Role of Hydrogen Peroxide in NF-κB Activation: From Inducer to Modulator

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Abstract

Hydrogen peroxide (H2O2) has been implicated in the regulation of the transcription factor NF-κB, a key regulator of the inflammatory process and adaptive immunity. However, no consensus exists regarding the regulatory role played by H2O2. We discuss how the experimental methodologies used to expose cells to H2O2 produce inconsistent results that are difficult to compare, and how the steady-state titration with H2O2 emerges as an adequate tool to overcome these problems. The redox targets of H2O2 in the NF-κB pathway—from the membrane to the post-translational modifications in both NF-κB and histones in the nucleus—are described. We also review how H2O2 acts as a specific regulator at the level of the single gene, and briefly discuss the implications of this regulation for human health in the context of κB polymorphisms. In conclusion, after near 30 years of research, H2O2 emerges not as an inducer of NF-κB, but as an agent able to modulate the activation of the NF-κB pathway by other agents. This modulation is generic at the level of the whole pathway but specific at the level of the single gene. Therefore, H2O2 is a fine-tuning regulator of NF-κB-dependent processes, as exemplified by its dual regulation of inflammation. Antioxid. Redox Signal. 11, 000–000.

The Classical NF-κB Activation Pathway

In 1986, the transcription factor nuclear factor-κB (NF-κB) was discovered by Sen and Baltimore in B cells (97). NF-κB forms homo- or heterodimers composed of members of the Rel subfamily—p65/RelA, c-Rel, p52/RelB, which contain C-terminal transactivation domains (TADs)—and the NF-κB subfamily: NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (20, 33, 65). The Rel-homology domain (RHD) is present in all NF-κB/Rel proteins and is responsible for dimerization, recognition and binding to DNA, and interaction with the inhibitory proteins, IκBs (51). The IκB proteins bind to NF-κB and prevent translocation of the latter to the nucleus and its binding to DNA. The IκB family is composed of IκB-α, IκB-β, IκB-ε, IκB-γ, and BCL-3 (B-cell lymphoma 3) and by the precursors NF-κB1 p105 and NF-κB2 p100 because they all possess the typical ankyrin repeats (51). Classically, NF-κB (p50/p65) is kept latent in the cytosol bound to IκBs. The prototypical activators tumor necrosis factor α (TNF-α), lipopolysaccharide (LPS), and interleukin 1 (IL-1) activate the IκB-kinase complex (IKK complex), which is responsible for phosphorylation of IκBs at specific regulatory amino acid residues: IκB-α (Ser32 and Ser36), IκB-β (Ser19 and Ser23) and IκB-ε (Ser18 and Ser22). Consequently, the IκBs are targeted for degradation by the 26S proteasome, thereby freeing NF-κB, which translocates to the nucleus and activates the target genes.

NF-κB Activation by Hydrogen Peroxide (H2O2): A History of Contradictions

NF-κB was shown to be redox-regulated in 1990, when Herzenberg and coworkers (102) described that intracellular thiols mediate NF-κB activation by TNF-α and phorbol 12-myristate 13-acetate (PMA). By lowering the levels of glutathione (GSH, γ-glutamyl-cysteinyl-glycine), the major redox buffer in cells, TNF-α-induced NF-κB activation increased, while by using the precursor of glutathione synthesis, N-acetyl-L-cysteine (NAC), NF-κB activation decreased (102). Similarly, the antioxidant pyrrolidine dithiocarbamate (PDTC) inhibits NF-κB activation by blocking NF-κB released from IκB-α in cells treated with IL-1 and TNF-α (94). Direct evidences of regulation by hydrogen peroxide (H2O2) came from Schreck et al. (95), who demonstrated for the first time that the NF-κB pathway is activated by adding 150 μM H2O2 to a subclone of Jurkat T cells, later named Wurzburg cells. These cells were infected with the human immunodeficiency virus type 1 (HIV-1), whose expression is dependent on

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NF-κB activation, and H₂O₂ was shown to increase expression of the virus (95). Interestingly, H₂O₂ does not activate the purified complex NF-κB/IkB in vitro, which indicates that the activation in cells is due to a consequent regulation by H₂O₂, such as changes in the redox state of the cell. These works indicated a possible convergent pathway for NF-κB activation by different stimuli, which is dependent on the intracellular redox level. Moreover, since NF-κB inducers, such as TNF-α and IL-1 induce the production of reactive oxygen species (ROS) (45, 81, 96, 105), ROS were pointed out as the universal mediators of the NF-κB activation pathway. In favor of this hypothesis was the fact that there was an inhibition of NF-κB activation caused by antioxidants like NAC and PDTC, and also by overexpression of catalase (92). This idea of a redox-regulated pathway was further supported by the proposal that the redox regulation of protein tyrosine phosphorylation is the common downstream point at which the responses triggered by the different agents that stimulate NF-κB converge (3).

However, soon after, several reports began to question the universal pathway of NF-κB activation mediated by H₂O₂. First, direct activation of NF-κB by H₂O₂ does not occur in all cell types, as observed in Table 1. Second, in H₂O₂-responsive cells, NF-κB activation proceeds slowly (hour range) after H₂O₂ treatment, contrasting with the rapid response (minute range) to cytokine stimuli (Table 1) (14). Third, re-analysis of the effects of NAC and PDTC showed that they attenuate NF-κB activation independently of their antioxidant potential (37). Hayakawa et al. (37) found that NAC selectively blocks TNF-α-induced signaling by lowering TNF-α affinity to its receptor TNF-R1 and that PDTC interferes with the ubiquitin ligase activity, thereby inhibiting IκB-α degradation. Brennan et al. (11) also identified a pro-oxidant character of PDTC capable of inhibiting NF-κB activation, which is reversed by addition of 2-mercaptoethanol. Fourth, conflicting data using antioxidant enzymes appeared to argue against a universal addition of 2-mercaptoethanol. Fourth, conflicting data using direct cell exposure to H₂O₂ treatment, contrasting with the rapid response (minute range) to cytokine stimuli (Table 1) (14). Third, re-analysis of the effects of NAC and PDTC showed that they attenuate NF-κB activation independently of their antioxidant potential (37). Hayakawa et al. (37) found that NAC selectively blocks TNF-α-induced signaling by lowering TNF-α affinity to its receptor TNF-R1 and that PDTC interferes with the ubiquitin ligase activity, thereby inhibiting IκB-α degradation. Brennan et al. (11) also identified a pro-oxidant character of PDTC capable of inhibiting NF-κB activation, which is reversed by addition of 2-mercaptoethanol. Fourth, conflicting data using antioxidant enzymes appeared to argue against a universal addition of 2-mercaptoethanol. However, many contradictory data have been published about H₂O₂ participation in the NF-κB pathway, either alone (Table 1) or in the presence of classical inducers (Table 2), impairing any conclusion about the role of H₂O₂ on the NF-κB pathway. In Tables 1 and 2, relevant details of the experimental setup are described (cell type, cell number or confluence, H₂O₂ and classical NF-κB inducers concentrations, incubation time used in the assay) together with key experimental observations and the overall effect exerted by H₂O₂ on NF-κB activation.

By itself, H₂O₂ is at best a weak NF-κB activator. In some cell lines NF-κB seems insensitive to H₂O₂, while in other cell lines where H₂O₂ activates NF-κB, H₂O₂ has little impact when compared with classical inducers (e.g., TNF-α) because H₂O₂ leads to lower levels of NF-κB activation and with slower kinetics (49, 76) (Table 1). Interestingly, in works where H₂O₂ induced NF-κB activation at significant levels, an alternative activation pathway for NF-κB activation was suggested, since typical upstream kinases or IκB-α modifications were not identified. Nevertheless, in the majority of the data presented in Table 1, an H₂O₂ concentration above 100 μM was necessary to observe any significant NF-κB activation, raising doubts over the in vivo relevance of NF-κB activation by H₂O₂. In addition, in some studies H₂O₂ inhibits the constitutive NF-κB activation in primary cell lines (23, 129).

The studies presented in Table 2 have in common the use of both H₂O₂ and classical NF-κB inducers (TNF-α, IL-1, and LPS) to analyze any possible modulatory or cooperative effects. Independently of the cell type (e.g., epithelial, endothelial, muscle cells, among others), the studies presented attribute to H₂O₂ either negative or positive effects, but also no effects on cells stimulated by classical NF-κB inducers. Even for the same cell type, such as in HeLa, MCF-7, and RLE cells, both positive and negative effects are described in the literature. Also, the target of H₂O₂ that leads to the overall effect described is not the same in all studies. Therefore, the lack of consistent results is probably due in part to cell-type specific biological responses, but the chemistry of H₂O₂ and the dynamic nature of H₂O₂ metabolism also make the common experimental setups applied to study the role of H₂O₂ inappropriate. Next, we will review how the basic biology of H₂O₂ undermines its study and how this can be overcome.

