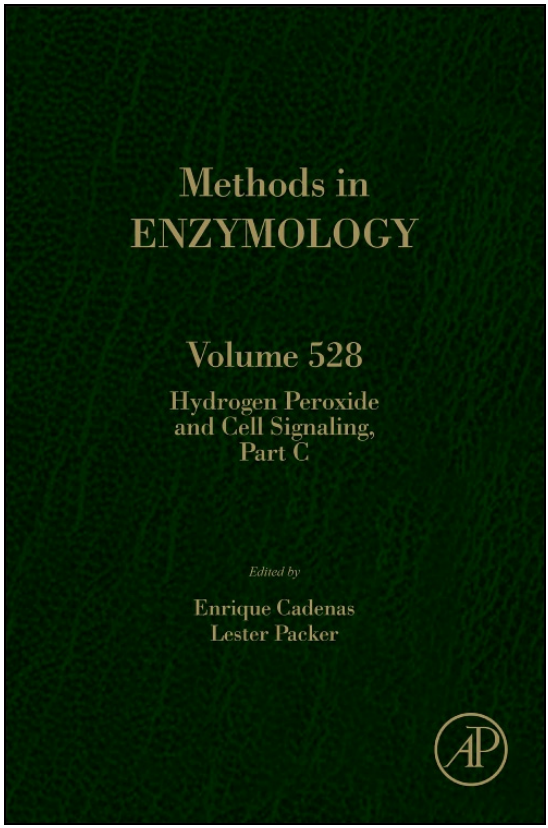

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CHAPTER TEN

H_2O_2 in the Induction of NF-κB-Dependent Selective Gene Expression

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Contents

Abstract

NF-κB is a transcription factor that plays key roles in health and disease. Learning how this transcription factor is regulated by hydrogen peroxide (H_2O_2) has been slowed down by the lack of methodologies suitable to obtain quantitative data. Literature is abundant with apparently contradictory information on whether H_2O_2 activates or inhibits NF-κB. There is increasing evidence that H_2O_2 is not just a generic modulator of transcription factors and signaling molecules but becomes a specific regulator of

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individual genes. Here, we describe a detailed protocol to obtain rigorous quantitative data on the effect of H_2O_2 on members of the NF-κB/Rel and IκB families, in which H_2O_2 is delivered as a steady-state addition instead of the usual bolus addition. Solutions, pilot experiments, and experimental set-ups are fully described. In addition, we outline a protocol to measure the impact of alterations in the promoter κB regions on the H_2O_2 regulation of the expression of individual genes. As important as evaluating the effects of H_2O_2 alone is the evaluation of the modulation elicited by this oxidant on cytokine regulation of NF-κB. We illustrate this for the cytokine tumor necrosis factor alpha.

1. INTRODUCTION

The NF-κB/Rel family of transcription factors consists of homo- and heterodimers of five distinct proteins p65 (RelA), RelB, c-Rel, p50 (and its precursor p105), and p52 (and its precursor p100). NF-κB has key regulatory roles in inflammation, innate and adaptive immune response, proliferation, and apoptosis (Chen & Greene, 2004; Ghosh, May, & Kopp, 1998). NF-κB activation leads to its translocation from the cytosol to the nucleus and has as an outcome an inflammatory response characterized by an increased expression of: proinflammatory cytokines, for example, tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6); chemokines, for example, monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8); adhesion molecules, for example, intercellular Adhesion Molecule-1 (ICAM-1), vascular cell adhesion protein-1 (VCAM-1) and E-selectin; growth factors; and, enzymes that produce secondary inflammatory mediators such as cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) (Brigelius-Flohé & Flohé, 2011). The role of H_2O_2 in NF-κB activation *in vivo* is highly controversial. Studies involving H_2O_2 either alone or in conjunction with cytokines do not show a consistent activation of NF-κB, and H_2O_2 can activate, inhibit, or have no effect on NF-κB (reviewed in Oliveira-Marques, Marinho, Cyrne, & Antunes, 2009b). Partly this is due to the fact that most studies on NF-κB do not apply calibrated and controlled methods of H_2O_2 delivery to cells, such as the steady-state titration (Antunes & Cadenas, 2001; Antunes, Cadenas, & Brunk, 2001). Recent studies using delivery of H_2O_2 in a steady state to cells (steady-state titration) have allowed the emergence of a new paradigm where H_2O_2 acts not as an inducer of NF-κB but as a modulator of the activation of the NF-κB pathway by other agents (Oliveira-Marques, Cyrne, Marinho, & Antunes, 2007; Oliveira-Marques et al., 2009b). In fact, by using the steady-state titration, we previously showed

