

## Role of Hydrogen Peroxide in NF- $\kappa$ B Activation: From Inducer to Modulator

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### Abstract

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) has been implicated in the regulation of the transcription factor NF- $\kappa$ B, a key regulator of the inflammatory process and adaptive immunity. However, no consensus exists regarding the regulatory role played by  $\text{H}_2\text{O}_2$ . We discuss how the experimental methodologies used to expose cells to  $\text{H}_2\text{O}_2$  produce inconsistent results that are difficult to compare, and how the steady-state titration with  $\text{H}_2\text{O}_2$  emerges as an adequate tool to overcome these problems. The redox targets of  $\text{H}_2\text{O}_2$  in the NF- $\kappa$ B pathway—from the membrane to the post-translational modifications in both NF- $\kappa$ B and histones in the nucleus—are described. We also review how  $\text{H}_2\text{O}_2$  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  $\kappa$ B polymorphisms. In conclusion, after near 30 years of research,  $\text{H}_2\text{O}_2$  emerges not as an inducer of NF- $\kappa$ B, but as an agent able to modulate the activation of the NF- $\kappa$ B 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,  $\text{H}_2\text{O}_2$  is a fine-tuning regulator of NF- $\kappa$ B-dependent processes, as exemplified by its dual regulation of inflammation. *Antioxid. Redox Signal.* 11, 000–000.

### The Classical NF- $\kappa$ B Activation Pathway

**I**N 1986, THE TRANSCRIPTION FACTOR NUCLEAR FACTOR- $\kappa$ B (NF- $\kappa$ B) was discovered by Sen and Baltimore in B cells (97). NF- $\kappa$ B forms homo- or heterodimers composed of members of the Rel subfamily—p65/RelA, c-Rel, RelB, which contain C-terminal transactivation domains (TADs)—and the NF- $\kappa$ B subfamily: NF- $\kappa$ B1 (p50 and its precursor p105) and NF- $\kappa$ B2 (p52 and its precursor p100) (20, 33, 65). The Rel-homology domain (RHD) is present in all NF- $\kappa$ B/Rel proteins and is responsible for dimerization, recognition and binding to DNA, and interaction with the inhibitory proteins, I $\kappa$ Bs (51). The I $\kappa$ B proteins bind to NF- $\kappa$ B and prevent translocation of the latter to the nucleus and its binding to DNA. The I $\kappa$ B family is composed of I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , I $\kappa$ B- $\epsilon$ , I $\kappa$ B- $\gamma$ , and BCL-3 (B-cell lymphoma 3) and by the precursors NF- $\kappa$ B1 p105 and NF- $\kappa$ B2 p100 because they all possess the typical ankyrin repeats (51). Classically, NF- $\kappa$ B (p50/p65) is kept latent in the cytosol bound to I $\kappa$ Bs. The prototypical activators tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), lipopolysaccharide (LPS), and interleukin 1 (IL-1) activate the I $\kappa$ B-kinase complex (IKK complex), which is responsible for phosphorylation of I $\kappa$ Bs at specific regulatory amino acid residues: I $\kappa$ B- $\alpha$  (Ser32 and Ser36), I $\kappa$ B- $\beta$  (Ser19 and Ser23) and I $\kappa$ B- $\epsilon$  (Ser18 and Ser22). Consequently,

the I $\kappa$ Bs are targeted for degradation by the 26S proteasome, thereby freeing NF- $\kappa$ B, which translocates to the nucleus and activates the target genes.

### NF- $\kappa$ B Activation by Hydrogen Peroxide ( $\text{H}_2\text{O}_2$ ): A History of Contradictions

NF- $\kappa$ B was shown to be redox-regulated in 1990, when Herzenberg and coworkers (102) described that intracellular thiols mediate NF- $\kappa$ B activation by TNF- $\alpha$  and phorbol 12-myristate 13-acetate (PMA). By lowering the levels of glutathione (GSH,  $\gamma$ -glutamyl-cysteinyl-glycine), the major redox buffer in cells, TNF- $\alpha$ -induced NF- $\kappa$ B activation increased, while by using the precursor of glutathione synthesis, N-acetyl-L-cysteine (NAC), NF- $\kappa$ B activation decreased (102). Similarly, the antioxidant pyrrolidine dithiocarbamate (PDTC) inhibits NF- $\kappa$ B activation by blocking NF- $\kappa$ B released from I $\kappa$ B- $\alpha$  in cells treated with IL-1 and TNF- $\alpha$  (94). Direct evidences of regulation by hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) came from Schreck *et al.* (95), who demonstrated for the first time that the NF- $\kappa$ B pathway is activated by adding 150  $\mu\text{M}$   $\text{H}_2\text{O}_2$  to a subclone of Jurkat T cells, later named Wurzberg cells. These cells were infected with the human immunodeficiency virus type 1 (HIV-1), whose expression is dependent on

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NF- $\kappa$ B activation, and H<sub>2</sub>O<sub>2</sub> was shown to increase expression of the virus (95). Interestingly, H<sub>2</sub>O<sub>2</sub> does not activate the purified complex NF- $\kappa$ B/I $\kappa$ B *in vitro*, which indicates that the activation in cells is due to a subsequent regulation by H<sub>2</sub>O<sub>2</sub>, such as changes in the redox state of the cell. These works indicated a possible convergent pathway for NF- $\kappa$ B activation by different stimuli, which is dependent on the intracellular redox level. Moreover, since NF- $\kappa$ B inducers, such as TNF- $\alpha$  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- $\kappa$ B activation pathway. In favor of this hypothesis was the fact that there was an inhibition of NF- $\kappa$ B 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- $\kappa$ B converge (3).

T1 ► However, soon after, several reports began to question the universal pathway of NF- $\kappa$ B activation mediated by H<sub>2</sub>O<sub>2</sub>. First, direct activation of NF- $\kappa$ B by H<sub>2</sub>O<sub>2</sub> does not occur in all cell types, as observed in Table 1. Second, in H<sub>2</sub>O<sub>2</sub>-responsive cells, NF- $\kappa$ B activation proceeds slowly (hour range) after H<sub>2</sub>O<sub>2</sub> 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- $\kappa$ B activation independently of their antioxidant potential (37). Hayakawa *et al.* (37) found that NAC selectively blocks TNF- $\alpha$ -induced signaling by lowering TNF- $\alpha$  affinity to its receptor TNF-R1 and that PDTC interferes with the ubiquitin ligase activity, thereby inhibiting I $\kappa$ B- $\alpha$  degradation. Brennan *et al.* (11) also identified a pro-oxidant character of PDTC capable of inhibiting NF- $\kappa$ B activation, which is reversed by addition of 2-mercaptoethanol. Fourth, conflicting data using antioxidant enzymes appeared to argue against a universal role for H<sub>2</sub>O<sub>2</sub> in NF- $\kappa$ B signaling. Overexpression of Mn-superoxide dismutase (SOD) in MCF-7 cells either inhibits (70) or potentiates (92) TNF- $\alpha$ -dependent NF- $\kappa$ B activation.

T2 ► Even though the universal role for H<sub>2</sub>O<sub>2</sub> in the NF- $\kappa$ B pathway was ruled out, studies using direct cell exposure to H<sub>2</sub>O<sub>2</sub> alone or together with other NF- $\kappa$ B stimulants continued because parts of the pathway are sensitive to redox changes. However, many contradictory data have been published about H<sub>2</sub>O<sub>2</sub> participation in the NF- $\kappa$ B pathway, either alone (Table 1) or in the presence of classical inducers (Table 2), impairing any conclusion about the role of H<sub>2</sub>O<sub>2</sub> on the NF- $\kappa$ B pathway. In Tables 1 and 2, relevant details of the experimental setup are described (cell type, cell number or confluence, H<sub>2</sub>O<sub>2</sub> and classical NF- $\kappa$ B inducers concentrations, incubation time used in the assay) together with key experimental observations and the overall effect exerted by H<sub>2</sub>O<sub>2</sub> on NF- $\kappa$ B activation.

By itself, H<sub>2</sub>O<sub>2</sub> is at best a weak NF- $\kappa$ B activator. In some cell lines NF- $\kappa$ B seems insensitive to H<sub>2</sub>O<sub>2</sub>, while in other cell lines where H<sub>2</sub>O<sub>2</sub> activates NF- $\kappa$ B, H<sub>2</sub>O<sub>2</sub> has little impact when compared with classical inducers (e.g., TNF- $\alpha$ ) because H<sub>2</sub>O<sub>2</sub> leads to lower levels of NF- $\kappa$ B activation and with slower kinetics (49, 76) (Table 1). Interestingly, in works where H<sub>2</sub>O<sub>2</sub> induced NF- $\kappa$ B activation at significant levels, an alternative activation pathway for NF- $\kappa$ B activation was suggested, since typical upstream kinases or I $\kappa$ B- $\alpha$  modifica-

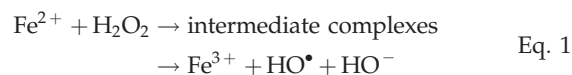
tions were not identified. Nevertheless, in the majority of the data presented in Table 1, an H<sub>2</sub>O<sub>2</sub> concentration above 100  $\mu$ M was necessary to observe any significant NF- $\kappa$ B activation, raising doubts over the *in vivo* relevance of NF- $\kappa$ B activation by H<sub>2</sub>O<sub>2</sub>. In addition, in some studies H<sub>2</sub>O<sub>2</sub> inhibits the constitutive NF- $\kappa$ B activation in primary cell lines (23, 129).

The studies presented in Table 2 have in common the use of both H<sub>2</sub>O<sub>2</sub> and classical NF- $\kappa$ B inducers (TNF- $\alpha$ , 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 H<sub>2</sub>O<sub>2</sub> either negative or positive effects, but also no effects on cells stimulated by classical NF- $\kappa$ B 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> and the dynamic nature of H<sub>2</sub>O<sub>2</sub> metabolism also make the common experimental setups applied to study the role of H<sub>2</sub>O<sub>2</sub> inappropriate. Next, we will review how the basic biology of H<sub>2</sub>O<sub>2</sub> undermines its study and how this can be overcome.

## Basic Biology of H<sub>2</sub>O<sub>2</sub>

### H<sub>2</sub>O<sub>2</sub> chemistry

In chemical terms, H<sub>2</sub>O<sub>2</sub> 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, H<sub>2</sub>O<sub>2</sub> is seen as a threat to organisms because of its interaction with transition metals, mostly reduced iron (Fe<sup>2+</sup>) or copper (Cu<sup>+</sup>) ions, and the consequent formation of the extremely reactive HO<sup>•</sup> 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 H<sub>2</sub>O<sub>2</sub>, but high concentrations of H<sub>2</sub>O<sub>2</sub> can induce the release of iron from some of these proteins (36). So, in general terms, regulatory roles by low levels of H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> is associated with HO<sup>•</sup> generation through the Fenton reaction:



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 pK<sub>a</sub> and are in the thiolate form (S<sup>-</sup>) at physiological pH (31). These cysteine residues are targeted by H<sub>2</sub>O<sub>2</sub>, 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

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TABLE 1. EFFECT OF H<sub>2</sub>O<sub>2</sub> ON NF- $\kappa$ B ACTIVATION

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

(Continued)

TABLE 1. (CONTINUED)

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

\*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<sup>lck</sup> and p72<sup>syk</sup> 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.

