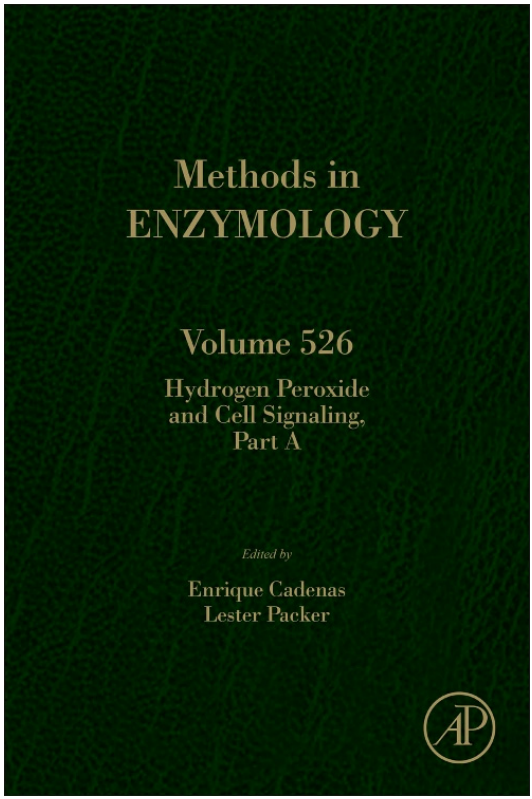

From: H. Susana Marinho, Luísa Cyrne, Enrique Cadenas, Fernando Antunes, H_2O_2 Delivery to Cells: Steady-State Versus Bolus Addition. In Enrique Cadenas and Lester Packer, editors: *Methods in Enzymology,* Vol. 526,
Burlington: Academic Press, 2013, pp. 159-173.
ISBN: 978-0-12-405883-5

CHAPTER TEN

H_2O_2 Delivery to Cells: Steady-State Versus Bolus Addition

H. Susana Marinho*, Luísa Cyrne*, Enrique Cadenas†, Fernando Antunes*,1

*Departamento de Química e Bioquímica and Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal
†Pharmacology & Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, California, USA
1Corresponding author: e-mail address: fantunes@fc.ul.pt

Contents

Abstract

Hydrogen peroxide (H_2O_2) is a ubiquitous biological molecule whose wide range of biological functions depends on its concentration. In this chapter, we compare the delivery of H_2O_2 to cells as (1) a single initial dose (bolus addition); (2) a continuous source using, for example, glucose oxidase; and (3) a steady state, in which H_2O_2 concentration is kept constant during the assay. Both the bolus addition and the use of a continuous source of H_2O_2 have as outcome concentration profiles of H_2O_2 that are dependent on experimental conditions and that are difficult to reproduce from the information that is usually

Methods in Enzymology, Volume 526
ISSN 0076-6879
http://dx.doi.org/10.1016/B978-0-12-405883-5.00010-7

revealed in the experimental section of most research articles. On the other hand, the outcome of delivering H_2O_2 as a steady state is a concentration profile that is independent of experimental conditions. The implementation of the steady state starts with the determination of the kinetics of H_2O_2 consumption in the system under study. Then, the amount of glucose oxidase needed to produce H_2O_2 at a rate that matches the rate of its consumption by cells at the desired H_2O_2 steady-state concentration is calculated. The setup of the steady state is initiated by adding this amount of glucose oxidase simultaneously with the desired concentration of H_2O_2. Because H_2O_2 consumption and delivery rates are matched, the initial H_2O_2 concentration added is kept constant during the assay. Detailed explanations on how to implement the steady state, including H_2O_2 measurements and adjustments in the amount of H_2O_2 or glucose oxidase during the assay, are described.

1. INTRODUCTION

Hydrogen peroxide (H_2O_2) is a small diffusible species produced in aerobic cells that acts as a mild oxidant *in vivo*. Depending on its concentration, H_2O_2 may oxidize sulfhydryl groups in proteins to different degrees and, as an outcome, changes in protein activity occur leading to important regulatory effects (Winterbourn & Hampton, 2008). In the presence of transition metals, H_2O_2 can be decomposed to the highly reactive hydroxyl radical that reacts virtually with the first species it collides in the vicinity of its production site (Koppenol, 1993). Thus, the biological effects of H_2O_2 are strongly dependent on its concentration (Antunes & Cadenas, 2001; De Oliveira-Marques, Cyrne, Marinho, & Antunes, 2007; Quinn et al., 2002) and opposite effects may be observed for a relative narrow range of concentrations (Matias, Marinho, Cyrne, Herrero, & Antunes, 2011). Another key characteristic of H_2O_2 metabolism is its rapid consumption by cellular antioxidant enzymes which include glutathione peroxidases, catalase, peroxiredoxins, cytochrome *c* peroxidase, and rubredoxins (Chance, Sies, & Boveris, 1979; Pedone, Bartolucci, & Fiorentino, 2004). When studying its cellular effects, researchers manipulate H_2O_2 concentrations either by influencing its cellular metabolism or by adding external H_2O_2 to the cells under study. In this chapter, we compare three alternative H_2O_2-delivering systems: H_2O_2 bolus addition, glucose oxidase (GO) addition, and steady-state incubation. We also describe in detail how to set up a steady-state incubation in cell cultures.