**Basic Biology of H₂O₂**

**H₂O₂ chemistry**

In chemical terms, H₂O₂ is poorly reactive: it can act as a mild oxidizing or as a mild reducing agent, but it does not oxidize most biological molecules, including lipids, DNA, and proteins, except for those proteins with highly reactive sulfhydryl groups (36). Nevertheless, H₂O₂ is seen as a threat to organisms because of its interaction with transition metals, mostly reduced iron (Fe²⁺) or copper (Cu²⁺) ions, and the consequent formation of the extremely reactive HO• radical (36). In vivo, iron is mostly present bound to heme proteins—such as hemoglobin, transferrin, ferritin, and lactoferrin—and so does not react with H₂O₂, but high concentrations of H₂O₂ can induce the release of iron from some of these proteins (36). So, in general terms, regulatory roles by low levels of H₂O₂ are associated with oxidation of sulfhydryl groups and consequently with signaling, while cell damage, including cell death either by necrosis or apoptosis, induced by high levels of H₂O₂ is associated with HO• generation through the Fenton reaction:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^• + \text{HO}^- \quad \text{Eq. 1}$$

Oxidation of sulfhydryl groups is not a simple process, and different levels of oxidation are reached, depending on the magnitude of the oxidative conditions imposed to the cell. Unlike the majority of cysteine residues on proteins, reactive cysteine residues have a low pKα and are in the thiolate form (5) at physiological pH (31). These cysteine residues are targeted by H₂O₂ and their oxidation can alter the protein structure and function. The sulfhydryl group (-SH) of a single cysteine residue of a protein may be oxidized to form a sulfenic acid (-SOH), which is generally unstable and can react...
### Table 1. Effect of H₂O₂ on NF-κB Activation

<table>
<thead>
<tr>
<th>H₂O₂</th>
<th>Cell type*</th>
<th>Incubation conditions</th>
<th>Observations</th>
<th>Global role of H₂O₂</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 3 mM bolus HeLa</td>
<td>Up to 1 h</td>
<td>Slight activation of IKK within 30 min, but with no effects on IκB-α phosphorylation, degradation and p65 translocation</td>
<td>No effect 49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM bolus HeLa and Raw 264.7</td>
<td>2–16 h</td>
<td>Slight ↑ of NF-κB binding to DNA (EMSA) with no effect on IKK activity or IκB-α levels.</td>
<td>Weak activator at longer exposures, through an alternative pathway 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM bolus RASMC</td>
<td>60 min (?)</td>
<td>No effects on NF-κB activation: IκB-α levels and DNA binding</td>
<td>No effect 113</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mM bolus HUVEC</td>
<td>15–90 min</td>
<td>No effects on IκB-α levels (no degradation); ↑ p65 translocation to the nucleus; ↑ NF-κB binding to DNA (EMSA)</td>
<td>Activator through an alternative pathway 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mM bolus Mice-derived neutrophils</td>
<td>Up to 60 min</td>
<td>No effect on NF-κB activation (EMSA)</td>
<td>No effect 104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25–1 mM bolus KBM-5 Jurkat T</td>
<td>1–4 h</td>
<td>↑ activity of Syk kinase; ↑ Tyr42 phosphorylation of IκB-α; No effects on IκB-α levels (no degradation); ↑ p65 phosphorylation and translocation to the nucleus; ↑ NF-κB binding to DNA (EMSA)</td>
<td>Activator through an alternative pathway involving Syk kinase 106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JCaM1 (Syk deficient) MCF-7 H1299</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0.2–1 mM</td>
<td>Rat primary astrocytes</td>
<td>0.5–3 h</td>
<td>↑ NF-κB binding to DNA (EMSA); ↑ NF-κB-dependent reporter expression</td>
<td>Inhibitor of constitutive NF-κB activation 23</td>
<td></td>
</tr>
<tr>
<td>0.25–0.5 mM bolus Rabbit lens epithelial cells</td>
<td>1 h</td>
<td>No effects on IκB-α, IκB-β, and IκB-ε levels; ↑ NF-κB binding to DNA (EMSA)</td>
<td>Activator through an alternative pathway 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25, 0.5 mM bolus; up to 20 mU mL⁻¹ Bone marrow neutrophils</td>
<td>Up to 2 h</td>
<td>↑ Proteasome activity (chymotrypsin and trypsin-like activities); ↑ cytosolic levels of IκB-α</td>
<td>Inhibitor of constitutive NF-κB activation 129</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO (10 mU generates 3.5 nmol H₂O₂ min⁻¹ mL⁻¹)</td>
<td>MH-S</td>
<td>No effect on chymotrypsin-like activity; No effect on NF-κB activation</td>
<td>No effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3 mM bolus EL4</td>
<td>Up to 4 h</td>
<td>↑ Tyr42 phosphorylation of IκB-α; ↑ IκB-α degradation (partially proteasome independent); ↑ DNA binding (EMSA)—max at 1 h</td>
<td>Activator through an alternative pathway 93</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
with a nearby thiol, such as GSH. GSH reduces the sulfenic acid by S-glutathionylation, a particular case of S-thiolation, which consists in the formation of a mixed disulfide involving the SH group of GSH and the SH group of the oxidized protein. It is a reversible process that occurs under physiological conditions, but it is an early cellular response to oxidative stress and affects the cellular redox state (91, 109). Protein mixed disulfides are efficiently reduced by the enzyme glutaredoxin, a reaction dependent on the NADPH pool (109). The best example of regulation through sulfenic acid

<table>
<thead>
<tr>
<th>$H_2O_2$</th>
<th>Cell type*</th>
<th>Incubation conditions</th>
<th>Observations</th>
<th>Global role of $H_2O_2$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1, 0.2 mM</td>
<td>HMEC-1</td>
<td>0.5–1 h</td>
<td>No effects on p65 phosphorylation (essential for DNA transactivation); Slight ↑ of NF-κB binding to DNA (ICAM-1 promoter, EMSA); No effects on ICAM-1 expression nor reporter gene assay</td>
<td>No effect</td>
<td>116</td>
</tr>
<tr>
<td>0.2 mM bolus 5 U mL$^{-1}$ GO (~0.3 mM)</td>
<td>C10 RLE</td>
<td>5–90 min IKK activity; 5 and 15 min IxB-z levels; 15 min DNA binding; 6 h reporter expression</td>
<td>No effect</td>
<td>No effect</td>
<td>54</td>
</tr>
<tr>
<td>0.1; 0.5 mM bolus</td>
<td>NHBE BEAS-2B</td>
<td>5 min IKK activity; 30 min IxB-z levels; 1 h and 8 h for mRNA levels and protein expression</td>
<td>↑ IKK activity in a dose-dependent manner (5 min incubation); No effect on IxB-z levels nor NF-κB DNA transactivation</td>
<td>No effect</td>
<td>47</td>
</tr>
<tr>
<td>0.1, 0.2 mM bolus</td>
<td>RLE</td>
<td>2–24 h</td>
<td>↑ activity of Ras and MEKK1; No effect on IxB-z levels; Slight ↑ on NF-κB-dependent reporter expression (8 and 16 h incubation)</td>
<td>Weak activator through an alternative pathway involving Ras</td>
<td>46</td>
</tr>
<tr>
<td>0.05, 0.1 and 0.2 mM bolus</td>
<td>SMC from human colon</td>
<td>Up to 1 h</td>
<td>No effects of IxB-z levels, but increased degradation of IxB-B; ↑ p65, p50 and c-Rel translocation to the nucleus; ↑ NF-κB binding to DNA (EMSA)</td>
<td>Activator, IxB-β-dependent</td>
<td>100</td>
</tr>
<tr>
<td>Up to 0.1 mM bolus</td>
<td>HeLa</td>
<td>4 h</td>
<td>No effect on NF-κB-dependent gene expression (IL-8)</td>
<td>No effect</td>
<td>29</td>
</tr>
<tr>
<td>25 μM s.s. 1 mM bolus</td>
<td>MCF-7</td>
<td>Up to 1 h (bolus) and 4 h (s.s.)</td>
<td>Slight ↑ of nuclear p65 levels</td>
<td>Weak activator, at longer exposures</td>
<td>76</td>
</tr>
<tr>
<td>40 mU mL$^{-1}$ GO (50–100 μM)</td>
<td>HLEC</td>
<td>GO for 4 h</td>
<td>No effects on NF-κB activation: IxB-z levels and DNA binding</td>
<td>No effect</td>
<td>123</td>
</tr>
</tbody>
</table>