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 2. EFFECT OF H<sub>2</sub>O<sub>2</sub> ON NF- $\kappa$ B ACTIVATION INDUCED BY CLASSICAL INDUCERS

Classical inducer	H <sub>2</sub> O <sub>2</sub>	Incubation conditions*	Cell type†	Cell number/confluence	Observations‡					Overall effect	Ref.
					IKK activation	I $\kappa$ B- $\alpha$ degradation	NF- $\kappa$ B translocation	NF- $\kappa$ B-dependent gene expression	Other		
20 ng mL <sup>-1</sup> TNF- $\alpha$	2–5 $\mu$ M bolus	0–60 min	RBEC	Confluent		N.E.	↓		↓ DNA transactivation	Negative	34
	0.1 mM bolus	Pre-exposure to H <sub>2</sub> O <sub>2</sub> for 3 h + TNF- $\alpha$ for 4 h	Jurkat T	10 <sup>6</sup> cell mL <sup>-1</sup>		↓		↓	↓ I $\kappa$ B- $\alpha$ Phosphorylation	Negative	58
	0.1 mM bolus	Simultaneous addition for 4 h	Jurkat T	10 <sup>6</sup> cell mL <sup>-1</sup>		↑		↑	Sustained degradation of I $\kappa$ B- $\alpha$	Positive	58
	0.1–3 mM bolus	Pre-exposure to H <sub>2</sub> O <sub>2</sub> for 10 min + TNF- $\alpha$ (IKK, 5 min), (I $\kappa$ B- $\alpha$ and DNA binding, 15 min), (reporter expression, 4 h)	HeLa and Raw 264.7 (transfected cells)	2 × 10 <sup>5</sup> cells/well (12-well plate); 3 × 10 <sup>6</sup> cells/100-mm dish <sup>§</sup>	↓	↓		↓		Negative	15
10 ng mL <sup>-1</sup> TNF- $\alpha$	Up to 3 mM bolus	10, 20, 30 min for IKK activity; 60 min for NF- $\kappa$ B binding to DNA	HeLa	Transfected cells: 5 × 10 <sup>5</sup> cells/35-mm dishes	↑				Sustained IKK activation for longer times (P of Ser in the activation loops); ↑DNA transactivation	Positive	49
	10 mM bolus	10, 20, 30 min for IKK activity; 60 min for NF- $\kappa$ B binding to DNA	HeLa	Transfected cells: 5 × 10 <sup>5</sup> cells/35-mm dishes					↓ DNA transactivation	Negative – Cys179 of IKK $\beta$ is probably oxidized	49
	0.1 mM bolus	8 h	RLE	Subconfluent <sup>§</sup>				↑		Positive	46
	0.1, 0.2 mM bolus	Up to 2 h for p65 and I $\kappa$ B- $\alpha$ levels; 4 h for gene expression	HeLa A549	80% Confluent <sup>§</sup>		↑		↑	↓ Cezanne activity and prolongs poly-ubiquitination and thus activation of RIP1; ↓ p65 nuclear export (prolongs nuclear localization)	Positive	29
0.1–3 mM bolus	Pre-exposure to H <sub>2</sub> O <sub>2</sub> for 10 min + TNF- $\alpha$ (IKK, 5 min), (I $\kappa$ B- $\alpha$ and DNA binding, 15 min), (reporter expression, 4 h)	HeLa and Raw 264.7 (transfected cells)	2 × 10 <sup>5</sup> cells/well (12-well plate); 3 × 10 <sup>6</sup> cells/100-mm dish <sup>§</sup>	↓	↓			↓		Negative	15

(Continued)



TABLE 2. (CONTINUED)

Classical inducer	H <sub>2</sub> O <sub>2</sub>	Incubation conditions*	Cell type†	Cell number/confluence	Observations‡					Overall effect	Ref.
					IKK activation	I $\kappa$ B- $\alpha$ degradation	NF- $\kappa$ B translocation	NF- $\kappa$ B gene expression	Other		
0.1; 0.5 mM bolus	0.1; 0.5 mM bolus	5 min IKK activity; 30 min I $\kappa$ B- $\alpha$ levels; 1 h and 8 h for mRNA levels and protein expression	NHBE	90–100% confluence <sup>s</sup>	↑	↓	↓	↑I $\kappa$ B- $\alpha$ ubiquitination; ↓DNA transactivation		Negative – probably at the proteasome level	47
		5 min IKK activity; 5 and 15 min I $\kappa$ B- $\alpha$ levels;	BEAS-2B								
		15 min DNA binding (1 ng mL <sup>-1</sup> TNF- $\alpha$ ;	C10 RLE	70–90% confluence. phenol red-free DMEM/F12 with 0.5% FBS	↓	↓	↓	↓ DNA Binding		Negative – oxidation of cysteine residues of IKK	54
		6 h reporter expression	HL-60	0.5–1.0 × 10 <sup>6</sup> cells mL <sup>-1</sup> (0.1% FBS)	↓			↑Cleavage of TNFR1; ↓RIP levels		Negative subunits	79
0.3 and 0.9 mM bolus	0.3 and 0.9 mM bolus	Pre-exposure to H <sub>2</sub> O <sub>2</sub> for 16 h + TNF- $\alpha$ 10 min	HeLa	0.5–1.0 × 10 <sup>6</sup> cells/ well (6-well plate) (0.1% FBS)	N.E.			↓I $\kappa$ B- $\alpha$ phosphorylation; ↓ Proteasome activity; ↓ DNA binding		No effect	
8.5 ng mL <sup>-1</sup> TNF- $\alpha$ (2 nM)	1.9–500 $\mu$ M bolus	Pre-exposure to GO for 4 h + TNF- $\alpha$ up to 30 min	HLEC	Confluent; serum-, pyruvate- and phenol red-free medium, supplemented with D-glucose		↓				Negative	123
		Up to 60 min	Mice-derived neutrophils	4 × 10 <sup>6</sup> cells mL <sup>-1</sup>	N.E.	↓	↓	N.E. on I $\kappa$ B- $\alpha$ phosphorylation; ↑NF- $\kappa$ B translocation for [H <sub>2</sub> O <sub>2</sub> ] < 15 $\mu$ M		Negative/Positive	104
2.5 ng mL <sup>-1</sup> TNF- $\alpha$	1 mM bolus	Pre-exposure to H <sub>2</sub> O <sub>2</sub> for 5 min + TNF- $\alpha$ for 30 min (I $\kappa$ B- $\alpha$ and NF- $\kappa$ B levels) or 4 h (gene expression)	HUVEC	Confluent			↓	N.E. on I $\kappa$ B- $\alpha$ levels; ↓ of GSH level by H <sub>2</sub> O <sub>2</sub> ; ↓ p65 nuclear levels; ↓ Secretion of cytokines (IL-6; IL-8)		Negative	128
		Simultaneous bolus + TNF- $\alpha$ up to 1 h; Pre-exposure to H <sub>2</sub> O <sub>2</sub> s.s. for 3 h + TNF- $\alpha$ up to 2 h	MCF-7 HeLa	MCF-7: 1.8 × 10 <sup>6</sup> cells/100-mm dish HeLa: 1.5 × 10 <sup>6</sup> cells/100-mm dish			↓			Negative	76

20 ng mL <sup>-1</sup> IL-1 $\beta$	0.1, 0.2 mM bolus	Up to 2 h for p65 and I $\kappa$ B- $\alpha$ levels; 4 h for gene expression	MCF-7 HeLa	MCF-7: 1.8 $\times$ 10 <sup>6</sup> cells/100-mm dish HeLa: 1.5 $\times$ 10 <sup>6</sup> cells/100-mm dish 80% Confluent <sup>§</sup>	$\uparrow$	$\uparrow$	Positive	76
			HeLa A549		$\uparrow$		$\downarrow$ Cezanne activity and prolongs poly-ubiquitination and thus activation of RIP1; $\downarrow$ p65 nuclear exportation (prolongs nuclear localization) $\downarrow$ Binding to DNA	29
10 ng mL <sup>-1</sup> IL-1 $\beta$	0.2 mM bolus 5 U mL <sup>-1</sup> GO ( $\sim$ 0.5 mM)	5 min IKK activity; 5 and 15 min I $\kappa$ B- $\alpha$ levels; 15 min DNA binding; 6 h reporter expression	C10 RLE	70–90% confluence. phenol red-free DMEM/F12 with 0.5% FBS	$\downarrow$	$\downarrow$	Negative – oxidation of cysteine residues of IKK	54
100 g mL <sup>-1</sup> LPS	0.3–1 mM bolus	Pre-exposure 30 min to H <sub>2</sub> O <sub>2</sub> + 30 min (?) with LPS	RASMC	No information	$\downarrow$		Negative – probably upstream IKK	113
1 $\mu$ g mL <sup>-1</sup> LPS	1.9–500 $\mu$ M bolus	Up to 60 min	Mice-derived neutrophils	4 $\times$ 10 <sup>6</sup> cells mL <sup>-1</sup> N.E.	$\downarrow$	$\downarrow$	$\uparrow$ NF- $\kappa$ B translocation for [H <sub>2</sub> O <sub>2</sub> ] < 15 $\mu$ M	104
	0.25, 0.5 mM bolus; up to 20 mU mL <sup>-1</sup> GO (10 mU generates 3.5 nmol H <sub>2</sub> O <sub>2</sub> min <sup>-1</sup> mL <sup>-1</sup> )	Up to 2 h	Bone marrow neutrophils	No information	$\downarrow$	$\downarrow$	$\downarrow$ Proteasome activity (chymotrypsin and trypsin-like activities); $\uparrow$ cytosolic levels of I $\kappa$ B- $\alpha$ ; $\downarrow$ nuclear accumulation of p65 N.E. on chymotrypsin-like activity	129
			MH-S			N.E.	No effect	

\*Unless otherwise specified, the incubation time column refers to the simultaneous addition of H<sub>2</sub>O<sub>2</sub> and the classical inducer.

<sup>†</sup>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).

<sup>‡</sup>Probable primary target of H<sub>2</sub>O<sub>2</sub> action is in bold face. N.E, No effect;  $\downarrow$ , inhibitory effect;  $\uparrow$ , stimulatory effect.

<sup>§</sup>Information provided by the authors.

formation is the inhibition of protein-tyrosine phosphatases (PTPs) by  $\text{H}_2\text{O}_2$ , 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  $\text{H}_2\text{O}_2$  concentrations, sulfinic groups are further oxidized to sulfinic ( $-\text{SO}_2\text{H}$ ) and sulfonic ( $-\text{SO}_3\text{H}$ ) 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).

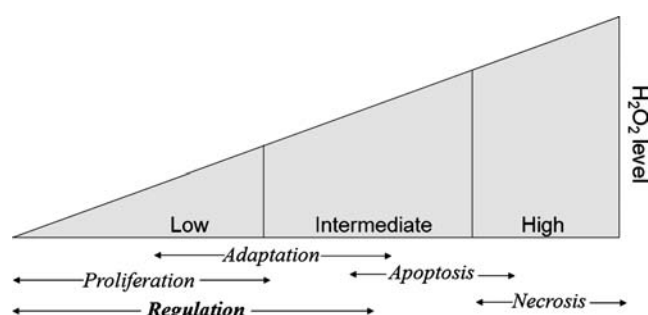
If there are two (or more) cysteine residues within the same protein, they may be oxidized by  $\text{H}_2\text{O}_2$  and form intramolecular disulfide bond(s), which can alter the conformation of the protein and consequently its function (109). GSH and Trx are able to directly reduce the disulfide bonds through their reversible oxidation.

Therefore, depending on the  $\text{H}_2\text{O}_2$  concentration applied, different levels of sulfhydryl oxidation of reactive cysteine residues are attained, leading to different conformational changes of the targeted proteins, with consequences to their biological activity. As the  $\text{H}_2\text{O}_2$  concentration increases, hydroxyl radical production also increases and damaging effects ensue. Figure 1 represents the multifunctionality of  $\text{H}_2\text{O}_2$ . 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,  $\text{H}_2\text{O}_2$  activates death pathways, such as apoptosis. Importantly, the biological effects of  $\text{H}_2\text{O}_2$  can change dramatically in a narrow range of concentrations: for example, in Jurkat T-cells intracellular concentrations below  $0.7 \mu\text{M}$  are regulatory, between  $0.7$  and  $3 \mu\text{M}$  induce apoptosis, and higher than  $3 \mu\text{M}$  induce necrosis (6). In conclusion, the concentration of  $\text{H}_2\text{O}_2$  during experiments should be rigorously controlled in order to obtain reproducible results.

#### $\text{H}_2\text{O}_2$ dynamic metabolism

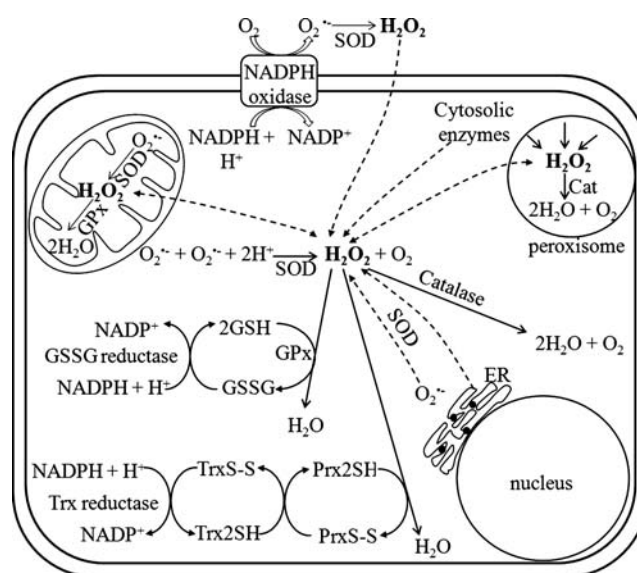
$\text{H}_2\text{O}_2$  metabolism is highly dynamic as a result of its continuous production, both intracellularly and extracellularly, and its constant removal by several enzymatic systems that can be found in virtually all aerobic cells. The half-life of  $\text{H}_2\text{O}_2$  is in the ms range and inside the cells  $\text{H}_2\text{O}_2$  exists in a steady-state level between  $10^{-9}$  and  $10^{-7} \text{ M}$  (17), while in an inflammatory situation a steady-state of  $15 \mu\text{M}$  can be reached (66, 108). Next, both  $\text{H}_2\text{O}_2$  production and removal systems are briefly described.