1.1. Bolus addition

The direct addition of H_2O_2 to the system under study at the desired concentration is the simplest approach to study the effect of H_2O_2. Because of the rapid consumption of H_2O_2 by cellular antioxidants, its concentration starts to decrease as soon as the dose is given. The concentration profile of H_2O_2 during the incubation is strongly dependent on the cellular density used and, for attached culture cells, the volume of the incubation media also affects the H_2O_2 profile (Fig. 10.1A). Therefore, this commonly used methodology is very dependent on the experimental setting and, consequently, experiments are difficult to reproduce. In addition, because H_2O_2 is consumed rapidly, in order to observe biological effects, often large initial concentrations of H_2O_2 are given, and so, the relevance of the results obtained is doubtful. This methodology helped to acquire useful data when the aim was studying damaging oxidative effects by large concentrations of H_2O_2, but to study fine regulatory roles exerted by H_2O_2, this methodology is clearly unsuitable.

1.2. Glucose oxidase

An alternative that avoids the need to add large initial doses of H_2O_2 is to add a system that continuously produces H_2O_2, thus compensating for its continuous consumption. GO, which uses glucose in the media to produce H_2O_2 (Eq. 10.1), is an example of such a system.

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-glucono-1,5-lactone} \quad [10.1]$$

Alternatively, if for some reason GO cannot be used, for example, if incubating a microorganism cell culture in the stationary phase of growth where glucose is absent, other H_2O_2-forming enzymes can be used. An example is amino acid oxidase that catalyzes H_2O_2 formation from amino acids present in the growth medium (Cyrne, Antunes, Sousa-Lopes, Diaz-Bérrio, & Marinho, 2010; Sousa-Lopes, Antunes, Cyrne, & Marinho, 2004).

After adding GO to the cell culture, the H_2O_2 concentration will increase initially until a steady state is reached where the production of H_2O_2 by the enzymatic system is compensated by its consumption by the cellular antioxidants. Both the value of this steady state and the time that it takes to reach it are dependent on the rate of H_2O_2 consumption in the experimental system. Often, both these values are unknown as the H_2O_2 concentration is not monitored during the assay, and the H_2O_2 incubation is reported as units of the enzyme added. This approach is also difficult

to reproduce as it is strongly dependent on the experimental conditions. If the amount of GO is adjusted based on the volume of the incubation media, the final steady state will depend on the number of cells present in the assay (Fig. 10.1B); if the amount of GO is adjusted based on the number of cells in the assay, the final H_2O_2 concentration will be independent of experimental conditions, but the time needed to reach the steady state will be dependent on these conditions (Fig. 10.1C). In this regard, notice that the H_2O_2 concentration profile obtained in an experiment with adherent cells seeded in a 100-mm dish with 10 mL of growth media differs from that obtained in a well of a 96-well plate with 0.1 mL of growth media (assuming the same cell density in both cases) (Fig. 10.1C).

Thus, for both bolus and GO additions, the H_2O_2 concentration profiles and, consequently, the results obtained have a low reproducibility.