*Cell type abbreviations: BEAS-2B, bronchoepithelial cell line; C10, spontaneously transformed alveolar type II cells; EL4, mouse T lymphocytic cell line; HeLa, human cervix adenocarcinoma epithelial cells; HLEC, human lens epithelial cells; HMEC-1, human dermal microvascular endothelial cells; HUVEC, human umbilical veins endothelial cells; JCaM1, p56$^{lck}$ and p72$^{sk}$-deficient T cells; Jurkat T-cells, human leukemia T-lymphocyte; KBM-5, leukemia cell line phenotypically myeloid with monocytic differentiation; MCF-7, human mammary gland adenocarcinoma epithelial cells; MH-S, mouse alveolar macrophages; RASMC, rat aortic smooth muscle cells; Raw cells, 264.7 macrophages; RLE, rat alveolar type II epithelial; SMC, smooth muscle cells.
<table>
<thead>
<tr>
<th>Classical inducer</th>
<th>( H_2O_2 )</th>
<th>Incubation conditions</th>
<th>Cell type</th>
<th>Cell number/confluence</th>
<th>IKK activation</th>
<th>IkB-( \alpha ) degradation</th>
<th>NF-( \kappa B )-dependent gene expression</th>
<th>Other</th>
<th>Overall effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ng mL(^{-1}) TNF-( \alpha )</td>
<td>2–5 ( \mu M ) bolus</td>
<td>0–60 min</td>
<td>RBEC</td>
<td>Confluent</td>
<td>N.E.</td>
<td>↓</td>
<td>↓</td>
<td>DNA transactivation</td>
<td>↓</td>
<td>Negative</td>
</tr>
<tr>
<td>0.1 mM bolus</td>
<td>Pre-exposure to ( H_2O_2 ) for 3 h + TNF-( \alpha ) for 4 h</td>
<td>Jurkat T</td>
<td>( 10^6 ) cell mL(^{-1})</td>
<td>↓</td>
<td>↓</td>
<td>Sustained degradation of IkB-( \alpha )</td>
<td>Positive</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM bolus</td>
<td>Simultaneous addition for 4 h</td>
<td>Jurkat T</td>
<td>( 10^6 ) cell mL(^{-1})</td>
<td>↑</td>
<td>↑</td>
<td>Sustained degradation of IkB-( \alpha )</td>
<td>Positive</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1–3 mM bolus</td>
<td>Pre-exposure to ( H_2O_2 ) for 10 min + TNF-( \alpha ) (IKK, 5 min), (IkB-( \alpha ) and DNA binding, 15 min), (reporter expression, 4 h)</td>
<td>HeLa and Raw 264.7 (transfected cells)</td>
<td>( 2\times10^5 ) cells/well (12-well plate); ( 3\times10^6 ) cells/100-mm dish(^6)</td>
<td>↓</td>
<td>↓</td>
<td>Sustained IKK activation for longer times (P of Ser in the activation loops); DNA transactivation</td>
<td>Positive</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Up to 3 mM bolus</td>
<td>10, 20, 30 min for IKK activity; 60 min for NF-( \kappa B ) binding to DNA</td>
<td>HeLa</td>
<td>Transfected cells: ( 5\times10^6 ) cells/35-mm dishes</td>
<td>↑</td>
<td>↑</td>
<td>Cezanne activity and prolongs poly-ubiquitination and thus activation of RIP1; p65 nuclear export (prolongs nuclear localization)</td>
<td>Positive</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM bolus</td>
<td>10, 20, 30 min for IKK activity; 60 min for NF-( \kappa B ) binding to DNA</td>
<td>HeLa</td>
<td>Transfected cells: ( 5\times10^5 ) cells/35-mm dishes</td>
<td>↑</td>
<td>↑</td>
<td>Cezanne activity and prolongs poly-ubiquitination and thus activation of RIP1; p65 nuclear export (prolongs nuclear localization)</td>
<td>Positive</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng mL(^{-1}) TNF-( \alpha )</td>
<td>0.1 mM bolus</td>
<td>8 h</td>
<td>RLE</td>
<td>Subconfluent(^6)</td>
<td>↑</td>
<td>↑</td>
<td>Cezanne activity and prolongs poly-ubiquitination and thus activation of RIP1; p65 nuclear export (prolongs nuclear localization)</td>
<td>Negative – Cys179 of IKK( \beta ) is probably oxidized</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>0.1, 0.2 ( \mu M ) bolus</td>
<td>Up to 2 h for p65 and IkB-( \alpha ) levels; 4 h for gene expression</td>
<td>HeLa A549</td>
<td>80% Confluent(^6)</td>
<td>↑</td>
<td>↑</td>
<td>Cezanne activity and prolongs poly-ubiquitination and thus activation of RIP1; p65 nuclear export (prolongs nuclear localization)</td>
<td>Positive</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1–3 mM bolus</td>
<td>Pre-exposure to ( H_2O_2 ) for 10 min + TNF-( \alpha ) (IKK, 5 min), (IkB-( \alpha ) and DNA binding, 15 min), (reporter expression, 4 h)</td>
<td>HeLa and Raw 264.7 (transfected cells)</td>
<td>( 2\times10^5 ) cells/well (12-well plate); ( 3\times10^6 ) cells/100-mm dish(^6)</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td>Negative</td>
<td>15</td>
<td></td>
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</table>

(Continued)
<table>
<thead>
<tr>
<th>Classical inducer</th>
<th>( H_2O_2 )</th>
<th>Incubation conditions*</th>
<th>Cell type</th>
<th>Cell number/ confluence</th>
<th>IKK activation</th>
<th>IκB- ( \alpha ) degradation</th>
<th>NF-κB translocation</th>
<th>Other</th>
<th>Overall effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1; 0.5 mM bolus</td>
<td>5 min IKK activity; 30 min IκB- ( \alpha ) levels; 1 h and 8 h for mRNA levels and protein expression</td>
<td>NHBE BEAS-2B</td>
<td>90-100% confluence</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>DNA transactivation</td>
<td>Negative – probably at the proteasome level</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>0.2 mM bolus 5 U mL(^{-1}) GO (( \sim 0.5 ) mM)</td>
<td>5 min IKK activity; 5 and 15 min IκB- ( \alpha ) levels; 15 min DNA binding (1 ng mL(^{-1}) TNF-( \alpha ); 6 h reporter expression</td>
<td>C10 RLE</td>
<td>70-90% confluence</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>DNA Binding</td>
<td>Negative – oxidation of cysteine residues of IKK subunits</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>0.3 and 0.9 mM bolus</td>
<td>Pre-exposure to ( H_2O_2 ) for 16 h + TNF-( \alpha ) 10 min</td>
<td>HL-60</td>
<td>0.5-1.0×10(^6) cells mL(^{-1}) (0.1% FBS)</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>Cleavage of TNFRI; RIP levels</td>
<td>Negative</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>20 mU mL(^{-1}) GO (30-50 ( \mu )M ( H_2O_2 ); 40 mU mL(^{-1}) GO (50-100 ( \mu )M)</td>
<td>Pre-exposure to GO for 4 h + TNF-( \alpha ) up to 30 min</td>
<td>HLEC</td>
<td>Confluent; serum-, pyruvate- and phenol red-free medium, supplemented with D-glucose</td>
<td>↓</td>
<td></td>
<td>↓</td>
<td>Cleavage of TNFRI; RIP levels</td>
<td>Negative</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>8.5 ng mL(^{-1}) (2 nM)</td>
<td>Up to 60 min</td>
<td>Mice-derived neutrophils</td>
<td>4×10(^6) cells mL(^{-1})</td>
<td>N.E.</td>
<td>↓</td>
<td>↓</td>
<td>Cleavage of TNFRI; RIP levels</td>
<td>Negative</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>2.5 ng mL(^{-1}) (1 mM)</td>
<td>Pre-exposure to ( H_2O_2 ) for 5 min + TNF-( \alpha ) for 30 min (IκB- ( \alpha ) and NF-κB levels) or 4 h (gene expression)</td>
<td>HUVEC</td>
<td>Confluent</td>
<td>↓</td>
<td></td>
<td></td>
<td>Negative</td>
<td>128</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.37 ng mL(^{-1}) (25 ( \mu )M s.s.)</td>
<td>Simultaneous bolus + TNF-( \alpha ) up to 1 h; Pre-exposure to ( H_2O_2 ) s.s. for 3 h + TNF-( \alpha ) up to 2 h</td>
<td>MCF-7 HeLa</td>
<td>MCF-7: 1.8×10(^6) cells/100-mm dish; HeLa: 1.5×10(^6) cells/100-mm dish</td>
<td>↓</td>
<td></td>
<td></td>
<td>Negative</td>
<td>76</td>
<td></td>
<td></td>
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</table>

Table 2. (Continued)
<table>
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<tr>
<th>Simultaneous s.s. + TNF-α up to 4 h</th>
<th>MCF-7</th>
<th>MCF-7: 1.8×10^6 cells/100-mm dish</th>
<th>HeLa</th>
<th>HeLa: 1.5×10^6 cells/100-mm dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ng mL⁻¹ IL-1β bolus</td>
<td>0.1, 0.2 mM bolus</td>
<td>Up to 2 h for p65 and IκB-α levels; 4 h for gene expression</td>
<td>HeLa</td>
<td>A549</td>
</tr>
<tr>
<td>10 ng mL⁻¹ IL-1β</td>
<td>0.2 mM bolus</td>
<td>5 min IKK activity; 5 and 15 min IκB-α levels; 15 min DNA binding; 6 h reporter expression</td>
<td>C10 RLE</td>
<td>70–90% confluence. phenol red-free DMEM/F12 with 0.5% FBS</td>
</tr>
<tr>
<td>100 g mL⁻¹ LPS</td>
<td>0.3–1 mM bolus</td>
<td>Pre-exposure 30 min to H₂O₂ + 30 min (7) with LPS</td>
<td>RASMC</td>
<td>No information</td>
</tr>
<tr>
<td>1 μg mL⁻¹ LPS</td>
<td>1.9–500 μM bolus</td>
<td>Up to 60 min</td>
<td>Bone marrow neutrophils</td>
<td>N.E.</td>
</tr>
</tbody>
</table>

MH-S | N.E. |

Positive 76

Positive 29

Positive 54

Negative – probably upstream IKK IκB translocation for [H₂O₂] < 15 μM Proteasome activity (chymotrypsin and trypsin-like activities); cytosolic levels of IκB-α; nuclear accumulation of p65

Negative 104

Negative 129

*Unless otherwise specified, the incubation time column refers to the simultaneous addition of H₂O₂ and the classical inducer. **A549, human alveolar basal epithelial cells; HL-60, human promyelocytic leukemia cells; NHBE, primary normal human bronchial epithelial cells; RBEC, rat brain capillary endothelial cells; (for other cell type abbreviations, see Table 1). **Probable primary target of H₂O₂ action is in bold face. N.E, No effect; ↓, inhibitory effect; ↑, stimulatory effect. **Information provided by the authors.
formation is the inhibition of protein-tyrosine phosphatases (PTPs) by H2O2, which results in increased levels of phosphorylated proteins at tyrosine residues (61). In eukaryotes, PTPs have a central role in controlling signaling events initiated in response to many stimuli, including growth factors and cytokines (119). At higher H2O2 concentrations, sulfenic groups are further oxidized to sulfinic (-SO2H) and sulfonic (-SO3H) acids. Sulfinic and sulfonic acids were viewed as irreversible protein modifications until the discovery of sulfiredoxin, first in Saccharomyces cerevisiae, which is able to reduce the cysteine-sulfinic acid in peroxiredoxin (Prx) (10, 19).

