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CHAPTER NINE

Activation of Nrf2 by H$_2$O$_2$: De Novo Synthesis Versus Nuclear Translocation

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Abstract

The most common mechanism described for the activation of the transcription factor Nrf2 is based on the inhibition of its degradation in the cytosol followed by its translocation to the nucleus. Recently, Nrf2 de novo synthesis was proposed as an additional mechanism for the rapid upregulation of Nrf2 by hydrogen peroxide (H$_2$O$_2$). Here, we describe a detailed protocol, including solutions, pilot experiments, and experimental setups, which allows exploring the role of H$_2$O$_2$, delivered either as a bolus or as a steady state, in endogenous Nrf2 translocation and synthesis. We also show experimental data, illustrating that H$_2$O$_2$ effects on Nrf2 activation in HeLa cells are strongly dependent both on the H$_2$O$_2$ concentration and on the method of H$_2$O$_2$ delivery. The de novo synthesis of Nrf2 is triggered within 5 min of exposure to low concentrations of H$_2$O$_2$. 

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preceding Nrf2 translocation to the nucleus which is slower. Evidence of de novo synthesis of Nrf2 is observed only for low H₂O₂ steady-state concentrations, a condition that is prevalent in vivo. This study illustrates the applicability of the steady-state delivery of H₂O₂ to uncover subtle regulatory effects elicited by H₂O₂ in narrow concentration and time ranges.

1. INTRODUCTION

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor of the leucine zipper family, and Keap1 (Kelch-like ECH-associated protein 1) is its specific repressor, responsible for Nrf2 sequestration in the cytoplasm (Itoh et al., 1999; Xue & Cooley, 1993) as well as for its proteosomal degradation pathway (Kobayashi et al., 2006; Zhang & Hannink, 2003). These two proteins mediate cellular response to oxidative stress and to electrophilic xenobiotics (Osburn & Kensler, 2008). Included among the target genes regulated by Nrf2 are antioxidant enzymes, involved in electrophile conjugation, glutathione homeostasis, production of reducing equivalents, proteasome function, and other (Hayes & McMahon, 2009).

In the cell, regulation of Nrf2 levels and its activity occurs at several levels, including transcription, translation, degradation, translocation, and posttranslational modifications such as phosphorylation (Huang, Nguyen, & Pickett, 2000, 2002; Kong et al., 2001; Nioi & Hayes, 2004; Zhang & Hannink, 2003).

One of the most important mechanisms determining the increase of Nrf2 protein levels, involves a decreased rate of Nrf2 protein degradation. In the absence of any stress conditions, the normally low cellular concentrations of Nrf2 are maintained by proteasomal degradation, through a Keap1–Cullin 3–Roc1-dependent mechanism, in which Keap1 serves as the substrate adaptor subunit in the E3 holoenzyme. Activation of Nrf2 allows it to escape proteolysis and to rapidly accumulate in the nucleus inducing its target genes (Kobayashi et al., 2004, 2006; Zhang, Lo, Cross, Templeton, & Hannink, 2004). Keap1 is a cysteine-rich protein (Human- and murine Keap1 contain 27 and 25 cysteine residues, respectively) and so modifications in sulfhydryl-containing residues of this protein result in conformational changes (Itoh et al., 1999). In fact, oxidative stress conditions, and many exogenous chemicals, alter the redox status of Keap1 cysteine residues. As a consequence, there is a destabilization of the Keap1/Nrf2 complex, preventing Nrf2 degradation, which allows Nrf2 translocation to the nucleus.
Recent work indicates that Nrf2 de novo synthesis is an important mechanism for the rapid Nrf2 upregulation by oxidative stress (Purdom-Dickinson, Sheveleva, Sun, & Chen, 2007). Purdom-Dickinson, Lin, et al. (2007) and Purdom-Dickinson, Sheveleva, et al. (2007) found that in rat cardiomyocytes, treatment with low to mild doses of H$_2$O$_2$ caused a rapid increase in endogenous Nrf2 protein levels, by a process that is independent of Nrf2 protein stabilization. The authors suggested that H$_2$O$_2$ stress can cause selective protein translation, resulting in a rapid increase of Nrf2 protein.