**$\text{H}_2\text{O}_2$  sources.** The biological sources of  $\text{H}_2\text{O}_2$  are diverse, however it is mostly produced from the dismutation of  $\text{O}_2^{\cdot-}$  by superoxide dismutases (SODs) (Fig. 2) (127).  $\text{O}_2^{\cdot-}$  in turn is produced in different subcellular localizations, such as mitochondria (electron transport, cytochrome  $P_{450}$ ), endoplasmic reticulum (cytochrome  $P_{450}$ ), cytosol (xanthine oxidoreductase), and plasma membrane (NADPH oxidase) (17, 109). Oxidases, with the exception of cytochrome  $c$  oxidase, are able to produce directly  $\text{H}_2\text{O}_2$ , for example, oxidases present in the peroxisomes (D-amino-acid oxidase, L- $\alpha$ -hydroxyacid oxidase, fatty acyl-CoA oxidase, and urate oxidase) (17), sulfhydryl oxidases present in the endoplasmic reticulum (110), and mono or polyamine oxidases, present in



**FIG. 1. Metabolic processes dependent on  $\text{H}_2\text{O}_2$  concentration.** The intracellular function of  $\text{H}_2\text{O}_2$  is determined by its concentration. Concentration is roughly divided in high (mM range) to low (nM to  $\mu\text{M}$  range).

the outer membrane of the mitochondria and cytosol, respectively (112).  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  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



**FIG. 2. Biological sources and sinks of  $\text{H}_2\text{O}_2$ .**  $\text{H}_2\text{O}_2$  is mostly formed through dismutation of  $\text{O}_2^{\cdot-}$  by superoxide dismutases (SODs), present in mitochondria (MnSOD), cytosol (CuZnSOD) and extracellularly.  $\text{O}_2^{\cdot-}$  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  $P_{450}$  (endoplasmic reticulum, mitochondria), xanthine oxidase (cytosol), and NADPH oxidase (plasma membrane). Direct  $\text{H}_2\text{O}_2$  formation occurs inside peroxisomes, but also in the endoplasmic reticulum, where sulfhydryl oxidases insert disulfide bonds during protein folding.  $\text{H}_2\text{O}_2$  intracellular level is tightly controlled by the enzymes catalase, GPxs and Prx.  $\text{H}_2\text{O}_2$  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.



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neutrophils, eosinophils, monocytes, and macrophages (Fig. 2). Exposure of phagocytes to microorganisms or inflammatory mediators results in the activation of NADPH oxidase, in which extracellular  $O_2$  is reduced at the expense of intracellular NADPH, resulting in the production of  $O_2^{\bullet-}$  (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  $HO^{\bullet}$ , constituting a line of defense against pathogens (52, 60). Importantly, part of the  $H_2O_2$  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  $H_2O_2$ , 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  $H_2O_2$ , where NF- $\kappa$ B activation is considered a crucial point of the inflammatory process.

**$H_2O_2$  sinks.** In order to avoid oxidative stress and because of the diverse biological sources of  $H_2O_2$ , cells are equipped with efficient enzymes that catalyze the reduction of excess  $H_2O_2$ . The first enzyme discovered to have the capacity to decompose  $H_2O_2$  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  $H_2O_2$  (73). GPx catalyzes the reduction by GSH of  $H_2O_2$  or organic hydroperoxides to  $H_2O$  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 distrib-

uted in the cytosol, the mitochondrial matrix, endoplasmic reticulum, and nucleus (12).

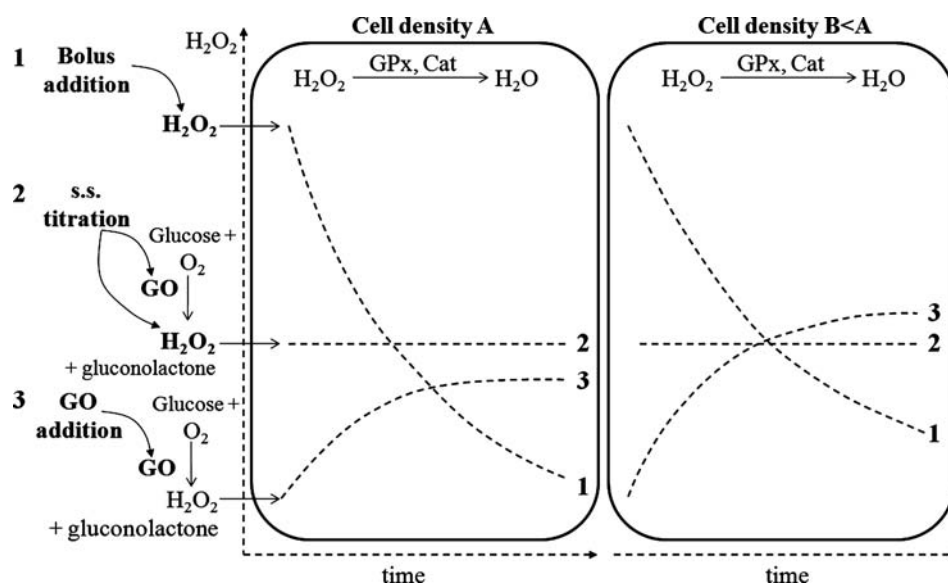
Catalase and GPx are the "classical" enzymes for  $H_2O_2$  detoxification, but a new class of enzymes, the Prxs, may also contribute for the total removal of  $H_2O_2$ . Prx have a reaction mechanism different from that of GPx (Fig. 2). While GSH is a substrate for GPx, a typical Prx contains two reduced 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  $H_2O_2$ , when in experiments cells are exposed to  $H_2O_2$  this metabolite is rapidly consumed, which makes it difficult to know for how long and to which levels of  $H_2O_2$  cells are exposed. When comparing experiments from different laboratories, in which cells are probably exposed to different  $H_2O_2$  concentrations, it is likely that a variety of cellular responses is observed, because  $H_2O_2$  elicits a variety of cellular responses in a narrow range of concentrations. This justifies the lack of consistent results regarding NF- $\kappa$ B activation by  $H_2O_2$ , as seen in Table 2. Next we will discuss how experiments can be set up in order to overcome these obstacles.

### Methodology of $H_2O_2$ Delivery

Figure 3 illustrates three possible methodologies of  $H_2O_2$  delivery to cells. A bolus addition (curve 1) of  $H_2O_2$  consists of a single addition of  $H_2O_2$  at the beginning of the experiment and represents the most commonly used method to expose culture cells to  $H_2O_2$ . This addition of  $H_2O_2$  does not result in a constant exogenous  $H_2O_2$  level during the course of an experiment because of its cellular consumption catalyzed by the

**FIG. 3. Comparison of methods of  $H_2O_2$  delivery to cells.** After the exogenous addition of  $H_2O_2$ ,  $H_2O_2$  diffuses inside cells and it is rapidly consumed due to the action of antioxidant enzymes, such as GPx and catalase. Due to this consumption, in the bolus addition method (1) high  $H_2O_2$  concentrations have to be used in order to induce cell responses. In the s.s. titration (2), the  $H_2O_2$  concentration is kept constant during the assay due to the addition of glucose oxidase (GO), simultaneously with an initial dose of  $H_2O_2$  at the desired assay concentration. The addition of GO alone (3) leads to a progressive increase of  $H_2O_2$  concentration over time, tending to a steady-state. When cell density decreases, the profile of  $H_2O_2$  concentration changes when either the bolus or the GO method are used, while with the s.s. titration the  $H_2O_2$  profile is the same, because it is based on a daily calibration (see text for more details).



◀F3

antioxidant enzymes (mainly GPx and catalase). This cellular consumption requires the use of high initial doses of  $\text{H}_2\text{O}_2$  (typically  $100\ \mu\text{M}$ – $1\ \text{mM}$ ), which may affect the redox homeostasis of cells and cause oxidative stress. Excessive oxidative stress is a possible negative modulator of NF- $\kappa$ B activation as seen in Table 2. While the simultaneous addition of  $\text{H}_2\text{O}_2$  and a classical inducer (e.g., TNF- $\alpha$ ) leads to a variety of effects, in all studies where a pretreatment of  $\text{H}_2\text{O}_2$  was performed before the addition of the inducer, a negative effect in NF- $\kappa$ B activation is observed, independently of the method of  $\text{H}_2\text{O}_2$  delivery (Table 2). Probably the accumulation of oxidative modifications impairs the proper activation of NF- $\kappa$ B when the inducer is finally added.

Moreover, the bolus addition is not a controlled method, because during the assay the concentration of  $\text{H}_2\text{O}_2$  is not monitored and not adjusted to the desired levels. The bolus addition also lacks calibration, because for the same initial  $\text{H}_2\text{O}_2$  dose, cells are effectively subjected to different concentrations of  $\text{H}_2\text{O}_2$ , depending on the specific conditions of the assay. For example, in a study describing an exposure to  $100\ \mu\text{M}$   $\text{H}_2\text{O}_2$  for 6 h, in reality it should be noted that  $100\ \mu\text{M}$  is the initial concentration. Depending on the cell type and density (Fig. 3),  $\text{H}_2\text{O}_2$  will be consumed at different rates and, often, after just 1–2 h of assay cells are no longer exposed to any  $\text{H}_2\text{O}_2$ , as all  $\text{H}_2\text{O}_2$  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  $100\ \mu\text{M}$   $\text{H}_2\text{O}_2$  corresponds to  $1\ \mu\text{mol}$ , while if the volume is 5 mL, the same initial concentration corresponds to  $0.5\ \mu\text{mol}$  of  $\text{H}_2\text{O}_2$ . At the end of the incubation period, if all  $\text{H}_2\text{O}_2$  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  $100\ \mu\text{M}$   $\text{H}_2\text{O}_2$  concentration given in a total volume of  $100\ \mu\text{L}$  growth medium in a well of a 96-well plate (growth area  $0.35\ \text{cm}^2$ ), corresponds to  $0.01\ \mu\text{mol}\ \text{H}_2\text{O}_2/0.35\ \text{cm}^2 = 0.0286\ \mu\text{mol}\ \text{H}_2\text{O}_2/\text{cm}^2$ . This is 60 % higher (on a cell basis) than a  $100\ \mu\text{M}$   $\text{H}_2\text{O}_2$  concentration given in 10 mL growth medium in a 100-mm-diameter (growth area  $56\ \text{cm}^2$ ) culture dish, which corresponds to  $1\ \mu\text{mol}\ \text{H}_2\text{O}_2/56\ \text{cm}^2 = 0.0179\ \mu\text{mol}\ \text{H}_2\text{O}_2/\text{cm}^2$  (assuming cells are seeded at the same density in both dishes). To have the same  $\text{H}_2\text{O}_2$  concentration delivered on a cell basis, we would need to perform the experiment in the 96-well plate with a concentration of  $100\ \mu\text{M}$   $\text{H}_2\text{O}_2$  delivered in  $62.5\ \mu\text{L}$  of medium or, alternatively, perform the experiment in the 100-mm culture dish with  $100\ \mu\text{M}$   $\text{H}_2\text{O}_2$  delivered in 16 mL.

To overcome these various difficulties, in our laboratory,  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  dose is added to cells, simultaneously with the enzyme glucose oxidase (GO). The activity of added GO will produce  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$ , 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  $\text{H}_2\text{O}_2$ , because the activity of GO is initially adjusted to compensate for that fact. Likewise, the  $\text{H}_2\text{O}_2$  steady-state obtained is not affected by the volume of growth medium. In addition, the concentration of  $\text{H}_2\text{O}_2$  is monitored during the assay, by measuring with an  $\text{O}_2$  electrode  $\text{O}_2$  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  $\text{H}_2\text{O}_2$  is the same during the whole period of the experiment. Experiments in which the  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  concentration. Since the desired  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  to cells, because the activity of the enzyme is not adjusted to the specific experimental conditions. Different  $\text{H}_2\text{O}_2$  concentration profiles are obtained for different cell densities (76) and, once again, the real  $\text{H}_2\text{O}_2$  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- $\kappa$ B inducers, such as IL-1, TNF- $\alpha$ , and LPS, have been pointed to induce the production of  $\text{O}_2^{\bullet-}$ , and consequently  $\text{H}_2\text{O}_2$ , probably through interaction of their receptors with NADPH oxidase components (22, 64, 81), although mitochondria may also be a source of  $\text{H}_2\text{O}_2$  (96), thereby linking  $\text{H}_2\text{O}_2$  with NF- $\kappa$ B activation. These recent mechanisms of ROS involvement on NF- $\kappa$ B activation are inducer- and cell type-independent (24). Moreover, there is a delay between the exposure to the NF- $\kappa$ B-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  $\text{H}_2\text{O}_2$ , although it is difficult to know the  $\text{H}_2\text{O}_2$  concentration during the assay. Extracellular levels of  $\text{H}_2\text{O}_2$  produced by NADPH oxidase can be measured using the horseradish peroxidase (HRP)-linked assay, where in the presence of  $\text{H}_2\text{O}_2$ , a probe is oxidized by HRP and fluorescence is monitored (107). The accuracy of  $\text{H}_2\text{O}_2$  quantification is limited by: (a) competition for  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  generated from  $\text{O}_2^{\bullet-}$ , are usually measured using the component 2',7'-dichlorofluorescein 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  $\text{H}_2\text{O}_2$  and it depends on unknown endogenous

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

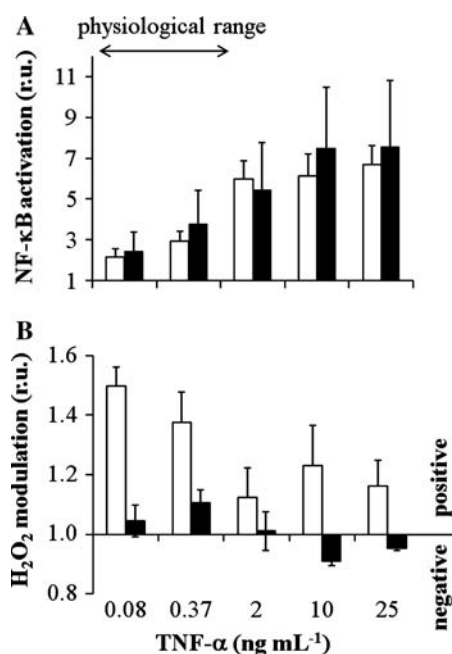
Whatever the strategy used to delivery  $\text{H}_2\text{O}_2$ , in order to study signaling pathways, such as the NF- $\kappa$ B pathway, it is imperative to use a controlled and calibrated method of exposure to  $\text{H}_2\text{O}_2$ .