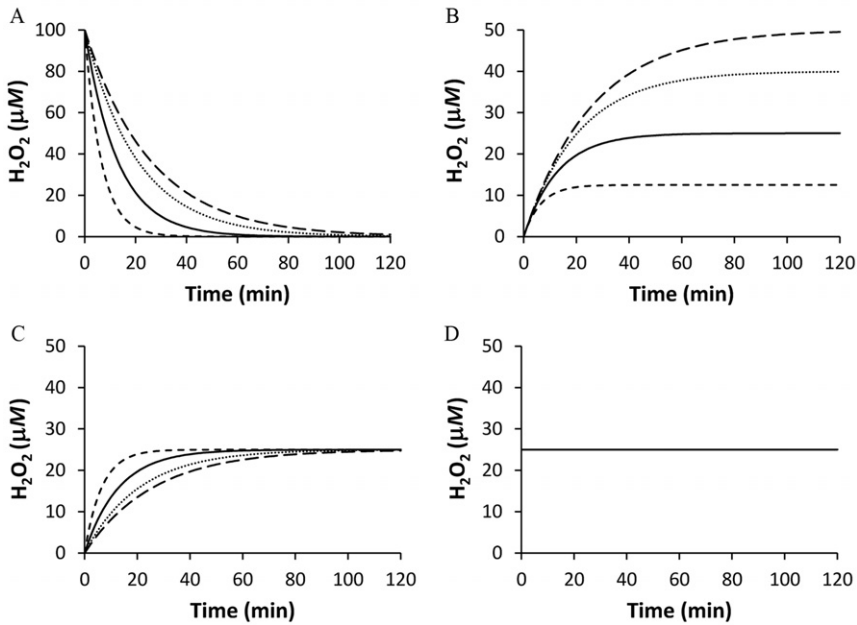

Figure 10.1 Different methods of H_2O_2 delivery to cells. MCF-7 cells were seeded at a density of 3.2×10^4 cells/cm^2 in a 100-mm dish in 10 mL of growth media (——), 1.6×10^4 cells/cm^2 in a 100-mm dish in 10 mL of growth media (– – –), 3.2×10^4 cells/cm^2 in a 100-mm dish in 5 mL of growth media (- - -), and 3.2×10^4 cells/cm^2 in 96-well plate in 0.1 mL of growth media (· · · · · ·). Cells were exposed to H_2O_2 by adding a 100 μ*M* bolus addition (A), by adding GO that produces H_2O_2 at 1.9 μ*M*/min (B), by adding GO that produces H_2O_2 at 0.011 μmol/10^6 cells (C), and by adding a steady state of 25 μ*M* H_2O_2 (D). Curves are simulations based on a first-order rate constant 0.43 min$^{-1}\times$ mL/10^6 cells for the consumption of H_2O_2 by MCF-7 cells.

Moreover, often, it is impossible to reproduce the experimental H_2O_2 concentration profile applied in a given work, because details such as the volume of the growth media and the number of cells used are usually absent in publications.

1.3. Steady state

In the steady-state approach, the addition of an initial concentration of H_2O_2 is combined with the addition of either GO or another H_2O_2-producing system. The dose given corresponds to the H_2O_2 concentration of interest, and the amount of GO is adjusted in order to balance the consumption of H_2O_2 at the concentration of interest, thus keeping the H_2O_2 concentration steady during the experiment. A key aspect of this methodology is that the H_2O_2 concentration is monitored during the assay and its level is adjusted in order to keep the concentration constant. Thus, this approach implies a higher effort when compared with either the bolus or the GO addition, but on the other hand, the H_2O_2 concentration profile obtained does not depend on the experimental conditions (Fig. 10.1D) facilitating experimental reproducibility, either in the same laboratory or between laboratories.

2. EXPERIMENTAL COMPONENTS AND CONSIDERATIONS

2.1. Theoretical consideration on how to set up a steady state

A steady state for H_2O_2 is obtained when the rates of H_2O_2 production and consumption match each other. At this stage, the concentration of H_2O_2 does not change and a steady state is reached. Mathematically, this is obtained by setting the differential equation that describes H_2O_2 dynamics to zero (Eq. 10.2).

$$\frac{d[H_2O_2]}{dt} = V_{production} - V_{consumption} = 0 \Rightarrow V_{production} = V_{consumption} \quad [10.2]$$

When setting up a steady state experimentally, the first step is to determine $V_{consumption}$. Once this is determined, $V_{production}$ can be obtained from Eq. (10.2), and the amount of GO, or of another H_2O_2-generating system, that is necessary to add to the system can be calculated. $V_{consumption}$ varies with the H_2O_2 concentration. Fortunately, for sublethal H_2O_2 concentrations, the kinetics of H_2O_2 consumption can be often approximated to first-order kinetics (Antunes & Cadenas, 2000; Branco, Marinho, Cyrne, &

Antunes, 2004; Gülden, Jess, Kammann, Maser, & Seibert, 2010; Makino, Mochizuki, Bannai, & Sugita, 1994). This is consistent with the known catalase and glutathione peroxidase kinetics (Chance et al., 1952, 1979; Flohe, Loschen, Gunzler, & Eichele, 1972). Under these conditions, $V_{production}$ can be calculated from Eq. (10.3), where k is the first-order rate constant for the consumption of H_2O_2.

$$V_{production} = k \times [H_2O_2] \quad [10.3]$$

The rate of H_2O_2 production is calculated by replacing in Eq. (10.3) the first-order rate constant k obtained experimentally and the desired steady-state H_2O_2 concentration to be established in the experiment ($[H_2O_2]$). If the kinetics of H_2O_2 consumption is more complex, an equivalent of Eq. (10.3) is obtained, and by replacing the desired H_2O_2 concentration and the determined kinetics constants, the rate of production can be calculated.