Here, we describe a detailed protocol, which allows exploring the role of H$_2$O$_2$, in endogenous Nrf2 translocation and synthesis, by exposing HeLa cells to a wide range of H$_2$O$_2$ concentrations, delivered either as a bolus or as a steady state.

### 2. EXPERIMENTAL CONDITIONS AND CONSIDERATIONS

**2.1. Cell culture**

HeLa cells (American Type Culture Collection, Manassas, VA, USA) grown in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μg/mL of Streptomycin, and 2 mM of L-glutamine at 37 °C and 5% (v/v) CO$_2$ should be kept at exponential phase and in monolayer growth by periodic replanting every 2–3 days.

Cell culture conditions are of paramount importance for the reproducibility of the experiments. In the preparation of the biological material for the experimental procedures described here, the following precautions should be taken:

1. Cells must be seeded at a density of 0.5 million cells in a 100-mm dish. It is important to be accurate in the cell counting and to distribute homogeneously cells in the dish.
2. Cells should be incubated for 46–48 h at 37 °C and 5% (v/v) CO$_2$. Reproducibility of the experiments is significantly affected if cells are incubated only overnight or for 24 h after seeding. At the day of the experiment, cells should show a confluence of about 60% (∼1.5 million cells in a 100-mm dish).
3. Growth media must be renewed 1 h prior to the experimental procedures, using prewarmed and CO$_2$ preequilibrated media. These conditions are to be used when delivering H$_2$O$_2$ either as bolus addition or as a steady-state addition.
2.2. Reagents

1. H$_2$O$_2$—Make fresh every day the solution using concentrated Perhydrol 30% (m/m) H$_2$O$_2$, density 1.11 g/mL, MW = 34.02, 9.79 M. To obtain the stock solution of H$_2$O$_2$ (~9–10 mM), dilute 1/1000 the concentrated H$_2$O$_2$ solution in water and confirm the concentration by reading the absorbance at 240 nm (ε = 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 mM sodium acetate, 40 mM 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.

5. Phosphate buffered saline (PBS)—1.5 mM KH$_2$PO$_4$, pH 7.4, 137 mM NaCl, 3.0 mM KCl, and 8.0 mM Na$_2$HPO$_4$.

6. Cytosolic lysis buffer—50 mM HEPES, pH 7.2, 2 mM EDTA, 10 mM NaCl, 250 mM sucrose, 2 mM DTT, 10% (v/v) Nonidet P40, and protease inhibitors (Sigma-Aldrich, Inc., St. Louis, MO, USA): 1 mM PMSF, 1.5 mg/mL benzamidine, 10μg/mL leupeptin, and 1 μg/mL pepstatin, all freshly added.

7. Nuclear lysis buffer—Identical to the cytosolic proteins buffer except that 250 mM sucrose was replaced by 20% (v/v) glycerol and NaCl was 400 mM.

8. RIPA buffer—50 mM Tris–HCl pH 7.4, 400 mM NaCl, 1% (v/v) Nonidet P-40, 0.25% (w/v) Na–deoxycholate, and protease inhibitors (Sigma-Aldrich, Inc., Saint Louis, MO, USA): 1 mM PMSF, 1.5 mg/mL benzamidine, 10μg/mL leupeptin, and 1 μg/mL pepstatin, all freshly added.

2.3. H$_2$O$_2$ measurement

H$_2$O$_2$ is followed by the formation of O$_2$ after the addition of catalase (Eq. 9.1) using an oxygen electrode, as explained in Marinho, Cyrne, Cadenas, and Antunes (2013b):

$$2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} \text{O}_2 + 2\text{H}_2\text{O}$$

[9.1]
We use a chamber oxygen electrode (Oxygraph system, Hansatech Instruments, Ltd, Norfolk, UK), with a magnetic stirrer and temperature control. All measurements are performed at room temperature and with a final volume of 800 μL. The electrode should be giving a stable baseline, which is particularly important when measuring low concentrations of H₂O₂. For that, it is recommended to add 800 μL of distilled water and to connect the stirring a few hours before the measurements.

