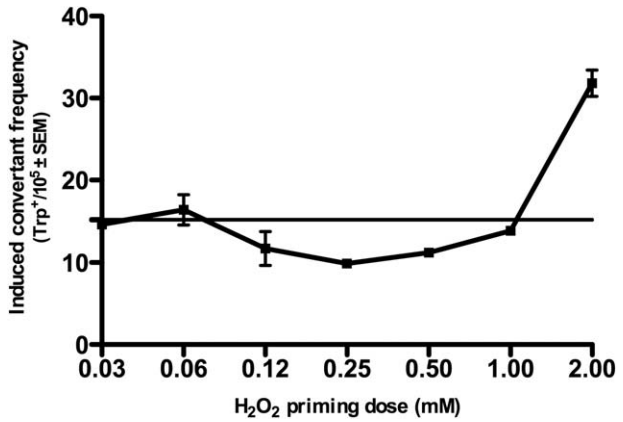

Fig. 1. Induction of *trp5* gene conversion in stationary phase cells of *Saccharomyces cerevisiae* strain D7 by 8 mM H_2O_2 after priming doses of H_2O_2 ranging from 0.03125 to 2 mM. The priming dose, given after 14-hr growth in YEPD, was followed by a 1-hr challenge at 18 hr. The convertant frequency induced by the 8 mM challenge with no prior H_2O_2 exposure was 15.2×10^{-5}, indicated by the horizontal line on the graph. The spontaneous convertant frequency was 0.85×10^{-5}. All values are induced convertant frequencies (spontaneous subtracted) ± SEM. If no error bars are shown, they are smaller than the points.

The interaction ratio between the combined treatment and the sum of treatments did not differ significantly from 1.0 for either convertants or revertants with priming doses up to 0.03125 mM. At 0.0625 mM and higher doses there were significant interactions between the priming dose and the challenge dose. The interaction ratios (combined/sum) for convertants were 0.89^{NS}, 0.78*, 0.36***, 0.17***, 0.23***, 0.46***, and 0.72** at 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, and 2 mM, respectively. The corresponding values for revertants were 0.87^{NS}, 0.68^{NS}, 0.30**, 0.26**, 0.36***, 0.48**, and 0.92^{NS}, respectively. These values are consistent with the primary data in Table IV, indicating that the induction of the adaptive response is biphasic with an optimal induction at roughly 0.25 mM. Figure 2 illustrates the biphasic nature of the response. The smallest priming doses were ineffective, in that the convertant frequencies did not differ significantly from that of the challenging dose alone. Priming doses from 0.0625 to 0.5 mM triggered a significant reduction in the convertant frequency induced by the challenging dose. The optimal priming dose was ~0.25 mM in repeat experiments.

To determine the time course of the induction and the persistence of the adaptive response, a priming dose of 0.125 mM H_2O_2 was given to an exponential culture at various times before a challenge dose of 1 mM. The challenge time was held constant at 10 hr of culture, and cultures were terminated 8 hr later. At all time points, the

TABLE III. Genotoxic Effects of H_2O_2 in Exponential Cultures of *Saccharomyces cerevisiae* Strain D7 With and Without a Prior Exposure to a Lower Dose of H_2O_2

H_2O_2 priming (mM)[a]	H_2O_2 challenge (mM)[a]	Toxicity (relative cell density)	Ilv^+ revertants per 10^6 cells ± SEM[b]	Trp^+ convertants per 10^5 cells ± SEM[b]
Replicate 1				
0	0	1.00	0.07 ± 0.03	1.53 ± 0.18
0.25	0	0.99	1.41 ± 0.25^{NS}	1.85 ± 0.50^{NS}
0	1	0.25	10.79 ± 0.80***	39.86 ± 2.41***
0.25	1	0.60	8.56 ± 1.20***;*	12.61 ± 0.71***;***
Interaction ratio (combined/Σ)			0.70^{NS}	0.31***
Replicate 2				
0	0	1.00	0.10 ± 0.06	1.80 ± 0.32
0.25	0	0.79	0.12 ± 0.00^{NS}	1.69 ± 0.35^{NS}
0	1	0.36	7.95 ± 0.80***	24.49 ± 0.89***
0.25	1	0.72	3.30 ± 0.74**;***	11.34 ± 0.09***;***
Interaction ratio (combined/Σ)			0.41*	0.42***
Replicate 3				
0	0	1.00	0.78 ± 0.06	1.17 ± 0.21
0.25	0	1.74	0.31 ± 0.09^{NS}	1.30 ± 0.06^{NS}
0	1	0.43	5.75 ± 0.37***	18.01 ± 1.26***
0.25	1	0.60	2.97 ± 0.43*;**	11.17 ± 0.98***;***
Interaction ratio (combined/Σ)			0.49**	0.59*

[a]Treatments were in YEPD with priming doses after 4 hr of culture, challenge doses at 10 hr, and plating at 18 hr. The cell densities in the untreated control cultures were 1.0, 1.0, and 1.1×10^8 cells per ml in replicates 1 to 3, respectively.

[b]The significance of differences was determined by ANOVA with Bonferroni multiple comparisons tests. The difference from the untreated control is shown for all treatments; for the combined treatments, it is followed by the difference from the same challenge dose with no priming exposure (^{NS}nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Means and error terms generated by resampling statistics were used to evaluate whether Interaction Ratios were significantly <1.0.

TABLE IV. Genotoxic Effects in Exponential Cultures of *Saccharomyces cerevisiae* Strain D7 Exposed to a Challenging Dose of H_2O_2 After a Broad Range of Priming Doses