2.2. Reagents

1. *H_2O_2*. Make fresh every day the solution using concentrated *Perhydrol* [30% (m/m) H_2O_2, density 1.11 g/mL, MW = 34.02, 9.79 *M*] from Merck. The original *Perhydrol*, 30% concentrated solution, should be aliquoted in order to avoid its frequent manipulation. To obtain the stock solution of H_2O_2 (approximately 9–10 m*M*), dilute 1/1000 the concentrated H_2O_2 solution. For that make an initial dilution of 1/100 in water (10 μL + 990 μL water or, alternatively, two sequential 1/10 dilutions) in an *Eppendorf* tube. Then perform a 1/10 dilution using 500 μL of H_2O_2 diluted 1/100 and adding 4.5 mL of water. Confirm the concentration by reading the absorbance of this 1/1000 diluted stock solution at 240 nm ($\varepsilon = 43.4\ M^{-1}cm^{-1}$). Keep on ice.
2. *Catalase* (bovine liver, Sigma C-1345, 2000–5000 U/mg protein) 1 mg/mL (in water). It can be stored for weeks.
3. *GO* from *Aspergillus niger*, Sigma G-0543, ≥200 U/mg protein, ≤0.1 U/mg catalase, buffered aqueous solution (in 100 m*M* sodium acetate, 40 m*M* KCl, with 0.004% thimerosal), pH 4.5, low catalase activity. Storage temperature, 2–8 °C. A working diluted solution (1/100, 1/1000, or 1/10,000 dilution in water) should be made daily.
4. *0.1 M Potassium phosphate buffer, pH 6.5.*

3. PILOT EXPERIMENTS

Pilot experiments consist of the determination of the kinetics of H_2O_2 consumption and the rate of formation of H_2O_2 catalyzed by GO under the

experimental conditions to be used for the steady-state incubation. For each cell type or for each new GO batch, the following procedures should be carried out.

3.1. H_2O_2 calibration curve

H_2O_2 is assayed by the formation of O_2 after the addition of catalase (Eq. 10.4) using an oxygen electrode.

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O \quad [10.4]$$

We use a chamber oxygen electrode (Oxygraph system, Hansatech Instruments Ltd., Norfolk, UK) with a magnetic stirrer and temperature control. All measurements are either performed at room temperature (for cell lines) or at 30 °C (for yeast) and with a final volume of 800 μL. The electrode should be given a stable baseline. For that, it is recommended to add 800 μL of distilled water and to connect the stirring a few hours before the measurements.

A H_2O_2 calibration curve within the range of 10–90 μ*M*, in which the O_2 electrode we use has a linear response, should be made. For that, do the following (see also Marinho, Cyrne, Cadenas, & Antunes, 2013):

1. Pipet from 10 μL up to 100 μL of the 9 m*M* H_2O_2 stock solution to different test tubes and add distilled water to a final volume of 5 mL. Keep the test tubes at the same temperature of the electrode chamber.
2. Add 400 μL of H_2O_2 from one of the test tubes, starting with the lowest concentration, to 400 μL of 0.1 *M* potassium phosphate buffer, pH 6.5, already in the electrode chamber. Readings can also be done without using the buffer, but the oxygen electrode has a more stable output when the buffer is used.
3. Start recording and, when a baseline is established, rapidly add 15 μL of catalase using a Hamilton syringe (being careful not to add air bubbles as they interfere in the measurement). This addition should cause a rapid increase in the reading as O_2 is formed from the H_2O_2 present by the action of catalase. After a new baseline is established, stop the recording. The value of the difference between the two baselines should be used to make a plot versus H_2O_2 concentration.
4. Remove the content of the oxygen electrode chamber and clean thoroughly with distilled water (fill the chamber up until the middle at least four times and then up until the top four times) in order to be sure to remove all the catalase before the next H_2O_2 assay.

3.2. Determination of kinetics of H_2O_2 consumption by cells

The consumption of H_2O_2 in cells should be done under the same conditions (cell density, media used, and incubation conditions) of the H_2O_2 steady-state assay.

1. The experiment is initiated with the addition of 100 μ*M* H_2O_2 (bolus addition) to the medium.
2. Take 800 μL aliquots at different times, add to the electrode chamber, and measure O_2 formation after adding 15 μL of catalase.
3. Remove the content of the oxygen electrode chamber and clean thoroughly with distilled water as described in the previous section in order to be sure to remove all the catalase before the next H_2O_2 assay.

At least six time points should be recorded. Use the calibration curve to calculate the concentrations. Time between aliquots depends on how fast H_2O_2 is being consumed in the assay, but data points spanning the range between 10 and 90 μ*M* should be obtained.

3.3. Determination of H_2O_2 production by GO

The determination of the rate of H_2O_2 production by GO should be done under the same conditions (media used and incubation conditions) of the H_2O_2 steady-state assay. For example, for cell lines, we use the following procedure:

1. Add 10 μL of GO to 990 μL of water (1/100 dilution of the original stock solution).
2. Add 10 μL of the previous solution to a 100-mm cell-culture dish with 8 mL of prewarmed and CO_2-preequilibrated media.
3. Put dish in the cell incubator.
4. Take 800 μL aliquots at different times, add to the electrode chamber, and measure O_2 formation after adding 15 μL of catalase.

At least five time points should be recorded. Use the calibration curve to calculate the H_2O_2 concentrations. Time between aliquots depends on glucose activity, but typically under these conditions, we take aliquots every 4–5 min. The maximum concentration recorded should be lower than 90 μ*M* and points should follow a linear increase (Fig. 10.2).