If there are two (or more) cysteine residues within the same protein, they may be oxidized by H2O2 and form intramolecular disulfide bond(s), which can alter the conformation of the protein and consequently its function (109). GSH and Trx are able to directly reduce the disulfide bonds through their reversible oxidation.

Therefore, depending on the H2O2 concentration applied, different levels of sulhydryl oxidation of reactive cysteine residues are attained, leading to different conformational changes of the targeted proteins, with consequences to their biological activity. As the H2O2 concentration increases, hydroxyl radical production also increases and damaging effects ensue. Figure 1 represents the multifunctionality of H2O2. For example, to study signaling pathways low to moderate concentrations are required, whereas for adaptation studies higher concentrations are needed and, finally, with further increases in concentration, H2O2 activates death pathways, such as apoptosis. Importantly, the biological effects of H2O2 can change dramatically in a narrow range of concentrations: for example, in Jurkat T-cells intracellular concentrations below 0.7 μM are regulatory, between 0.7 and 3 μM induce apoptosis, and higher than 3 μM induce necrosis (6). In conclusion, the concentration of H2O2 during experiments should be rigorously controlled in order to obtain reproducible results.

**H2O2 dynamic metabolism**

H2O2 metabolism is highly dynamic as a result of its continuous production, both intracellularly and extracellularly, and its constant removal by several enzymatic systems that can be found in virtually all aerobic cells. The half-life of H2O2 is in the ms range and inside the cells H2O2 exists in a steady-state level between 10−9 and 10−7 M (17), while in an inflammatory situation a steady-state of 15 μM can be reached (66, 108). Next, both H2O2 production and removal systems are briefly described.

**H2O2 sources.** The biological sources of H2O2 are diverse, however it is mostly produced from the dismutation of O2− by superoxide dismutases (SODs) (127). O2− in turn is produced in different subcellular localizations, such as mitochondria (electron transport, cytochrome P450), endoplasmic reticulum (cytochrome P450), cytosol (xanthine oxidase), and plasma membrane (NADPH oxidase) (17). Oxidases, with the exception of cytochrome c oxidase, are able to produce directly H2O2, for example, oxidases present in the peroxisomes (D-amino-acid oxidase, L-α-hydroxyacid oxidase, fatty acyl-CoA oxidase, and urate oxidase) (17), sulhydryl oxidases present in the endoplasmic reticulum (110), and mono or polyamine oxidases, present in the outer membrane of the mitochondria and cytosol, respectively (112). O2− and H2O2 are mostly formed as side products of normal metabolic reactions. The first enzyme identified as producing deliberately ROS was the phagocyte NADPH oxidase present in the plasma membrane of the cell.

**FIG. 1. Metabolic processes dependent on H2O2 concentration.** The intracellular function of H2O2 is determined by its concentration. Concentration is roughly divided in high (μM range) to low (nM to mM range).

**FIG. 2. Biological sources and sinks of H2O2.** H2O2 is mostly formed through dismutation of O2− by superoxide dismutases (SODs), present in mitochondria (MnSOD), cytosol (CuZnSOD) and extracellularly. O2− is in turn produced during normal metabolic processes, such as respiration in the mitochondrial electron transport chain, and also by the activity of enzymes such as cytochrome P450 (endoplasmic reticulum, mitochondria), xanthine oxidase (cytosol), and NADPH oxidase (plasma membrane). Direct H2O2 formation occurs inside peroxisomes, but also in the endoplasmic reticulum, where sulphydryl oxidases insert disulfide bonds during protein folding. H2O2 intracellular level is tightly controlled by the enzymes catalase, GPXs and Prxs. H2O2 reduction catalyzed by GPX and Prx is dependent on GSH and reduced Trx, respectively, which in turn are kept in the reduced form through reactions catalyzed by the NADPH-dependent enzymes GSSG reductase and Trx reductase. ER, endoplasmic reticulum.
NF-κB ACTIVATION BY HYDROGEN PEROXIDE

NF-κB activation is considered a crucial point of the inflammatory process. Exposure of phagocytes to microorganisms or inflammatory mediators results in the activation of NADPH oxidase, in which extracellular O₂ is reduced at the expense of intracellular NADPH, resulting in the production of O₂⁻¹ (60). After the integration of the pathogen by the membrane and the formation of the phagosome, phagocyte granules containing CuZn-SOD and myeloperoxidase will fuse and contribute for the formation of the highly toxic hypochlorous acid and HOCl, constituting a line of defense against pathogens (52, 60). Importantly, part of the H₂O₂ formed diffuses out of the phagosome into the extracellular compartment (108). This leakage was first interpreted as a local damaging process, contributing for tissue inflammation. However, the discovery of analogous subunits to the phagocyte NADPH oxidase in nonimmune cells has linked ROS, and especially H₂O₂, with signaling processes (119). Importantly, endothelial and epithelial cells present in the inflammatory site will be subject to inflammatory mediators, such as cytokines, but also to H₂O₂, where NF-κB activation is considered a crucial point of the inflammatory process.

H₂O₂ sinks. In order to avoid oxidative stress and because of the diverse biological sources of H₂O₂, cells are equipped with efficient enzymes that catalyze the reduction of excess H₂O₂. The first enzyme discovered to have the capacity to decompose H₂O₂ was catalase. Catalase is present in virtually all cells, and in mammalian cells is mainly localized in peroxisomes (7, 17) (Fig. 2). Gordon C. Mills discovered the classical GPx (GPx1) in 1957, as an enzyme able to prevent the oxidation of hemoglobin by H₂O₂ (73). GPx catalyzes the reduction by GSH of H₂O₂ or organic hydroperoxides to H₂O or alcohols, respectively (85) (Fig. 2). Apart from the classical GPx, there are three other major GPx isoenzymes: gastrointestinal GPx (GPx2), plasma GPx (GPx3), and phospholipid hydroperoxide GPx (GPx4) (12). GPx1 and GPx4 are distributed in the cytosol, the mitochondrial matrix, endoplasmic reticulum, and nucleus (12).

Catalase and GPx are the “classical” enzymes for H₂O₂ detoxification, but a new class of enzymes, the Prxs, may also contribute for the total removal of H₂O₂. Prx have a reaction mechanism different from that of GPx (Fig. 2). While GSH is a substrate for GPx, a typical Prx contains two reduced cysteine residues in its active center, which are responsible for hydroperoxide reduction. The intramolecular disulfide formed is then reduced by thiols, such as thioredoxin (Trx). Prx are primarily located in the cytosol, but can also be found in mitochondria, peroxisomes, and in the nucleus. Six isoforms have already been identified in mammalian cells (122).

In conclusion, because cells are equipped with an array of enzymes that efficiently remove H₂O₂, when in experiments cells are exposed to H₂O₂ this metabolite is rapidly consumed, which makes it difficult to know for how long and to which levels of H₂O₂ cells are exposed. When comparing experiments from different laboratories, in which cells are probably exposed to different H₂O₂ concentrations, it is likely that a variety of cellular responses is observed, because H₂O₂ elicits a variety of cellular responses in a narrow range of concentrations. This justifies the lack of consistent results regarding NF-κB activation by H₂O₂, as seen in Table 2. Next we will discuss how experiments can be set up in order to overcome these obstacles.

Methodology of H₂O₂ Delivery

Figure 3 illustrates three possible methodologies of H₂O₂ delivery to cells. A bolus addition (curve 1) of H₂O₂ consists of a single addition of H₂O₂ at the beginning of the experiment and represents the most commonly used method to expose culture cells to H₂O₂. This addition of H₂O₂ does not result in a constant exogenous H₂O₂ level during the course of an experiment because of its cellular consumption catalyzed by the...
addition also lacks calibration, because for the same initial concentration of H\textsubscript{2}O\textsubscript{2} delivery (Table 2). Probably the accumulation of oxidant stress is a possible negative modulator of NF-κB activation is observed, independently of the method of H\textsubscript{2}O\textsubscript{2} delivery (Table 2). Probably the accumulation of oxidant stress is a possible negative modulator of NF-κB activation is observed, independently of the method of H\textsubscript{2}O\textsubscript{2} delivery (Table 2). Probably the accumulation of oxidant stress is a possible negative modulator of NF-κB activation is observed, independently of the method of H\textsubscript{2}O\textsubscript{2} delivery (Table 2). Probably the accumulation of oxidant stress is a possible negative modulator of NF-κB activation.