### How Much TNF- $\alpha$ or IL-1?

TNF- $\alpha$  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- $\alpha$  is thus commonly used to study NF- $\kappa$ B activation, often at concentrations above  $10 \text{ ng mL}^{-1}$  (15, 47, 49, 54), which is far higher than the *in vivo* concentrations (less than  $1.5 \text{ ng mL}^{-1}$  (2)). In fact, only in acute episodes of septic shock can TNF- $\alpha$  serum levels attain a level of  $3 \text{ ng mL}^{-1}$  (26). These are overall concentrations, but it is possible that local TNF- $\alpha$  concentrations reach much higher values, particularly in the periphery of activated macrophages. Nevertheless, even in studies *in vitro* with activated monocytes with LPS, TNF- $\alpha$  production and secretion does not attain  $10 \text{ ng mL}^{-1}$ : in human monocytes ( $10^6 \text{ cells mL}^{-1}$ ) a maximal concentration of  $1.2 \text{ ng mL}^{-1}$  of TNF- $\alpha$  after 8 h of incubation is measured (57) and in murine peritoneal macrophages ( $10^6 \text{ cells mL}^{-1}$ ) this level is about  $0.2 \text{ ng mL}^{-1}$  (24 h incubation) (115). TNF- $\alpha$  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 histiocytic lymphoma cell line,  $5 \text{ ng mL}^{-1}$  of TNF- $\alpha$  does not induce ROS production, while  $15 \text{ ng mL}^{-1}$  generates a significant increase of ROS (45), indicating that a physiological TNF- $\alpha$  concentration either does not trigger ROS production or does it at low rates.

**F4** ▶ Figure 4 shows how NF- $\kappa$ B translocation to the nucleus and the expression of a NF- $\kappa$ B-reporter plasmid both respond to different concentrations of TNF- $\alpha$ , alone or together with  $\text{H}_2\text{O}_2$ . Both NF- $\kappa$ B translocation and the reporter gene expression increase with the concentration of TNF- $\alpha$ , but the simultaneous addition of a constant  $\text{H}_2\text{O}_2$  dose leads to divergent results. For NF- $\kappa$ B translocation to the nucleus, a synergism that is more significant for lower and physiological TNF- $\alpha$  concentrations is observed. Similarly, there is a synergism in the expression of the reporter gene, but for higher TNF- $\alpha$  doses, an antagonism is observed instead. These observations indicate that results obtained with high TNF- $\alpha$  concentration may be unrelated, or even opposed, to results obtained with low concentrations of TNF- $\alpha$ .

IL-1 is also an important mediator of the inflammatory process and it is usually used at doses between  $1\text{--}10 \text{ ng mL}^{-1}$  (63, 64, 79, 101), which are higher than the IL-1 levels detected in serum from patients with multiple myeloma ( $0.01 \text{ ng mL}^{-1}$ ) (55), Lyme arthritis ( $0.014 \text{ ng mL}^{-1}$ ) (83), or measured from



**FIG. 4.** The effects of  $\text{H}_2\text{O}_2$  signaling on NF- $\kappa$ B activation depend on the levels of TNF- $\alpha$ . Nuclear p65 (□) and reporter plasmid expression (■) levels, for 3 h and 4 h respectively, were analyzed in HeLa cells treated with the indicated concentrations of TNF- $\alpha$  (A) alone or (B) simultaneously with  $25 \mu\text{M}$  s.s.  $\text{H}_2\text{O}_2$ . p65 levels of the nuclear protein fraction were measured by Western blot, using the ImageJ software to quantify the band intensity (antibody sc-372, Santa Cruz Biotechnology, Santa Cruz, CA). HeLa cells were transfected (FugeneHD) with a plasmid containing the  $\kappa$ B site of the MHC class I gene coupled to a luciferase reporter gene and luminescence levels were measured using the Dual-Luciferase Reporter Assay System (Promega). (B) Modulation by  $\text{H}_2\text{O}_2$  is calculated as the ratio between the observed response for the simultaneous addition of  $\text{H}_2\text{O}_2$  and TNF- $\alpha$  over the response observed for TNF- $\alpha$  alone; in HeLa cells the effect of  $\text{H}_2\text{O}_2$  alone is null (76).

murine peritoneal macrophages ( $10^6 \text{ cells}$ ) stimulated with LPS ( $0.014 \text{ ng mL}^{-1}$ ) (115). Nevertheless, in a study with LPS-activated human monocytes, IL-1 attained a maximum of  $3 \text{ ng mL}^{-1}$  after 4 h incubation (57).

### NF- $\kappa$ B Regulation by $\text{H}_2\text{O}_2$

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

#### Receptors

TNF- $\alpha$  exerts its biological activity through binding to its cellular receptors, the ubiquitously expressed TNF-R1 and

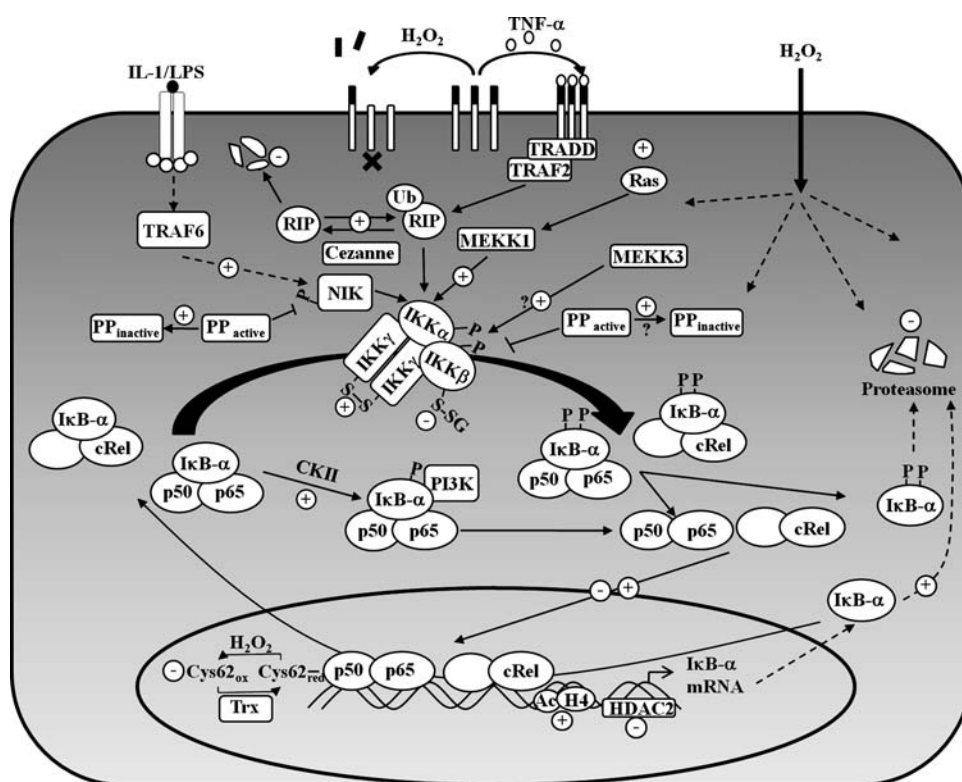
◀AU1

◀AU2



F5 ▶ also TNF-R2. Soluble TNF receptors (sTNF-R) are released by cleavage of the extracellular domain of the TNF-Rs and can compete for TNF- $\alpha$  binding, thereby serving as physiological attenuators of TNF- $\alpha$  activity (2). In A549 cells, a human pulmonary epithelial cell line, H<sub>2</sub>O<sub>2</sub> accelerates the shedding of TNF-R1 in a dose- (between 100  $\mu$ M and 1 mM) and time-dependent fashion (40), thereby downregulating TNF- $\alpha$ -dependent pathways, such as NF- $\kappa$ B activation (Fig. 5). The mechanism of this shedding does not involve modulation of TNF-R1 expression, but correlates with activation of protein kinase C and metalloproteinases. In addition, in C10 cells, a spontaneously transformed mouse alveolar type II epithelial cell line, H<sub>2</sub>O<sub>2</sub> (300  $\mu$ M) inhibits signal transduction at the level of TNF-R1 by inducing the degradation of receptor-interacting protein (RIP), a protein essential for IKK activation (80). Nevertheless, other processes dependent on the binding of TNF- $\alpha$  to TNF-R1, such as activation of c-Jun-N-terminal kinase (JNK) are activated by H<sub>2</sub>O<sub>2</sub>. For example, in C10 cells exposed to 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>, the adapter proteins TNF receptor-associated death domain (TRADD) and TNF-R associated factor 2 (TRAF2) are recruited to TNF-R1, leading to activation of JNK (80), showing that H<sub>2</sub>O<sub>2</sub> can have opposite effects in signaling pathways starting at the same receptor.

Lipid rafts are thought to be involved in recruiting the machinery of NF- $\kappa$ B activation in the plasma membrane, and represent a potential target for H<sub>2</sub>O<sub>2</sub> control. In effector T cells, most of the molecular machinery of NF- $\kappa$ B activation, including the adaptor proteins RIP1, TRAF2, and TRAF6, the members of the IKK complex, IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$ , and the upstream NF- $\kappa$ B regulators protein kinase C  $\theta$  (PKC  $\theta$ ), 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 H<sub>2</sub>O<sub>2</sub> regulates NF- $\kappa$ B activation at this level is unknown but recent evidences suggests that H<sub>2</sub>O<sub>2</sub> may control lipid raft formation: (a) raft-disrupting agents block the H<sub>2</sub>O<sub>2</sub>-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, H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M) 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- $\kappa$ B (99); and (d), in yeast the molecular mechanisms by which H<sub>2</sub>O<sub>2</sub> modulates lipid raft formation started to be uncovered, and they involve



**FIG. 5. Regulation of NF- $\kappa$ B activation by H<sub>2</sub>O<sub>2</sub>.** Schematic representation of the global events in NF- $\kappa$ B regulation described in the literature (view main text for details). Symbols  $\oplus$  and  $\ominus$  represent H<sub>2</sub>O<sub>2</sub> actions that either stimulate or inhibit the NF- $\kappa$ B pathway, respectively. H<sub>2</sub>O<sub>2</sub> promotes receptor shedding and, probably, clustering of the NF- $\kappa$ B machinery in the plasma membrane. H<sub>2</sub>O<sub>2</sub> is able to induce upstream kinases, such as Ras, MAPK family, and NIK, modulating the activity of the IKK complex. Increase of IKK activity is correlated with phosphorylation of Ser180 and Ser181 on the activation loops of IKK $\alpha$ , and IKK $\beta$  and with dimerization of IKK $\gamma$  proteins through disulfide bond formation, whereas S-glutathionylation of Cys179 in IKK $\beta$  inhibits IKK activity. Potentiating of NIK and IKK phosphorylation by H<sub>2</sub>O<sub>2</sub> is

also proposed as a result of protein phosphatase (PP) inhibition, such as PP2A. H<sub>2</sub>O<sub>2</sub> is able to prolong RIP poly-ubiquitination, and consequently IKK activity, by inhibiting the deubiquitinating protein Cezanne, but it may also stimulate RIP degradation, which will generate an opposite effect on IKK activity. IKK activation results in a higher translocation of NF- $\kappa$ B dimers to the nucleus, dependent or independently of I $\kappa$ B- $\alpha$  degradation. On the other hand, H<sub>2</sub>O<sub>2</sub> may decrease the activity of the proteasome, inhibiting NF- $\kappa$ B translocation. In an alternative pathway identified in lymphocytes, H<sub>2</sub>O<sub>2</sub> promotes I $\kappa$ B- $\alpha$  phosphorylation through CKII and PI3K, independently of IKK. H<sub>2</sub>O<sub>2</sub> is also able to inhibit the nuclear import of newly synthesized I $\kappa$ B- $\alpha$ , which will be degraded by the proteasome, thus maintaining NF- $\kappa$ B inside the nucleus for a longer time. Once in the nucleus, Cys62 of p50 needs to be reduced to enable NF- $\kappa$ B transactivation of the DNA, which is facilitated by H<sub>2</sub>O<sub>2</sub> through acetylation of histone H4 and inhibition of HDAC2. A more detailed description of the redox regulation of NF- $\kappa$ B is presented elsewhere in this Forum (35).