3.4. Data handling

3.4.1 Determination of k

The data obtained in Section 3.2 is plotted as ln [H_2O_2] versus time. If H_2O_2 is consumed as a first-order process, this plot should be linear with a negative

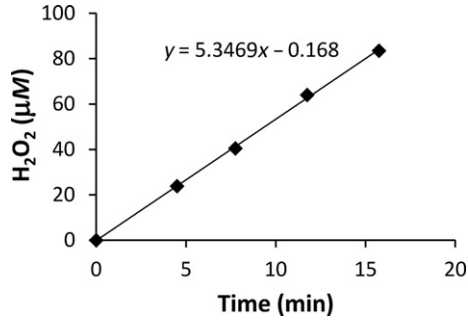

Figure 10.2 Determination of glucose oxidase activity. Production of H_2O_2 was followed in an oxygen electrode by measuring the oxygen formed after the addition of catalase. 10 μL of a 1/100 dilution of the original GO solution is added to 8 mL of prewarmed RPMI-1640 media.

slope. The first-order rate constant is determined from the slope ($k = -$slope), and it will have units of time^{-1}. If the determination is done in suspension cells, this value has to be divided by the cell density (usually million of cells/mL or, in the case of microorganisms, OD_{600}).

If the determination is done in attached cells, a plot of the ln [H_2O_2] versus time cannot be done directly from the experimental data, because after taking each 800 μL aliquot, the reaction volume covering the cells decreases, and H_2O_2 will be consumed faster as the number of the cells in the assay remains constant. So, before plotting the data, data points should be corrected according to the procedure shown in Fig. 10.3. The rate constant can be obtained from the slope of the semilogarithmic plot of the corrected H_2O_2 concentrations versus time. The rate constant is equal to slope × (reaction volume)/(number of cells), where reaction volume unit is mL and the number of cells comes in million. Formally, this is identical to the rate constant described above for the suspension cells (slope/cell density). Taking as an example the data in Fig. 10.3 for HT-4 cells, slope is −0.0239 min^{-1}, so the rate constant is

$$0.0239\,\mathrm{min}^{-1} \times 10\mathrm{mL}/\left(1.8 \times 10^6 \mathrm{cells}\right) = 0.13\,\mathrm{min}^{-1}\mathrm{mL}/10^6\mathrm{cells}$$

3.4.2 Calculation of GO activity

The slope determined from the plot in Fig. 10.2 was 5.35 μM min^{-1}. Multiplying by the volume of the assay in which the activity was determined (8 mL) and dividing by the volume of GO 1/100 dilution added (10 μL),

A

	B	C	D
1	H_2O_2 experimental	Correction	Corrected H_2O_2
2	(μM)	factor	(μM)
3	100.0		100.0
4	93.0	1	93.0
5	65.0	0.9	67.4
6	47.1	0.8	52.0
7	29.1	0.7	37.2
8	17.9	0.6	27.8
9			
10	D4: =EXP(LN(B4/B3)*C4)*D3		
11	D4 from Row 4 through Row 8		

B

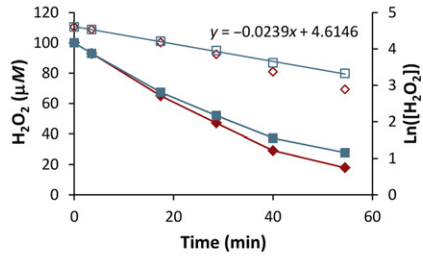

Figure 10.3 Calculation of the first-order rate constant for H_2O_2 consumption by attached HT-4 cells (1.8 million cells in a 100-mm dish). (A) The experimental H_2O_2 values (Column B) are corrected to account for the removal of 1 mL aliquots at each time point (Column D); the initial volume of incubation media was 10 mL. The correction factor (Column C) is calculated as the ratio between the volume in which H_2O_2 consumption was measured and the initial reaction volume. At 3.5 min, the first 1 mL aliquot is taken and, in this case, the correction factor is one, that is, no correction is needed because consumption during the first 3.5 min was done in the original volume of 10 mL. At 17.4 min, a second 1 mL aliquot was taken and a correction of factor of 9/10 was introduced because H_2O_2 consumption between 3.5 and 17.4 min was done in 9 mL (and not in the original 10 mL). (B) Plot of uncorrected (◆) and corrected (■) H_2O_2 concentrations in normal (closed symbols) and semilogarithmic scale (open symbols). Note the deviation from linearity for the uncorrected H_2O_2 concentration in the semilogarithmic plot.

we obtain the number of moles produced per unit time per μL of the 1/100 dilution of GO:

$$\left(5.35\,\mu M\,\mathrm{min}^{-1}\right) \times 8\,\mathrm{mL}/10\,\mu\mathrm{L} = 4.28\,\mathrm{nmol\ of\ H_2O_2\ min^{-1}\mu L^{-1}\ of\ 1/100\ GO\ solution}$$