Moreover, the bolus addition is not a controlled method, because during the assay the concentration of H\textsubscript{2}O\textsubscript{2} is not monitored and not adjusted to the desired levels. The bolus addition also lacks calibration, because for the same initial H\textsubscript{2}O\textsubscript{2} dose, cells are effectively subjected to different concentrations of H\textsubscript{2}O\textsubscript{2} depending on the specific conditions of the assay. For example, in a study describing an exposure to 100 μM H\textsubscript{2}O\textsubscript{2} for 6 h, in reality it should be noted that 100 μM is the initial concentration. Depending on the cell type and density (Fig. 3), H\textsubscript{2}O\textsubscript{2} will be consumed at different rates and, often, after just 1–2 h assay cells are no longer exposed to any H\textsubscript{2}O\textsubscript{2} as all H\textsubscript{2}O\textsubscript{2} is already consumed. Another example of how a bad experimental design leads to inconsistent results is caused by the volume of growth medium used when working with attached cells. If the incubation volume on top of the attached cells is, for example 10 mL, the initial concentration of 100 μM H\textsubscript{2}O\textsubscript{2} corresponds to 1 μmol, while if the volume is 5 mL, the same initial concentration corresponds to 0.5 μmol of H\textsubscript{2}O\textsubscript{2}. At the end of the incubation period, if all H\textsubscript{2}O\textsubscript{2} is consumed, cells will be subjected to different levels of oxidative stress. In practice, this question is particularly relevant when using different culture dishes. For example, a 100 μM H\textsubscript{2}O\textsubscript{2} concentration given in a total volume of 100 μL growth medium in a well of a 96-well plate (growth area 0.35 cm\textsuperscript{2}), corresponds to 0.01 μmol H\textsubscript{2}O\textsubscript{2}/0.35 cm\textsuperscript{2} = 0.0286 μmol H\textsubscript{2}O\textsubscript{2}/cm\textsuperscript{2}. This is 60 % higher (on a cell basis) than a 100 μM H\textsubscript{2}O\textsubscript{2} concentration given in 10 mL growth medium in a 100 mm-diameter (growth area 56 cm\textsuperscript{2}) culture dish, which corresponds to 1 μmol H\textsubscript{2}O\textsubscript{2}/56 cm\textsuperscript{2} = 0.0179 μmol H\textsubscript{2}O\textsubscript{2}/cm\textsuperscript{2} (assuming cells are seeded at the same density in both dishes). To have the same H\textsubscript{2}O\textsubscript{2} concentration delivered on a cell basis, we would need to perform the experiment in the 96-well plate with a concentration of 100 μM H\textsubscript{2}O\textsubscript{2} delivered in 62.5 μL of medium or, alternatively, perform the experiment in the 100-mm culture dish with 100 μM H\textsubscript{2}O\textsubscript{2} delivered in 16 mL.

To overcome these various difficulties, in our laboratory, H\textsubscript{2}O\textsubscript{2} is delivered to cells using the steady-state (s.s.) titration (Fig. 3, curve 2). In the s.s. titration, at the beginning of the experiment, the desired H\textsubscript{2}O\textsubscript{2} dose is added to cells, simultaneously with the enzyme glucose oxidase (GO). The activity of added GO will produce H\textsubscript{2}O\textsubscript{2} at the same rate that it is being consumed by both the cells and the incubation medium in the specific conditions of the assay, which include the cell density, the small day to day differences in cell behavior and the type of incubation medium used. The outcome is that cells are exposed to a steady-state concentration of H\textsubscript{2}O\textsubscript{2} until the end of the experiment, and that this exposure is calibrated on a daily basis. This is represented in Fig. 3, where a different known cell density is not affecting the s.s. level of H\textsubscript{2}O\textsubscript{2} because the activity of GO is initially adjusted to compensate for that fact. Likewise, the H\textsubscript{2}O\textsubscript{2} steady-state obtained is not affected by the volume of growth medium. In addition, the concentration of H\textsubscript{2}O\textsubscript{2} is monitored during the assay, by measuring with an O\textsubscript{2} electrode O\textsubscript{2} production in aliquots taken from the incubation medium, after subjecting them to an excess of catalase (5). Any necessary adjustments in GO activity are made assuring that the concentration of H\textsubscript{2}O\textsubscript{2} is the same during the whole period of the experiment. Experiments in which the H\textsubscript{2}O\textsubscript{2} concentration measured deviates significantly (more than 20%) from the desired concentration, a situation which occurs rarely, are discarded. A potential disadvantage of the s.s. titration technique is that each assay requires additional measurements, but in our hands this is compensated by the higher reproducibility of the experiments, and therefore a lower number of experiments is needed to attain statistically significant results.

It is important to note that the s.s. titration differs from the simple addition of GO to cells (Fig. 3, curve 3). Besides the bolus addition method, the addition of GO is also a common method (54, 129), characterized by a gradual increase in H\textsubscript{2}O\textsubscript{2} concentration. Since the desired H\textsubscript{2}O\textsubscript{2} concentration is not obtained immediately, this method is not useful for experiments involving short exposures. Moreover, just like the bolus addition method, the GO method represents an uncalibrated and uncontrolled way of delivering H\textsubscript{2}O\textsubscript{2} to cells, because the activity of the enzyme is not adjusted to the specific experimental conditions. Different H\textsubscript{2}O\textsubscript{2} concentration profiles are obtained for different cell densities (76) and, once again, the real H\textsubscript{2}O\textsubscript{2} concentration that induces the observed effects is not known, because it is not monitored during the assay.

The discovery of NADPH oxidase isoforms in nonimmune cells brought a new methodology for ROS signaling studies. Typical NF-κB inducers, such as IL-1, TNF-α, and LPS, have been pointed to induce the production of O\textsubscript{2}\textsuperscript{•−}, and consequently H\textsubscript{2}O\textsubscript{2}, probably through interaction of their receptors with NADPH oxidase components (22, 64, 81), although mitochondria may also be a source of H\textsubscript{2}O\textsubscript{2} (96), thereby linking H\textsubscript{2}O\textsubscript{2} with NF-κB activation. These recent mechanisms of ROS involvement on NF-κB activation are inducer- and cell type-dependent (24). Moreover, there is a delay between the exposure to the NF-κB-inducer and the significant production of ROS and, therefore, long exposure periods are required. Also, often nonphysiological levels of the inducers are needed, which may be problematic as discussed in the next section. Nevertheless, this technique has the advantage of an endogenous production of H\textsubscript{2}O\textsubscript{2}, although it is difficult to know the H\textsubscript{2}O\textsubscript{2} concentration during the assay. Extracellular levels of H\textsubscript{2}O\textsubscript{2} produced by NADPH oxidase can be measured using the horseradish peroxidase (HRP)-linked assay, where in the presence of H\textsubscript{2}O\textsubscript{2}, a probe is oxidized by HRP and fluorescence is monitored (107). The accuracy of H\textsubscript{2}O\textsubscript{2} quantification is limited by: (a) competition for H\textsubscript{2}O\textsubscript{2} by other enzymes, such as catalase; (b) other biological substrates of HRP (thiol compounds); and (c) quenching of fluorescent signals by cell and tissue components (107). Intracellular ROS levels, including H\textsubscript{2}O\textsubscript{2} generated from O\textsubscript{2}\textsuperscript{•−}, are usually measured using the probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) that enters and stays trapped into cells, and becomes fluorescent when it is oxidized to DCF. This method is not specific for H\textsubscript{2}O\textsubscript{2} and it depends on unknown endogenous antioxidants enzymes (mainly GPx and catalase). This cellular consumption requires the use of high initial doses of H\textsubscript{2}O\textsubscript{2} (typically 100 μM–1 mM), which may affect the redox homeostasis of cells and cause oxidative stress. Excessive oxidative stress is a possible negative modulator of NF-κB activation as seen in Table 2. While the simultaneous addition of H\textsubscript{2}O\textsubscript{2} and a classical inducer (e.g., TNF-α) leads to a variety of effects, in all studies where a pretreatment of H\textsubscript{2}O\textsubscript{2} was performed before the addition of the inducer, a negative effect in NF-κB activation is observed, independently of the method of H\textsubscript{2}O\textsubscript{2} delivery (Table 2).
NF-κB ACTIVATION BY HYDROGEN PEROXIDE

peroxidases that catalyze the oxidation of the probe (32). Recently, a genetic fluorescent probe specific for H$_2$O$_2$ (HyPer) that solves many of these problems was developed, representing a promising tool to investigate the effects of H$_2$O$_2$ in the cell. Cells are transfected and express a protein containing the regulatory domain of the OxyR transcription factor, which is sensitive to H$_2$O$_2$, linked to a circularly permuted fluorescence protein. Oxidation of OxyR by H$_2$O$_2$ leads to a conformational change of the protein, and consequently to the emission of fluorescence. It is both a sensitive and a specific method for H$_2$O$_2$ (8).

Whatever the strategy used to delivery H$_2$O$_2$, in order to study signaling pathways, such as the NF-κB pathway, it is imperative to use a controlled and calibrated method of exposure to H$_2$O$_2$.

How Much TNF-α or IL-1?

TNF-α is one of the first cytokines produced by activated macrophages at the beginning of inflammation, promoting the spread of the inflammatory response, but subsequently it also helps the resolution of inflammation (114). TNF-α is thus commonly used to study NF-κB activation, often at concentrations above 10 ng mL$^{-1}$ (15, 47, 49, 54), which is far higher than the in vitro concentrations (less than 1.5 ng mL$^{-1}$ (2)). In fact, only in acute episodes of septic shock can TNF-α serum levels attain a level of 3 ng mL$^{-1}$ (26). These are overall concentrations, but it is possible that local TNF-α concentrations reach much higher values, particularly in the periphery of activated macrophages. Nevertheless, even in studies in vitro with activated monocytes with LPS, TNF-α production and secretion does not attain 10 ng mL$^{-1}$: in human monocytes (10$^6$ cells mL$^{-1}$) a maximal concentration of 1.2 ng mL$^{-1}$ of TNF-α after 8 h of incubation is measured (57) and in murine peritoneal macrophages (10$^6$ cells mL$^{-1}$) this level is about 0.2 ng mL$^{-1}$ (24 h incubation) (115). TNF-α concentrations used in experimental designs should be chosen in order to avoid an excessive or unphysiological ROS generation by NADPH oxidase. In the human monocyte-like histioytic lymphoma cell line, 5 ng mL$^{-1}$ of TNF-α does not induce ROS production, while 15 ng mL$^{-1}$ generates a significant increase of ROS (45), indicating that a physiological TNF-α concentration either does not trigger ROS production or does it at low rates.