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  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  dose and cell type.

#### *$\text{H}_2\text{O}_2$ and the IKK complex*

The ability of  $\text{H}_2\text{O}_2$  to regulate IKK activity has been investigated by multiple groups. In mouse alveolar epithelial cells,  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ ) 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  $\text{mL}^{-1}$  TNF- $\alpha$ , preventing both I $\kappa$ B- $\alpha$  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  $\text{H}_2\text{O}_2$  (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  $\text{H}_2\text{O}_2$  slightly activates IKK, although at a later time than TNF- $\alpha$ , and observed a sustained activation of NF- $\kappa$ B when  $\text{H}_2\text{O}_2$  and TNF- $\alpha$  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  $\text{H}_2\text{O}_2$ -mediated IKK activation (Fig. 5, Table 2) (49). A recent study reported that IKK $\gamma$  dimers are linked through disulfide bonds formed between Cys54 and Cys347, a requirement for a correct NF- $\kappa$ B activation. In mouse fibroblasts,  $\text{H}_2\text{O}_2$  (50–500  $\mu\text{M}$ ) induces IKK $\gamma$  dimerization, but a pretreatment with 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  before the addition of TNF- $\alpha$  prevents IKK activation, probably by interfering with the IKK $\beta$  subunit (39).

Alternatively,  $\text{H}_2\text{O}_2$  may act upstream of IKK. In favor of this hypothesis, IL-1 $\beta$ -stimulated MCF-7 cells induce  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  (63).

#### *$\text{H}_2\text{O}_2$ , I $\kappa$ Bs, and NF- $\kappa$ B translocation to the nucleus*

In cells where  $\text{H}_2\text{O}_2$  activates NF- $\kappa$ B, there are somewhat conflicting views on the fate of I $\kappa$ B- $\alpha$  after the activation. In EL4 mouse lymphoma cells, phosphorylation of Ser32 and Ser36 residues in I $\kappa$ B- $\alpha$  is not required for NF- $\kappa$ B activation by  $\text{H}_2\text{O}_2$  alone (300  $\mu\text{M}$ ), whereas independent-IKK phosphorylation of both Tyr42 and PEST is essential (93). Phosphorylation of Tyr42 and PEST on I $\kappa$ B- $\alpha$  relies probably on casein kinase II (CKII) activity, with the Syk upstream-kinase as mediator (93,106). In fact,  $\text{H}_2\text{O}_2$  is a known inhibitor of protein tyrosine phosphatases (61), which can account for the increased I $\kappa$ B- $\alpha$  phosphorylation observed. Whether I $\kappa$ B- $\alpha$  phosphorylated on Tyr42 is subsequently degraded is not yet consensual (93, 106). It is important to note that I $\kappa$ B- $\alpha$  phosphorylation at Tyr42 residue induced by  $\text{H}_2\text{O}_2$  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 I $\kappa$ B- $\alpha$ , through its Src homology 2 domains, thereby sequestering I $\kappa$ B- $\alpha$  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  $\text{H}_2\text{O}_2$  is able to induce PI3K (86) and c-Src (1), these kinases may be important in  $\text{H}_2\text{O}_2$ -induced Tyr42 phosphorylation of I $\kappa$ B- $\alpha$ . NF- $\kappa$ B activation by hypoxia is probably dependent on the stimulation of ROS production by mitochondria (18,67). The importance of  $\text{H}_2\text{O}_2$  on this activation is not well characterized, but it is known that hypoxia also activates NF- $\kappa$ B through an  $\text{H}_2\text{O}_2$ -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 I $\kappa$ B- $\alpha$  at the typical serine residues (25).

In cells exposed to classical NF- $\kappa$ B inducers,  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  alone does not cause I $\kappa$ B- $\alpha$  degradation, but 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  together with 10 ng  $\text{mL}^{-1}$  TNF- $\alpha$  lead to a co-operative activation of NF- $\kappa$ B (Table 2) (46).  $\text{H}_2\text{O}_2$  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- $\alpha$  signaling through IKK. Similar results were obtained by us in HeLa and MCF-7 cells treated with 0.37 ng  $\text{mL}^{-1}$  TNF- $\alpha$ , in which 25  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in s.s. markedly increases I $\kappa$ B- $\alpha$  degradation (unpublished observation), resulting in an increased p65 translocation to the nucleus (Table 2) (76).

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

The differences in the results described above may be a consequence of the method of  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  delivery methods on p65 translocation to the nucleus, the s.s. titration method using a s.s. of 25  $\mu\text{M}$   $\text{H}_2\text{O}_2$  versus a bolus addition of 1 mM  $\text{H}_2\text{O}_2$  (76). Interestingly, in opposition to the s.s. method, where  $\text{H}_2\text{O}_2$  increases p65 translocation in TNF- $\alpha$ -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-exposition to  $\text{H}_2\text{O}_2$  in s.s. followed by the addition of TNF- $\alpha$  also inhibits NF- $\kappa$ B activation. Inhibitory effects on NF- $\kappa$ B activation were also observed in other studies that used pre-exposures to  $\text{H}_2\text{O}_2$  delivered either as a bolus (58) or by using glucose oxidase (123) (Table 2). So, oxidative modifications in molecular



mediators of the NF- $\kappa$ B pathway or in the global redox state of the cells are probably dictating the inhibition of signaling response.

#### *H<sub>2</sub>O<sub>2</sub> and DNA transactivation by NF- $\kappa$ B*

Sulfhydryl oxidizing agents, such as diamide, inhibit NF- $\kappa$ B binding to DNA *in vitro*, while reducing agents increase DNA binding (111). Jornot *et al.* (48) illustrated the ability of H<sub>2</sub>O<sub>2</sub> (1 mM) to induce NF- $\kappa$ B translocation to the nucleus in human endothelial cells, but without further transactivation of DNA. The Cys62 residue on the p50 subunit of NF- $\kappa$ B 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- $\kappa$ B binding to DNA (84). In favor of such mechanism, overexpression of Trx reverses Cys62 oxidation (72). Endogenous Trx is responsible for the maintenance of reducing conditions within the nucleus, so that NF- $\kappa$ B is able to bind to DNA (38). Therefore, different redox requirements in the cytosol and in the nucleus are necessary for an appropriate NF- $\kappa$ B activation. In the cytosol, a pro-oxidant signal may activate NF- $\kappa$ B and lead to its translocation to the nucleus but, when in the nucleus, NF- $\kappa$ B 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), H<sub>2</sub>O<sub>2</sub> modulates positively the expression of NF- $\kappa$ B-dependent genes (46, 76). This indicates that this modulation by H<sub>2</sub>O<sub>2</sub> is probably dependent on the cell type and on H<sub>2</sub>O<sub>2</sub> concentrations. Also, NF- $\kappa$ B 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 H<sub>2</sub>O<sub>2</sub> is a good topic to explore in order to understand the alterations of gene expression induced by H<sub>2</sub>O<sub>2</sub>.

Besides inducing modifications of NF- $\kappa$ B subunits, H<sub>2</sub>O<sub>2</sub> may also induce post-translational modifications of other proteins such as histones. In alveolar epithelial cells, H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) 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- $\kappa$ B-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- $\kappa$ B activation is increased.

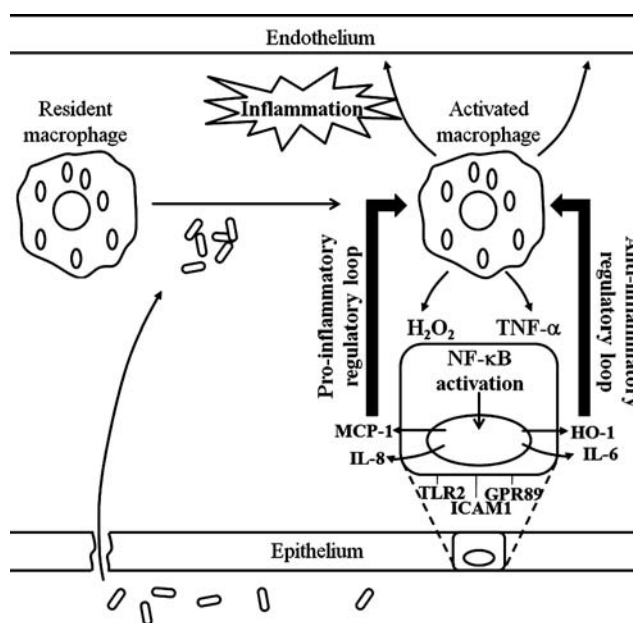
A recent work from Enesa *et al.* reported that H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) stimulates IL-1- and TNF- $\alpha$ -dependent activation of NF- $\kappa$ B transcription in HeLa cells by suppressing p65 export, thus prolonging its nuclear localization and binding to DNA (29). This effect resulted from the enhanced degradation of the newly synthesized I $\kappa$ B- $\alpha$ , which normally enters the nucleus in order to remove NF- $\kappa$ B from the DNA (Fig. 5).

#### **Dual Regulation of Inflammation by H<sub>2</sub>O<sub>2</sub>**

Besides the established germicide role for H<sub>2</sub>O<sub>2</sub> during the inflammatory response, a signaling role for H<sub>2</sub>O<sub>2</sub> has been hypothesized. Whether H<sub>2</sub>O<sub>2</sub> stimulates or inhibits inflammation has been under dispute because opposite results have been reported. We have found recently that H<sub>2</sub>O<sub>2</sub> at concen-

trations (12.5  $\mu$ M) close to the ones produced by leukocytes during an inflammatory response is able to have a synergistic effect with TNF- $\alpha$ , also at low concentrations (0.37 ng mL<sup>-1</sup>), 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- $\kappa$ B-dependent (76) (Fig. 6). This dual effect of H<sub>2</sub>O<sub>2</sub>, 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 H<sub>2</sub>O<sub>2</sub> has a crucial dual role during the inflammatory process (76).

The anti-inflammatory role of H<sub>2</sub>O<sub>2</sub> 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- $\alpha$ , and higher expression of NF- $\kappa$ B proteins, such as p65, c-Rel, and



**FIG. 6. Dual regulation of inflammation by H<sub>2</sub>O<sub>2</sub>.** Pathogens may enter into the tissues through a wound in the epithelium. After recognition of surface receptors of pathogens, resident macrophages become activated and liberate different cytokines, namely TNF- $\alpha$ , and chemokines to trigger inflammation and activate local endothelial and epithelial cells, and simultaneously produce ROS to attack the pathogen. Neighborhood epithelial cells are then exposed simultaneously to H<sub>2</sub>O<sub>2</sub> and to TNF- $\alpha$ . TNF- $\alpha$  alone is capable of activating NF- $\kappa$ B, but the presence of H<sub>2</sub>O<sub>2</sub> is important to upregulate NF- $\kappa$ B activation levels and therefore a specific set of genes, as indicated in the figure. The set of activated genes shows that H<sub>2</sub>O<sub>2</sub> has a fine-tuning regulation role, as it includes both pro-inflammatory genes, important to spread and increase the inflammatory levels, and anti-inflammatory genes, important to avoid an exacerbated inflammatory response.

◀ F6

p50, than healthy monocytes (13). Accordingly, in this disease I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , and I $\kappa$ B- $\epsilon$  are not upregulated, and thus an inflammatory condition with an aberrant NF- $\kappa$ B 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  $\mu$ M H<sub>2</sub>O<sub>2</sub> 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<sup>phox</sup> and gp91<sup>phox</sup>) decreases ROS, but in opposition to the expected, it increases the inflammatory response, due to an exacerbated NF- $\kappa$ B activation. Although the mechanism of action was not entirely elucidated, these results support the anti-inflammatory role of ROS (126).

On the other hand, H<sub>2</sub>O<sub>2</sub> is also related with pro-inflammatory conditions, since there are several studies reporting an upregulation of chemokines and adhesion molecules in the presence of H<sub>2</sub>O<sub>2</sub> (27, 59, 87).

