Redox Biology 1 (2013) 210–217

Contents lists available at SciVerse ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Method

An assay for the rate of removal of extracellular hydrogen peroxide by cells ☆

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ARTICLE INFO

Article history:
Received 3 January 2013
Accepted 10 January 2013

Keywords:
Hydrogen peroxide
Kinetics
Erythrocyte
Quantitative redox biology

ABSTRACT

Cells have a wide range of capacities to remove extracellular hydrogen peroxide. At higher concentrations of extracellular H_2O_2 (micromolar) the rate of removal can be approximated by a rate equation that is first-order in the concentration of H_2O_2 and cell density. Here we present a method to determine the observed rate constant for the removal of extracellular H_2O_2 on a per cell basis. In the cells examined, when exposed to 20 μM H_2O_2, these rate constants (k_{cell}) range from 0.46×10^{-12} s^{-1} $cell^{-1}$ L for Mia-PaCa-2 cells (human pancreatic carcinoma) to 10.4×10^{-12} s^{-1} $cell^{-1}$ L for U937 cells (human histiocytic lymphoma). For the relatively small red blood cell $k_{cell} = 2.9 \times 10^{-12}$ s^{-1} $cell^{-1}$ L. These rate constants, k_{cell}, can be used to compare the capacity of cells to remove higher levels of extracellular H_2O_2, as often presented in cell culture experiments. They also provide a means to estimate the rate of removal of extracellular H_2O_2, rate $= -k_{cell}[H_2O_2]$ (cells L^{-1}), and the half-life of a bolus of H_2O_2. This information is essential to optimize experimental design and interpret data from experiments that expose cells to extracellular H_2O_2.

Introduction

Considerable research is now focused on the basic biology associated with the cellular production of free radicals, related oxidants, and antioxidants. There is a growing consensus that these species are not just associated with various pathologies and aging, but rather are central to the biology of normal cells and tissues [1–5]. Unfortunately, much of what we know about oxidants and antioxidants in biology is observational in nature due to the high reactivity and low levels of the initial oxidative intermediates [6]. Many popular assays provide relative changes that may not be specific or have a linear response in the readout [7,8]. In addition, once formed these highly reactive species can rapidly react with multiple targets, disappearing into the cellular milieu, resulting in a vanishingly small steady-state level, far below lower-limits-of-detection of most analytical approaches.

Although many kinetic rate constants for the reactions of free radicals, related oxidants and antioxidants, as well as antioxidant enzymes are available, quantitative integration into our understanding of more complex biological systems has been challenging and slow [2,9–14]. Modeling of complex systems with the integration of physics, chemistry, and biology will allow more thorough analyses, yielding better predictions and understanding of fundamental redox processes and consequences in biology [6,9–17]. Currently, most analyses are presented as qualitative assessments with limited predictive abilities. To establish better mathematical models of biological redox systems we need to develop new approaches to gather quantitative information on fundamental components of the redox circuits that comprise biologic systems.

The integration of free radical and oxidant/antioxidant chemistry and biology are being addressed in the burgeoning field of redox biology, more specifically in the newly developing field of Quantitative Redox Biology (QRB) [17]. To gain the next level of understanding of cellular redox processes, quantitative information on the generation and removal of superoxide and hydrogen peroxide by cells and tissues must be in hand. Here we address the kinetics of the removal of extracellular H_2O_2 by intact cells. For example, even though red blood cells produce a low flux of superoxide and H_2O_2 intracellularly [18,19], they also efficiently remove extracellular H_2O_2 [20–22]. Removal of extracellular H_2O_2 of course is not restricted to erythrocytes, many different types of cells are able to remove extracellular H_2O_2 [23–32]. Many different enzyme systems are involved in this removal process, and new pathways are still being discovered. For some of the known reactions involved in the removal of H_2O_2 the kinetic rate constants have been determined with in vitro experiments using purified enzymes. As a result there is a beginning understanding of their potential contributions to the maintenance of a normal steady-state level of H_2O_2 as well as their roles in pathological

Abbreviations: QRB, Quantitative Redox Biology; RBC, Red blood cells

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http://dx.doi.org/10.1016/j.redox.2013.01.011

settings. However, there is no one assay that can experimentally determine the overall rate of removal of extracellular H_2O_2 by all systems combined as described here.

Many studies on the mechanisms and consequences of exposure of cells to H_2O_2 use a bolus addition of H_2O_2 to the culture media over the cells. However, cells can remove this extracellular H_2O_2; thus, the concentration of H_2O_2 will vary with time. The observed consequences of exposure are not only a function of the concentration of H_2O_2, but also how fast it is removed. It is reasonable to assume that the greater the "cell density" the more rapidly the added H_2O_2 will be removed [23,24]. Thus, the actual cellular consequences observed may also change with cell density [28,30,33]. The possibility of significant changes in the concentration of H_2O_2 with time adds another complicating factor to the interpretation of the data from these types of experiments.

To maintain a constant level of H_2O_2 Antunes et al. developed a new approach, the cellular steady-state titration of H_2O_2 [30,31,34]. In this approach a stable flux of H_2O_2 is produced, e.g. by glucose/glucose oxidase. This production is balanced by the cellular removal of this extracellular H_2O_2 to achieve a desired extracellular and intracellular steady-state level of H_2O_2 over a long period of time. The cellular consequences can be quite different than experiments employing bolus additions of H_2O_2 and may be much more relevant to the in vivo biology of cells and tissues [31].

Here we present a method to determine an observed rate constant for the removal of extracellular H_2O_2 on a per cell basis. This rate constant provides a quantitative means to compare the capacity of cells to remove extracellular H_2O_2; results can then be coupled with cell density to determine the rate of removal of H_2O_2 in experiments, allowing for better design and interpretation of data. In addition this information is required to mathematically model redox processes in systems to advance the new field of Quantitative Redox Biology.