3.4.3 Calculation of the amount of GO to be added

The amount of GO to be added to the steady-state assay is calculated in three steps. The rate of H_2O_2 production needed is calculated by applying Eq. (10.3), where the k obtained in Section 3.4.1 and the desired steady state ($[H_2O_2]$ss) are replaced; the conditions of the assay (cell density for suspension cells or number of cells and reaction volume for attached cells) are also included:

$$V_{\mathrm{production}} = k \times [\mathrm{H_2O_2}]\mathrm{ss} \times \mathrm{cell\ density} \quad \text{or}$$
$$V_{\mathrm{production}} = k \times [\mathrm{H_2O_2}]\mathrm{ss} \times \mathrm{number\ of\ cells/reaction\ volume}$$

1. $V_{production}$ obtained has units of concentration × time^{-1}. For example, if a 25 μ*M* steady state is to be established in an assay with 1.8 million HT-4 cells attached in a 100-mm dish with 10 mL of reaction volume, $V_{production}$ would be as follows:

$$\left(0.13\,\text{min}^{-1}\text{mL}/10^6\,\text{cells}\right) \times 25\,\mu M \times 1.8 \times 10^6\,\text{cells}/10\,\text{mL} = 0.585\,\mu M\,\text{min}^{-1}.$$

2. The number of molecules of H_2O_2 produced is obtained by multiplying the value obtained in step 1 by the reaction volume. Continuing with the previous example,

$$\left(0.585\,\mu M\,\text{min}^{-1}\right) \times 10\text{mL} = 5.85\,\text{nmol}\,\text{min}^{-1}.$$

3. By dividing the number obtained in the previous step by the activity calculated in Section 3.4.2, we obtain the volume in μL of the 1/100 glucose dilution needed to obtain the desired steady state. In our example,

$$\left(5.85\,\text{nmol}\,\text{min}^{-1}\right)/\left(4.28\,\text{nmol}\,\text{min}^{-1}\mu\text{L}^{-1}\right) = 1.37\,\mu\text{L}$$

We would need 1.37 μL of a 1/100 dilution of the original glucose oxidation. To minimize pipetting error, a 13.7 μL of a 1/1000 diluted solution would be advisable.

4. STEADY-STATE EXPERIMENTAL IMPLEMENTATION

4.1. H_2O_2 calibration curve

A H_2O_2 calibration curve as described in Section 3.1 should be made daily in a range around the steady state of interest. For example, for 25 μ*M*, we usually use concentrations of 15, 20, 25, 30, and 35 μ*M*.

4.2. Steady-state implementation

The incubating media should be replaced with prewarmed media 1 h before experiment. Used media may have an inherent H_2O_2 consumption rate, because many cells export glutathione (Bannai & Tsukeda, 1979; Griffith et al., 1979). To start the steady state, add simultaneously the desired amount of H_2O_2 and an amount of GO calculated as in Section 3.4.3. The steady state should be checked every hour, or for short incubations, an aliquot should be taken just before the end of the experiment to measure the real

H_2O_2 concentration. To this end, take an 800 μL aliquot and measure the H_2O_2 concentration as described before. Adjust the H_2O_2 concentration in the following way:

1. If the H_2O_2 concentration measured is lower than the desired steady state, correct by adding the missing H_2O_2 and GO. GO addition should be calculated as a direct proportion to the deviation from the desired steady state observed. For example, if a steady state of 25 μ*M* was the aim, and a 22.5 μ*M* concentration is observed, that is, 10% lower than the aim, then add 10% of both the GO and H_2O_2 amounts added initially.
2. If the H_2O_2 concentration measured is higher than the desired steady state, in case of a cell suspension, nothing can be done. If working with attached cells, remove a volume of incubation medium calculated as a direct proportion to the deviation from the desired steady state observed. If the observed concentration is 10% superior to the desired one, remove 10% of the incubation media and replace with fresh prewarmed and CO_2-preequilibrated media. By doing this, the excess of both GO and H_2O_2 is removed.
3. In the case of attached cells, GO additions and media removal have to take into account the volume of the aliquot taken to check the steady state. For example, if the incubation media is 8 mL and a 800 μL aliquot is taken and the steady state matches exactly the desired steady state, add, in 800 μL of fresh media, 10% of GO and 10% of H_2O_2 amounts that were added initially to compensate for the removal of the aliquot. If the steady state measured is 10% higher than the desired steady state, just add 800 μL of fresh media. If the steady state is 10% lower than the desired, it is necessary to add 20% of the initially added GO and H_2O_2 amounts.
4. For cells growing in suspension, the considerations in the previous point do not apply, because when taking aliquots, only the overall volume is decreasing, while relative GO activity, H_2O_2 concentration, and number of cells remain constant.