DNA transactivation of NF- $\kappa$ B-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 different subunits of the NF- $\kappa$ B/Rel 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 H<sub>2</sub>O<sub>2</sub> in inflammation signaling has been also demonstrated by the regulation of IL-12, since addition of H<sub>2</sub>O<sub>2</sub> in the range of 62–250  $\mu$ M inhibits IL-12 p40 subunit expression in LPS-IFN- $\gamma$ -stimulated macrophages (53). This inhibition results from a differential regulation of NF- $\kappa$ B dimers, since H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> s.s. increases nuclear c-Rel in TNF- $\alpha$ -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 H<sub>2</sub>O<sub>2</sub> and TNF- $\alpha$  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- $\alpha$ , and other inflammatory mediators, with H<sub>2</sub>O<sub>2</sub>. NF- $\kappa$ B, as the principal regulator of inflammation, is targeted for both pro- and anti-inflammatory modulation by H<sub>2</sub>O<sub>2</sub>, which contributes for a more effective and controlled response.

### Selective Gene Expression by H<sub>2</sub>O<sub>2</sub>

From the previous section, it is clear that the modulating effect of H<sub>2</sub>O<sub>2</sub> on NF- $\kappa$ B dependent genes is not indiscriminate, as H<sub>2</sub>O<sub>2</sub> modulation is restricted to a subset of genes. This selective role of H<sub>2</sub>O<sub>2</sub> in gene expression is a general

property being also observed for genes that are NF- $\kappa$ B independent and in several organisms (118). The ability of H<sub>2</sub>O<sub>2</sub> to selectively stimulate a subset of genes while leaving others unchanged further supports its potential fine-tuning role as a regulator of NF- $\kappa$ B-dependent processes. The molecular basis for this selective stimulation is unknown, but several potential mechanisms have been suggested.

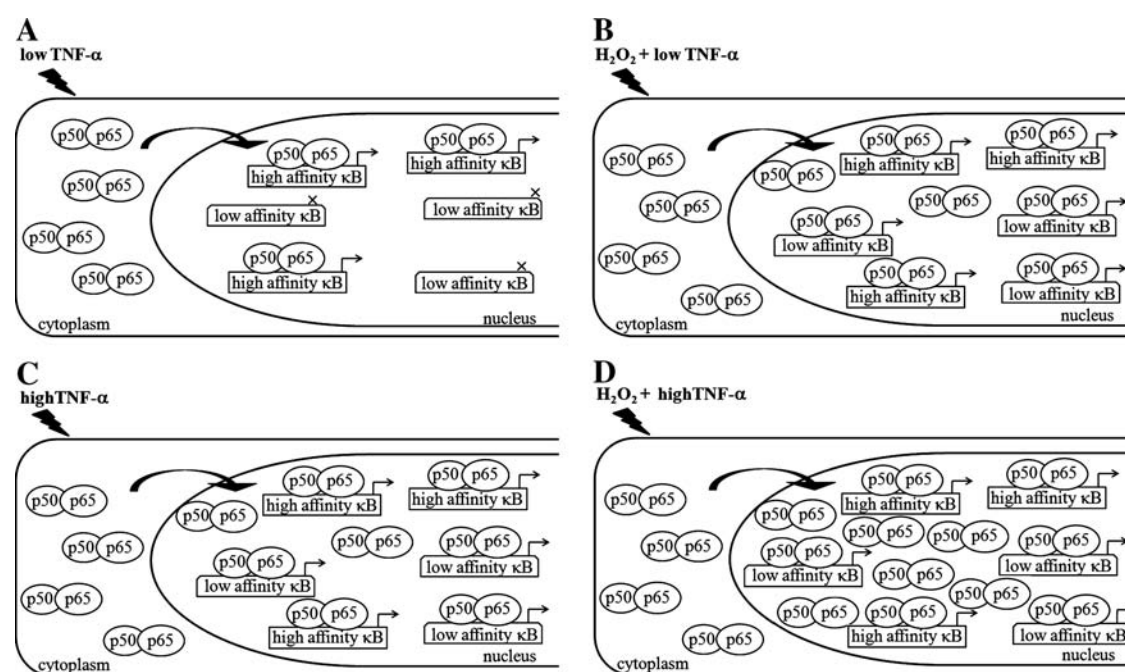
The control of the expression of a single gene is often done by several transcription factors and the expression pattern is a combination of the activation state of each transcription factor. Therefore, H<sub>2</sub>O<sub>2</sub> selective modulation of one gene could be attributed to the action of H<sub>2</sub>O<sub>2</sub> on a transcription factor, other than NF- $\kappa$ B. The induction of the HO-1 gene may represent such an example. This gene is under the control of both NF- $\kappa$ B and NF-E2-related factor-2 (NRF-2), but probably H<sub>2</sub>O<sub>2</sub> 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, H<sub>2</sub>O<sub>2</sub> 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- $\kappa$ B is subjected to many post-translational modifications (35). How these NF- $\kappa$ B modifications modulate gene expression depends on the  $\kappa$ B 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  $\kappa$ B sites that fit a GGRWWYYYYY 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  $\kappa$ B site). A second category that includes constructs with the KGRAHWTYCC 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  $\kappa$ B site), ICAM-1, IL-6, and I $\kappa$ B- $\alpha$ , and in endothelial cells, the failure of H<sub>2</sub>O<sub>2</sub> to induce ICAM-1 expression was attributed to a failure of H<sub>2</sub>O<sub>2</sub> to induce p65 phosphorylation (116). A third category is formed by constructs containing the GGGRATTYCC 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  $\kappa$ B promoter sequences may also lead to a differential gene expression in the presence of H<sub>2</sub>O<sub>2</sub>, due to changes in NF- $\kappa$ B affinity towards  $\kappa$ B promoter sites (Fig. 7). To investigate this hypothesis we transfected HeLa cells with three reporter genes bearing  $\kappa$ B sites with different affinities for NF- $\kappa$ B (77). Genes containing high affinity sites are less sensitive to a H<sub>2</sub>O<sub>2</sub> stimulatory effect on NF- $\kappa$ B-dependent activation than those containing the lower affinity site. Likewise, the stimulatory effect induced by H<sub>2</sub>O<sub>2</sub> on the expression of endogenous NF- $\kappa$ B-dependent genes is predominantly observed at low levels of TNF- $\alpha$ , while at higher levels of this cytokine the H<sub>2</sub>O<sub>2</sub> effects are small (77). These observations can be explained by considering that high affinity  $\kappa$ B

◀F7



**FIG. 7. H<sub>2</sub>O<sub>2</sub> is involved in the regulation of genes containing low-affinity κB sequences.** In the *left panels*, the translocation of NF-κB (p50/p65) to the nucleus in response to a low and a high concentration of TNF-α is represented. In the *right panels*, the contribution of H<sub>2</sub>O<sub>2</sub> to gene expression is shown. (A) Physiological concentrations of TNF-α (low TNF-α) are predicted to trigger the translocation of a few NF-κB molecules, just enough to induce the expression of NF-κB-dependent genes bearing high affinity κB sequences. (B) If H<sub>2</sub>O<sub>2</sub> is simultaneously present with the low TNF-α concentration, it will induce a higher concentration of nuclear NF-κB and therefore insure the expression of low-affinity NF-κB dependent genes, a situation predicted for the inflammatory process. (C) In experiments, cells are often subjected to higher and nonphysiological concentrations of TNF-α and thus more NF-κB molecules will translocate to the nucleus and κB sites will be filled until saturation. (D) Consequently, the higher nuclear levels of p50/p65 induced by H<sub>2</sub>O<sub>2</sub> are not important for the overall gene expression.

sites will be fully occupied even at moderate levels of NF-κB in the nucleus and so the higher NF-κB translocation induced by H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>. 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> whereas high affinity κB sites or conditions of strong NF-κB activation make genes relatively insensitive to the positive modulation by H<sub>2</sub>O<sub>2</sub>, while low affinity κB sites or conditions of weak NF-κB activation turn genes sensitive to the positive modulation by H<sub>2</sub>O<sub>2</sub>.

In conclusion, post-translation modifications of both p65 and histones, κB affinity towards NF-κB, and the combinatory action of NF-κB with other transcription factors provide a molecular arsenal by which H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> will be potentiated, attenuating at least partially the deleterious effect of the genetic alteration. Nevertheless, this enhanced regulating role of H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> depend on the dose and the method used for the delivery, any appropriate method should control the H<sub>2</sub>O<sub>2</sub> dose given per cell over time, and it should also mimic the endogenous cellular H<sub>2</sub>O<sub>2</sub> production. Steady-state titration with H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>. At



the expected  $\text{H}_2\text{O}_2$  intracellular levels attained in cells during normal aerobic metabolism—up to  $0.1\ \mu\text{M}$  (17)—NF- $\kappa$ B activation should be insensitive to any modulatory effect by  $\text{H}_2\text{O}_2$ . 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  $\text{H}_2\text{O}_2$  levels, as both these processes are activated by extracellular  $\text{H}_2\text{O}_2$  bolus concentrations between  $0.1$  and  $10\ \mu\text{M}$ . These concentrations probably lead to an  $\text{H}_2\text{O}_2$  intracellular steady-state near the predicted *in vivo*  $0.1\ \mu\text{M}$  range, if we consider both the gradients formed between extracellular and intracellular  $\text{H}_2\text{O}_2$  concentrations when  $\text{H}_2\text{O}_2$  is added externally (5) and the fast consumption of external  $\text{H}_2\text{O}_2$  by cells. At levels reached in the vicinity of inflammatory sites (around  $15\ \mu\text{M}$  (66, 108)),  $\text{H}_2\text{O}_2$  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,  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  on each gene is a formidable challenge, but this characterization is necessary to understand how  $\text{H}_2\text{O}_2$  modulates biological processes. It is of particular relevance to know the impact  $\text{H}_2\text{O}_2$  has on pathologies associated with polymorphisms and whether a redox intervention would be an appropriate strategy for such conditions. While in this review we have focused on the control of inflammation by  $\text{H}_2\text{O}_2$ , other biological processes controlled by NF- $\kappa$ B such as apoptosis could also be the target of  $\text{H}_2\text{O}_2$  control.

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### References

1. Abe J, Takahashi M, Ishida M, Lee JD, and Berk BC. c-Src is required for oxidative stress-mediated activation of big mitogen-activated protein kinase 1. *J Biol Chem* 272: 20389–20394, 1997.
2. Aderka D, Sorkine P, Abu-Abid S, Lev D, Setton A, Cope AP, Wallach D, and Klausner J. Shedding kinetics of soluble tumor necrosis factor (TNF) receptors after systemic TNF leaking during isolated limb perfusion. Relevance to the pathophysiology of septic shock. *J Clin Invest* 101: 650–659, 1998.
3. Anderson MT, Staal FJ, Gitler C, Herzenberg LA, and Herzenberg LA. Separation of oxidant-initiated and redox-regulated steps in the NF-kappa B signal transduction pathway. *Proc Natl Acad Sci USA* 91: 11527–11531, 1994.
4. Anrather J, Racchumi G, and Iadecola C. Cis-acting, element-specific transcriptional activity of differentially phosphorylated nuclear factor-kappa B. *J Biol Chem* 280: 244–252, 2005.
5. Antunes F and Cadenas E. Estimation of  $\text{H}_2\text{O}_2$  gradients across biomembranes. *FEBS Lett* 475: 121–126, 2000.
6. Antunes F and Cadenas E. Cellular titration of apoptosis with steady state concentrations of  $\text{H}_2\text{O}_2$ : submicromolar levels of  $\text{H}_2\text{O}_2$  induce apoptosis through Fenton chemistry independent of the cellular thiol state. *Free Radic Biol Med* 30: 1008–1018, 2001.
7. Antunes F, Han D, and Cadenas E. Relative contributions of heart mitochondria glutathione peroxidase and catalase to  $\text{H}_2\text{O}_2$  detoxification in *in vivo* conditions. *Free Radic Biol Med* 33: 1260–1267, 2002.
8. Belousov VV, Fradkov AF, Lukyanov KA, Staroverov DB, Shakhbazov KS, Tersikh AV, and Lukyanov S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat Methods* 3: 281–286, 2006.
9. Beraud C, Henzel WJ, and Baeuerle PA. Involvement of regulatory and catalytic subunits of phosphoinositide 3-kinase in NF-kappaB activation. *Proc Natl Acad Sci USA* 96: 429–434, 1999.
10. Biteau B, Labarre J, and Toledano MB. ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* 425: 980–984, 2003.
11. Brennan P and O'Neill LA. 2-mercaptoethanol restores the ability of nuclear factor kappa B (NF kappa B) to bind DNA in nuclear extracts from interleukin 1-treated cells incubated with pyrrolidine dithiocarbamate (PDTC). Evidence for oxidation of glutathione in the mechanism of inhibition of NF kappa B by PDTC. *Biochem J* 320: 975–981, 1996.
12. Brigelius-Flohe R. Tissue-specific functions of individual glutathione peroxidases. *Free Radic Biol Med* 27: 951–965, 1999.
13. Brown KL, Bylund J, Macdonald KL, Song-Zhao GX, Elliott MR, Falsafi R, Hancock RE, and Speert DP. ROS-deficient monocytes have aberrant gene expression that correlates with inflammatory disorders of chronic granulomatous disease. *Clin Immunol* 129: 90–102, 2008.
14. Bubici C, Papa S, Dean K, and Franzoso G. Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene* 25: 6731–6748, 2006.
15. Byun MS, Jeon KI, Choi JW, Shim JY, and Jue DM. Dual effect of oxidative stress on NF-kappaB activation in HeLa cells. *Exp Mol Med* 34: 332–339, 2002.
16. Canty TG, Jr., Boyle EM, Jr., Farr A, Morgan EN, Verrier ED, and Pohlman TH. Oxidative stress induces NF-kappaB nuclear translocation without degradation of IkappaB-alpha. *Circulation* 100: II361–II364, 1999.
17. Chance B, Sies H, and Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527–605, 1979.
18. Chandel NS, Trzyna WC, McClintock DS, and Schumacker PT. Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. *J Immunol* 165: 1013–1021, 2000.
19. Chang TS, Jeong W, Woo HA, Lee SM, Park S, and Rhee SG. Characterization of mammalian sulfiredoxin and its