A typical measurement is as follows:

1. Take an 800 μL aliquot from the incubation media and add to the electrode chamber.
2. 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 since they interfere in the measurement). After a new baseline is established, stop the recording. The value of the difference between the two baselines is converted to H₂O₂ concentration with the help of a calibration curve.
3. 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 also) in order to be sure to remove all the catalase before the next H₂O₂ assay.

A H₂O₂ calibration curve should be made daily as described in Marinho et al. (2013b) briefly:

1. Make H₂O₂ solutions in water with known concentrations. Keep at room temperature.
2. Add 400 μL of H₂O₂ 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 we found that with the buffer the oxygen electrode has a more stable output.
3. Measure H₂O₂ by adding catalase as explained earlier.

2.4. Protein sample preparation

In order to prevent protein denaturation and/or degradation all following procedures must be done on ice and with precooled reagents.

2.4.1 Cytosol/nucleus differential protein extraction

The differential protein extraction from the cytosol and nucleus is performed according to the method described by Roebuck, Rahman, Lakshminarayanan, Janakidevi, and Malik (1995):
1. After exposing cells to H₂O₂, the incubation media in the 100-mm plates is removed, and cells are washed twice with 1 mL of PBS.

2. Afterward lysis is promoted by addition of 300 μL of cytosolic lysis buffer and scrapping. The lysate is transferred to a cooled Eppendorf tube, and the cells remaining in the dish are collected with additional 100 μL of cytosolic lysis buffer. Note that in order to prevent variation on the total time cells are exposed to H₂O₂, the wash and breaking step must be as brief and reproducible as possible. It is not advisable to process a large number of samples simultaneously.

3. The cytosolic protein samples are obtained as the supernatant after centrifugation at 3000 × g for 4 min at 4 °C.

4. The pellet is used for extraction of nuclear proteins. It is washed by resuspending it in 300 μL of cytosolic lysis buffer and centrifuged again at 3000 × g for 4 min at 4 °C. Next, the pellets are resuspended in 30 μL of nuclear lysis buffer and allowed to incubate 20 min on ice, with vortexing every 5 min. After that, the nuclear protein samples are obtained by centrifugation at 10,000 × g for 15 min at 4 °C.

5. Samples are stored at −80 °C.

2.4.2 Total protein extraction

Total protein extracts are obtained according to the method described by Luo et al. (2004):

1. Washing and lysis are as described in the previous section, except that RIPA buffer is used instead of lysis buffer.

2. After incubating for 10 min at 4 °C with RIPA buffer, the total protein extracts are obtained from the supernatant of a centrifugation at 10,000 × g at 4 °C for 10 min.

2.4.3 Detection and protein quantification by Western blot

1. Protein samples are quantified using the Bradford method (Bradford, 1976).

2. 50 μg of sample is resolved in a 12.5% (w/v) SDS-PAGE gel and transferred to a nitrocellulose membrane by semidyry electroblotting.

3. Membranes are stained with Ponceau S red, in order to confirm protein loading, before being blocked by incubation for 1 h with a solution of 5% (w/v) lyophilized fat-free cow milk.

4. Antibody incubations are carried at room temperature. The primary antibody against Nrf2 (1/600; clone 383727 from R&D Systems,
Inc., USA) is incubated for 2 h, and the secondary antibody (1/2000; Sc2005 from Santa Cruz Biotechnology Inc., USA) is incubated for 1 h. Membranes are washed three times with a PBS solution of 0.1% (v/v) Tween-20 for 15 min after incubation with the primary antibody and three times with PBS for 15 min after incubation with the secondary antibody.