using MCF-7 and HeLa cells that H_2O_2, at concentrations close to those occurring during an inflammatory situation, that is, 5–15 μ*M* (Liu & Zweier, 2001; Test & Weiss, 1984), has a synergistic effect on TNF-α-dependent translocation of p65 from the cytosol to the nucleus. This increased nuclear translocation of p65 in the presence of H_2O_2 and TNF-α has as outcome the enhanced gene expression of a subset of NF-κB-dependent genes, including proinflammatory genes, for example, IL-8; MCP-1; TLR2; and TNF-α, and anti-inflammatory genes, for example, heme oxygenase-1 (Oliveira-Marques et al., 2007). NF-κB, once inside the nucleus, binds to the promoter/enhancer regions of target genes (Moynagh, 2005), the κB sites, which have the general consensus sequence GGGR NNYYCC (R is purine, Y is pyrimidine, and N is any base). The differential gene regulation caused by H_2O_2 depends, among other factors, on the apparent affinity of κB sites in the gene-promoter regions toward NF-κB and, the lower the affinity, the higher the range of TNF-α concentrations where H_2O_2 upregulates gene expression (Oliveira-Marques, Marinho, Cyrne, & Antunes, 2009a). This selective gene expression has potential implications in personalized medicine because many single-nucleotide polymorphisms are found in the κB sites of the human genome (Kasowski et al., 2010).

Here, we present a detailed protocol that allows exploring the role of H_2O_2 as a modulator of NF-κB-dependent gene expression by exposing cells to H_2O_2 and cytokines in inflammatory-like conditions.

2. EXPERIMENTAL COMPONENTS AND CONSIDERATIONS

2.1. 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*. To obtain the stock solution of H_2O_2 (~9–10 m*M*), dilute 1/1000 the concentrated H_2O_2 in water and confirm the concentration by reading the absorbance at 240 nm ($\varepsilon = 43.4\ M^{-1}cm^{-1}$). Keep on ice.
2. Catalase (bovine liver, Sigma C-1345, 2000–5000 units/mg protein) 1 mg/mL (in water). Can be stored for weeks.
3. Glucose oxidase from *Aspergillus niger*, Sigma G-0543, ≥200 units/mg protein, ≤0.1 units/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. Optionally needed for the H_2O_2 calibration curve.
5. Phosphate-buffered saline (PBS)—1.5 m*M* KH_2PO_4 pH 7.4, 137 m*M* NaCl, 3.0 m*M* KCl, and 8.0 m*M* Na_2HPO_4.
6. Cytosolic proteins buffer—50 m*M* HEPES, pH 7.2, 2 m*M* EDTA, 10 m*M* NaCl, 250 m*M* sucrose, with freshly added protease inhibitor cocktail protease inhibitors (1 m*M* PMSF, 1.5 μg/mL benzamidine, 10 μg/mL leupeptin, and 1 μg/mL pepstatin), 2 m*M* dithiothreitol, and the detergent IGEPAL CA-630 0.1% (v/v), all from Sigma, Saint Louis, MO, USA.
7. Nuclear proteins buffer—Identical to the cytosolic proteins buffer except that 250 m*M* sucrose was replaced by 20% (v/v) glycerol and NaCl was 400 m*M*.