Methods

Cell culture and enumeration

HL-60, U937, MIA-PaCa-2, MDA-MB-231 (ATCC, Manassas, VA) and BAEC (isolated from bovine heart [35]) were maintained in RPMI 1640 media supplemented with 10% FBS and 85 U/mL penicillin and 85 μg/mL streptomycin (Invitrogen, Grand Island, NY). MCF-7 cells were maintained in MEM with 10% FBS with the same penicillin and streptomycin levels as above. The MCF-7 p51 (GPx-4) stable overexpressing cells [36] with a selectable antibiotic resistance marker were grown in the same media supplemented with Geneticin® (G418, 350 μg/mL) (Research Products International Corp, Prospect, IL) and 30 nM sodium selenite. All cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

Human RBC's obtained from donors with informed consent were isolated after removal of WBC's with Histopaque (Sigma-Aldrich, St. Louis, MO). The isolated RBC's were washed 2 × in PBS pH 7.4, counted and then suspended in HBSS (Invitrogen).

Cells were detached with trypsin (0.25%)–EDTA (0.038%) (Invitrogen) that had been supplemented with 0.1% disodium EDTA. Cells were then washed in media and counted with a Z2™ Coulter Counter® Cell and Particle Counter (Beckman-Coulter, Miami, FL). Typically, cells were seeded with cold media (5 °C) and allowed to attach overnight (< 12 h) in 96-well tissue culture plates incubated at 37 °C with 5% CO_2. This experimental approach allowed the cells to attach overnight with little growth. Typically, the number of cells in a well the following day was within 3% of the intended cell-number. Cells that grew as suspension cultures or cell lines that typically grow as monolayers, but were to be used in a "detached" state, were washed, suspended in HBSS, and then counted with the Z2™ Coulter Counter®. Cells attached as monolayers were rinsed three times with HBSS before being used in experiments: all cells were in wells of clear 96-well microplates (Costar, Corning, NY, or Nunc, Roskilde, Denmark) covered with 50 μL HBSS.

pHPA extracellular hydrogen peroxide assay

Overview of assay

In this assay a known number of cells is introduced into the wells of a 96-well plate. Then extracellular H_2O_2 is added to subsets of the wells at different defined times. The cells commence to remove this extracellular H_2O_2. The system is quenched at predetermined times and the concentration of extracellular H_2O_2 remaining is determined. The information on number of cells, amount of extracellular H_2O_2 remaining, total volume of media, and the different times of exposure of cells to extracellular H_2O_2 allows the determination of the capacity of cells to remove extracellular H_2O_2.

Pilot studies with the various cell lines were run to establish the range of optimal experimental parameters, *i.e.* appropriate cell-number per well required and time-frame for removal of extracellular peroxide, which is cell-type and cell density dependent. These experiments established general conditions for each cell line, allowing cell-number and time of exposure to extracellular H_2O_2 as variables in our determinations of the rate of removal extracellular H_2O_2. For most adherent cell lines we established the highest number of cells that can attach overnight and cover 90% of the 96-well bottom surface. Cell size influences this experimental parameter. Under conditions in which suspended cells or freshly trypsinized cells were to be used, the cells were counted and placed immediately in HBSS before the assay. The protocol below is for an experiment where the cells are exposed to 20.0 μM H_2O_2 in the media with a maximum total time of exposure of 10 min. Both of these parameters can be varied as needed. The final concentration of H_2O_2 in the analysis is $\leq$ 10.0 μM.

Solutions

Standard solutions of H_2O_2

Stock solutions of H_2O_2 standards are prepared just before use. These stock solutions are added to the designated wells of the 96-well plate while the cells are being exposed to H_2O_2. Standard stock solutions are prepared at 0.0, 2.0, 4.0, 8.0, 12, 16, 20, 24, 32, and 40 μM in HBSS using appropriately diluted 30% v/v (≈ 10 M) H_2O_2 (Sigma) stock in HBSS. The actual concentration of the original stock is determined using $\varepsilon_{240}=39.4\ M^{-1}\ cm^{-1}$ [37]. The wells to be used for standards are pre-loaded with 50 μL of HBSS; then, the standards are added to wells as 50 μL additions, resulting in H_2O_2 standards of 0.0, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12, 14, 16, 18, and 20 μM, (100 μL): note that at the end of the experiment, 100 μL of the stopping solution is added producing final concentrations in the wells of 0.0, 0.50, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 μM H_2O_2. All standards are run in triplicate and corrected to the 0.0 μM (blank) wells. These standard curves allow absolute determination of the concentrations of the remaining H_2O_2 in the wells where cells have been exposed to H_2O_2.

Stopping solution: The stopping solution is made as a mixture of: 20 mL HBSS (Invitrogen); 20 μL 1 M 4(-2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), pH 7.2–7.5; 10 mg $NaHCO_3$ (3 mM), 5 mg *para*-hydroxyphenylacetic acid (*p*HPA; alias 2-(4-hydroxyphenyl)acetic acid, CAS Nr 156-38-7) (0.8 mM) (Sigma, St Louis, MO); and 2 mg HRP (horseradish peroxidase

Type 1)(Sigma): this solution was prepared fresh immediately before use.