5. Immunoreactivity is detected using the ECL kit (GE Healthcare Life Sciences, USA) according to supplier’s instructions.

3. PILOT EXPERIMENTS

To implement the delivery of H$_2$O$_2$ as a steady state, two pilot experiments are needed:

1. The determination of the kinetics of H$_2$O$_2$ consumption by cells.
2. The rate of formation of H$_2$O$_2$ catalyzed by glucose oxidase under the experimental conditions to be used for the steady-state incubation.

As described in detail in this volume (Marinho, Cyrne, Cadenas, & Antunes, 2013a), to set up the steady state, the concentration of H$_2$O$_2$ added initially is maintained constant by adding a source of H$_2$O$_2$ that matches the cellular consumption of this oxidant. Glucose oxidase is an excellent candidate because it uses the glucose present in the growth media to produce H$_2$O$_2$.

3.1. Glucose oxidase activity

The determination of the rate of H$_2$O$_2$ production by glucose oxidase is done in growth media in the cell incubator:

1. Add 10 µL of glucose oxidase 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 medium.
3. Put dish in the cell incubator.
4. Take 800 µL aliquots at different times to measure H$_2$O$_2$.

A plot of H$_2$O$_2$ concentration versus time should be linear. From the slope calculate glucose oxidase activity as nanomolar of H$_2$O$_2$ produced per minute per microliter of the 1/100 glucose oxidase solution. The activity of the vial of glucose oxidase used in the experiments described here was 4.28 nmol/(min µL).
3.2. Kinetics of \( \text{H}_2\text{O}_2 \) consumption by HeLa cells

Cells are seeded as described in Section 2.1.

1. To start the experiment add 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (bolus addition) to the medium.

2. Take 800 \( \mu \text{L} \) aliquots at different times to measure \( \text{H}_2\text{O}_2 \) in the range 10–90 \( \mu \text{M} \). Initially, when consumption is faster, take aliquots every 5 min then, when consumption is slower, 10 min between aliquots is appropriate. At least six time points should be recorded. Use the calibration curve to calculate \( \text{H}_2\text{O}_2 \) concentrations and make a plot of \( \ln[\text{H}_2\text{O}_2] \text{corrected} \) versus time. A correction of the \( \text{H}_2\text{O}_2 \) concentrations is needed to take into account the decrease in the incubation volume caused by the removal of the aliquots to measure \( \text{H}_2\text{O}_2 \). The correction is described in detail in Marinho et al. (2013a) and is calculated as the ratio between the volume in which \( \text{H}_2\text{O}_2 \) consumption is measured (Vol\text{measurement}) over the initial reaction volume (Vol\text{initial}) as shown in Eq. (9.2):

\[
[\text{H}_2\text{O}_2]_{\text{corrected}} = [\text{H}_2\text{O}_2]_{\text{experimental}} \times \frac{\text{Vol}\text{measurement}}{\text{Vol}\text{initial}} \quad [9.2]
\]

The rate constant obtained from the slope of the plot of \( \ln[\text{H}_2\text{O}_2] \text{corrected} \) versus time was 0.50 \( \text{min}^{-1} \) mL/10\(^6\) cells.

4. EXPERIMENTAL H\( \text{2O}_2 \) EXPOSURE

After determining the kinetics of \( \text{H}_2\text{O}_2 \) consumption by cells and the glucose oxidase activity, a full set of \( \text{H}_2\text{O}_2 \) experiments can be carried out.

4.1. Bolus addition

When delivering \( \text{H}_2\text{O}_2 \) as a bolus addition, no pilot experiments are needed. The \( \text{H}_2\text{O}_2 \) profiles observed upon a bolus addition are strongly dependent on experimental conditions, such as the number of cells and the volume of incubation media. For all experiments, the conditions described in Section 2.1 are used for the cell culture. Indicated initial concentrations of \( \text{H}_2\text{O}_2 \) (50, 100, and 200 \( \mu \text{M} \)) are added to cells, and no further measurements to monitor \( \text{H}_2\text{O}_2 \) concentrations are done.