2.2. Cell culture preparation

To ensure that a reproducible and stable H_2O_2 steady state is obtained, it is fundamental to standardize cell culture preparation, especially when using adherent cells. When the experiment is performed, adherent cells should have completely recovered from splitting, for example, recovered their normal shape with no signs of toxicity, with a uniform distribution throughout the plate/flask and with a confluence between 60% and 80%. This means that the characteristics of the adherent cell lines being used should be known in advance and that conditions should be rigorously maintained for all experiments to have reproducible results. Too many cells in a plate/flask imply a higher H_2O_2 consumption and decrease in the steady state while lower cell numbers or unrecovered cells will be more sensitive to H_2O_2, causing an increase in H_2O_2 concentration and possibly inducing oxidative stress. Therefore, we recommend counting and plating the cells 46 h before the experiment. The number of cells plated is entirely dependent on the cells doubling time to achieve 60–80% confluence at the day of the experiment. As an example, to achieve the desired confluence in a 100-mm Petri dish, 0.9×10^6 MCF-7 (European Collection of Cell Cultures, Salisbury Wiltshire, UK) and 0.5×10^6 HeLa (American Type Culture Collection, Manassas, VA, USA) cells are plated to get 1.8×10^6 and 1.5×10^6 cells, respectively, after 46 h.

For an experiment of protein or mRNA extraction, we recommend using 100-mm Petri dishes to have enough extracted material per condition and at the same time to have enough cell culture volume to not disturb the system when taking aliquots for steady-state confirmation (Section 4). For other types of experiments, such as viability assays that do not require so many cells, it is possible to use multi-well plates (e.g., 96-well, 24-well plates), and in such situations, the number of cells has to be adjusted to achieve a 60–80% confluence at the day of the experiment.

2.3. Methodological considerations

When working with H_2O_2, it is important to understand that cells are equipped with mechanisms to rapidly consume this molecule. Although H_2O_2 is a mild oxidant, high H_2O_2 levels can cause oxidative stress and cytotoxicity. Only at the low/moderate levels occurring *in vivo* does H_2O_2 fulfill its potential as a signaling molecule. The steady-state titration arises exactly from this need of studying signaling pathways and works with doses close to the physiological ones. The addition of a single low H_2O_2 initial dose—bolus addition—might not be enough to switch-on signaling pathways because of the rapid H_2O_2 consumption within cells. For example, in our experiments using MCF-7 cells, a single bolus addition of 100 μ*M* H_2O_2 is consumed in less than 30 min of incubation with a confluence of 60% in a 100-mm Petri dish with 10 mL of incubation media. To overcome this issue, many have opted to increase the initial H_2O_2 dose up to the millimolar range that is far from the physiological relevance required. Again, in our hands, more than 90% of H_2O_2 is consumed during the first hour after a 1-m*M* bolus addition to 1.8×10^6 MCF-7 cells in 10 mL incubation media (Oliveira-Marques et al., 2007).

When using the steady-state titration, H_2O_2 consumption is balanced by its production via glucose oxidase, an enzyme that catalyzes the formation of H_2O_2 through the oxidation of glucose present in cell culture medium. When combining the addition of glucose oxidase to the addition of H_2O_2 at the desired concentration, the assay starts and keeps running at the same concentration of H_2O_2. This method allows using low/moderate concentrations of H_2O_2, which is fundamental to study signaling pathways, for example, NF-κB (Marinho, Cyrne, Cadenas, & Antunes, 2013a). When MCF-7 cells are exposed to a typical bolus addition of 1 m*M* H_2O_2 or to a steady state of 25 μ*M* H_2O_2 for 2 h, there is a translocation of NF-κB into the nucleus (Oliveira-Marques et al., 2007). However, as shown in

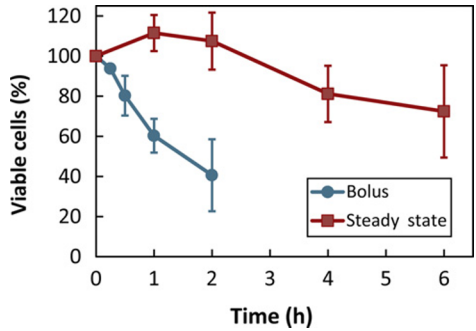

Figure 10.1 Comparison of cell viability of MCF-7 cells upon incubation with 1 m*M* H_2O_2 bolus dose (•) or a 25 μ*M* H_2O_2 steady state (■). Cell viability was assessed by following MTT reduction (McGahon et al., 1995).