H_2O_2 priming (mM)[a]	H_2O_2 challenge (mM)[a]	Relative cell density	Ilv^+ revertants per plate	Trp^+ convertants per plate	Revertants per 10^6 cells[b]	Convertants per 10^5 cells[b]
0	0	1.00	1.2	10.8	0.09 ± 0.04	0.85 ± 0.13
0.000975	0	1.01	1.0	10.0	0.08 ± 0.04^{NS}	0.78 ± 0.29^{NS}
0.00195	0	0.85	4.3	7.7	0.40 ± 0.03^{NS}	0.70 ± 0.08^{NS}
0.0039	0	0.87	2.3	7.3	0.21 ± 0.08^{NS}	0.66 ± 0.11^{NS}
0.0078	0	0.83	3.0	7.7	0.25 ± 0.11^{NS}	0.72 ± 0.08^{NS}
0.0156	0	0.81	1.0	5.3	0.10 ± 0.06^{NS}	0.52 ± 0.03^{NS}
0.03125	0	0.96	1.3	7.7	0.11 ± 0.07^{NS}	0.63 ± 0.13^{NS}
0.0625	0	0.86	2.7	7.3	0.24 ± 0.15^{NS}	0.66 ± 0.13^{NS}
0.125	0	0.78	2.3	7.6	0.23 ± 0.12^{NS}	0.76 ± 0.12^{NS}
0.25	0	0.89	3.3	7.3	0.29 ± 0.08^{NS}	0.64 ± 0.11^{NS}
0.5	0	0.73	15.0	35.3	1.60 ± 0.12^{NS}	3.76 ± 0.50**
1	0	0.49	39.3	68.3	6.32 ± 0.54***	10.99 ± 0.05***
2	0	0.18	37.3	102.3	16.09 ± 1.01***	44.11 ± 2.49***
0	1	0.62	25.5	70.0	3.20 ± 0.17	8.77 ± 0.24
0.000975	1	0.66	21.3	78.3	2.53 ± 0.20^{NS}	9.28 ± 0.31^{NS}
0.00195	1	0.58	25.0	69.7	3.39 ± 0.24^{NS}	9.44 ± 0.72^{NS}
0.0039	1	0.59	24.3	76.7	3.22 ± 0.48^{NS}	10.14 ± 0.42^{NS}
0.0078	1	0.58	27.7	63.0	3.71 ± 0.65^{NS}	8.44 ± 0.48^{NS}
0.0156	1	0.54	18.0	64.7	2.62 ± 0.42^{NS}	9.40 ± 0.34^{NS}
0.03125	1	0.66	23.7	64.3	2.82 ± 0.46^{NS}	7.66 ± 0.73^{NS}
0.0625	1	0.66	19.3	58.3	2.29 ± 0.38^{NS}	6.91 ± 0.40*
0.125	1	0.67	9.3	31.7	1.08 ± 0.20***	3.66 ± 0.20***
0.25	1	0.83	10.0	22.7	0.94 ± 0.14***	2.13 ± 0.03***
0.5	1	0.67	15.0	28.3	1.74 ± 0.12^{NS}	3.29 ± 0.70***
1	1	0.55	32.3	64.0	4.58 ± 0.74^{NS}	9.06 ± 0.67^{NS}
2	1	0.23	53.0	112.3	17.78 ± 3.63***	37.70 ± 1.14***

[a]Treatment of an 8-hr culture with a priming dose of H_2O_2 in YEPD at 28°C in a shaker. After 2 hr, D7 was given a challenging dose of H_2O_2 in YEPD. Plating was in triplicate except that there were six replicate plates for the untreated control. The cell density of the untreated control culture was 1.3×10^8 cells ml^{-1}.

[b]Frequencies are means ± SEM. Bonferroni multiple comparisons tests were used to evaluate the significance of differences between the priming doses and the untreated control and to compare the 1 mM challenge with and without a prior subgenotoxic (<0.5 mM) priming dose (^{NS}nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

convertant and revertant frequencies did not differ significantly from the control in yeast given the priming dose alone. Table V shows that the adaptive response was expressed within 10–20 min after exposure to the priming dose. It was optimal within 1 hr, and the yeast returned to the nonadapted state after ~4–6 hr.

It has been proposed that adaptive responses are a manifestation of hormesis, a phenomenon characterized by a biphasic dose–response relationship in which effects at low doses are opposite to those at high doses [Calabrese et al., 2007]. In light of the proposed association, we explored whether the low doses of H_2O_2 that induce an adaptive response in strain D7 are hormetic in the original sense of the term, such that they reduce the spontaneous frequency of genotoxic effects. We measured convertant frequencies using several low doses and multiple replicates at each dose to provide better resolution of differences from controls than that afforded by a typical experiment with plating in triplicate.

Table VI shows data from an experiment in which toxicity and the induction of gene conversion were measured in 18 replicate plates from six control cultures and in 12 replicate plates from four cultures for each of four low doses of H_2O_2. Higher doses of H_2O_2 were included as positive controls with three replicate plates. The low doses were those that effectively induced an adaptive response when administered as a priming dose (Table IV). Recombinagenicity was used as the sole indicator of genotoxicity because the higher spontaneous frequency of *trp5* gene conversion ($\sim 10^{-5}$) than *ilv1-92* reversion ($\sim 10^{-7}$) makes it practical to measure a reduction in convertant frequency below the spontaneous level if there is a hormetic effect. Table VI shows the expected dose-dependent toxicity and induction of gene conversion by high doses of H_2O_2. There was an apparent threshold at or slightly below 0.125 mM. There was no evidence of hormesis for either toxicity ($P = 0.54$ for the difference between the control and 0.03125 mM) or the recombinagenic effect at subgenotoxic doses of H_2O_2.

Figure 3 shows the dose–response relationship graphically for an independent experiment of similar design. The induction of gene conversion at the *trp5* locus was

accompanied by toxicity at high doses (relative cell density $\approx$ 1.0 at $\leq$ 0.25 mM and 0.76, 0.57, and 0.11 at 0.5, 1, and 2 mM H_2O_2, respectively). As in Table VI, there were no statistically significant differences in convertant frequency between control values (mean = 1.208 $\pm$ 0.051 $\times$ 10^{-5}) and H_2O_2 treatments between 0.03125 and 0.125 mM. Genotoxicity was detected at 0.25 mM. There was an apparent threshold for the genotoxic effect of H_2O_2 at or slightly below 0.125 mM. The pattern in Figure 3 closely resembles that of the independent experiment in Table VI.