Figure 4 shows how NF-κB translocation to the nucleus and the expression of a NF-κB-reporter plasmid both respond to different concentrations of TNF-α, alone or together with H$_2$O$_2$. Both NF-κB translocation and the reporter gene expression increase with the concentration of TNF-α, but the simultaneous addition of a constant H$_2$O$_2$ dose leads to divergent results. For NF-κB translocation to the nucleus, a synergism that is more significant for lower and physiological TNF-α concentrations is observed. Similarly, there is a synergism in the expression of the reporter gene, but for higher TNF-α doses, an antagonism is observed instead. These observations indicate that results obtained with high TNF-α concentration may be unrelated, or even opposed, to results obtained with low concentrations of TNF-α.

IL-1 is also an important mediator of the inflammatory process and it is usually used at doses between 1–10 ng mL$^{-1}$ (63, 64, 79, 101), which are higher than the IL-1 levels detected in serum from patients with multiple myeloma (0.014 ng mL$^{-1}$) (55), Lyme arthritis (0.014 ng mL$^{-1}$) (83), or measured from murine peritoneal macrophages (10$^6$ cells) stimulated with LPS (0.014 ng mL$^{-1}$) (115). Nevertheless, in a study with LPS-activated human monocytes, IL-1 attained a maximum of 3 ng mL$^{-1}$ after 4 h incubation (57).

NF-κB Regulation by H$_2$O$_2$

The regulation of NF-κB by H$_2$O$_2$ is probably mostly done by modulating the action of cytokines. This modulation is biologically relevant because during inflammation there is simultaneously a high H$_2$O$_2$ production by phagocytes together with pro-inflammatory cytokines, such as TNF-α and IL-1. Nevertheless, whether H$_2$O$_2$ stimulates or inhibits the NF-κB activation pathway and what species within this pathway are subjected to H$_2$O$_2$-regulation remains to be elucidated (Table 2). Next we review the effect of H$_2$O$_2$ in each component of the NF-κB pathway. Unless otherwise referred, the exogenous additions of H$_2$O$_2$ described were made using bolus additions.

Receptors

TNF-α exerts its biological activity through binding to its cellular receptors, the ubiquitously expressed TNF-R1 and
F5

Lipid rafts are thought to be involved in recruiting the machinery of NF-κB activation in the plasma membrane, and represent a potential target for H2O2 control. In effector T cells, most of the molecular machinery of NF-κB activation, including the adaptor proteins RIP1, TRAF2, and TRAF6, the members of the IKK complex, IKKα, IKKβ, and IKKγ, and the upstream NF-κB regulators protein kinase C (PKC) and caspase recruitment domain family 11 (CARD11), mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1), and Bcl-10 were isolated in the lipid raft fraction (74). Whether H2O2 regulates NF-κB activation at this level is unknown but recent evidences suggests that H2O2 may control lipid raft formation: (a) raft-disrupting agents block the H2O2-induced pro-survival pathway that is dependent on Akt and ERK1/2 phosphorylation in bovine aortic endothelial cells (125); (b) ROS modulate the formation of lipid rafts in T lymphocytes during the immune response (68); (c) in mouse embryonic fibroblasts, H2O2 (500 μM) induces clustering of GM1, a protein marker of lipid rafts, and co-localization of GM1 with RIP, as well as formation of a complex between RIP and TRAF2, but without activating NF-κB (99); and (d), in yeast the molecular mechanisms by which H2O2 modulates lipid raft formation started to be uncovered, and they involve...
the modulation of a set of genes that control lipid and sterol metabolism, resulting in modulation of lipid composition and organization of the plasma membrane (82).

The overall effect of H$_2$O$_2$ at the receptor level, either inhibition by receptor shedding and RIP degradation, or activation by promoting clustering of the NF-$\kappa$B machinery in the membrane lipid rafts, will probably be dependent on the H$_2$O$_2$ dose and cell type.

**H$_2$O$_2$ and the IKK complex**

The ability of H$_2$O$_2$ to regulate IKK activity has been investigated by multiple groups. In mouse alveolar epithelial cells, H$_2$O$_2$ (200 $\mu$M) by itself does not lead to IKK and NF-$\kappa$B activation, but it markedly decreases the activation of the IKK complex by 10 ng/mL $^{-1}$ TNF-$\zeta$, preventing both IxB-$\zeta$ degradation and NF-$\kappa$B activation (54) (Fig. 5, Table 2). This effect was independent of phosphoinositide 3-kinase (PI3K) and tyrosine kinases, which are regulated by H$_2$O$_2$ (54). Inhibition of IKK was associated with a direct oxidation of the Cys179 in IKK$\beta$ (Fig. 5). In opposition to these findings, Kamata et al. (49) using HeLa cells showed that 3 mM H$_2$O$_2$ slightly activates IKK, although at a later time than TNF-$\zeta$, and observed a sustained activation of NF-$\kappa$B when H$_2$O$_2$ and TNF-$\zeta$ were added together. Moreover, phosphorylation of IKK serine residues, namely Ser180 of IKK$\alpha$ and Ser181 of IKK$\beta$ which are located in the activation loops, is essential for H$_2$O$_2$-mediated IKK activation (Fig. 5, Table 2) (49). A recent study reported that IKK$\gamma$ dimers are linked through disulphide bonds formed between Cys54 and Cys347, a requirement for a correct NF-$\kappa$B activation. In mouse fibroblasts, H$_2$O$_2$ (50–500 $\mu$M) induces IKK$\gamma$ dimerization, but a pretreatment with 200 $\mu$M H$_2$O$_2$ before the addition of TNF-$\zeta$ prevents IKK activation, probably by interfering with the IKK$\gamma$ subunit (39).

Alternatively, H$_2$O$_2$ may act upstream of IKK. In favor of this hypothesis, IL-1$\beta$-stimulated MCF-7 cells induce H$_2$O$_2$ production in a NOX-dependent manner, which in turn facilitates NF-$\kappa$B-inducing kinase (NIK) activation through inhibition of phosphatases. The consequent phosphorylation of IKK$\alpha$ by NIK was confirmed by exposing cells with 1 mM H$_2$O$_2$ (63).

**H$_2$O$_2$, IxBs, and NF-$\kappa$B translocation to the nucleus**

In cells where H$_2$O$_2$ activates NF-$\kappa$B, there are somewhat conflicting views on the fate of IxB-$\zeta$ after the activation. In EL4 mouse lymphoma cells, phosphorylation of Ser32 and Ser36 residues in IxB-$\zeta$ is not required for NF-$\kappa$B activation by H$_2$O$_2$ alone (300 $\mu$M), whereas independent-IKK phosphorylation of both Tyr42 and PEST is essential (93). Phosphorylation of Tyr42 and PEST on IxB-$\zeta$ relies probably on casein kinase II (CKII) activity, with the Syk upstream-kinase as mediator (93,106). In fact, H$_2$O$_2$ is a known inhibitor of protein tyrosine phosphatases (61), which can account for the increased IxB-$\zeta$ phosphorylation observed. Whether IxB-$\zeta$ phosphorylated on Tyr42 is subsequently degraded is not yet consensual (93, 106). It is important to note that IxB-$\zeta$ phosphorylation at Tyr42 residue induced by H$_2$O$_2$ seems restricted to T cells, as in other cell lines this modification is not observed. Interestingly, this alternative NF-$\kappa$B activation pathway also occurs in the presence of the tyrosine phosphatase inhibitor pervanadate and during hypoxia or hypoxia followed by reoxygenation (9,43,67). PI3K and c-Src have been pointed out as the mediators of this alternative activation of NF-$\kappa$B (9, 30, 67). More specifically, the regulatory subunit p85$\alpha$ of PI3K is able to bind Tyr42-phosphorylated IxB-$\zeta$, through its Src homology 2 domains, thereby sequestering IxB-$\zeta$ from NF-$\kappa$B, which is then free to translocate to the nucleus. Also, the catalytic activity of PI3K appears to be critical for NF-$\kappa$B activity in those conditions (9). Since H$_2$O$_2$ is able to induce PI3K (86) and c-Src (1), these kinases may be important in H$_2$O$_2$-induced Tyr42 phosphorylation of IxB-$\zeta$. NF-$\kappa$B activation by hypoxia is probably dependent on the stimulation of ROS production by mitochondria (18,67).

The importance of H$_2$O$_2$ on this activation is not well characterized, but it is known that hypoxia also activates NF-$\kappa$B through an H$_2$O$_2$-independent mechanism, dependent on prolyl hydroxylases (PHDs). PHDs are responsible for the degradation of hypoxia-inducible factor-1 under normoxia conditions, but are inhibited during hypoxia. Interestingly, PHDs inhibition leads to IKK$\beta$ activation and, consequently, phosphorylation of IxB-$\zeta$ at the typical serine residues (25).

In cells exposed to classical NF-$\kappa$B inducers, H$_2$O$_2$ may act on the classical activation pathway and contribute for the overall NF-$\kappa$B response (Fig. 5). For example, in rat lung epithelial cells H$_2$O$_2$ alone does not cause IxB-$\zeta$ degradation, but 100 $\mu$M H$_2$O$_2$ together with 10 ng/mL $^{-1}$ TNF-$\zeta$ lead to a cooperative activation of NF-$\kappa$B (Table 2) (46). H$_2$O$_2$ acted through Ras, known to be a sensor of oxidative stress, and the engagement of MEKK1 and JNK was indicated as the convergent point downstream of TNF-$\zeta$ signaling through IKK.

Similar results were obtained by us in HeLa and MCF-7 cells treated with 0.37 ng/mL $^{-1}$ TNF-$\zeta$, in which 25 $\mu$M H$_2$O$_2$ in s.s. markedly increases IxB-$\zeta$ degradation (unpublished observation), resulting in an increased p65 translocation to the nucleus (Table 2) (76).