Fig. 10.1, there is a significant difference in cell viability, and so translocation of NF-κB into the nucleus under bolus addition conditions occurs when cell viability is already lost. This clearly illustrates the advantages of using a H_2O_2 steady state, where it is possible to adjust the concentration of H_2O_2, even for long incubations, with no loss in cell viability.

2.4. H_2O_2 measurement

To have the steady-state method working properly, it is necessary to measure the actual concentration of H_2O_2 present in the cell culture medium so that the H_2O_2 concentration is not just an assumed value based on the amounts of glucose oxidase and H_2O_2 added. With either an oxygen (O_2) or a H_2O_2 electrode, it is possible to measure the actual concentration of H_2O_2 present in the cell culture medium at a certain incubation point. An O_2 electrode allows time point measurements by adding few microliters of 1 mg/mL catalase (10–15 μL) to convert H_2O_2 present in the medium to O_2. We use the Oxygraph system (Hansatech Instruments Ltd., Norfolk, UK), directly linked to a PC for registration. The O_2 permeable membrane is replaced every week and tested for quality before an experiment. When not in use, the chamber is always filled with distilled H_2O so that the membrane does not dry.

The following generic method describes the typical H_2O_2 measurements in cell culture medium of adherent cell lines using an O_2 electrode (see also Marinho, Cyrne, Cadenas, & Antunes, 2013b):

1. Approximately, 12 h before starting the experiment, leave the O_2 electrode on, with H_2O in the chamber, so that the system is fully stabilized in the day of the experiment;
2. Measure H_2O_2 present in the medium the following way:
   a. Aspirate H_2O from the electrode chamber;
   b. Add the medium/solution with H_2O_2 and cover with the electrode chamber cap;
   c. Start recording a baseline that should be horizontal and when stable add 10–15 μL of catalase to convert H_2O_2 into O_2. A steep change of the slope should be observed, if any H_2O_2 is present in the medium;
   d. When H_2O_2 is fully converted, the slope should return to baseline type and recording can be stopped;
   e. Aspirate medium from the chamber and clean thoroughly by filling the chamber up until the middle at least four times and up until the top four times also with distilled H_2O to ensure that all catalase is removed before the next assay.

The difference between both baselines represents the quantity of H_2O_2 present in the medium and can be converted in concentration by making a calibration curve of H_2O_2. We prepare daily a 9-m*M* stock solution of H_2O_2 to assess the real concentration in a spectrophotometer at 240 nm ($\varepsilon_{240nm} = 43.4\ M^{-1}cm^{-1}$) and make a calibration curve of H_2O_2 solutions (in H_2O) ranging from 9 to 90 μ*M* as described in Marinho et al. (2013b). Each measurement is performed as explained earlier.

3. PILOT EXPERIMENTS

Pilot experiments are required to calibrate the system every time there is a change in components of the steady-state method, such as the cell line to be used, the cell culture medium, or the batch of glucose oxidase.

3.1. Calibrating the system: Cellular H_2O_2 consumption

To have a continuous H_2O_2 source working properly, preliminary studies to calibrate the system are required. Removal of added H_2O_2 depends on several factors, such as the cellular capacity to remove H_2O_2, cell density, and consumption of H_2O_2 by the growth medium (Oliveira-Marques et al., 2009b). Therefore, in order to know the amount of glucose oxidase to add to the medium studies to estimate H_2O_2 consumption by cells need to be performed. Every time the conditions change, such as new medium or the cell number in the experiment, we recommend recalibrating the

system. We typically use in our cell culture experiments the following incubation medium: RPMI 1640 medium supplemented with 10% (v/v) of fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 m*M* L-glutamine (Lonza, Basel, Switzerland), which does not interfere with the H_2O_2 steady-state method. Unless otherwise explained, throughout this chapter, we use as medium the described supplemented RPMI 1640 medium.