Protocol

1. Standard solutions (50 μL) having ten different concentrations of H_2O_2 (0.0–40 μM; 0.0, 2.0, 4.0, 8.0, 12, 16, 20, 24, 32, 40 μM) are placed into designated wells of a clear plastic 96-well tissue culture plate, for example wells B2–B11, C2–C11, D2–D11, for triplicate determinations. To these wells HBSS (50 μL) is also added, a total volume of 100 μL in each well. Final concentrations of H_2O_2 in the wells for these standards are 0.0, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, 16, 20 μM. Note: to faithfully mimic the exposure of cells to extracellular H_2O_2, the freshly prepared H_2O_2 standards are added to the standard wells while the cells are being exposed to H_2O_2, *i.e.* in parallel with step 3 below.
2. Counted cells in 50 μL of HBSS are placed in other wells of the clear plastic 96-well tissue culture plate; for example into wells E2–E11, F2–F11, G2–G11 to provide triplicate determinations for each time point; or for different cell lines or treatment within a row.
3. Experiments are initiated by adding 50 μL of freshly prepared 40 μM H_2O_2 stock solution in HBSS to achieve 20 μM final concentration of H_2O_2 in each well with cells; 100 μL total volume in each well; additions and progression to new wells is typically done in 1.0-min intervals over a 10-min exposure period. For example, H_2O_2 stock solution is added to wells E2, F2, G2, then one minute later to wells E3, F3, G3, etc. for triplicate determinations at each time point. In some circumstances additions may be at 5-min intervals over 45 min, e.g. low number of cells or low H_2O_2 removal capacity, or some other appropriate combination of concentration and time.
4. Immediately after the addition of H_2O_2 to the final set of three wells (within 10 s), *i.e.* the end of the experiment and the last time point, 100 μL of a stopping solution is added to each well; this addition is made to both sets of wells, i.e. wells with cells and wells with standards with the aid of a 12-channel multi pipette. The horseradish peroxidase in the stopping solution reacts with the remaining H_2O_2 in the wells. The activated HRP then oxidizes *p*HPA resulting in formation of the fluorescent *p*HPA dimer, which is used as the readout for the amount of H_2O_2 in the wells. Note: because the volume of solution in each well is now doubled, all concentrations of H_2O_2 are now halved, here 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 μM for the standards with the concentration of the remaining H_2O_2 in wells with cells also halved.
5. Inhibition of catalase with 3-AT (optional): Cellular catalase activity was irreversibly inhibited using 3-amino-1,2,4-triazole (Sigma; CAS 61-82-5) before cells were examined in a peroxide-removal assay [38]. Briefly, attached or suspended cells were incubated in full media with 20 mM 3-AT for 1 h at 37 °C, the media was removed, and cells were washed three times with HBSS before addition of the buffer for the peroxide-removal assay: suspended cells were counted before placing them into the peroxide-removal assay buffer and assigned wells of the assay plate. 3-AT must be removed from the media before initiation of the peroxide-removal assay, as it will react with compound I of HRP, interfering with the quantitation of the remaining H_2O_2.
6. At room temperature the assay color/fluorescence is sufficiently developed and can be read in a fluorescence plate reader after 5 min. We routinely run the assay at room temperature and read the plates using a Tecan SPECTRAfluor PLUS (Tecan US, Research Triangle Park, NC) fluorescence plate reader with excitation at 345 nm while monitoring emission at 425 nm from above the wells. Typically, the assay plate is read every 5 min over 30 min. This ensures that the chemistry of the assay for the detection of the remaining H_2O_2 in the wells has come to completion. Actually, only one reading is needed; the additional readings are to ensure that there are no anomalies. If an anomaly is noted in one of the plate-readings, another can be used to arrive at a best result. Note that both the experimental wells (with cells) and the standards are "stopped" at the same time; reading of all the wells is done essentially simultaneously, i.e. as fast as the plate reader allows; this is essential so that all measurements are internally consistent. This is one of the most important aspects of this assay: All samples are treated the same and analyzed the same way, with no temporal differences in standards and cell samples.
7. For the data analysis it is important to subtract the appropriate "blank" from all other readings, both standards and cell samples. In our assay the blank is the mean (or median) value of the fluorescence from the standards with no H_2O_2. It is important to examine all the readings from the plate to ensure there are no anomalies, especially the blank wells. Note that the blank is prepared so that it matches the media over the cells during the exposure to extracellular H_2O_2.
8. First-order rate constant calculations: The rate of removal of extracellular H_2O_2 by cells under the experimental conditions of this assay is approximated well by a first-order rate law, $d[H_2O_2]/dt = -k_{obs}\,[H_2O_2]$, where k_{obs} is the observed first-order rate constant as provided by the slope of: $\ln[H_2O_2]$ vs. time (in seconds), i.e. time is the abscissa and $\ln[H_2O_2]$ is the ordinate; because the removal of H_2O_2 is typically first-order, the readout in arbitrary fluorescent units (corrected for the blank) will allow determination of k_{obs}, *i.e.* ln(fluorescence units) vs. time; units for k_{obs} are s^{-1}. The observed rate constant for each cell will be, $k_{cell} = k_{obs}/(\text{cell } L^{-1})$, where (cell L^{-1}) is the number of cells in the well divided by the total volume of media in the well, in liters. This is the volume of media during the time the cells are exposed to the bolus of extracellular H_2O_2, *i.e.* before the addition of the stopping solution. This rate constant, k_{cell}, is not only a measure of the capacity of cells to remove extracellular H_2O_2, but it is also a tool to design and interpret data from experiments in which extracellular H_2O_2 is employed, either as bolus or as a flux. The rate of removal of extracellular H_2O_2 in an experiment with a known cell density will be: $\text{rate} = -k_{cell}\,[H_2O_2]$ (number of cells L^{-1}). The average contribution of one cell to this overall rate will be: $\text{rate}_{cell} = -k_{cell}\,[H_2O_2]$ (1 cell L^{-1}). Very useful for both experimental design and data interpretation is the half-life of a bolus addition of H_2O_2 in an experiment with a specific cell density: $t_{1/2} = 0.693/(k_{cell}\,(\text{number of cells } L^{-1}))$.