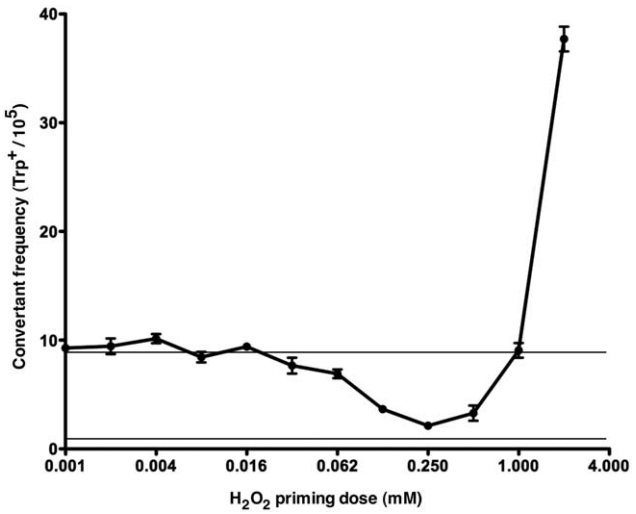

Fig. 2. Biphasic adaptive response induced by a priming dose of H_2O_2 given to an exponential culture of *S. cerevisiae* strain D7 2 hr before a challenging dose of 1 mM H_2O_2. Experimental conditions were as in Table IV. If no error bars are shown, they are smaller than the points. The horizontal lines show the spontaneous convertant frequency (0.85 $\times$ 10^{-5}) and the convertant frequency for the 1 mM challenge without a priming dose (8.77 $\times$ 10^{-5}).

In the experiments shown in Table VI and Figure 3, nonlinear regression gave only a slight increase in the coefficient of determination (r^2) relative to the already high r^2 from linear regression (i.e., from 0.942 to 0.973 and from 0.969 to 0.976, respectively). However, a comparison of slopes at low doses (0–0.125 mM) and high doses (0.25–2 mM) supports a threshold model, in that the former do not differ significantly from zero (P = 0.91^{NS} and 0.092^{NS}) whereas the latter are described by a significant positive slope (P = 0.016* and P = 0.014*). The differences between the slopes in the low-dose range and those in the high-dose range are highly significant (P < 0.0001****).

DISCUSSION

Three dose–response models have generated debate with respect to effects of toxicants and radiation at low doses—the LNT model, threshold model, and hormesis. The threshold model is well established in most of toxicology, but the LNT model has often been assumed in genetic toxicology [Doak et al., 2007; Gocke and Müller, 2009; Hoffmann, 2009; Lutz and Lutz, 2009; Bryce et al., 2010] except in the case of aneugens, for which there is

TABLE V. Time Course of the Induction of an Adaptive Response to H_2O_2 in Exponential Cultures of *Saccharomyces cerevisiae* Strain D7: Frequencies of *trp5* Convertants and Revertants of *ilv1-92*

Priming-challenge interval (h : min)[a]	H_2O_2 doses priming-challenge (mM)[b]	Relative cell density	Revertant frequency ($Ilv^+/10^6$)[c]	Convertant frequency ($Trp^+/10^5$)[c]
—	0–0	1.00	0.06 ± 0.03	0.80 ± 0.16
—	0–1	0.54	8.57 ± 0.59	18.34 ± 0.47
0:03	0.125–1	0.47	9.71 ± 0.94^{NS}	18.54 ± 0.24^{NS}
0:05	0.125–1	0.49	8.40 ± 0.46^{NS}	21.09 ± 0.74**
0:10	0.125–1	0.55	7.04 ± 0.30^{NS}	17.17 ± 0.74^{NS}
0:20	0.125–1	0.70	3.80 ± 0.78***	9.20 ± 0.53***
0:30	0.125–1	0.71	1.13 ± 0.11***	3.89 ± 0.35***
0:45	0.125–1	1.18	0.56 ± 0.17***	2.23 ± 0.36***
1:00	0.125–1	0.91	0.82 ± 0.21***	2.57 ± 0.31***
1:30	0.125–1	0.79	1.47 ± 0.31***	5.40 ± 0.17***
2:00	0.125–1	0.79	1.79 ± 0.48***	4.96 ± 0.92***
3:00	0.125–1	0.88	2.05 ± 0.36***	5.45 ± 0.19***
4:00	0.125–1	0.55	5.84 ± 0.89*	14.66 ± 0.55***
5:00	0.125–1	0.61	6.17 ± 0.76*	13.84 ± 0.27***
6:00	0.125–1	0.52	6.10 ± 0.91*	18.18 ± 0.70^{NS}

[a]Priming doses of H_2O_2 were added to YEPD cultures at the specified intervals before a challenge dose of H_2O_2 at 10 hr of an 18-hr incubation. Plating was in triplicate except that there were six plates in the controls with no priming dose. The cell density of the untreated control was 1.07 $\times$ 10^8 cells per ml.

[b]The convertant and revertant frequencies in yeast exposed to the priming dose alone did not differ significantly from the control at any of the 13 measured time points.

[c]Frequencies are means ± SEM. The significance of differences from the challenge dose alone was determined by ANOVA with a Bonferroni multiple comparisons test (NS: nonsignificant; *P < 0.05; **P < 0.01; ***P < 0.001).

TABLE VI. Frequencies of Gene Conversion at the *trp5* Locus After Treatment of Exponentially Growing Cells of *Saccharomyces cerevisiae* Strain D7 With Low Doses of H_2O_2 in Multiple Replicates

H_2O_2 (mM)[a]	Relative cell density	Mean relative cell density	Trp^+ convertants per plate	Convertants per 10^5 cells[b]	Mean convertant frequency[b]
0	1.098	1.000	49.7	1.038 ± 0.061	1.086 ± 0.048
0	0.945		50.7	1.230 ± 0.066	
0	0.983		53.7	1.254 ± 0.214	
0	1.013		45.0	1.019 ± 0.092	
0	0.868		47.0	1.242 ± 0.122	
0	1.105		38.0	0.789 ± 0.055	
0.03125	0.964	1.031	47.3	1.127 ± 0.117	1.092 ± 0.056^{NS}
0.03125	1.109		51.3	1.062 ± 0.060	
0.03125	0.929		58.0	1.433 ± 0.087	
0.03125	1.120		39.3	0.806 ± 0.036	
0.0625	0.922	0.979	40.7	1.013 ± 0.082	1.106 ± 0.062^{NS}
0.0625	1.006		41.7	0.950 ± 0.046	
0.0625	1.010		46.0	1.045 ± 0.026	
0.0625	0.979		60.3	1.415 ± 0.087	
0.125	0.994	0.913	61.0	1.407 ± 0.093	1.343 ± 0.074^{NS}
0.125	0.883		59.0	1.533 ± 0.113	
0.125	0.795		40.0	1.155 ± 0.088	
0.125	0.979		53.7	1.259 ± 0.082	
0.25	0.918	0.988	111.3	2.783 ± 0.203	2.354 ± 0.104**
0.25	0.933		90.7	2.231 ± 0.175	
0.25	1.082		95.7	2.027 ± 0.151	
0.25	1.017		107.7	2.429 ± 0.256	
0.5	0.719	0.719	256.0	8.163 ± 0.584	8.163 ± 0.584***
1.0	0.440	0.440	242.7	12.640 ± 0.542	12.640 ± 0.542***
2.0	0.042	0.042	69.0	37.500 ± 3.261	37.500 ± 3.261***