The following generic method describes the typical calibration for cellular H_2O_2 consumption (see also Marinho et al., 2013a):

1. Seed cells approximately 46 h before the experiment at two/three different final numbers. We recommend using 100-mm Petri dishes because they are easier to handle than flasks;
2. Approximately 12 h before the experiment, leave the oxygen electrode on, with H_2O in the chamber so that the system is fully stabilized in the day of the experiment;
3. On the day of the experiment, replace cells medium with prewarmed new medium (9 mL) and wait for 1 h. For experiments that start in the morning and total volumes above 50 mL, medium can be left inside the incubator overnight; otherwise, it will take several hours to attain 37 °C;
4. During this time, prepare H_2O_2 stock solution and make the calibration curve as described in Section 2.4;
5. Add 100 μ*M* of H_2O_2 to cells culture medium, swirl gently, and start counting time (cells are kept inside the incubator);
6. After 5 min, take the first aliquot of medium (800 μL minimum) and measure H_2O_2 concentration with the O_2 electrode as described in Section 2.4;
7. Continue the process every 5/7 min until no H_2O_2 is detectable;
8. Calculate the rate of H_2O_2 consumption for the different conditions and use this value to calibrate the steady-state titration experiments. Take into consideration that when using attached cells a correction for the medium volume is needed. The correction is calculated as the ratio between the volume in which H_2O_2 consumption was measured ($Vol_{measurement}$) over the initial reaction volume ($Vol_{initial}$) as shown in Equation 10.1

$$[H_2O_2]_{corrected} = [H_2O_2]_{experimental} \times Vol_{measurement}/Vol_{initial}; \qquad [10.1]$$

9. The rate constant is obtained from the slope of the plot of ln $[H_2O_2]_{corrected}$ versus time. For HeLa and MCF-7 cells, rate constants obtained were 0.50 and 0.43 min^{-1} mL/10^6 cells, respectively.

3.2. Calibrating the system: Glucose oxidase activity

In the presence of O_2, glucose oxidase uses glucose present in the culture medium to produce H_2O_2 and D-glucono-1,5-lactone. Glucose oxidase (*A. niger*) activity should be tested when using a new commercial flask since it is a fundamental parameter to establish accurate H_2O_2 steady states. And the activity will depend on the assay conditions. So the correct way to calculate glucose oxidase activity is keeping constant the exact experimental conditions such as the cells culture medium, the incubation temperature, and the oxygen electrode for H_2O_2 measurements (see Marinho et al., 2013a).

1. Warm up 10 mL of incubation medium for 1 h in the cells incubator;
2. Dilute 1/100 glucose oxidase in H_2O_2 and add to the medium 10 μL;
3. Measure the actual H_2O_2 concentration produced every 5–7 min with the O_2 electrode as described in Section 2.4.

A plot of H_2O_2 concentration versus time should be linear. From the slope, calculate glucose oxidase activity as nanomole of H_2O_2 produced per minute per microliter of the 1/100 glucose oxidase solution.

4. STEADY-STATE TITRATION EXPERIMENTS

With the system fully calibrated (Section 3) and cell cultures prepared as explained in Section 2.1, everything is set up for a steady-state titration experiment. Prepare the number of plates for each condition to be used in the experiment plus an extra plate for a steady-state pretest of the day. Although all parameters are previously calculated, day-by-day errors will be corrected with this pretesting to be sure that the H_2O_2 concentration is the desired one during the assay.

Repeat points 1–4 from Section 3.1, with the exception that 7 mL of medium is used, and continue through the following steps:

1. Add to the pretest dish the calculated volumes for H_2O_2 and glucose oxidase to achieve the desired steady-state concentration; see (Covas, Marinho, Cyrne, & Antunes, 2013; Marinho et al., 2013a) for examples of calculations;
2. After 1 h of incubation, measure the external H_2O_2 concentration in a 800-μL aliquot. If the H_2O_2 concentration is not the desired one,

recalculate the amount of glucose oxidase to be added to the assays, assuming that the difference between the H_2O_2 concentration measured and the wanted one is directly proportional to the correction needed. For example, if the calculated amount of glucose oxidase is 50 μL, and the steady state measured in the pretest dish is 10% higher than the desired one, add 90% of 50 μL to the assays;

3. Start the experiments by adding the glucose oxidase units taking into account the result obtained in the previous step;
4. Monitor H_2O_2 concentration every hour and at the end of the experiment;
5. For experiments up to 1 h, no corrections are introduced. For longer experiments, correct steady states taking into account the deviations from the target steady state assuming a direct proportionality between the deviation and the correction. Take also into account the removal of H_2O_2 and glucose oxidase in the aliquots. A detailed calculation is shown in Covas et al. (2013);
6. Importantly, do not make additional measurements than the needed ones to avoid disturbing unnecessarily the system.