Statistics

The lower limits of detection (LLD) for standard curves were estimated as described by Anderson [39].

Results and discussion

Standards for H_2O_2

Our goal was to develop a simple and flexible microplate reader assay for measuring H_2O_2 reliably while being compatible with cells in vitro. We have adapted the flexible H_2O_2 assay in which *p*HPA in the presence of H_2O_2 and HRP is oxidized to a dimeric fluorophore $(p\text{HPA})_2$ [40–42]. This assay is for use in measuring H_2O_2 in media and not intended for measuring intracellular generation of H_2O_2 or its extracellular accumulation

in media from cells. The assay can measure H_2O_2 in a convenient and flexible format utilizing clear tissue culture plates and fluorescence microplate readers with monochromator/grating or commonly used filter sets (Fig. 1**A**). The fluorescence assay for H_2O_2 based on formation of *p*HPA dimer is linear in the range of interest, 0–30 μM. The assay is responsive, but nonlinear at higher levels of H_2O_2, *i.e.* >30 μM (This is the final concentration after addition of stopping solution.), Fig. 1**A** and **B**. As seen in both Fig. 1**A** and **B** the color/fluorescence assay continues to develop over time and doesn't reach a true end point until at least 30 min. However, the assay is linear from 0 to 30 μM of H_2O_2, whether read at 5 or 25 min after the addition of the stopping solution; valid estimates of H_2O_2 concentration are possible at any time between 5 and 30 min. The assay protocol demonstrated the potential for broad applicability: especially in timing with multiple samples in a 96-well plate format.

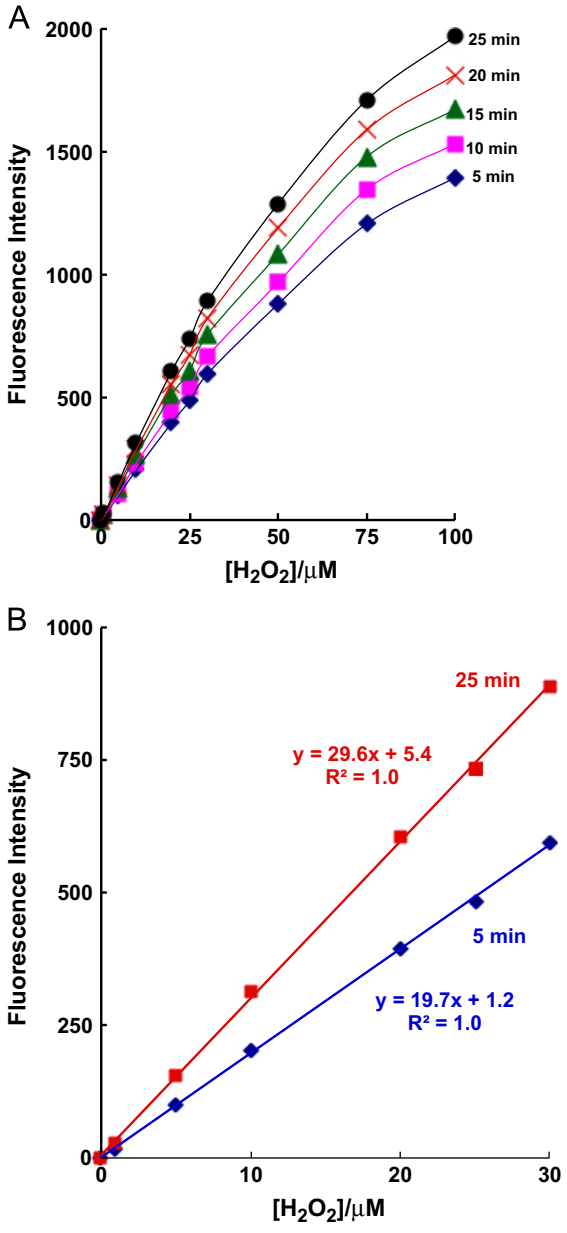

Fig. 1. Standard curves for the HRP/*p*HPA assay for H_2O_2 change with time and are nonlinear at higher concentrations of H_2O_2. **(A)** Standards for H_2O_2 (5–100 μM, final concentration in the wells) in the HRP/*p*HPA-based assay were read at 5, 10, 15, 20, and 25 min after addition of the stopping solution; see Methods. Assays were run in clear 96-well tissue culture plates (200 μL total volume) and monitored for fluorescence with band-pass filters for λ_{ex}=345 nm, λ_{em}=425 nm; **(B)** The initial linear portion of the standard curves from 0 to 30 μM H_2O_2 shown at 5 and 25 min. Each reading of the plate produced a linear response. This demonstrates that for this assay, standards and samples must be run in parallel to ensure the same timeframe. This is a representative assay from one experiment, plotting the average from the triplicate wells of each concentration. It is important in the analysis to subtract the "blank", i.e. wells with no cells and no H_2O_2, but all other components the same as in the wells that contain cells.

We further explored the usefulness of the assay with cultured cells by demonstrating that the assay is linear at low H_2O_2 concentrations, i.e. at levels that can be reliably measured, while low enough to prevent rapid and overt cellular toxicity. Fig. 2**A** is a standard curve from a representative experiment with 0.5 to 10 μM H_2O_2 (final concentration in the wells), using our standard protocol. The assay shows linearity when read at either 5 or 25 min with a lower limit of detection (LLD) of 0.4 μM at 25 min. The H_2O_2 concentration range we have used is the range anticipated for most of our cell experiments. As seen in Fig. 1**A**, standard curves are initially linear with increasing slopes over time; Fig. 2**B** presents this quantitatively. This demonstrates that the assay does not reach a true end point at the temperatures and times we have employed, and that a low level, systemic oxidation occurs. This low rate of background oxidation highlights and enforces the importance that standard curves and experimental samples must be developed and analyzed simultaneously. Additionally, the assay could be modified and is applicable to kinetic-based assays for estimates of H_2O_2 concentration based on rates of $(p\text{HPA})_2$ fluorescent dimer formation.

pHPA, caveats

The upper limit of H_2O_2 concentration that can be used in the 96-well plate reader assay is 30 μM (final concentration; 60 μM during cell-exposure), which is a limit similar to the 40 μM, using fluorimeters, as reported by Hyslop and Sklar [41]. The loss of linear responses above 30–40 μM H_2O_2 in standards curves is probably due to the absorbance of $(p\text{HPA})_2$, which will affect excitation intensity [41] as well as other photo-induced fluorescence reactions [43]. The lower limit of detection (LLD) can approach 0.4 μM: this of course varies depending on the quality of the standard curve produced when running the assay.