[a]Strain D7 was grown for 8 hr from an inoculum of $\sim 4 \times 10^6$ cells per 5 ml YEPD at 28°C in a shaker. The 8-hr cultures were pooled and then divided into 1 ml cultures. H_2O_2 was added, and incubation at 28°C was continued for 10 hr before culture termination for plating. Plating was in triplicate, using approximately 8×10^6 cells per plate to select for convertants and 160 cells on supplemented medium to determine cell density as a measure of toxicity. The average cell density of the six untreated control cultures was $1.09 \pm 0.016 \times 10^8$ cells per ml.

[b]Frequencies are means ± SEM. The significance of differences between the treatments and the untreated control was determined by ANOVA with a Dunnett multiple comparisons test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). The means represent $n = 18$ for the control; $n = 12$ for the four lowest doses, and $n = 3$ for the three highest doses.

general agreement that thresholds exist [Bryce et al., 2010]. Resolution of the shape of dose-response curves in the low-dose zone for genotoxicants is impeded by the difficulty of measuring small changes in events that occur at a low spontaneous frequency. LNT has persisted for conceptual and historical reasons and because experimental data supported linearity at moderate-to-high doses. Nevertheless, mutagenesis is not a unitary interaction between agent and target, and deviations from linearity may arise through such factors as uptake and metabolism of mutagens, direct and indirect interactions with DNA, processing of damage in repair and recombination, and conditions for mutant expression [Hoffmann, 2009]. Moreover, accumulating experimental evidence supports sublinear dose responses [Lutz and Lutz, 2009; Dobo et al., 2011] and the existence of thresholds for some genotoxic effects [Doak et al., 2007; Gocke and Müller, 2009; Lutz and Lutz, 2009; Bryce et al., 2010; Gollapudi et al., 2013; Thomas et al., 2013], while rigorous statistical analysis confirms that LNT best describes genotoxicity results for other endpoints and agents [Bryce et al., 2010; Spassova et al., 2013].

Although persuasive evidence has been presented for the existence of hormesis [Davis and Svendsgaard, 1994; Calabrese and Baldwin, 2001; Calabrese et al., 2006; Hoffmann, 2009], it is less clear that hormesis is broadly generalizable to dose–response relationships independently of agents, biological endpoints, organisms, and individual variation [Mushak, 2007], as is sometimes argued [Calabrese, 2008, 2010]. In the case of genotoxicity, there are examples of genetic effects that exhibit biphasic responses in the low-dose zone [Hooker et al., 2004; Gocke and Müller, 2009; Thomas et al., 2013], but there is little evidence that hormesis is widespread. It has also been reported that responses to many chemicals in large-scale bacterial mutagenicity testing are hormetic [Calabrese et al., 2011], but the methods underlying this conclusion have been contested [Zeiger and Hoffmann, 2012].

There are parallels between hormesis and adaptive responses, but it is unclear whether they are manifestations

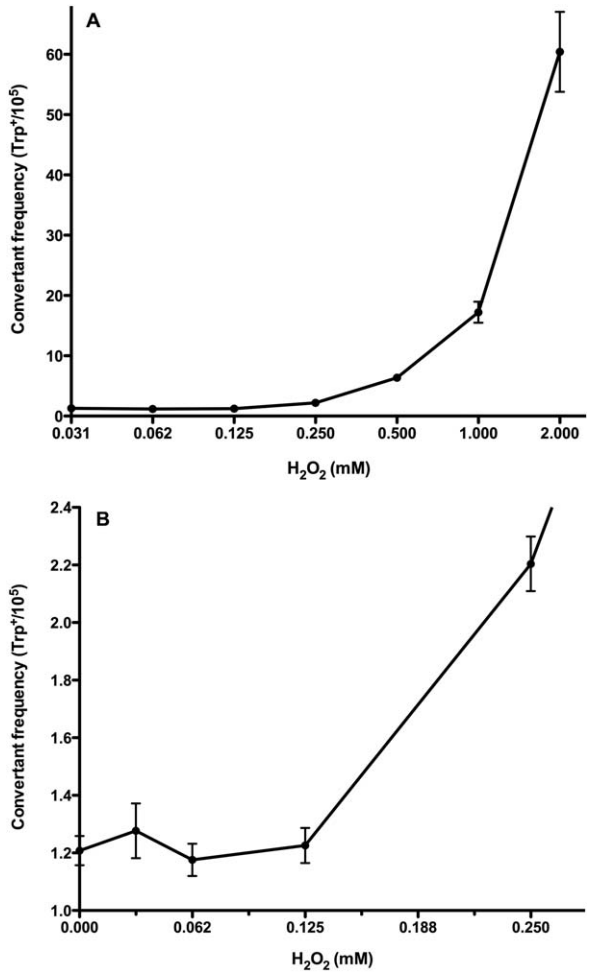

Fig. 3. Frequencies of gene conversion at the *trp5* locus after treatment of exponentially growing cells of yeast strain D7 with low doses of H_2O_2: (**A**) convertant frequencies after treatment of 8-hr cultures, using 18 replicate plates from 6 control cultures, 12 plates from 4 cultures at each of 4 low doses, and 3 plates from single cultures at the 3 highest doses. The plating cell density was $\sim 8 \times 10^6$ cells per plate to select for convertants. The average cell density of the untreated controls was $1.22 \pm 0.029 \times 10^8$ cells per ml, and the spontaneous convertant frequency was $1.208 \pm 0.051 \times 10^{-5}$. Frequencies are means ± SEM. If no error bars are shown, they are smaller than the points. (**B**) expansion of scale for the control and four lowest doses.

of the same phenomenon or whether the similarities are superficial. Adaptive responses occur in phylogenetically diverse organisms [Samson and Cairns, 1977, Olivieri et al., 1984, Calabrese et al., 2007, Hoffmann, 2009], and previous studies have reported adaptive responses of yeast to H_2O_2 on the basis of changes in viability after priming and challenging doses [Collinson and Dawes, 1992; Jamieson, 1992; Davies et al., 1995; Temple et al., 2005; Morano et al., 2012]. There is overlap between adaptive responses, in that exposure to H_2O_2 can confer resistance to other inducers of oxidative stress, and exposure to heat shock, products of lipid peroxidation, or NaCl can confer resistance to H_2O_2 [Temple et al., 2005; Guan et al., 2012]. Cross-resistance is not always reciprocal, however, and its occurrence forms a complex pattern among oxidants, suggesting the existence of several adaptive systems with overlapping components [Temple et al., 2005; Morano et al., 2012].