4.2. Steady-state method

A \( \text{H}_2\text{O}_2 \) steady state—[\( \text{H}_2\text{O}_2 \]ss)—can be obtained by adding the desired concentration of \( \text{H}_2\text{O}_2 \) simultaneously with a quantity of glucose oxidase
that counters the cellular H$_2$O$_2$ consumption by producing H$_2$O$_2$, and thereby, the concentration of this compound is kept constant throughout the test.

The rate of H$_2$O$_2$ production needed is calculated by applying Eq. (9.3), where the desired steady state, the rate constant $k$ obtained in Section 3.2, and the conditions of the assay are used:

$$V_{\text{production}} = k \times [\text{H}_2\text{O}_2]_{\text{ss}} \times \text{number of cells/reaction volume} \quad [9.3]$$

1. The $V_{\text{production}}$ obtained has units of concentration $\times$ time$^{-1}$. For example, to establish a 12.5 $\mu$M steady state in an assay with 1.5 million HeLa cells in a 100-mm dish with 10 mL of reaction volume, $V_{\text{production}}$ is: (0.50 min$^{-1}$ mL/10$^6$ cells) $\times$ 12.5 $\mu$M $\times$ 1.5 $\times$ 10$^6$ cells/10 mL = 0.938 $\mu$M/min.

2. The number of molecules of H$_2$O$_2$ produced is obtained by multiplying the value obtained in step 1 by the reaction volume: (0.938 $\mu$M/min) $\times$ 10 mL = 9.38 nmol/min.

3. By dividing the number obtained in the previous step by the activity calculated in Section 3.1, we obtain the volume in microliter of the 1/100 glucose dilution needed to obtain the desired steady state: (9.38 nmol/min)/(4.28 nmol/(min $\mu$L)) = 2.2 $\mu$L.

We need 2.2 $\mu$L of a 1/100 dilution of the original glucose oxidation. To minimize pipetting error, a 22.0 $\mu$L of a 1/1000 diluted solution would be advisable.

4.2.1 Monitoring cell exposure to H$_2$O$_2$

For long exposure times (longer than 1 h), the H$_2$O$_2$ concentration must be monitored and corrected. To this end, the H$_2$O$_2$ concentration is measured every hour by removing an 800 $\mu$L aliquot of the medium. Only assays in which the H$_2$O$_2$ concentration variation to the desired value is not higher than 20% should be accepted.

Table 9.1 depicts an example of the monitoring and corrections made for a 12.5 $\mu$M H$_2$O$_2$ steady state lasting for 6 h. After 1 h, a H$_2$O$_2$ concentration of 11.8 $\mu$M was measured. The difference from the desired concentration (12.5 $\mu$M) is (11.8–12.5)/12.5 $\times$ 100 = $-6\%$. In order to correct the H$_2$O$_2$ steady state to the desired value, glucose oxidase and H$_2$O$_2$ must be added. For that, we consider that the difference between the H$_2$O$_2$ concentration measured and the target one is directly proportional to the
correction needed. However, an additional correction is needed. Because the steady-state concentration of H$_2$O$_2$ is maintained from a balance between H$_2$O$_2$ consumption, by the cells, and H$_2$O$_2$ formation by glucose oxidase, when an aliquot is taken from the media, given that the cells are attached to the bottom of the culture dish, an unbalance is introduced by the removal of the glucose oxidase present in the aliquot. Therefore, since the H$_2$O$_2$ concentration was 6% lower than the desired one and that 8% of the initial glucose oxidase and H$_2$O$_2$ were removed with the 800 mL aliquot (i.e., 800 mL represent 8% of the original 10 mL incubation volume), we need to add 14% of the original glucose oxidase addition, that is, 3.1 mL of glucose oxidase 1/1000 dilution, plus 14% of the original H$_2$O$_2$ addition, that is, 1.9 mL of 9 mM H$_2$O$_2$, in 800 mL of incubation media to restore original volume. For practical reasons, we would add 31 mL of a 1/10,000 glucose oxidase solution and 19 mL of 900 mM H$_2$O$_2$.