Antagonistic effects of H$_2$O$_2$ on NF-$\kappa$B activation have also been described. In TNF-$\zeta$-induced human bronchial epithelial cells, H$_2$O$_2$ (500 $\mu$M) stimulates IKK activity, IxB-$\zeta$ phosphorylation and ubiquitination, but without IxB-$\zeta$ degradation resulting in inhibition of NF-$\kappa$B transactivation (47). This suggests inhibition of the proteasome activity by H$_2$O$_2$ (Fig. 5, Table 2). In accordance, in two different works with LPS-treated neutrophils, H$_2$O$_2$ (250–1000 $\mu$M) decreases NF-$\kappa$B-dependent gene expression by attenuating IxB-$\zeta$ degradation. No effect on IKK phosphorylation was observed (104) but inhibition of the proteasome activity explained the impaired IxB-$\zeta$ degradation (129) (Table 2).

The differences in the results described above may be a consequence of the method of H$_2$O$_2$ delivery, and also of the type and number of cells used. Excessive oxidant conditions can originate different results. For instance, we have compared the effects of two H$_2$O$_2$ delivery methods on p65 translocation to the nucleus, the s.s. titration method using a s.s. of 25 $\mu$M H$_2$O$_2$ versus a bolus addition of 1 mM H$_2$O$_2$ (76). Interestingly, in opposition to the s.s. method, where H$_2$O$_2$ increases p65 translocation in TNF-$\zeta$-treated cells, the bolus addition inhibits this translocation. The differences were attributed to an excessive oxidative load introduced by the bolus addition, since a pre-exposure to H$_2$O$_2$ in s.s. followed by the addition of TNF-$\zeta$ also inhibits NF-$\kappa$B activation. Inhibitory effects on NF-$\kappa$B activation were also observed in other studies that used pre-exposures to H$_2$O$_2$ delivered either as a bolus (58) or by using glucose oxidase (123) (Table 2). So, oxidative modifications in molecular
mediators of the NF-κB pathway or in the global redox state of the cells are probably dictating the inhibition of signaling response.

**H₂O₂ and DNA transactivation by NF-κB**

Sulfhydryl oxidizing agents, such as diamide, inhibit NF-κB binding to DNA in vitro, while reducing agents increase DNA binding (111). Jornot et al. (48) illustrated the ability of H₂O₂ (1 mM) to induce NF-κB translocation to the nucleus in human endothelial cells, but without further transactivation of DNA. The Cys62 residue on the p50 subunit of NF-κB was identified as being responsible for this dependence of DNA transactivation on redox conditions (Fig. 5) (71, 72). The probable modification of Cys62 is the formation of a sulfenic acid in the presence of oxidants, followed by S-glutathionylation, which inhibits NF-κB binding to DNA (84). In favor of such mechanism, overexpression of Trx repressor, Cysteine oxidase 1 (72), and endogenous Trx is required for the maintenance of reducing conditions within the nucleus, so that NF-κB is able to bind to DNA (38). Therefore, different redox requirements in the cytosol and in the nucleus are necessary for an appropriate NF-κB activation. In the cytosol, a pro-oxidant signal may activate NF-κB and lead to its translocation to the nucleus but, when in the nucleus, NF-κB proteins must remain reduced in order for DNA binding to occur (41). Nevertheless, in cells treated with classical inducers, and in opposition to the study of Jornot et al. (48), H₂O₂ modulates positively the expression of NF-κB-dependent genes (46, 76). This indicates that this modulation by H₂O₂ is probably dependent on the cell type and on H₂O₂ concentrations. Also, NF-κB subunits, such as p65 and c-Rel, can be phosphorylated (20, 69, 120) or acetylated (21), modifications that usually increase their transactivation potential. Therefore, regulation of these modifications by H₂O₂ is a good topic to explore in order to understand the alterations of gene expression induced by H₂O₂.

Besides inducing modifications of NF-κB subunits, H₂O₂ may also induce post-translational modifications of other proteins such as histones. In alveolar epithelial cells, H₂O₂ (100 μM) enhances acetylation of histone H4 proteins and decreases both the activity and the expression of histone deacetylase 2 (HDAC2) (Fig. 5), permitting an increased NF-κB-dependent transcription rate (75). These observations may have relevance in a variety of chronic inflammatory diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and rheumatoid arthritis (44), in which NF-κB activation is increased.

A recent work from Enesa et al. reported that H₂O₂ (100 μM) stimulates IL-1- and TNF-α-dependent activation of NF-κB transcription in HeLa cells by suppressing p65 export, thus prolonging its nuclear localization and binding to DNA (29). This effect resulted from the enhanced degradation of the newly synthesized IkB-α, which normally enters the nucleus in order to remove NF-κB from the DNA (Fig. 5).

**Dual Regulation of Inflammation by H₂O₂**

Besides the established germicide role for H₂O₂ during the inflammatory response, a signaling role for H₂O₂ has been hypothesized. Whether H₂O₂ stimulates or inhibits inflammation has been under dispute because opposite results have been reported. We have found recently that H₂O₂ at concentrations (12.5 μM) close to the ones produced by leukocytes during an inflammatory response is able to have a synergistic effect with TNF-α, also at low concentrations (0.37 ng mL⁻¹), and simultaneously upregulates a set of pro-inflammatory (MCP-1, IL-8, ICAM-1) and anti-inflammatory (HO-1, IL-6) genes that are NF-κB-dependent (76) (Fig. 6). This dual effect of H₂O₂ upregulating the expression of proteins important to spread inflammation, but at the same time leading to the production of proteins that will lower the inflammatory response level (HO-1) (78) or even contributing for its resolution (IL-6) (124), allows a more efficient response and indicates that H₂O₂ has a crucial dual role during the inflammatory process (76).

The anti-inflammatory role of H₂O₂ may be viewed as protecting from chronic inflammation or sepsis and is supported by studies on the chronic granulomatous disease (CGD). This condition results from mutations in the components of the phagocytic NADPH oxidase originating a non-functional NADPH oxidase defective in ROS production. When compared with healthy monocytes, CGD-deriving monocytes respond to infection with LPS with higher levels of pro-inflammatory mediators, such as IL-8 and TNF-α, and higher expression of NF-κB proteins, such as p65, c-Rel, and...
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p50, than healthy monocytes (13). Accordingly, in this disease IκB-α, IκB-β, and IκB-ε are not upregulated, and thus an inflammatory condition with an aberrant NF-κB activation prevails (13). Also, in neutrophils from the blood of patients with CGD, IL-8 levels are highly increased and the addition of an exogenous dose of 100 μM H₂O₂ is able to decrease IL-8 levels (62). Cigarette smoke induces the recruitment of inflammatory cells in the lungs and consequently the generation of ROS. The ablation of components of the NADPH oxidase (p47-phox and gp91-phox) decreases ROS, but in opposition to the expected, it increases the inflammatory response, due to an exacerbated NF-κB activation. Although the mechanism of action was not entirely elucidated, these results support the anti-inflammatory role of ROS (126).

On the other hand, H₂O₂ is also related with pro-inflammatory conditions, since there are several studies reporting an upregulation of chemokines and adhesion molecules in the presence of H₂O₂ (27, 59, 87).

DNA transactivation of NF-κB-dependent genes usually involves the p65 subunit and the p50/p65 dimer, which is the most abundant in cells and therefore the most studied. However, the different phenotypes obtained in knockout mice for most abundant in cells and therefore the most studied. How-ever, the different subunits of the NF-κB family indicate specific gene expression. For example, IL-12 is a pro-inflammatory cytokine only produced by activated macrophages and it is important for induction of helper T cells and thus to make the bridge between innate and adaptive inflammation (90). IL-12 is composed of two subunits, and interestingly the expression of one of them, p40, is dependent on c-Rel and not on p65 (90). The involvement of H₂O₂ in inflammation signaling has been also demonstrated by the regulation of IL-12, since addition of H₂O₂ in the range of 62–250 μM inhibits IL-12 p40 subunit expression in LPS-IFN-γ-stimulated macrophages (53). This inhibition results from a differential regulation of NF-κB dimers, since H₂O₂ induces p65 and p50 but inhibits c-Rel translocation to the nucleus (53). At a first glance this role can be viewed as negative, because it can block the triggering of the adaptive inflammatory response. However, it can also contribute to avoid an exacerbated inflammatory response since c-Rel dimers are also involved in the activation of other pro-inflammatory genes, such as MCP-1 and IL-8, together with p65 dimers (42, 56). In fact, we have observed that H₂O₂ s.s. increases nuclear c-Rel in TNF-α-treated MCF-7 cells, but the same conditions have an inhibitory effect in HeLa cells (unpublished observation). As a consequence, we found high levels of synergism between H₂O₂ and TNF-α for the mRNA expression of MCP-1 (63%) and IL-8 (93%) in MCF-7 cells, but a more moderate synergism in HeLa cells (11% for MCP-1 and 34% in IL-8) where only p65 dimers contribute to this synergism (76).

From all the information available about the inflammatory environment, it is expected the simultaneous presence of TNF-α, and other inflammatory mediators, with H₂O₂, NF-κB, as the principal regulator of inflammation, is targeted for both pro- and anti-inflammatory modulation by H₂O₂, which contributes for a more effective and controlled response.

Selective Gene Expression by H₂O₂

From the previous section, it is clear that the modulating effect of H₂O₂ on NF-κB dependent genes is not indiscriminate, as H₂O₂ modulation is restricted to a subset of genes. This selective role of H₂O₂ in gene expression is a general property being also observed for genes that are NF-κB independent and in several organisms (118). The ability of H₂O₂ to selectively stimulate a subset of genes while leaving others unchanged further supports its potential fine-tuning role as a regulator of NF-κB-dependent processes. The molecular basis for this selective stimulation is unknown, but several potential mechanisms have been suggested.