Tables I–IV extend the analysis of adaptive responses in yeast to the induction by H_2O_2 of gene conversion and point mutations. As in earlier studies [Jamieson, 1992; Stephen et al., 1995], cells in stationary phase (Tables I and II) were less susceptible to the toxicity of H_2O_2 than those in exponential phase (Tables III and IV). Nevertheless, an adaptive response occurs in both growth phases, as evidenced by the weaker induction of convertants and revertants when a challenging dose of H_2O_2 is preceded by a smaller priming dose. The same interpretation is supported by interaction ratios significantly <1 when induced convertant and revertant frequencies (spontaneous subtracted) in combined treatments are compared to the sum of the induced frequencies from the separate priming and challenge doses.

Our data suggest that the induction of an adaptive response to H_2O_2 in yeast occurs within a window of dosage (Table IV; Figs. 1 and 2), above and below which the adaptive response is not observed. Some studies of adaptive responses to radiation have supported a similar interpretation while others have not. An adaptive response of human lymphocytes based on the induction of chromosomal aberrations by X rays was effectively triggered by low priming doses (0.5–20 cGy) but not by high doses [Shadley and Wolff, 1987]. An optimal priming dose (13 cGy) was similarly reported for an adaptive response measured by growth of human embryonic fibroblasts after a challenge dose of 2 Gy X rays [Ishii and Watanabe, 1996]. These studies suggest that the induction of adaptive responses is biphasic. In contrast, other studies report similar adaptive responses over a broad range of priming doses, such as a 500-fold range of γ-ray doses at a low-dose-rate for the induction of micronuclei by γ rays in human fibroblasts [Broome et al., 2002] and a 1000-fold range of X-ray priming doses for the induction of chromosomal inversions by X rays in pKZ1 mice [Day et al., 2007]. Such factors as dose rate, other stressors, physiological conditions and genotype may underlie these differences among studies [Mitchel, 2010].

Like the data from stationary-phase cultures (Fig. 1), convertant and revertant frequencies in exponentially growing cells show the induction of the adaptive response to H_2O_2 to be biphasic (Table IV and Fig. 2). The lowest H_2O_2 priming doses for which there were statistically significant differences between the combined treatment and the sum of the separate treatments were 0.0625 mM for convertants and 0.125 mM for revertants. Doses between 0.975 and 31.25 μM H_2O_2 were apparently too low to induce the adaptive response because none of the interaction ratios differed significantly from 1.0 (the mean ± SEM was 1.06 ± 0.05 for

convertants and 0.92 ± 0.05 for revertants). Thus, there is an apparent threshold for the induction of the adaptive response, suggesting that a small amount of damage is required before a measurable response is triggered. The interaction ratios were lowest at 0.25 mM H_2O_2 (0.17 for convertants and 0.26 for revertants), indicating an optimal adaptive response.

The time course of the induction of the adaptive response in exponential yeast shows that the response to priming time (Table V), like that to priming dose (Table IV), is biphasic. A priming dose of 0.125 mM administered as early as 5 hr and as late as 20 min prior to a 1 mM challenge resulted in convertant and revertant frequencies significantly lower than those induced by the challenge alone. No adaptive response was evident when the priming dose was added less than 10 min before the challenge, and cultures had essentially returned to the ground state if the challenge was delayed until 6 hr after priming. The maximal adaptive response occurred 30–60 min after the priming exposure. A study of salt-induced tolerance to H_2O_2 in yeast similarly showed a maximum adaptive response within 60 min, followed by a slow decay in the level of resistance extending to roughly 6 hr [Guan et al., 2012]. In WTK1 human lymphoblasts, the time intervals were longer than in yeast, in that an adaptive response to mutagenesis by 2-Gy γ-rays was not yet evident 2 hr after a priming dose of 0.05 Gy, was fully expressed at 4 hr, and the adapted state persisted at least 24 hr [Zhang et al., 2009].

Adaptive responses may arise by mechanisms working at several levels to prevent damage and enhance repair [Miura, 2004; Hoffmann, 2009; Morano, 2012]. Mechanisms for coping with oxidative stress involve cell-cycle alterations and enhanced antioxidant defenses, including the production of endogenous scavengers, quenching agents, and enzymes of detoxication [Miura, 2004; Arumugam et al., 2006; Morano et al., 2012]. The response of yeast to oxidative stress entails transcriptional, translational, and post-translational regulation that brings about a reorganization of gene expression and metabolic functions [Shenton et al., 2006; Temple et al., 2005; Morano et al., 2012]. The transcription factor Yap1 plays a central role in the antioxidant response in yeast, controlling the expression of at least 32 proteins [Stephen et al., 1995; Morano et al., 2012].

The biphasic nature of the induction of an adaptive response to H_2O_2 (Table IV; Figs. 1 and 2) may be ascribable to a minimal amount of damage being required to induce stress responses, while larger amounts obscure the protective effects by contributing to damage. Alternatively, the processes underlying adaptive responses may be regulated in opposite directions with low and high doses. The latter interpretation is consistent with the finding that H_2O_2 triggers a general inhibition of protein synthesis in yeast, but that certain RNAs, including those encoding proteins that protect against stress, increase in association with ribosomes under H_2O_2 stress [Shenton et al., 2006]. Moreover, for certain mRNAs, protein production is increased by low concentrations of H_2O_2 but not by high concentrations [Shenton et al., 2006].