4.3. Additional points

Steady-state incubations are usually carried out in RPMI, a growth medium that consumes H_2O_2 at a very low rate. Other media consume H_2O_2 at significant amounts, which can be compensated by giving an additional amount of GO (Clément, Ramalingam, Long, & Halliwell, 2001; Hoffman, Pine, & Bell, 1983; Martín-Romero, Miguel-Lasobras, Domínguez-Arroyo, González-Carrera, & Alvarez, 2008). However, if

H_2O_2 is consumed extracellularly at significant rates, one can question whether the cellular H_2O_2 effects are due to the interaction of H_2O_2 with cellular components, or due to the products being formed extracellularly from the reactions of H_2O_2 with medium components.

The generation of H_2O_2 catalyzed by GO is based on the oxidation of glucose to D-glucono-1,5-lactone (Eq. 10.1). It is important to test that D-glucono-1,5-lactone does not interfere in the system. One simple control that can be done is to add catalase. If the biological effects triggered by the steady-state incubation are absent in the presence of catalase, then D-glucono-1,5-lactone is not interfering with the measurements. The consumption of O_2 by GO can also affect the results, and in this case, the addition of catalase does not provide a definitive answer because half of the O_2 consumed in the reaction catalyzed by GO is released in the catalase-catalyzed reaction. If the cellular consumption of H_2O_2 by the cells is much lower than the cellular O_2 consumption, the depletion of O_2 caused by GO is not a problem. However, if cellular H_2O_2 and O_2 consumptions are of the same order of magnitude, the O_2 concentration in solution should be checked.

After the steady state is set up, a good reproducibility is usually achieved. When the H_2O_2 concentration measured deviates more than 20% of the desired concentration, a correction as described in Section 4.2 is not attempted and the experiment is discarded.

Further information on the application of the steady-state delivery in specific situations is described in detail in Covas, Marinho, Cyrne, and Antunes (2013) and Cyrne, Oliveira-Marques, Marinho, and Antunes (2013).

5. SUMMARY

In this chapter, the exposure of cells to H_2O_2 steady states was described in detail. Compared with the common bolus addition, in which H_2O_2 is delivered as a single initial dose, the steady-state incubation is much more complex to implement. However, the addition of H_2O_2 as a steady state provides more reproducible results and, by providing a rigorous control of H_2O_2 delivery, it allows to observe cellular effects that occur in narrow H_2O_2 concentration ranges that would be difficult to observe with the bolus addition. In our experience, after an initial learning curve, the higher complexity of the experimental procedure involved in the implementation of a H_2O_2 steady state is largely compensated by the higher experimental reproducibility that allows achieving statistically significant results with a lower number of experimental replicates. Moreover, the initial setup, in which

the kinetics of H_2O_2 consumption for the system under study is determined, may also provide useful information and unexpected findings. For example, the discovery of the regulation of plasma membrane permeability by H_2O_2 was triggered by the observation that H_2O_2-adapted yeast cells, which have higher levels of antioxidant enzymes, have kinetics of H_2O_2 consumption similar to control cells (Branco et al., 2004). In the field of nitric oxide research, it was soon recognized that the use of donors that provide continuous release of nitric oxide is more appropriate than the use of bolus additions of nitric oxide. Unfortunately, after several decades of research, in the field of H_2O_2, the use of a bolus addition is still the method of choice by a vast majority of researchers. The use of a H_2O_2 bolus addition, and the inherent lack of standardization, is responsible for apparently contradictory findings and a lack of rigorous quantitative data, which has delayed the development of the field (Brigelius-Flohé & Flohé, 2011). We suggest that the delivery of H_2O_2 to cells as a steady state should be the method of choice when studying regulatory effects of H_2O_2.

ACKNOWLEDGMENTS

Supported by Fundação para a Ciência e a Tecnologia (FCT), Portugal (Grants PTDC/QUI/69466/2006, PTDC/QUI-BIQ/104311/2008, and PEst-OE/QUI/UI0612/2011) and by NIH Grants RO1AG016718 (to E. C.) and PO1AG026572 (to Roberta D. Brinton).

REFERENCES

Antunes, F., & Cadenas, E. (2000). Estimation of H_2O_2 gradients across biomembranes. *FEBS Letters*, *475*, 121–126.

Antunes, F., & Cadenas, E. (2001). Cellular titration of apoptosis with steady-state concentrations of H_2O_2. Sub-micromolar levels of H_2O_2 induce apoptosis through Fenton chemistry independent of cellular thiol state. *Free Radical Biology & Medicine*, *30*, 1008–1018.

Bannai, S., & Tsukeda, H. (1979). The export of glutathione from human diploid cells in culture. *The Journal of Biological Chemistry*, *254*(9), 3444–3450.

Branco, M. R., Marinho, H. S., Cyrne, L., & Antunes, F. (2004). Decrease of H_2O_2 plasma membrane permeability during adaptation to H_2O_2 in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry*, *279*, 6501–6506.