For incubations of 1 h or shorter, corrections are not introduced, although H$_2$O$_2$ is monitored. Figure 9.1 shows typical H$_2$O$_2$ concentration profiles obtained either for short or long steady-state incubations.

### Table 9.1 Monitoring H$_2$O$_2$ concentration during incubation with a steady state of 12.5 μM

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>[H$_2$O$_2$] (μM)</th>
<th>Difference to desired concentration (%)</th>
<th>H$_2$O$_2$ or GO removed in aliquot (%)</th>
<th>GO and H$_2$O$_2$ correction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.8</td>
<td>−6</td>
<td>+8</td>
<td>+14</td>
</tr>
<tr>
<td>2</td>
<td>15.0</td>
<td>+20</td>
<td>+8</td>
<td>−12</td>
</tr>
<tr>
<td>3</td>
<td>15.0</td>
<td>+20</td>
<td>+8</td>
<td>−12</td>
</tr>
<tr>
<td>4</td>
<td>11.8</td>
<td>−6</td>
<td>+8</td>
<td>+14</td>
</tr>
<tr>
<td>5</td>
<td>10.1</td>
<td>−19</td>
<td>+8</td>
<td>+27</td>
</tr>
<tr>
<td>6</td>
<td>15.0</td>
<td>+20</td>
<td>+8</td>
<td>−12</td>
</tr>
</tbody>
</table>

The steady state is initiated by delivering 22 μL of a 1/1000 glucose oxidase (GO) dilution and 13.9 μL of 9 mM H$_2$O$_2$ to a 100-mm dish containing 1.5 million HeLa cells and 10 mL incubation media. Correction is calculated as: (Difference to desired concentration (%) − % of H$_2$O$_2$ or GO removed).

---

5. DATA HANDLING AND ANALYSIS

Following cell exposure to H$_2$O$_2$, Western blot data concerning activation of Nrf2 are processed:

1. The films are scanned, and the intensity of bands obtained is determined relative to the control band (protein extract from cells that were not exposed to any treatment), using the ImageJ software (Rasband, 1997).
2. In order to correct for differences in the total amount of protein applied to each well of the gel, we use as correction factor the relative intensity of staining with Ponceau S red area corresponding to each well.

3. Data are plotted according to Figs. 9.2–9.4. When delivered as a bolus addition, H₂O₂ caused, in a concentration-dependent way, an increase in the nuclear, but not in the cytosolic levels of Nrf2 within 30 min of incubation (Fig. 9.2). This is consistent with the model in which Keap1 senses H₂O₂, causing the termination of both Nrf2 ubiquitination and degradation, allowing accumulation of Nrf2 in the nucleus. The absence of variation in the cytosolic levels of Nrf2 can be explained by a balance resulting from its decreased degradation and its increased nuclear translocation. This scenario changed when H₂O₂ was delivered as a steady state. A fast significant accumulation of cytosolic Nrf2 was observed in the first 15 min, while nuclear levels only changed significantly after 2 h (Fig. 9.3). The increase in cytosolic levels was mirrored by total Nrf2 cellular levels (Fig. 9.3). These observations are consistent with the hypothesis that the exposure of HeLa cells to a 12.5 μM H₂O₂ steady state led to a triggering of Nrf2 de novo synthesis, as described by Purdom-Dickinson, Lin, et al. (2007) and Purdom-Dickinson, Sheveleva, et al. (2007).

These results are confirmed if a concentration study is done (Fig. 9.4). At 15-min time, a bolus addition within a wide range of H₂O₂ concentrations—12.5–400 μM—did not increase Nrf2 cytosolic concentration, while a nuclear accumulation of Nrf2 was observed for 50 and 100 μM H₂O₂. The increase in cytosolic Nrf2 levels was only observed for 12.5 μM H₂O₂.