There is a background oxidation of *p*HPA that occurs in standard curves (Fig. 2**A** and **B**) as well as cell samples. Using the protocol presented here overcomes this issue in quantitation due to the simultaneous development and analysis of the produced *p*HPA fluorophore. Erroneous results will occur if this background oxidation is not accounted for in experiments where samples and standards are not assayed and run simultaneously. This low level oxidation would be misinterpreted in samples in which cells are being evaluated as the apparent generation or accumulation of H_2O_2 from cells.

To simplify the analysis of an experiment the number of cells in each well of the 96-well plate should be the same. The observed kinetics for the rate of removal of extracellular H_2O_2 is approximated by a first-order rate equation. Thus, the actual concentration of the H_2O_2 remaining in each of the wells with cells, in principle, need not be known. The value of k_{obs} can simply be determined by a plot of ln(net fluorescence, in arbitrary units) vs. time, where net fluorescence=(fluorescence in well)–(fluorescence of blank). The standards are run because: they take very little extra time; the cost of reagents is minimal; the actual concentration in the wells is known and can be reported with confidence; but most important, the standards provide quality control as many (but not all) possible abnormalities can identified.

Removal of extracellular H_2O_2

The kinetics of the removal of extracellular H_2O_2 is approximated well by a simple first-order rate equation, first-order in $[H_2O_2]$,

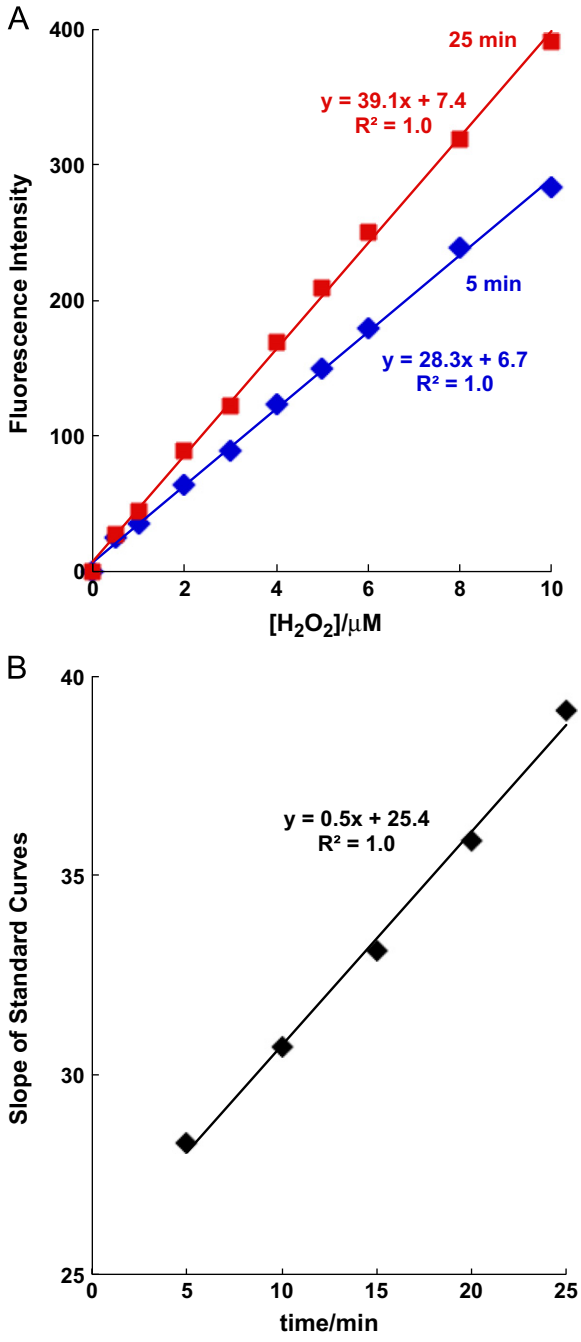

Fig. 2. A typical standard curve performed under the conditions of the assay for removal of extracellular H_2O_2 by cells. (A) Representative standard curve from a HRP/*p*HPA-based assay for H_2O_2 read with a fluorescence plate reader. The data are the average of triplicate samples on a plate read 5 and 25 min after addition of the stopping solution. The lower limit of detection (LLD) for the 25-min standard curve was 0.43 μM. **(B)** The slope of a standard curve changes linearly with time. Here the plate with the standard samples was read at five different times (5–25 min) after addition of the stopping solution. Again, this demonstrates that standards and samples must be run in parallel with steps in the protocol being congruent as appropriate.