Brigelius-Flohé, R., & Flohé, L. (2011). Basic principles and emerging concepts in the redox control of transcription factors. *Antioxidants & Redox Signaling*, *15*(8), 2335–2381.

Chance, B., Greenstein, D. S., & Roughton, F. J. W. (1952). The mechanism of catalase action. I. Steady-state analysis. *Archives of Biochemistry and Biophysics*, *37*, 301–339.

Chance, B., Sies, H., & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews*, *59*, 527–605.

Clément, M.-V., Ramalingam, J., Long, L. H., & Halliwell, B. (2001). The in vitro cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. *Antioxidants & Redox Signaling*, *3*(1), 157–163.

Covas, G., Marinho, H., Cyrne, L., & Antunes, F. (2013). Activation of Nrf2 by H_2O_2: De novo synthesis versus nuclear translocation. *Methods in Enzymology*, *526*.

Cyrne, L., Antunes, F., Sousa-Lopes, A., Diaz-Bérrio, J., & Marinho, H. S. (2010). Glyceraldehyde-3-phosphate dehydrogenase is largely unresponsive to low regulatory levels of hydrogen peroxide in *Saccharomyces cerevisiae*. *BMC Biochemistry*, *11*, 49.

Cyrne, L., Oliveira-Marques, V., Marinho, H., & Antunes, F. (2013). Activation of Nrf2 by H_2O_2: De novo synthesis versus nuclear translocation. *Methods in Enzymology*, *526*.

De Oliveira-Marques, V., Cyrne, L., Marinho, H., & Antunes, F. (2007). A quantitative study of NF-kappa B activation by H_2O_2: Relevance in inflammation and synergy with TNF-alpha. *The Journal of Immunology*, *178*, 3893–3902.

Flohe, L., Loschen, G., Gunzler, A., & Eichele, E. (1972). Glutathione peroxidase, V. The kinetic mechanism. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*, *353*, 987–999.

Griffith, O. W., Novogrodsky, A., & Meister, A. (1979). Translocation of glutathione from lymphoid cells that have markedly different γ-glutamyl transpeptidase activities. *Proceedings of the National Academy of Sciences*, *76*(5), 2249–2252.

Gülden, M., Jess, A., Kammann, J., Maser, E., & Seibert, H. (2010). Cytotoxic potency of H_2O_2 in cell cultures: Impact of cell concentration and exposure time. *Free Radical Biology & Medicine*, *49*(8), 1298–1305.

Hoffman, P. S., Pine, L., & Bell, S. (1983). Production of superoxide and hydrogen peroxide in medium used to culture *Legionella pneumophila*: Catalytic decomposition by charcoal. *Applied and Environmental Microbiology*, *45*(3), 784–791.

Koppenol, W. H. (1993). The centennial of the Fenton reaction. *Free Radical Biology & Medicine*, *15*, 645–651.

Makino, N., Mochizuki, Y., Bannai, S., & Sugita, Y. (1994). Kinetic studies on the removal of extracellular hydrogen peroxide by cultured fibroblasts. *The Journal of Biological Chemistry*, *269*(2), 1020–1025.

Marinho, H., Cyrne, L., Cadenas, E., & Antunes, F. (2013). The cellular steady-state of H_2O_2: Latency concepts and gradients. *Methods in Enzymology*, *526*.

Martín-Romero, F. J., Miguel-Lasobras, E. M., Domínguez-Arroyo, J. A., González-Carrera, E., & Alvarez, I. S. (2008). Contribution of culture media to oxidative stress and its effect on human oocytes. *Reproductive Biomedicine Online*, *17*(5), 652–661.

Matias, A. C., Marinho, H. S., Cyrne, L., Herrero, E., & Antunes, F. (2011). Biphasic modulation of fatty acid synthase by hydrogen peroxide in *Saccharomyces cerevisiae*. *Archives of Biochemistry and Biophysics*, *515*, 107–111.

Pedone, E., Bartolucci, S., & Fiorentino, G. (2004). Sensing and adapting to environmental stress: The archaeal tactic. *Frontiers in Bioscience*, *9*, 2909–2926.

Quinn, J., Findlay, V. J., Dawson, K., Millar, J. B. A., Jones, N., Morgan, B. A., et al. (2002). Distinct regulatory proteins control the graded transcriptional response to increasing H(2)O(2) levels in fission yeast *Schizosaccharomyces pombe*. *Molecular Biology of the Cell*, *13*(3), 805–816.

Sousa-Lopes, A., Antunes, F., Cyrne, L., & Marinho, H. S. (2004). Decreased cellular permeability to H_2O_2 protects *Saccharomyces cerevisiae* cells in stationary phase against oxidative stress. *FEBS Letters*, *578*(1–2), 152–156.

Winterbourn, C. C., & Hampton, M. B. (2008). Thiol chemistry and specificity in redox signaling. *Free Radical Biology & Medicine*, *45*(5), 549–561.