The hormesis model holds that low levels of toxic chemicals or radiation elicit responses that are opposite to those at larger doses of the same agent [Calabrese and Baldwin, 2001; Calabrese, 2008; Hoffmann, 2009]. Hormetic curves are therefore biphasic, often described as J-shaped when describing an adverse effect or as an inverted U when applied to the decline in a normal biological function [Hoffmann, 2009]. Mechanisms that may contribute to hormetic effects have been reviewed [Conolly and Lutz, 2004; Arumugam et al., 2006; Calabrese et al., 2007; Calabrese, 2008; Rattan, 2008; Hoffmann, 2009]. Overlapping mechanisms, agents, and dose dependence have led to the hypothesis that hormesis, adaptive responses, and preconditioning represent a broad family of evolutionarily conserved biological responses to stress. An extension of this hypothesis is that adaptive responses are a manifestation of hormesis [Calabrese et al., 2007]. Adaptive responses and preconditioning differ from hormesis as initially defined because they depend on an earlier low-dose exposure, whereas hormesis, per se, does not [Calabrese and Baldwin, 2001; Miura, 2004; Calabrese, 2008; Hoffmann, 2009]. The demonstration of hormesis for genetic effects would therefore require clear evidence that the damage at low doses is less than the spontaneous level.

To explore the hypothesized correspondence of adaptive responses to hormesis, we treated yeast with low doses of H_2O_2 under conditions that are effective in inducing the adaptive response. Hormetic effects are inherently difficult to quantify because they are modest departures from control values [Calabrese and Baldwin, 2001, Hoffmann, 2009], and measuring small differences is problematic for effects that occur at low spontaneous frequencies. Although typical tests sometimes include points that give an impression of hormesis, the putative hormetic effect may also be ascribable to random variation. To compensate for these difficulties we included multiple replicates and several low doses, and we used convertants as the genetic endpoint because mitotic recombination occurs at a higher frequency ($\sim 10^{-5}$) than mutation ($\sim 10^{-7}$ for *ilv1-92* reversion). The data do not show hormesis under these conditions (Table VI; Fig. 3), despite the fact that the same low doses induce an adaptive response to H_2O_2 (Table IV), and higher doses show the expected genetic effects.

A difficulty in evaluating evidence for hormesis is that one may evaluate specific elements of a hormetic response, such as whether certain points differ significantly from control values, but there is no statistical test for hormesis that is generally accepted as valid [Crump,

2001]. Data consistent with a threshold model (Table VI; Fig. 3) cannot unequivocally exclude a small hormetic effect (Crump, 2001). Even experiments with no deviation from the control in the direction predicted for hormesis may be interpreted as compatible with a hormetic model if they happen to fall in the lowest percentiles of a family of responses that is compatible with hormesis [Calabrese et al., 2008]. Therefore, our data cannot exclude the possibility of a small hormetic effect. However, a deviation of 30–60% from the control has often been reported as typical of hormesis [Calabrese et al., 1999; Calabrese and Blain, 2005; Calabrese, 2008], and the magnitude of the stimulatory response has been described as "the most consistent quantitative feature of the hormetic dose response" [Calabrese, 2010]. Our data are not compatible with a hormetic effect of this magnitude.

Reliable negative controls are essential when attempting to measure a small reduction in the frequency of genetic alterations. An atypically high control may give the illusion of hormesis when it is absent [Thayer et al., 2005; Hoffmann, 2009], and an atypically low control may impede the detection of hormesis if it is present. The controls in all our experiments had spontaneous *trp5* convertant frequencies consistent with the frequencies reported for strain D7 in the literature and historic controls in our laboratory [Zimmermann, 1975, 1992; Hoffmann et al., 1999, 2011]. Control frequencies were further constrained in our dose-response experiments by growing independent cultures for 8 hr and then pooling them before dividing them into 1 ml cultures for H_2O_2 treatment. The replicate cultures were therefore identical in spontaneous convertant frequency at the time of treatment. Using these conditions and several replicates favors the ability to measure a decrease in convertant frequency below the spontaneous level if it occurred. The data do not show such a hormetic decrease.

We conclude that low levels of oxidative stress caused by H_2O_2 in yeast induce an adaptive response for genotoxicity that is observed in sequential treatments, but we saw no evidence of hormesis, as it is usually defined, in single exposures. A possible alternative explanation is that subtle differences between the adaptive response and hormesis may have favored our detection of the former but not the latter. A single low-dose treatment with H_2O_2 may produce a low level of genotoxicity prior to the expression of a hormetic effect. When we terminated H_2O_2 treatments with catalase at various times, we found that most damage occurs in the first 10 min of treatment, which is too short for the expression of the adaptive response (Table V). This leaves open the possibility that a weak genotoxic effect of the low doses is offset by hormetic protection expressed shortly thereafter, giving a composite effect equivalent to no response. A weakness of this explanation is that it relies on a serendipitous counterbalancing of two opposite effects to give a slope of zero at low doses. Another possible explanation for the lack of a hormetic response is that low levels of ROS may not contribute to the spontaneous convertant frequency. In order to cause a hormetic response, the low-dose exposure would have to eliminate damage that leads to spontaneous convertants. Antioxidant defenses provide effective protection against ROS in cells that are not under oxidative stress [Halliwell, 2006; Morano et al., 2012], and such protection may be sufficient under typical conditions. These findings lead us to suspect that the proposed linkage between adaptive responses and hormesis [Calabrese et al., 2007] is an excessively broad application of the hormesis concept [Jonas, 2010].

Our data are consistent with a threshold in the range of 0.0625–0.125 mM for the genotoxic effect of H_2O_2 in exponentially growing yeast. The evidence is that convertant frequencies at low doses do not differ significantly from control frequencies in repeat experiments with multiple replicates. The slopes of the responses in the low-dose range (<0.125 mM) do not differ significantly from zero, while higher doses show positive slopes. The difference between the slopes in the low- and high-dose ranges is highly significant ($P < 0.0001$****). A similar rationale underlies the hockey-stick model developed by Lutz and Lutz [2009], which has provided support for thresholds in alkylating-agent-induced micronuclei [Doak et al., 2007; Bryce et al., 2010] and *hprt* mutations [Doak et al., 2007] in cultured mammalian cells, micronuclei in mouse bone marrow, and mutations in *lacZ* transgenic mice [Gocke and Müller, 2009]. Such thresholds in yeast and mammalian systems are contrary to the longstanding expectation of linearity.

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AUTHORS CONTRIBUTIONS

G. R. Hoffmann is the corresponding author and has primary responsibility for this work. Andrew Moczula, Amanda Laterza, Lindsey MacNeil and Jason Tartaglione all contributed to the acquisition of the data and preparation of the manuscript as undergraduate research participants. There are no conflicts of interest.