It is worth mentioning that if using 96-well plates it is important to have several replicates to be able to pool the medium from several wells, for example, four wells with 200 μL each, and measure an average for H_2O_2 steady-state concentration.

5. NF-κB FAMILY PROTEIN LEVELS

Cells treated with H_2O_2 in steady state can be processed to analyze signaling pathways. For the NF-κB/Rel and IκB families, we typically perform the analysis of protein levels by immunoblot. We set up the H_2O_2 steady-state conditions as explained in previous sections in the range of 5–25 μ*M* H_2O_2. Petri dishes of 100-mm usually give enough protein material for good immunoblot signals and are adequate and easy to handle for H_2O_2 steady-state methodology.

We recommend organizing your samples in groups of treatment that should be directly compared and keep in mind having time to measure all H_2O_2 concentrations in the O_2 electrode before protein extraction. Have an untreated control plate for each time point. For the NF-κB family members' protein levels, we use four conditions per time: control; steady-state H_2O_2; TNF-α; steady-state H_2O_2 plus TNF-α added simultaneously.

TNF-α (Human Recombinant, Sigma, Saint Louis, MO, USA) is used at 0.37 ng/mL and does not interfere with the steady-state H_2O_2 level.

We next describe the steps for cellular sub-fractioning to collect both cytosolic and nuclear proteins from the same samples.

5.1. Protein extraction

Several protein extraction protocols might be used. Here, we detail a fractionated protocol we have been using to analyze NF-κB/Rel- and IκB family members levels separately in cytosolic and nuclear compartments. The following procedure should be made in cold (Oliveira-Marques et al., 2007).

1. At the end of the incubation time, check the H_2O_2 concentration as explained before and follow to extraction procedure. If the H_2O_2 concentration differs more than 20% from the desired one, the experiment is discarded;
2. Aspirate the medium and wash the 100-mm plates with cold PBS;
3. Add 500 μL of cytosolic proteins buffer, scrape cells, and transfer the mixture to a sterile 1.5-mL tube;
4. Repeat the procedure with 100 μL of cytosolic proteins buffer and pool mixtures;
5. Centrifuge the tubes at 3000 *g* for 4 min at 4 °C;
6. Collect the supernatant that contains the cytosolic proteins to new tubes;
7. Wash the pellet with 300 μL of cytosolic buffer and centrifuge as in step 4;
8. Discard supernatant and resuspend the pellet with 30 μL of nuclear proteins buffer;
9. Keep tubes on ice for 20–25 min and extract proteins by vortexing three times during that period;
10. Centrifuge samples at 10,000 g for 10 min at 4 °C;
11. Collect to new tubes the enriched supernatant with nuclear proteins;
12. Quantify protein concentration by the Bradford method, which has low interferences.

5.2. Western blot

The following conditions have been used by us for studying the effect of H_2O_2 on NF-κB/Rel and IκB families. All proteins are analyzed on either 8% or 12.5% polyacrylamide gels. LMW-SDS protein markers from GE Healthcare Life Sciences (Uppsala, Sweden) or LMW protein markers from

NZYTech (Lisboa, Portugal) are used. Antibodies sc-372 (1:1000), sc-70 (1:300), sc-371 (1:800), sc-945 (1:400), and sc-7156 (1:800) are incubated for 2 h and used to identify p65, c-Rel, IκB-α, IκB-β, and IκB-ε, respectively (all from Santa Cruz Biotechnology, Santa Cruz, California, USA). The bands corresponding to each protein are then quantified by signal intensity analysis, with normalization to the protein loading (membrane stained with Ponceau S). We use the *ImageJ* software for band intensity quantification (Rasband, 1997).

