

Modulation of NF- κ B–Dependent Gene Expression by H₂O₂: A Major Role for a Simple Chemical Process in a Complex Biological Response

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Abstract

We recently observed that H₂O₂ regulates inflammation *via* upexpression of a few NF- κ B–dependent genes, while leaving expression of most NF- κ B–dependent genes unaltered. Here we test the hypothesis that this differential gene expression depends on the apparent affinity of κ B sites in the gene-promoter regions toward NF- κ B. Accordingly, cells were transfected with three reporter plasmids containing κ B sequences with different affinities for NF- κ B. It was observed that the lower the affinity, the higher the range of TNF- α concentrations where H₂O₂ upregulated gene expression. Mathematical models reproduced the key experimental observations indicating that H₂O₂ upregulation ceased when NF- κ B fully occupied the κ B sites. *In vivo*, it is predicted that genes with high-affinity sites remain insensitive to H₂O₂, whereas genes with lower-affinity sites are upregulated by H₂O₂. In conclusion, a simple chemical mechanism is at the root of a complex biologic process such as differential gene expression caused by H₂O₂. *Antioxid. Redox Signal.* 11, 2043–2053.

Introduction

HYDROGEN PEROXIDE (H₂O₂), the most abundant reactive oxygen species *in vivo*, has a central role in cell signaling (15, 31). The tight control of intracellular H₂O₂ levels and mild oxidative properties of H₂O₂ support such a role. H₂O₂ shows specificity to its redox-sensitive targets, particularly to cysteine residues in proteins, which on oxidation activate transcription factors such as OxyR in bacteria (41) and Yap1 in yeast (12); it also modulates signaling enzymes such as tyrosine phosphatases, which are inhibited (13), and tyrosine kinases, which are activated (8, 34). Other transcription factors and signaling molecules that are modulated by H₂O₂ include activator protein-1 (AP-1), hypoxia-inducible factor-1 (HIF-1), NF-E2–related factor-2 (NRF-2), and nuclear factor κ B (NF- κ B) (14), but the precise molecular oxidative events are not known.

In addition to these global actions on the cellular signaling network, H₂O₂ selectively modulates the expression of specific individual genes, leaving the expression of other genes unchanged, even when they share common transcription factors. For example, we recently observed that a low dose of H₂O₂ modulates the activation of only a handful set of genes

among 100 genes whose expression is dependent on NF- κ B (28). The molecular basis for this selectivity constitutes the topic of this work, and we use the modulation of NF- κ B–dependent genes activated by the pro-inflammatory cytokine tumor necrosis factor- α (TNF- α) as a test model. NF- κ B is a transcription factor with a key regulatory role in inflammation, adaptive immune response and apoptosis. The prototypical NF- κ B is a heterodimer composed of two proteins, the p65 and p50 subunits, which remains inactive in the cytosol bound to its inhibitory proteins, the I κ Bs. After a specific signal, such as the binding of TNF- α to its cell receptors, I κ Bs are phosphorylated by the I κ B kinase (IKK) complex and subsequently degraded by the 26S proteasome. Free NF- κ B migrates to the nucleus to activate the target genes. The biologic role of H₂O₂ as a regulator of NF- κ B activation is biphasic, because, in HeLa and MCF-7 cells, NF- κ B translocation into the nucleus is inhibited by high levels of H₂O₂ (millimolar range), whereas low doses of H₂O₂ (micromolar range) similar to those found *in vivo*, stimulate this translocation (28). Once inside the nucleus, NF- κ B binds to the promoter/enhancer regions of the target genes (27), the κ B sites, which have a general consensus sequence of GGGRNNYYCC (R is purine, Y is pyrimidine, and N is any base).

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The modulation of specific genes leaving others unchanged, even with an increase in the levels of nuclear NF- κ B, can be achieved in a number of ways. The transactivation potential of NF- κ B depends on modifications in the NF- κ B subunits and DNA, such as acetylations (10) and phosphorylations (3, 40) and also on the efficient recruitment of all the transcriptional machinery to form the enhanceosome, which is a dynamic binding-release process (5). All these processes depend on co-activators, such as histone acetyltransferases (HATs) and co-repressors, such as histone deacetylases (HDACs), whose recruitment is essential to coordinate transcription (32). Leung *et al.* (23) showed that even the conformation of the complex (NF- κ B| κ B), which can be changed by modification of only one nucleotide in the κ B site, determines the recruitment of the essential co-activators, controlling the initiation of transcription. All together, transcription is a tightly regulated and complex event, and each of these processes could be selectively targeted by H₂O₂, causing the upregulation of some genes but not others. However, ultimately, the interaction of NF- κ B with κ B binding sites is determined by the equilibrium between free NF- κ B, free κ B sites, and the complex (NF- κ B| κ B), and can be regarded as a simple chemical process. We hypothesized that for each κ B binding site (a) H₂O₂ is able to shift this equilibrium toward a higher degree of κ B site occupancy, by increasing NF- κ B translocation into the cell nucleus, and (b) the magnitude of this shift is dependent on the affinity of the κ B binding site to NF- κ B. It is important to note that a single modification of a nucleotide within a κ B site can change abruptly its affinity toward NF- κ B (38). According to our hypothesis, genes containing low- or medium-affinity κ B sites should be positively modulated by H₂O₂, whereas genes with high-affinity κ B sites should be mostly insensitive to H₂O₂, because occupancy of their κ B regions is already maximal in the presence of low TNF- α levels.