- reactivation of hyperoxidized peroxiredoxin through reduction of cysteine sulfinic acid in the active site to cysteine. *J Biol Chem* 279: 50994–51001, 2004.
20. Chen LF and Greene WC. Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* 5: 392–401, 2004.
  21. Chen L, Fischle W, Verdin E, and Greene WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science* 293: 1653–1657, 2001.
  22. Chen XL, Zhang Q, Zhao R, and Medford RM. Superoxide, H<sub>2</sub>O<sub>2</sub>, and iron are required for TNF-alpha-induced MCP-1 gene expression in endothelial cells: Role of Rac1 and NADPH oxidase. *Am J Physiol Heart Circ Physiol* 286: H1001–H1007, 2004.
  23. Choi JJ, Choi J, Kang CD, Chen X, Wu CF, Ko KH, and Kim WK. Hydrogen peroxide induces the death of astrocytes through the down-regulation of the constitutive nuclear factor-kappaB activity. *Free Radic Res* 41: 555–562, 2007.
  24. Clark RA and Valente AJ. Nuclear factor kappa B activation by NADPH oxidases. *Mech Ageing Dev* 125: 799–810, 2004.
  25. Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F, Godson C, Nielsen JE, Moynagh P, Pouyssegur J, and Taylor CT. Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. *Proc Natl Acad Sci USA* 103: 18154–18159, 2006.
  26. Damas P, Ledoux D, Nys M, Vrindts Y, De Groote D, Franchimont P, and Lamy M. Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. *Ann Surg* 215: 356–362, 1992.
  27. DeForge LE, Preston AM, Takeuchi E, Kenney J, Boxer LA, and Remick DG. Regulation of interleukin 8 gene expression by oxidant stress. *J Biol Chem* 268: 25568–25576, 1993.
  28. Dudek EJ, Shang F, and Taylor A. H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress activates NF-kappa B in lens epithelial cells. *Free Radic Biol Med* 31: 651–658, 2001.
  29. Enesa K, Ito K, Luong IA, Thorbjornsen I, Phua C, To Y, Dean J, Haskard DO, Boyle J, Adcock I, and Evans PC. Hydrogen peroxide prolongs nuclear localization of NF-kappaB in activated cells by suppressing negative regulatory mechanisms. *J Biol Chem* 283: 18582–18590, 2008.
  30. Fan C, Li Q, Ross D, and Engelhardt JF. Tyrosine phosphorylation of I kappa B alpha activates NF kappa B through a redox-regulated and c-Src-dependent mechanism following hypoxia/reoxygenation. *J Biol Chem* 278: 2072–2080, 2003.
  31. Finkel T. Redox-dependent signal transduction. *FEBS Lett* 476: 52–54, 2000.
  32. Forman HJ. Use and abuse of exogenous H<sub>2</sub>O<sub>2</sub> in studies of signal transduction. *Free Radic Biol Med* 42: 926–932, 2007.
  33. Gilmore TD. Introduction to NF-kappaB: Players, pathways, perspectives. *Oncogene* 25: 6680–6684, 2006.
  34. Ginis I, Hallenbeck JM, Liu J, Spatz M, Jaiswal R, and Shohami E. Tumor necrosis factor and reactive oxygen species cooperative cytotoxicity is mediated via inhibition of NF-kappaB. *Mol Med* 6: 1028–1041, 2000.
  35. Gloire G and Piette J. Redox regulation of nuclear post-translational modifications during NF-kappaB activation. *Antioxid Redox Signal* xx: 000–000, 2009. [this issue]
  36. Halliwell B, Clement MV, and Long LH. Hydrogen peroxide in the human body. *FEBS Lett* 486: 10–13, 2000.
  37. Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, and Kikugawa K. Evidence that reactive oxygen species do not mediate NF-kappaB activation. *EMBO J* 22: 3356–3366, 2003.
  38. Hayashi T, Ueno Y, and Okamoto T. Oxidoreductive regulation of nuclear factor kappa B. Involvement of a cellular reducing catalyst thioredoxin. *J Biol Chem* 268: 11380–11388, 1993.
  39. Herscovitch M, Comb W, Ennis T, Coleman K, Yong S, Armstead B, Kalaitzidis D, Chandani S, and Gilmore TD. Intermolecular disulfide bond formation in the NEMO dimer requires Cys54 and Cys347. *Biochem Biophys Res Commun* 367: 103–108, 2008.
  40. Hino T, Nakamura H, Abe S, Saito H, Inage M, Terashita K, Kato S, and Tomoike H. Hydrogen peroxide enhances shedding of type I soluble tumor necrosis factor receptor from pulmonary epithelial cells. *Am J Respir Cell Mol Biol* 20: 122–128, 1999.
  41. Hirota K, Murata M, Sachi Y, Nakamura H, Takeuchi J, Mori K, and Yodoi J. Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *J Biol Chem* 274: 27891–27897, 1999.
  42. Hoffmann A, Leung TH, and Baltimore D. Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. *EMBO J* 22: 5530–5539, 2003.
  43. Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auburger P, Baeuerle PA, and Peyron JF. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell* 86: 787–798, 1996.
  44. Ito K, Hanazawa T, Tomita K, Barnes PJ, and Adcock IM. Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration. *Biochem Biophys Res Commun* 315: 240–245, 2004.
  45. Jamaluddin M, Wang S, Boldogh I, Tian B, and Brasier AR. TNF-alpha-induced NF-kappaB/RelA Ser(276) phosphorylation and enhanceosome formation is mediated by an ROS-dependent PKAc pathway. *Cell Signal* 19: 1419–1433, 2007.
  46. Janssen-Heininger YM, Macara I, and Mossman BT. Cooperativity between oxidants and tumor necrosis factor in the activation of nuclear factor (NF)-kappaB: Requirement of Ras/mitogen-activated protein kinases in the activation of NF-kappaB by oxidants. *Am J Respir Cell Mol Biol* 20: 942–952, 1999.
  47. Jaspers I, Zhang W, Fraser A, Samet JM, and Reed W. Hydrogen peroxide has opposing effects on IKK activity and IkappaBalpha breakdown in airway epithelial cells. *Am J Respir Cell Mol Biol* 24: 769–777, 2001.
  48. Jornot L, Petersen H, and Junod AF. Modulation of the DNA binding activity of transcription factors CREP, NFkappaB and HSF by H<sub>2</sub>O<sub>2</sub> and TNF alpha. Differences between *in vivo* and *in vitro* effects. *FEBS Lett* 416: 381–386, 1997.
  49. Kamata H, Manabe T, Oka S, Kamata K, and Hirata H. Hydrogen peroxide activates IkappaB kinases through phosphorylation of serine residues in the activation loops. *FEBS Lett* 519: 231–237, 2002.
  50. Kang S, Kim YB, Kim MH, Yoon KS, Kim JW, Park NH, Song YS, Kang D, Yoo KY, Kang SB, and Lee HP. Polymorphism in the nuclear factor kappa-B binding promoter region of cyclooxygenase-2 is associated with an increased risk of bladder cancer. *Cancer Lett* 217: 11–16, 2005.
  51. Karin M and Ben Neriah Y. Phosphorylation meets ubiquitination: The control of NF-[kappa]B activity. *Annu Rev Immunol* 18: 621–663, 2000.



52. Karlsson A and Dahlgren C. Assembly and activation of the neutrophil NADPH oxidase in granule membranes. *Antioxid Redox Signal* 4: 49–60, 2002.
53. Khan N, Rahim SS, Boddupalli CS, Ghousunnissa S, Padma S, Pathak N, Thiagarajan D, Hasnain SE, and Mukhopadhyay S. Hydrogen peroxide inhibits IL-12 p40 induction in macrophages by inhibiting c-rel translocation to the nucleus through activation of calmodulin protein. *Blood* 107: 1513–1520, 2006.
54. Korn SH, Wouters EF, Vos N, and Janssen-Heininger YM. Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of IkappaB kinase. *J Biol Chem* 276: 35693–35700, 2001.
55. Kuku I, Bayraktar MR, Kaya E, Erkurt MA, Bayraktar N, Cikim K, and Aydogdu I. Serum proinflammatory mediators at different periods of therapy in patients with multiple myeloma. *Mediators Inflamm* 2005: 171–174, 2005.
56. Kunsch C and Rosen CA. NF-kappa B subunit-specific regulation of the interleukin-8 promoter. *Mol Cell Biol* 13: 6137–6146, 1993.
57. Kwak DJ, Augustine NH, Borges WG, Joyner JL, Green WF, and Hill HR. Intracellular and extracellular cytokine production by human mixed mononuclear cells in response to group B streptococci. *Infect Immun* 68: 320–327, 2000.
58. Lahdenpohja N, Savinainen K, and Hurme M. Pre-exposure to oxidative stress decreases the nuclear factor-kappa B-dependent transcription in T lymphocytes. *J Immunol* 160: 1354–1358, 1998.
59. Lakshminarayanan V, Beno DW, Costa RH, and Roebuck KA. Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by H<sub>2</sub>O<sub>2</sub> and tumor necrosis factor-alpha in endothelial and epithelial cells. *J Biol Chem* 272: 32910–32918, 1997.
60. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 4: 181–189, 2004.
61. Lee SR, Kwon KS, Kim SR, and Rhee SG. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273: 15366–15372, 1998.
62. Lekstrom-Himes JA, Kuhns DB, Alvord WG, and Gallin JL. Inhibition of human neutrophil IL-8 production by hydrogen peroxide and dysregulation in chronic granulomatous disease. *J Immunol* 174: 411–417, 2005.
63. Li Q and Engelhardt JF. Interleukin-1beta induction of NFkappaB is partially regulated by H<sub>2</sub>O<sub>2</sub>-mediated activation of NFkappaB-inducing kinase. *J Biol Chem* 281: 1495–1505, 2006.
64. Li Q, Harraz MM, Zhou W, Zhang LN, Ding W, Zhang Y, Eggleston T, Yeaman C, Banfi B, and Engelhardt JF. Nox2 and Rac1 regulate H<sub>2</sub>O<sub>2</sub>-dependent recruitment of TRAF6 to endosomal interleukin-1 receptor complexes. *Mol Cell Biol* 26: 140–154, 2006.
65. Li Q and Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2: 725–734, 2002.
66. Liu X and Zweier JL. A real-time electrochemical technique for measurement of cellular hydrogen peroxide generation and consumption: Evaluation in human polymorphonuclear leukocytes. *Free Radic Biol Med* 31: 894–901, 2001.
67. Lluis JM, Buricchi F, Chiarugi P, Morales A, and Fernandez-Checa JC. Dual role of mitochondrial reactive oxygen species in hypoxia signaling: activation of nuclear factor-kappaB via c-SRC and oxidant-dependent cell death. *Cancer Res* 67: 7368–7377, 2007.
68. Lu SP, Lin Feng MH, Huang HL, Huang YC, Tsou WI, and Lai MZ. Reactive oxygen species promote raft formation in T lymphocytes. *Free Radic Biol Med* 42: 936–944, 2007.
69. Madrid LV, Mayo MW, Reuther JY, and Baldwin AS, Jr. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem* 276: 18934–18940, 2001.
70. Manna SK, Zhang HJ, Yan T, Oberley LW, and Aggarwal BB. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J Biol Chem* 273: 13245–13254, 1998.
71. Matthews JR, Kaszubska W, Turcatti G, Wells TN, and Hay RT. Role of cysteine62 in DNA recognition by the P50 subunit of NF-kappa B. *Nucleic Acids Res* 21: 1727–1734, 1993.
72. Matthews JR, Wakasugi N, Virelizier JL, Yodoi J, and Hay RT. Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res* 20: 3821–3830, 1992.
73. Mills GC. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. *J Biol Chem* 229: 189–197, 1957.
74. Misra RS, Russell JQ, Koenig A, Hinshaw-Makepeace JA, Wen R, Wang D, Huo H, Littman DR, Ferch U, Ruland J, Thome M, and Budd RC. Caspase-8 and c-FLIPL associate in lipid rafts with NF-kappaB adaptors during T cell activation. *J Biol Chem* 282: 19365–19374, 2007.
75. Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bauter MR, Kilty I, and Rahman I. Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. *FASEB J* 18: 1897–1899, 2004.
76. Oliveira-Marques V, Cyrne L, Marinho HS, and Antunes F. A quantitative study of NF- $\kappa$ B activation by H<sub>2</sub>O<sub>2</sub>: Relevance in inflammation and synergy with TNF- $\alpha$ . *J Immunol* 178: 3893–3902, 2007.
77. Oliveira-Marques V, Marinho HS, Cyrne L, and Antunes F. Modulation of NF-kappaB-dependent gene expression by H<sub>2</sub>O<sub>2</sub>: A major role for a simple chemical process in a complex biological response. *Antioxid Redox Signal* 11: 2009.
78. Otterbein LE, Bach FH, Alam J, Soares M, Tao LH, Wysk M, Davis RJ, Flavell RA, and Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6: 422–428, 2000.
79. Panopoulos A, Harraz M, Engelhardt JF, and Zandi E. Iron-mediated H<sub>2</sub>O<sub>2</sub> production as a mechanism for cell type-specific inhibition of tumor necrosis factor alpha-induced but not interleukin-1beta-induced IkappaB kinase complex/nuclear factor-kappaB activation. *J Biol Chem* 280: 2912–2923, 2005.
80. Pantano C, Shrivastava P, McElhinney B, and Janssen-Heininger Y. Hydrogen peroxide signaling through tumor necrosis factor receptor 1 leads to selective activation of c-Jun N-terminal kinase. *J Biol Chem* 278: 44091–44096, 2003.
81. Park HS, Jung HY, Park EY, Kim J, Lee WJ, and Bae YS. Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-kappa B. *J Immunol* 173: 3589–3593, 2004.