6. NF-κB-DEPENDENT GENE EXPRESSION

The H_2O_2 steady-state method can also be applied to study gene expression regulated by NF-κB. All current methods to assess gene expression, such as real-time PCR and gene expression microarrays, can be adapted for cell culture exposure to H_2O_2 in steady state by following the steps extensively described in previous sections. One of the first decisions to make is the quantity of material that will be needed to measure gene expression and set up the system with the appropriate number of cells for the Petri dishes or multi-well plates chosen.

To exemplify, we describe the study of NF-κB regulation of gene expression using HeLa cells transiently transfected with a reporter plasmid containing different κB regions. As before, we use TNF-α as a classical NF-κB inducer and analyze the modulatory effects elicited by a H_2O_2 steady state.

6.1. Plasmid constructs

Experimental κB reporter plasmids are generated using common molecular biology techniques by inserting a minimal promoter in the pGL3-basic vector (Promega, Madison, WI, USA) with *Bgl*II (5′end) and *Hin*dIII (3′end) restriction enzymes (New England Biolabs, Ipswich, England): 5′-GATCTGGGTATATAATGGATCCCCGGGTACGCAGCTCA-3′. The κB sequences (Udalova, Mott, Field, & Kwiatkowski, 2002) are inserted upstream the minimal promoter, between the KpnI/SacI restriction site, with the following general sequence: 5′-GCT-κB-CTGGCTCCT-κB-CTCAGCT-3′. We tested three different κB sequences: κB1-GGGGACTTCC; κB2-GGGGATTCCC and κB3-GGGAATTTCC.

6.2. Cellular transfection and reporter gene assays

For reporter gene assays, we use the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) where cells are cotransfected with the

experimental plasmid that has the firefly luciferase and a second control plasmid pRL-SV40 (Promega, Madison, WI, USA) that bears the renilla luciferase, important to normalize luminescence. Setting up the right amount of DNA to transfect is important for transfection efficiency, taking also in consideration the ratio between the plasmids to avoid interferences between promoters. An excess of cytotoxicity that might be introduced by transfection methods should be avoided, even if at the cost of a lower transfection efficiency, since H_2O_2 steady state could become toxic if cells are not healthy when starting the experiment. The first step is to choose the appropriate type of transfection reagent. Lipofectamine (Invitrogen, Carlsbad, California, USA) is widely used, but here we tested fugeneHD (Roche, Mannheim, Germany), which gave a good balance between transfection efficiency and toxicity. As recommended by the manufacturer, we do several tests to choose the appropriate transfection reagent quantity to use, but also test for the time to let cells recover from transfection, before starting the steady state. We recommend at this stage to run viability assays, such as MTT (McGahon et al., 1995), or alamar blue (O'Brien, Wilson, Orton, & Pognan, 2000), before initiating with luminescence experiments. For example, we observed that transfection *per se* was leading to approximately 50% loss in cell viability. Importantly, we had to reduce glucose oxidase volume to 50% of the usual volume to maintain the desired H_2O_2 steady state (Oliveira-Marques et al., 2009a).

The following procedure exemplifies a typical transfection experiment and H_2O_2 steady-state treatment to assess NF-κB-dependent gene expression.

1. Plate HeLa cells onto 24-well plates at a density of 4.5×10^4 cells/well in 500 μL of medium;
2. Let cells recover for 24 h;
3. Prepare the transfection mixture of fugeneHD: DNA 5:2 (v/m) in Opti-MEM medium (Invitrogen, Carlsbad, California, USA) and incubate for 20 min at room temperature;
4. Replace cells medium with 500 μL of fresh medium without antibiotics;
5. Add in a drop-wise manner 18 μL of the transfection mixture. This mixture contains 180 ng of kB experimental plasmid, 9 ng of pRL-SV40 control plasmid, and 171 ng of pGL3-basic plasmid and 0.9 μL of fugeneHD;
6. Swirl the wells to ensure distribution over the entire plate surface. Perform the assay within 24 h;
7. Expose cells to steady-state H_2O_2 as explained in Section 4 and TNF-α concentrations ranging from 0.18 to 50 ng/mL. Use 800 μL of medium

per well to allow an accurate measurement of H_2O_2 with the oxygen electrode;