REFERENCES

Arumugam TV, Gleichmann M, Tang S-C, Mattson MP. 2006. Hormesis/preconditioning mechanisms, the nervous system and aging. Ageing Res Rev 5:165–178.

Axelrod D, Burns K, Davis D, Von Larebeke N. 2004. "Hormesis"—An inappropriate extrapolation from the specific to the universal. Int J Occup Environ Health 10:335–339.

Broome EL, Brown DL, Mitchel REJ. 2002. Dose responses for adaptation to low doses of ^{60}Co γ rays and ^{3}H ß particles in normal human fibroblasts. Radiat Res 158:181–186.

Bryce SM, Avlasevich SL, Bemis JC, Phonethepswath S, Dertinger SD. 2010. Miniaturized flow cytometric in vitro micronucleus assay represents an efficient tool for comprehensively characterizing genotoxicity dose-response relationships. Mutat Res 703: 191–199.

Calabrese EJ. 2008. Hormesis: Why it is important to toxicology and toxicologists. Environ Toxicol Chem 27:1451–1474.

Calabrese EJ. 2009. Hormesis: A conversation with a critic. Environ Health Perspect 117:1339–1343.

Calabrese EJ. 2010. Hormesis is central to toxicology, pharmacology and risk assessment. Hum Exp Toxicol 29:249–261.

Calabrese EJ, Baldwin LA. 2001. The frequency of U-shaped dose responses in the toxicological literature. Toxicol Sci 62:330–338.

Calabrese EJ, Baldwin LA, Holland CD. 1999. Hormesis: A highly generalizable and reproducible phenomenon with important implications for risk assessment. Risk Anal 19:261–281.

Calabrese EJ, Blain R. 2005. The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: An overview. Toxicol Appl Pharmacol 202:289–301.

Calabrese EJ, Staudenmayer JW, Stanek EJ, Hoffmann GR. 2006. Hormesis outperforms threshold model in NCI anti-tumor drug screening database. Toxicol Sci 94:368–378.

Calabrese EJ, Bachmann KA, Bailer AJ, Bolger PM, Borak J, Cai L, Cedergreen N, Cherian MG, Chiueh CC, Clarkson TW, Cook RR, Diamond DM, Doolittle DJ, Dorato MA, Duke SO, Feinendegen L, Gardner DE, Hart RW, Hastings KL, Hayes AW, Hoffmann GR, Ives JA, Jaworowski Z, Johnson TE, Jonas WB, Kaminski NE, Keller JG, Klaunig JE, Knudsen TB, Kozumbo WJ, Lettieri T, Liu SZ, Maisseu A, Maynard KI, Masoro EJ, McClellan RO, Mehendale HM, Mothersill C, Newlin DB, Nigg HN, Oehme FW, Phalen RF, Philbert MA, Rattan SI, Riviere JE, Rodricks J, Sapolsky RM, Scott BR, Seymour C, Sinclair DA, Smith-Sonneborn J, Snow ET, Spear L, Stevenson DE, Thomas Y, Tubiana M, Williams GM, Mattson MP. 2007. Biological stress response terminology: Integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework. Toxicol Appl Pharmacol 222:122–128.

Calabrese EJ, Stanek EJ, Nascarella MA, Hoffmann GR. 2008. Hormesis predicts low-dose responses better than threshold models. Int J Toxicol 27:369–378.

Calabrese EJ, Hoffmann GR, Stanek EJ, Nascarella MA. 2010. Hormesis in high-throughput screening of antibacterial compounds in *E. coli*. Hum Exptl Toxicol 29:667–677.

Calabrese EJ, Stanek EJ III, Nascarella MA. 2011. Evidence for hormesis in mutagenicity dose–response relationships. Mutat Res 726:91–97.

Chen J, Stubbe J. 2005. Bleomycins: Towards better therapeutics. Nat Rev Cancer 5:102–112.

Collinson LP, Dawes IW. 1992. Inducibility of the response of yeast cells to peroxide stress. J Gen Microbiol 138:329–335.

Conolly RB, Lutz WK. 2004. Nonmonotonic dose-response relationships: Mechanistic basis, kinetic modeling, and implications for risk assessment. Toxicol Sci 77:151–157.

Cook R, Calabrese EJ. 2006a. The importance of hormesis to public health. Environ Health Perspect 114:1631–1635.

Cook RR, Calabrese EJ. 2006b. Hormesis is biology, not religion. Environ Health Perspect 114:A688.

Crump K. 2001. Evaluating the evidence for hormesis: A statistical perspective. Crit Rev Toxicol 31:669–679.

Davies JMS, Lowry CV, Davies KJA. 1995. Transient adaptation to oxidative stress in yeast. Arch Biochem Biophys 317:1–6.

Davis JM, Svendsgaard DJ. 1994. Nonmonotonic dose-response relationships in toxicological studies. In: Calabrese EJ, editor. Biological Effects of Low Level Exposures: Dose-Response Relationships. Boca Raton: Lewis Publishers/CRC Press. Chapter 5, pp 67–85.

Day TK, Zeng G, Hooker AM, Bhat M, Turner DR, Sykes PJ. 2007. Extremely low doses of x-radiation can induce adaptive responses in mouse prostate. Dose-Response 5:315–322.

Doak SH, Jenkins GJS, Johnson GE, Quick E, Parry EM, Parry JM. 2007. Mechanistic influences for mutation induction curves after exposure to DNA-reactive carcinogens. Cancer Res 67: 3904–3911.

Dobo KL, Fiedler RD, Gunther WC, Thiffeault CJ, Cammerer Z, Coffing SL, Shutsky T, Schuler M. 2011. Defining EMS and ENU dose-response relationships using the Pig-a mutation assay in rats. Mutat Res 725:13–21.

Elliott KC. 2011. Is a Little Pollution Good for You? Incorporating Societal Values in Environmental Research. New York: Oxford University Press. 246 p.

Gocke E, Müller L. 2009. *In vivo* studies in the mouse to define a threshold for the genotoxicity of EMS and ENU. Mutat Res 678:101–107.

Gollapudi BB, Johnson GE, Hernandez LG, Pottenger LH, Dearfield KL, Jeffrey AM, Julien E, Kim JH, Lovell DP, MacGregor JT, Moore MM, van Benthem J, White PA, Zeiger E, Thybaud V. 2013. Quantitative approaches for assessing dose-response relationships in genetic toxicology studies. Environ Mol Mutagen 54:8–18.