The control of the expression of a single gene is often done by several transcription factors and the expression pattern is a combination of the activation state of each transcription factor. Therefore, H₂O₂ selective modulation of one gene could be attributed to the action of H₂O₂ on a transcription factor, other than NF-κB. The induction of the HO-1 gene may represent such an example. This gene is under the control of both NF-κB and NF-E2-related factor-2 (NRF-2), but probably H₂O₂ exerts its effect via NRF-2 (89).

The state of chromatin is an important variable controlling the accessibility of the transcription factor to the gene, and this often controls the expression of genes with a slow response, such as RANTES (i.e., genes whose expression is not immediate after activation of the controlling transcription factor). So, by controlling the state of chromatin, H₂O₂ could also modulate the kinetics of gene expression causing gene-specific effects. The post-translation modification of histones observed in alveolar cells (75) supports this possibility.

As discussed in detail in this Forum, NF-κB is subjected to many post-translational modifications (35). How these NF-κB modifications modulate gene expression depends on the κB promoter site, and three sets of regions have been defined according to the dependence of gene expression on the state of phosphorylation of p65 (4). Constructs with highly asymmetric κB sites that fit a GGRWWWYYYY consensus sequence are only efficiently induced by wild-type p65. Examples of endogenous genes that harbor this sequence are E-selectin, IL-2 receptor-alpha, human IL-8, and VCAM-1 (first κB site). A second category that includes constructs with the KGRAHWYCC consensus sequence are activated by p65 serine 205, 276, and 281 mutants, although at a weaker level than by wild-type p65. Endogenous genes that harbor this sequence include VCAM-1 (second κB site), ICAM-1, IL-6, and IκB-α, and in endothelial cells, the failure of H₂O₂ to induce ICAM-1 expression was attributed to a failure of H₂O₂ to induce p65 phosphorylation (116). A third category is formed by constructs containing the GGRATTHYCC consensus sequence, whose expression is induced by wild-type p65 and p65 mutants at a similar extent. Endogenous genes that harbor these sequences include the MHC class I and human E-selectin (4).

Variations in the κB promoter sequences may also lead to a differential gene expression in the presence of H₂O₂, due to changes in NF-κB affinity towards κB promoter sites (Fig. 7). To investigate this hypothesis we transfected HeLa cells with three reporter genes bearing κB sites with different affinities for NF-κB (77). Genes containing high affinity sites are less sensitive to a H₂O₂ stimulatory effect on NF-κB-dependent activation than those containing the lower affinity site. Likewise, the stimulatory effect induced by H₂O₂ on the expression of endogenous NF-κB-dependent genes is predominately observed at low levels of TNF-α, while at higher levels of this cytokine the H₂O₂ effects are small (77). These observations can be explained by considering that high affinity κB
sites will be fully occupied even at moderate levels of NF-κB in the nucleus and the higher NF-κB translocation induced by H₂O₂ will not lead to a further increase in the occupancy of the κB sites, justifying the lack of sensitivity of genes with high-affinity κB sequences to H₂O₂. On the contrary, low-affinity κB sites will be susceptible to an increase of NF-κB levels in the nucleus caused by a synergistic effect of H₂O₂ together with a cytokine (77). In summary, a simple association-dissociation equilibrium between NF-κB and the κB sites in DNA can explain the differential gene expression by H₂O₂ whereas high affinity κB sites or conditions of strong NF-κB activation make genes relatively insensitive to the positive modulation by H₂O₂ whereas high affinity κB sites or conditions of strong NF-κB activation make genes relatively insensitive to the positive modulation by H₂O₂, while low affinity κB sites or conditions of weak NF-κB activation turn genes sensitive to the positive modulation by H₂O₂.

In conclusion, post-translation modifications of both p65 and histones, κB affinity towards NF-κB, and the combinatorial action of NF-κB with other transcription factors provide a molecular arsenal by which H₂O₂ may exert modulator effects at the level of a single gene.

A single change in a nucleotide can affect dramatically the affinity of a κB site towards NF-κB (117), so the regulation of a single NF-κB-dependent gene by H₂O₂ may be relevant for polymorphisms within κB sites of NF-κB-target genes, some of which have been correlated with human disease. For example, polymorphisms within the NF-κB binding site of the interferon-γ (IFN-γ) and of the cyclooxygenase-2 (COX-2) gene have been associated with the susceptibility to tuberculosis (88) and with an increased risk of bladder cancer (50), respectively. The impact of H₂O₂ on the regulation of these polymorphisms is still unknown. It can be speculated that if a polymorphism decreases the apparent affinity of a high-affinity κB site towards NF-κB, the stimulatory role of H₂O₂ will be potentiated, attenuating at least partially the deleterious effect of the genetic alteration. Nevertheless, this enhanced regulating role of H₂O₂ may be deleterious because it makes the expression of the affected gene less protected from fluctuations of the cellular redox state. If the polymorphism increases the apparent affinity of a low-affinity κB site towards NF-κB, the H₂O₂ regulatory role will be attenuated or even lost, disrupting the fine-regulation of the gene, potentially triggering the pathology.

Conclusions and Perspectives

There is a lack of standardization in the way H₂O₂ activation of NF-κB is studied. This has introduced many conflicting observations and has slowed down the development of the field. Because even qualitative effects by H₂O₂ depend on the dose and the method used for the delivery, any appropriate method should control the H₂O₂ dose given per cell over time, and it should also mimic the endogenous cellular H₂O₂ production. Steady-state titration with H₂O₂ has these characteristics and is of simple experimental application.

Compared with other signal transduction pathways, the NF-κB pathway is not the most sensitive pathway to H₂O₂. At
the expected H$_2$O$_2$ intracellular levels attained in cells during normal aerobic metabolism—up to 0.1 $\mu$M (17)—NF-$\kappa$B activation should to be insensitive to any modulatory effect by H$_2$O$_2$. This contrasts with the activation of NRF-2 (98, 121) or with the phosphorylation of the heterogeneous nuclear ribonucleoprotein C1/C2 (103), both of which are expected to work as molecular sensors of redox changes at these H$_2$O$_2$ levels, as both these processes are activated by extracellular H$_2$O$_2$ bolus concentrations between 0.1 and 10 $\mu$M. These concentrations probably lead to an H$_2$O$_2$ intracellular steady-state near the predicted in vivo 0.1 $\mu$M range, if we consider both the gradients formed between extracellular and intracellular H$_2$O$_2$ concentrations when H$_2$O$_2$ is added externally (5) and the fast consumption of external H$_2$O$_2$ by cells. At levels reached in the vicinity of inflammatory sites (around 15 $\mu$M (66, 108)), H$_2$O$_2$ by itself probably does not exert any significant effect on the modulation of NF-$\kappa$B-dependent processes, but, together with other agents, may have an important fine-tuning modulatory role. For example, H$_2$O$_2$ produced during inflammation probably does not act only as a germicide, but also as a fine-tuning signaling molecule that exacerbates inflammation, increasing pathogen removal, and simultaneously attenuates possible adverse effects through induction of an anti-inflammatory control loop. This fine-tuning role of H$_2$O$_2$ in the NF-$\kappa$B pathway may be important to keep the normal inflammatory events under control, thus avoiding the onset of a pathological event.

The mechanisms by which H$_2$O$_2$ is able, under different cellular conditions, to selectively modulate NF-$\kappa$B-dependent genes started to be uncovered only recently. To have a detailed picture of the effects of H$_2$O$_2$ on each gene is a formidable challenge, but this characterization is necessary to understand how H$_2$O$_2$ modulates biological processes. It is of particular relevance to know the impact H$_2$O$_2$ has on heart mitochondria glutathione peroxidase and catalase to H(2)O(2) detoxification in in vivo conditions. Free Radic Biol Med 33: 1260–1267, 2002.

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Abbreviations Used

CARCl11 ¼ caspase recruitment domain family 11  
CKII ¼ casein kinase II  
COX-2 ¼ cyclooxygenase-2  
GO ¼ glucose oxidase  
GPx ¼ glutathione peroxidase  
GSH ¼ reduced form of glutathione  
GSSG ¼ oxidized form of glutathione  
H2O2 ¼ hydrogen peroxide  
HAT ¼ histone acetyltransferase  
HDAC ¼ histone deacetylases  
HRP ¼ horseradish peroxidase  
IFN-γ ¼ interferon-γ  
IKK ¼ IκB kinase  
IL-1 ¼ interleukin 1  
iNOS ¼ inducible nitric oxide synthase  
LPS ¼ lipopolysaccharide  
MALTI ¼ mucosa associated lymphoid tissue  
NF-E2-related factor-2 ¼ lymphoma translocation gene 1  
NAC ¼ N-acetyl-L-cysteine  
NF-κB ¼ nuclear factor κB  
(NF-κB1xκB) ¼ NF-κB and κB site complex  
NIK ¼ NF-κB-inducing kinase  
NRF-2 ¼ NF-E2-related factor-2  
PHD ¼ prolyl hydroxylase  
P3K ¼ phosphoinositide 3-kinase  
PKC ¼ protein kinase C  
PMA ¼ phorbol 12-myristate 13-acetate  
PRX ¼ peroxiredoxins  
RIP ¼ receptor interacting protein  
ROS ¼ reactive oxygen species  
SOD ¼ superoxide dismutase  
TAD ¼ transactivation domain  
TNF-α ¼ tumor necrosis factor-α  
TNF-R ¼ TNF-α receptor  
TRADD ¼ TNF receptor-associated death domain  
TRAF ¼ TNF-R associated factor  
Trx ¼ thioredoxin
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