8. Incubate cells for 4 h. Exceptionally, intermediate corrections of H_2O_2 should not be made, because they would imply the use of several replicate wells, as all medium from one well is needed to measure H_2O_2. This is a cost/quality balance choice. For a system calibrated to achieve 25 μ*M* H_2O_2 steady state, we normally measured 21 μ*M* of H_2O_2 after a 4 h incubation;
9. Lysis and luciferase analysis were assayed accordingly to the manufacturer instructions. Luminescence is read with the luminometer Zenyth 3100 with 1 s of integration time, one sample at a time because of the rapid decreased of the renilla signal. Each sample is read in triplicate.

Figure 10.2 shows the effect of H_2O_2 on NF-κB-dependent gene expression as a function of the κB site. The effect of H_2O_2 is dependent on the affinity of the κB site toward NF-κB, with genes with high-affinity sites (κB2) being modulated by H_2O_2 at lower levels of TNF-α, while genes with low-affinity sites (κB1) are modulated by H_2O_2 at higher levels of TNF-α (Oliveira-Marques et al., 2009a). The medium-affinity site (κB3) gives an intermediate response.

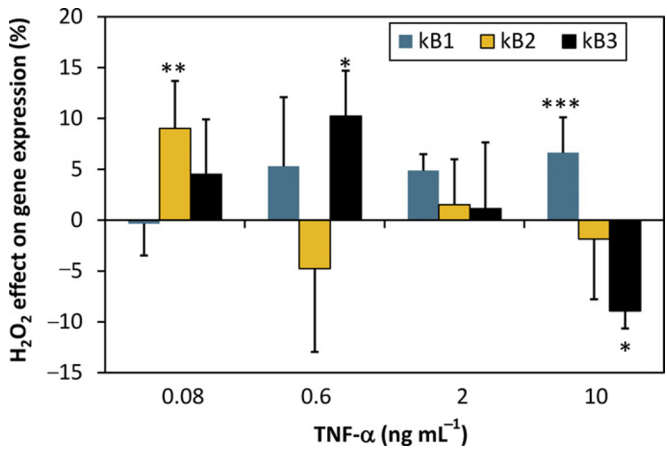

Figure 10.2 Effect of H_2O_2 on gene expression of NF-κB-dependent-reporter genes. The effect shown is the change elicited by H_2O_2 on TNF-α-dependent expression. $^{*}p < 0.001$; $^{**}p = 0.012$; $^{***}p = 0.002$. Data are replotted from Oliveira-Marques et al. (2009a).

7. SUMMARY

This chapter presents an description of experimental components necessary to study NF-κB activation by H_2O_2 in a rigorous quantitative way. For that, cells are exposed to H_2O_2 steady states, by balancing the cellular H_2O_2 consumption with the production of H_2O_2 with glucose oxidase, which catalyzes the oxidation of glucose present in the growth media. H_2O_2 is monitored during the assays, and so the experimental H_2O_2 profiles are independent of the experimental conditions, facilitating the acquisition of reproducible data. Under these conditions, the variation of subtle biological responses as a function of H_2O_2 concentration can be obtained. This contrasts with experiments where H_2O_2 is delivered as a single initial dose—bolus addition—where H_2O_2 profiles and the amount of H_2O_2 delivered per cell are strongly dependent on the experimental conditions. We illustrated the advantage of the steady-state delivery methodology by showing that selective gene regulation by H_2O_2 occurs for genes that have κB sites in the promoter region with different affinity toward NF-κB. Thus H_2O_2 regulation may play an important role in the design of personal medicine drugs that target NF-κB, because single-nucleotide polymorphisms present in the κB sites are responsible for different gene expression patterns in humans.

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Conflicts of interest

VOM is a full-time employee of Thelial Technologies S.A. This present report precedes her current employment and there is no overlap in interests.

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