Guan Q, Haroon S, González Bravo D, Will JL, Gasch AP. 2012. Cellular memory of acquired stress resistance in *Saccharomyces cerevisiae*. Genetics 192:495–505.

Halliwell B. 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141:312–322.

Hoffmann GR. 2009. A perspective on the scientific, philosophical, and policy dimensions of hormesis. Dose-Response 7:1–51.

Hoffmann GR, Shorter RA, Quaranta JL, McMaster PD. 1999. Two mechanisms of antimutagenicity of the aminothiols cysteamine and WR-1065 in *Saccharomyces cerevisiae*. Toxicol In Vitro 13:1–9.

Hoffmann GR, Laterza AM, Sylvia KE, Tartaglione JP. 2011. Potentiation of the mutagenicity and recombinagenicity of bleomycin in yeast by unconventional intercalating agents. Environ Mol Mutagen 52:130–144.

Hooker AM, Bhat M, Day TK, Lane JM, Swinburne SJ, Morley AA, Sykes PJ. 2004. The linear no-threshold model does not hold for low-dose ionizing radiation. Radiat Res 162:447–452.

Ishii K, Watanabe M. 1996. Participation of gap-junctional cell communication on the adaptive response in human cells induced by low dose of X-rays. Int J Radiat Biol 69:291–299.

Jamieson DJ. 1992. *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. J Bacteriol 174:6678–6681.

Jonas WB. 2010. What dose metaphor? Hum Exp Toxicol 29:271–273.

Kupiec M. 2000. Damage-induced recombination in the yeast *Saccharomyces cerevisiae*. Mutat Res 451:91–105.

Laval F. 1988. Pretreatment with oxygen species increases the resistance of mammalian cells to hydrogen peroxide and gamma-rays. Mutat Res 201:73–79.

Lutz WK, Lutz RW. 2009. Statistical model to estimate a threshold dose and its confidence limits for the analysis of sublinear dose–response relationships, exemplified for mutagenicity data. Mutat Res 678:118–122.

Mitchel RE. 2010. The dose window for radiation-induced protective adaptive responses. Dose Response 8:192–208.

Miura Y. 2004. Oxidative stress, radiation-adaptive responses, and aging. J Radiat Res 45:357–372.

Morano KA, Grant CM, Moye-Rowley WS. 2012. The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. Genetics 190:1157–1195.

Mushak P. 2007. Hormesis and its place in nonmonotonic dose-response relationships: Some scientific reality checks. Environ Health Perspect 115:500–506.

Mushak P. 2009. Ad hoc and fast forward: The science of hormesis growth and development. Environ Health Perspect 117: 1333–1338.

Olivieri G, Bodycote J, Wolff S. 1984. Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. Science 223:594–597.

Prado F, Cortés-Ledesma F, Huertas P, Aguilera A. 2003. Mitotic recombination in *Saccharomyces cerevisiae*. Curr Genet 42:185–198.

Rattan SIS. 2008. Hormesis in aging. Ageing Res Rev 7:63–78.

Samson L, Cairns J. 1977. A new pathway for DNA repair in *Escherichia coli*. Nature 267:281–283.

Shackelford RE, Kaufmann WK, Paules RS. 2000. Oxidative stress and cell cycle checkpoint function. Free Radic Biol Med 28:1387–1404.

Shadley JD, Wolff S. 1987. Very low doses of X-rays can cause human lymphocytes to become less susceptible to ionizing radiation. Mutagenesis 2:95–96.

Shenton D, Smirnova JB, Selley JN, Carroll K, Hubbard SJ, Pavitt GD, Ashe MP, Grant CM. 2006. Global translational responses to oxidative stress impact upon multiple levels of protein synthesis. J Biol Chem 281:29011–29021.

Spassova MA, Miller DJ, Eastmond DA, Nikolova NS, Vulimiri SV, Caldwell J, Chen C, White PD. 2013. Dose-response analysis of bromate-induced DNA damage and mutagenicity is consistent with low-dose linear, nonthreshold processes. Environ Mol Mutagen 54:19–35.

Stephen DW, Rivers SL, Jamieson DJ. 1995. The role of the *YAP1* and *YAP2* genes in the regulation of the adaptive oxidative stress responses of *Saccharomyces cerevisiae*. Mol Microbiol 16: 415–423.

Temple MD, Perrone GG, Dawes IW. 2005. Complex cellular responses to reactive oxygen species. Trends Cell Biol 15:319–326.

Thayer KA, Melnick R, Burns K, Davis D, Huff J. 2005. Fundamental flaws of hormesis for public health decisions. Environ Health Perspect 113:1271–1276.

Thayer KA, Melnick R, Huff J, Burns K, Davis D. 2006. Hormesis: A new religion? Environ Health Perspect 114:A632–A633.

Thomas AD, Jenkins GJ, Kaina B, Bodger OG, Tomaszowski KH, Lewis PD, Doak SH, Johnson GE. 2013. Influence of DNA repair on nonlinear dose-responses for mutation. Toxicol Sci 132:87–95.

Zeiger E, Hoffmann GR. 2012. An illusion of hormesis in the Ames test: Statistical significance is not equivalent to biological significance. Mutat Res 746:89–93.

Zhang Y, Zhou J, Baldwin J, Held KD, Prise KM, Redmond RW, Liber HL. 2009. Ionizing radiation-induced bystander mutagenesis and adaptation: Quantitative and temporal aspects. Mutat Res 671: 20–25.

Zimmermann FK. 1975. Procedures used in the induction of mitotic recombination and mutation in the yeast *Saccharomyces cerevisiae*. Mutat Res 31:71–86.

Zimmermann FK. 1992. Tests for recombinagens in fungi. Mutat Res 284:147–158.

Zimmermann, FK, Kern R, Rasenberger H. 1975. A yeast strain for the simultaneous detection of induced mitotic crossing over, mitotic gene conversion and reverse mutation. Mutat Res 28:381–388.

Zimmermann FK, von Borstel RC, von Halle ES, Parry JM, Siebert D, Zetterberg G, Barale R, Loprieno N. 1984. Testing of chemicals for genetic activity with *Saccharomyces cerevisiae*: A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat Res 133:199–244.

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