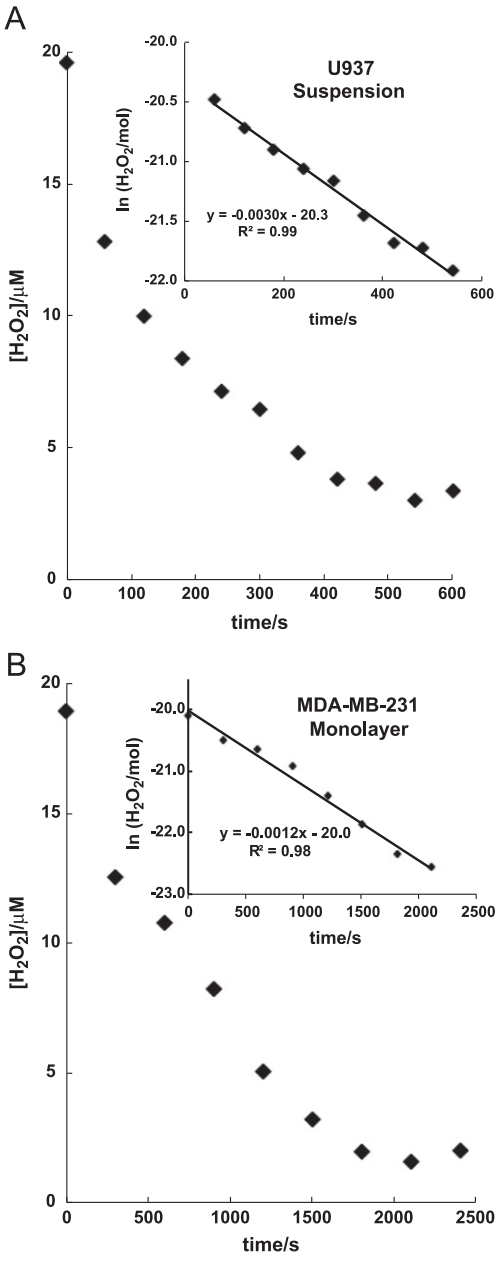

Fig. 3. Removal of extracellular H_2O_2 by cells grown in suspension culture or as adherent monolayers is a first-order process. (A) U937 cells (5.0×10^4 cells well^{-1}) in HBSS were incubated with 20 μM H_2O_2 for 0–10 min (100 μL total volume) at room temperature and then assayed for the remaining extracellular H_2O_2 using the *p*HPA assay. The observed first-order rate constant for the loss of extracellular H_2O_2 in this experiment was determined to be $k_{obs} = 3.0 \times 10^{-3}$ s^{-1}. The cell density in this experiment was 5.0×10^8 cells L^{-1}. This yields $k_{cell} = k_{obs}/(5.0 \times 10^8$ cell L$^{-1}) = 6.0 \times 10^{-12}$ s^{-1} cell^{-1} L. **(B)** MDA-MB-231 human breast cancer cells (2.1×10^5 cells well^{-1}) were allowed to attach overnight to the bottom of the tissue culture well, rinsed of old media, and washed with HBSS and covered with 50 μL HBSS. The cells were then treated with 20 μM H_2O_2 (100 μL total volume); because of their much lower capacity to remove extracellular H_2O_2 the time of maximum exposure was 40 min. The observed first-order rate constant for the loss of extracellular H_2O_2 in this experiment was determined to be $k_{obs} = 1.2 \times 10^{-3}$ s^{-1}. The cell density in this experiment was 2.1×10^9 cell L^{-1}. This yields $k_{cell} = k_{obs}/(2.1 \times 10^9$ cell L$^{-1}) = 0.57 \times 10^{-12}$ s^{-1} cell^{-1} L. Both experiments are representatives of typical observations seen in greater than 50 experiments with various cell lines.

Figs. 3 and 4. However, as seen in Fig. 3, the capacity of cells to remove extracellular H_2O_2 varies drastically between different cell lines; note that the observed first-order rate constant for the removal of extracellular H_2O_2 by the human histiocytic lymphoma suspension cell line U937 is approximately three times larger than the attached monolayer of human mammary adenocarcinoma MDA-MB-231 cells, despite there being approximately four times more MDA-MB-231 cells in the experiment than U937 cells; also note the difference in the time-scale for each abscissa of Fig. 3**A** and **B**. U937 cells grow as a suspension culture and were exposed to extracellular H_2O_2 while in

suspension, whereas the MDA-MB-231 cells are cultured as a monolayer and were exposed to extracellular H_2O_2 as attached cells. The difference in this physical aspect of cell culture may contribute to the very different capacities of these cells to remove extracellular H_2O_2.