![Figure 9.1](image_url)
steady state, while higher H₂O₂ steady-state concentrations did not trigger this accumulation. Conversely, the fast nuclear accumulation of Nrf2 was only observed for higher H₂O₂ steady-state concentrations (25 and 50 μM). It is important to note that a 25 μM steady-state concentration of H₂O₂ already elicits cell toxicity after 6 h (Oliveira-Marques, Cyrne, Marinho, & Antunes, 2007).

Figure 9.2 Establishment of adequate experimental conditions for the study of Nrf2 activation by H₂O₂ using a bolus addition. The indicated H₂O₂ concentrations were added as a bolus to HeLa cells and Nrf2 levels in (A) cytosol and (B) nucleus were determined by Western blot. Representative Western blot for cytosolic (C) and nuclear (D) extracts are shown. Quantification of Nrf2 protein levels was performed by signal intensity analysis using the ImageJ software and is shown in arbitrary units relative to control. Control of protein loading was performed by analysis of the membrane stained with Ponceau S red. Results are mean ± standard deviation of two to nine independent experiments. *P < 0.01 versus control using ANOVA followed by a Holm–Sidak post test.
Figure 9.3 The *de novo* synthesis of Nrf2 is triggered very rapidly by H$_2$O$_2$, while Nrf2 translocation to the nucleus is slower. HeLa cells were treated with 12.5 µM steady-state H$_2$O$_2$ for the indicated times, and Nrf2 levels were determined in cytosolic, nuclear, and total cellular extracts by Western blot. (A) Quantification of Nrf2 protein levels performed as described in Fig. 9.2. (B) Representative Western blot for total cellular extracts are shown. Results are mean ± standard deviation of 2–14 independent experiments. *P < 0.01, **P = 0.01, °P < 0.05 versus control using ANOVA followed by a Holm–Sidak post test.

Figure 9.4 Nrf2 activation by H$_2$O$_2$ is strongly dependent both on the concentrations of H$_2$O$_2$ and on the method of H$_2$O$_2$ delivery. Titration of the effect of exposing HeLa cells for 15 min to increasing H$_2$O$_2$ concentrations added either as a bolus or as a steady state on nuclear (A) and cytosolic (B) Nrf2 levels. Nrf2 levels in protein extracts were determined as described in Fig. 9.2. Results are mean ± standard deviation of two to nine independent experiments. *P < 0.01 versus control using ANOVA followed by a Holm–Sidak post test.
Overall, these results suggest that low sustained H$_2$O$_2$ concentrations trigger preferentially Nrf2 \textit{de novo} synthesis without nuclear translocation. For Nrf2 nuclear translocation to occur, an higher H$_2$O$_2$ concentration or, alternatively, cell exposure to a sustained low H$_2$O$_2$ concentration for a long period of time is needed. Fast nuclear accumulation of Nrf2 may only be triggered by H$_2$O$_2$ at high potentially toxic levels that cannot be coped by the cell for prolonged periods of time. Whether Nrf2 \textit{de novo} synthesis is blocked by high concentrations of H$_2$O$_2$ cannot be evaluated from the data presented here because an absence of Nrf2 accumulation in the cytosol at high H$_2$O$_2$ concentrations may be due to its fast translocation to the nucleus.

6. SUMMARY

This chapter describes the application of H$_2$O$_2$ steady-state incubations to the study of Nrf2 activation. H$_2$O$_2$ effects on Nrf2 activation are strongly dependent both on the H$_2$O$_2$ concentration and on the method of H$_2$O$_2$ delivery. The \textit{de novo} synthesis of Nrf2 is triggered within 5 min of exposure to low concentrations of H$_2$O$_2$, while Nrf2 translocation to the nucleus is slower. Evidence of \textit{de novo} synthesis of Nrf2 is observed only for low H$_2$O$_2$ steady-state concentrations, a condition that is prevalent \textit{in vivo}.

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