82. Pedroso N, Matias AC, Cyrne L, Antunes F, Borges C, Malho R, de Almeida RF, Herrero E, and Marinho HS. Modulation of plasma membrane lipid profile and microdomains by H(2)O(2) in *Saccharomyces cerevisiae*. *Free Radic Biol Med* 46: 289–298, 2009.
83. Pietruczuk A, Swierzbinska R, Pancewicz S, Pietruczuk M, and Hermanowska-Szpakowicz T. Serum levels of interleukin-18 (IL-18), interleukin-1beta (IL-1beta), its soluble receptor sIL-1RII and C-reactive protein (CRP) in patients with Lyme arthritis. *Infection* 34: 158–162, 2006.
84. Pineda-Molina E, Klatt P, Vazquez J, Marina A, Garcia dL, Perez-Sala D, and Lamas S. Glutathionylation of the p50 subunit of NF-kappaB: A mechanism for redox-induced inhibition of DNA binding. *Biochemistry* 40: 14134–14142, 2001.
85. Pinto RE and Bartley W. The effect of age and sex on glutathione reductase and glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates. *Biochem J* 112: 109–115, 1969.
86. Roberts ML and Cowser LM. Interleukin-1 beta and reactive oxygen species mediate activation of c-Jun NH2-terminal kinases, in human epithelial cells, by two independent pathways. *Biochem Biophys Res Commun* 251: 166–172, 1998.
87. Roebuck KA, Rahman A, Lakshminarayanan V, Janakidevi K, and Malik AB. H2O2 and tumor necrosis factor-alpha activate intercellular adhesion molecule 1 (ICAM-1) gene transcription through distinct cis-regulatory elements within the ICAM-1 promoter. *J Biol Chem* 270: 18966–18974, 1995.
88. Rossouw M, Nel HJ, Cooke GS, van Helden PD, and Hoal EG. Association between tuberculosis and a polymorphic NFkappaB binding site in the interferon gamma gene. *Lancet* 361: 1871–1872, 2003.
89. Rushworth SA and O'Connell MA. Haem oxygenase-1 in inflammation. *Biochem Soc Trans* 32: 1093–1094, 2004.
90. Sanjabi S, Hoffmann A, Liou HC, Baltimore D, and Smale ST. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc Natl Acad Sci USA* 97: 12705–12710, 2000.
91. Schafer FQ and Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30: 1191–1212, 2001.
92. Schmidt K, Amstad P, Cerutti P, and Baeuerle PA. The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-kB. *Chem Biol* 2: 13–22, 1995.
93. Schoonbroodt S, Ferreira V, Best-Belpomme M, Boelaert JR, Legrand-Poels S, Korner M, and Piette J. Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of I kappa B alpha in NF-kappa B activation by an oxidative stress. *J Immunol* 164: 4292–4300, 2000.
94. Schreck R, Meier B, Mannel DN, Droge W, and Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *J Exp Med* 175: 1181–1194, 1992.
95. Schreck R, Rieber P, and Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 10: 2247–2258, 1991.
96. Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, and Fiers W. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J Biol Chem* 267: 5317–5323, 1992.
97. Sen R and Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 46: 705–716, 1986.
98. Seng S, Avraham HK, Jiang S, Yang S, Sekine M, Kimelman N, Li H, and Avraham S. The nuclear matrix protein, NRP/B, enhances Nrf2-mediated oxidative stress responses in breast cancer cells. *Cancer Res* 67: 8596–8604, 2007.
99. Shen HM, Lin Y, Choksi S, Tran J, Jin T, Chang L, Karin M, Zhang J, and Liu ZG. Essential roles of receptor-interacting protein and TRAF2 in oxidative stress-induced cell death. *Mol Cell Biol* 24: 5914–5922, 2004.
100. Shi XZ, Lindholm PF, and Sarna SK. NF-kappa B activation by oxidative stress and inflammation suppresses contractility in colonic circular smooth muscle cells. *Gastroenterology* 124: 1369–1380, 2003.
101. Solt LA, Madge LA, Orange JS, and May MJ. Interleukin-1-induced NF-kappaB activation is NEMO-dependent but does not require IKKbeta. *J Biol Chem* 282: 8724–8733, 2007.
102. Staal FJ, Roederer M, Herzenberg LA, and Herzenberg LA. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proc Natl Acad Sci USA* 87: 9943–9947, 1990.
103. Stone JR and Collins T. Rapid phosphorylation of heterogeneous nuclear ribonucleoprotein C1/C2 in response to physiologic levels of hydrogen peroxide in human endothelial cells. *J Biol Chem* 277: 15621–15628, 2002.
104. Strassheim D, Asehnoune K, Park JS, Kim JY, He Q, Richter D, Mitra S, Arcaroli J, Kuhn K, and Abraham E. Modulation of bone marrow-derived neutrophil signaling by H2O2: Disparate effects on kinases, NF-kappaB, and cytokine expression. *Am J Physiol Cell Physiol* 286: C683–C692, 2004.
105. Suzuki YJ, Forman HJ, and Sevanian A. Oxidants as stimulators of signal transduction. *Free Radic Biol Med* 22: 269–285, 1997.
106. Takada Y, Mukhopadhyay A, Kundu GC, Mahabeleshwar GH, Singh S, and Aggarwal BB. Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: Evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. *J Biol Chem* 278: 24233–24241, 2003.
107. Tarpey MM and Fridovich I. Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ Res* 89: 224–236, 2001.
108. Test ST and Weiss SJ. Quantitative and temporal characterization of the extracellular H2O2 pool generated by human neutrophils. *J Biol Chem* 259: 399–405, 1984.
109. Thannickal VJ and Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 279: L1005–L1028, 2000.
110. Thorpe C, Hooper KL, Raje S, Glynn NM, Burnside J, Turi GK, and Coppock DL. Sulfhydryl oxidases: emerging catalysts of protein disulfide bond formation in eukaryotes. *Arch Biochem Biophys* 405: 1–12, 2002.
111. Toledano MB and Leonard WJ. Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction *in vitro*. *Proc Natl Acad Sci USA* 88: 4328–4332, 1991.

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112. Toninello A, Pietrangeli P, De Marchi U, Salvi M, and Mondovi B. Amine oxidases in apoptosis and cancer. *Biochim Biophys Acta* 1765: 1–13, 2006.
113. Torrie LJ, MacKenzie CJ, Paul A, and Plevin R. Hydrogen peroxide-mediated inhibition of lipopolysaccharide-stimulated inhibitory kappa B kinase activity in rat aortic smooth muscle cells. *Br J Pharmacol* 134: 393–401, 2001.
114. Tracey KJ. The inflammatory reflex. *Nature* 420: 853–859, 2002.
115. Tripathi S, Bruch D, and Kittur DS. Ginger extract inhibits LPS induced macrophage activation and function. *BMC Complement Altern Med* 8: 1, 2008.
116. True AL, Rahman A, and Malik AB. Activation of NF-kappaB induced by H<sub>2</sub>O<sub>2</sub> and TNF-alpha and its effects on ICAM-1 expression in endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 279: L302–L311, 2000.
117. Udalova IA, Mott R, Field D, and Kwiatkowski D. Quantitative prediction of NF-kappa B DNA-protein interactions. *Proc Natl Acad Sci USA* 99: 8167–8172, 2002.
118. Vandenbroucke K, Robbins S, Vandepoele K, Inze D, Van de PY, and Van Breusegem F. Hydrogen peroxide-induced gene expression across kingdoms: A comparative analysis. *Mol Biol Evol* 25: 507–516, 2008.
119. Veal EA, Day AM, and Morgan BA. Hydrogen peroxide sensing and signaling. *Mol Cell* 26: 1–14, 2007.
120. Viatour P, Merville MP, Bours V, and Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem Sci* 30: 43–52, 2005.
121. Wilson LA, Gemin A, Espiritu R, and Singh G. ets-1 is transcriptionally up-regulated by H<sub>2</sub>O<sub>2</sub> via an antioxidant response element. *FASEB J* 19: 2085–2087, 2005.
122. Wood ZA, Schroder E, Robin HJ, and Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 28: 32–40, 2003.
123. Wu M, Bian Q, Liu Y, Fernandes AF, Taylor A, Pereira P, and Shang F. Sustained oxidative stress inhibits NF-kappaB activation partially via inactivating the proteasome. *Free Radic Biol Med* 46: 62–69, 2009.
124. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, and Achong MK. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 101: 311–320, 1998.
125. Yang B, Oo TN, and Rizzo V. Lipid rafts mediate H<sub>2</sub>O<sub>2</sub> pro-survival effects in cultured endothelial cells. *FASEB J* 20: 1501–1503, 2006.
126. Yao H, Edirisinghe I, Yang SR, Rajendrasozhan S, Kode A, Caito S, Adenuga D, and Rahman I. Genetic ablation of NADPH oxidase enhances susceptibility to cigarette smoke-induced lung inflammation and emphysema in mice. *Am J Pathol* 172: 1222–1237, 2008.
127. Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol Rev* 74: 139–162, 1994.
128. Zahler S, Kupatt C, and Becker BF. Endothelial preconditioning by transient oxidative stress reduces inflammatory responses of cultured endothelial cells to TNF-alpha. *FASEB J* 14: 555–564, 2000.
129. Zmijewski JW, Zhao X, Xu Z, and Abraham E. Exposure to hydrogen peroxide diminishes NF-kappaB activation,

IkappaB-alpha degradation, and proteasome activity in neutrophils. *Am J Physiol Cell Physiol* 293: C255–C266, 2007.

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## Abbreviations Used

CARD11	= caspase recruitment domain family 11
CKII	= casein kinase II
COX-2	= cyclooxygenase-2
GO	= glucose oxidase
GPx	= glutathione peroxidase
GSH	= reduced form of glutathione
GSSG	= oxidized form of glutathione
H <sub>2</sub> O <sub>2</sub>	= hydrogen peroxide
HAT	= histone acetyltransferase
HDAC	= histone deacetylases
HRP	= horseradish peroxidase
IFN- $\gamma$	= interferon- $\gamma$
IKK	= I $\kappa$ B kinase
IL-1	= interleukin 1
iNOS	= inducible nitric oxide synthase
LPS	= lipopolysaccharide
MALT1	= mucosa associated lymphoid tissue lymphoma translocation gene 1
NAC	= N-acetyl-L-cysteine
NF- $\kappa$ B	= nuclear factor $\kappa$ B
(NF- $\kappa$ B) $\kappa$ B	= NF- $\kappa$ B and $\kappa$ B site complex
NIK	= NF- $\kappa$ B-inducing kinase
NRF-2	= NF-E2-related factor-2
PHD	= prolyl hydroxylase
PI3K	= phosphoinositide 3-kinase
PKC $\theta$	= protein kinase C $\theta$
PMA	= phorbol 12-myristate 13-acetate
Prx	= peroxiredoxins
RIP	= receptor interacting protein
ROS	= reactive oxygen species
SOD	= superoxide dismutase
s.s.	= steady-state
TAD	= transactivation domain
TNF- $\alpha$	= tumor necrosis factor- $\alpha$
TNF-R	= TNF- $\alpha$ receptor
TRADD	= TNF receptor-associated death domain
TRAF	= TNF-R associated factor
Trx	= thioredoxin



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