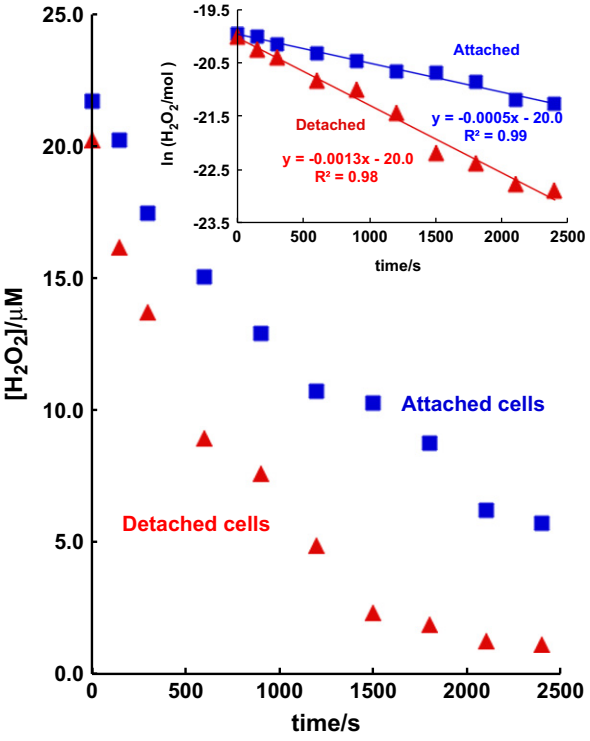

Fig. 4. The physical setting of cells affects the rate of extracellular H_2O_2 removal. Bovine aortic endothelial cells were allowed to attach overnight or were freshly trypsinized, counted, and then assayed for their ability to remove extracellular H_2O_2. The same cell densities (1.0×10^5 cells well^{-1}) were used for the assays for both attached and detached cells. The cells were exposed to a bolus addition of 20 μM H_2O_2 (100 μL total volume yielding a cell density of 1.0×10^9 cells L^{-1}). Because of the low capacity of BAECs to remove extracellular H_2O_2 the maximum time of exposure was increased to 40 min, before addition of stopping solution. Insets demonstrate the pseudo-first-order kinetics observed for H_2O_2 removal by BAECS. In these sets of experiments $k_{cell}=0.50\times10^{-12}$ s^{-1} cell^{-1} L for attached cells and $k_{cell}=1.3\times10^{-12}$ s^{-1} cell^{-1} L for suspended cells.

Physical constraints on removal of extracellular H_2O_2

Cells in an adherent culture will have proportionally less surface area available for the diffusion of extracellular H_2O_2 into the cell, and thus a possible slower rate of removal of extracellular H_2O_2. To address this possible physical constraint we examined the rate of removal of extracellular H_2O_2 by attached and detached bovine aortic endothelial cells (BAEC). BAECs that were attached as a monolayer or detached as a suspension were assayed simultaneously with equal cell numbers per well. The data of Fig. 4 demonstrate that detached BAECS remove bolus additions of extracellular H_2O_2 at a rate 2.6 times greater than the same cells when attached as a monolayer at the bottom of tissue culture wells; see also Table 1. In this experiment the cellular first-order rate constants (k_{cell}) for the removal of extracellular peroxide-removal by BAECs were 0.50×10^{-12} s^{-1} cell^{-1} L for attached cells and $k_{cell}=1.3\times10^{-12}$ s^{-1} cell^{-1} L for suspended cells (Fig. 4 inset). Thus, the "available" surface area influences the removal of extracellular H_2O_2.

Inhibition of catalase

In preliminary studies to explore the contribution of different peroxide-removal enzymes responsible for the removal of extracellular H_2O_2 in our experimental configuration, we treated cells with the catalase inhibitor 3-aminotriazole. This treatment specifically inhibits catalase irreversibly through a suicide inactivation mechanism dependent on H_2O_2 [44]. Because aminotriazole requires H_2O_2 to inactivate catalase it can also be used to detect H_2O_2 in vitro and in vivo [45–49]. In general we have found that cellular catalase activity can be inhibited to less than 20% of its original activity when incubated for 1 h with 20 mM 3-aminotriazol and that intracellular as well as extracellular H_2O_2 can facilitate this inactivation [49].

The inhibition of catalase decreased the rate of peroxide-removal from all the cells tested, but to varying degrees, Table 1. Other peroxide-removal enzymes and mechanisms exist and their contributions are apparent as k_{cell} with 3-AT is $>20\%$ of k_{cell} in the absence of 3-AT. These observations with 3-AT not only demonstrate that extracellular H_2O_2 can penetrate into the cytoplasm of cells but additionally into organelles such as peroxisomes, where catalase is located. Changes in k_{cell} following inhibition of catalase demonstrate that the assay and methodology reflect real and measurable changes in the ability of cells to remove extracellular H_2O_2.

Table 1
Rate constant, k_{cell}, for removal of extracellular H_2O_2 by cells.

Cell (Vol/pL)[b]	State	$k_{cell}/10^{-12}$ s^{-1} cell^{-1} L[a]			$k_{cell}/10^{-12}$ s^{-1} cell^{-1} L with 3-aminotraziole		
		Mean	± Std err	n[c]	Mean	± Std err	n[c]
HL-60 Human promyelocytic leukemia (0.64)	Suspended	**1.8**	0.4	5	**0.64**		1
U937 Human histiocytic lymphoma (0.93)	Suspended	**10.4**	1.7	6	**2.7**		1
MDA-MB-231 Human mammary adenocarcinoma (1.53)	Monolayer	**0.47**	0.03	8	**0.32**	0.04	5
MCF-7 Human mammary adenocarcinoma (1.70)	Monolayer	**0.64**	0.06	5	**0.49**	0.06	4
MCF-7p51 Human mammary adenocarcinoma (1.84)	Monolayer (GPx-4 Overex pressor)	**3.7**	1.5	5	**1.7**	0.5	3
MIA-PaCa-2 Human pancreatic carcinoma (2.03)	Monolayer	**0.46**	0.04	5	**0.38**	0.03	5
PC3 human prostate cancer *cell* line (2.93)	Monolayer	**2.0**	0.3	9			
BAEC Bovine Aortic Endothelial Cells (0.93)	Monolayer	**0.72**	0.16	11	**0.42**	0.09	5
BAEC Bovine Aortic Endothelial Cells (0.93)	Detached	**1.9**	0.2	13			
RBC's Human red blood cell (0.09)	Suspended	**2.9**	0.7	10	**1.1**		1
RBC's mouse red blood cell (0.05)	Suspended	**2.4**		2			
Jurkat[d] Clone E6−1 (0.78)	Suspended	**1.0**	0.1	16			

[a] The rate of removal of H_2O_2 will be: rate$=-k_{cell}$ [H_2O_2] (number of cells L^{-1}). The half-life of H_2O_2 in an experiment upon bolus addition of H_2O_2 will be approximately $t_{1/2}=0.693/(k_{cell}$ (number of cells L^{-1})).

[b] Cell volumes are from Ref. [57]; Jurkat volume from [58].

[c] n is the number of independent experiments. Each experiment had, as minimum, triplicate determinations at each time point and for each standard.

[d] This value of k_{cell} was estimated from the data in Ref. [30]. Note that k_{cell} of Antunes et al. is k_{obs} here. Their cell density was 1.0×10^9 cell L^{-1}.

Differences in cellular capacity to remove extracellular H_2O_2

As seen in Table 1, k_{cell} for removal of extracellular H_2O_2, as determined using our assay protocol, varies widely in the set of cells examined. The human pancreatic cancer cell line MIA-PaCa-2 and the human mammary adenocarcinoma cell line MDA-MB-231 have the lowest values of k_{cell}. In contrast the human histiocytic lymphoma cell line U937 has 20 times greater capacity to remove H_2O_2 as estimated by k_{cell}. Interestingly, the MCF-7 human breast cancer cell line that overexpresses GPx-4 [50,51] removes H_2O_2 at a rate 3.5 times faster than the normal wild-type MCF-7 counterpart. This difference is not due to changes in catalase, GPx1, or attributable to differences in GSH levels, as the GPx-4 over-expresser has similar levels to the parental MCF-7 wild type cell line [50]. As seen with lipid hydroperoxides [52], GPx-4 may be a major contributor to removal of H_2O_2.

Erythrocytes have a remarkable capacity to remove extracellular H_2O_2, $k_{cell}=2.9\times10^{-12}\,s^{-1}\,cell^{-1}\,L$, despite their relatively small size (0.090 pL) compared to the other cells examined (0.64–2.93 pL). Because RBCs do not have mitochondria, meeting their needs for ATP through glycolysis, the principal intracellular source of oxidants is thought to be the very slow autoxidation of oxyhemoglobin, producing methemoglobin and superoxide. The rate constant of this reaction for human oxyhemoglobin is estimated to be $\approx1\times10^{-7}\,s^{-1}$ [53,54]; if the concentration of oxyhemoglobin in RBCs is 5 mM, then the rate of superoxide production will be $5\times10^{-10}\,M\,s^{-1}$ yielding a rate for production of H_2O_2 of $2.5\times10^{-10}\,M\,s^{-1}$, assuming SOD reacts with the vast majority of superoxide produced. The flux of H_2O_2 in each RBC would then be 0.023 zmol s^{-1} or 13 molecules of $H_2O_2\,RBC^{-1}\,s^{-1}$. In our experiments to determine k_{cell} cells were exposed to 20 μM H_2O_2; the initial rate of removal of this extracellular H_2O_2 by a cell would be: $rate=-(2.9\times10^{-12}\,s^{-1}\,cell^{-1}\,L)\ (20\times10^{-6}\,M)\ (1\,cell\,L^{-1})=-58\times10^{-18}\,M\,s^{-1}$ or for each cell 58 amol s^{-1} (36×10^6 molecules $cell^{-1}\,s^{-1}$). Thus, the capacity to remove H_2O_2 by RBCs greatly exceeds the rate of intracellular production. The biconcave shape of RBCs yields a large surface-to-volume ratio that supports rapid exchange of oxygen and carbon dioxide; this also allows rapid diffusion of extracellular H_2O_2 into the RBC where the peroxide-removal system has a great capacity to remove this H_2O_2. In whole blood the density of RBCs is about 5×10^{12} cells L^{-1}; this would yield an estimate of $k_{obs}=(2.9\times10^{-12}\,s^{-1}\,cell^{-1}\,L)\ (5\times10^{12}\,cells\,L^{-1})=15\,s^{-1}$, yielding a half-life of H_2O_2 in whole blood of less than 50 ms.

This high capacity for removal of extracellular H_2O_2 by RBCs is thought to be a means for red blood cells to remove localized pulses of very high fluxes of H_2O_2, such as generated by activated immune cells, thereby protecting other cells and tissues from oxidative damage and yet not suffer extensive damage themselves that would cripple their function [55].

There are several peroxide-removal systems in cells, e.g. peroxiredoxins, glutathione peroxidases, catalase, and others. It must be kept in mind that the fraction of the flux of extracellular H_2O_2 removed by any one of these systems depends on the flux of H_2O_2 entering the cell and the availability of co-factors required for their individual function [11,12,18,23,24–27,56]. In addition, the biological response observed upon exposure will be a function of the exposure/dose (mole $cell^{-1}$) or flux (mole $cell^{-1}\,s^{-1}$) [28,31,32], and references therein. There are literally hundreds of reports where the exposure to a bolus of extracellular H_2O_2 is presented as concentration without information on the volume of media or cell density; because in a laboratory most experiments employ the same protocol, i.e. same number of cells and the same volume of media, the results reported are only relative within that protocol. Such results may not be universally interpretable or translatable to other experimental results that used different protocols. We encourage researchers to provide more quantitative information on the exposure of cells to extracellular H_2O_2, *i.e.* mole $cell^{-1}$ or mole $cell^{-1}\,s^{-1}$. We suggest information be provided on a per cell basis (a biophysical normalization) as well as per mg cell protein (a biochemical normalization). Use of both normalizations will maximize potential information from experiments. Expressing cell density in cell L^{-1} and the use of seconds as the unit for time (SI unit) allows direct use when connecting to standard chemical kinetics and mathematical modeling [57]. This also allows direct quantitative comparisons between different cell types, a goal of quantitative redox biology [17,57].

Declaration of interest

The authors declare that they have no competing interests. This research was supported by NIH grant R01 GM073929. TJvE had support from NIH P42 ES013661, Training Core; JRW had support from NIH T32CA078586. Core facilities were supported in part by P30 CA086862. The content is solely the responsibility of the authors and does not represent views of the National Institutes of Health.

Acknowledgments

We thank Eric E. Kelley for the kind gift of the BAECs and Cameron M. Cushing for helpful discussions. The University of Iowa ESR facility provided invaluable assistance.

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