To test these predictions, HeLa cells were transfected with three plasmids coupled to a luciferase reporter gene containing κ B sequences with three different affinities. After transfection, cells were exposed to TNF- α in the absence or presence of a controlled steady-state (s.s.) concentration of H₂O₂ to check whether the profile of luciferase expression in the presence of H₂O₂ was dependent on the apparent affinity of the κ B sequence toward NF- κ B. Results obtained were successfully simulated by two kinetic mathematic models based on simple chemical equilibrium reactions.

Materials and Methods

Cell culture and reagents

HeLa cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% of fetal bovine serum, penicillin 100 U/ml, streptomycin, 100 μ g/ml, and L-glutamine, 2 mM, all from Lonza (Basel, Switzerland). Glucose oxidase (*Aspergillus niger*), tumor necrosis factor- α (TNF- α) (human recombinant), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). H₂O₂ was obtained from Merck & Co., Inc. (Whitehouse Station, NJ). All plasmid reporter vectors were kindly provided by Dr. Helena Soares (Instituto Gulbenkian de Ciéncia, Portugal).

Cells incubations

HeLa cells were counted and plated \sim 46 h before the experiment. Fresh medium was added to the cells 1 h before the incubations. H₂O₂ exposure was performed by using the steady-state titration (4); the method is extensively explained in (28). In brief, a steady-state level of H₂O₂ is maintained during the entire assay by adding, simultaneous with an initial dose of H₂O₂, a quantity of glucose oxidase enough to counteract H₂O₂ consumption by cells. All experiments were performed with an initial concentration of 25 μ M, ending at \sim 21 μ M. TNF- α was used at a range of 0.036–75 ng/ml.

Protein extraction and immunoblot analysis

HeLa cells were plated onto 100-mm dishes to achieve 1.5×10^6 cells per dish on the day of the experiment. Preparation of nuclear extracts and immunoblot assays was performed as described previously (28). The p65 analysis was done with antibodies sc-372 and sc-109 (Santa Cruz Biotechnology, Santa Cruz, CA), and the band intensity was quantified by using the ImageJ software (30), normalized to the protein loading (membrane stained with Ponceau S). Contamination of the nuclear fraction with cytosolic components was ruled out by performing controls with the methyl green-pyronin dye and by estimating, *via* immunoblot analysis, that in nontreated cells, nuclear p65 amounts are \sim 3% of total p65. If endogenous NF- κ B activation is considered (6), we concluded that contamination of nuclear fraction is negligible.

Plasmid constructs

The κ B-reporter plasmids were generated by inserting a minimal promoter in the pGL3-basic vector (Promega, Madison, WI) with BglII (5' end) and HindIII (3' end) restriction enzymes (New England Biolabs, Ipswich, England): 5'-GATCTGGGTATATAATGGATCCCCGGGTACGCAGCTCA-3'. Three different κ B sequences, which were chosen based on the work of Udalova *et al.* (38), were inserted upstream of the minimal promoter, between the *KpnI/SacI* restriction site, with the following general sequence: 5'-GCT- κ B-CTGGCTCCT- κ B-CTCAGCT-3' (Table 1).

Reporter gene assays

Cells were distributed onto 24-well plates at a density of 4.5×10^4 cells/well. After 24 h, the medium was replaced by RPMI 1640 without antibiotics, and the cells were transfected with 18 μ l of Opti-MEM containing a total of 360 ng of DNA (180 ng of κ B experimental plasmid, 9 ng of pRL-SV40 control plasmid, and 171 ng of pGL3-basic plasmid) and 0.9 μ l of fu-geneHD (Roche, Mannheim, Germany). Cells recovered for

TABLE 1. κ B SEQUENCES INSERTED IN THE PLASMID CONSTRUCT

Plasmid	Affinity	κ B sequence (5'-3')
κ Bl	Low	GGGACTTCC
κ Bm	Medium	GGGATTCCC
κ Bh	High	GGGAATTCC

24 h and then were exposed to different conditions for 4 h. Lysis and luciferase analyses were assayed by using the Dual-Luciferase Reporter Assay System (Promega), accordingly to manufacturer instructions. The κ B experimental plasmids are coupled with the firefly (F) luciferase gene, whereas the control vector used (pRL-SV40) contains the renilla (R) luciferase gene.

Luminescence was read with the luminometer Zenyth 3100 with 1 s of integration time, one sample at a time, because of the rapid decrease of the renilla signal. Each sample was read in triplicate, and firefly luciferase activity was normalized to renilla luciferase activity (F/R).

Viability assays

Cells were distributed in 96-well plates at a density of 7,500 cells/well. The procedure used for transfection was the same as described earlier, adding 3 μ l of the complex prepared. After exposure to TNF- α or H₂O₂ or both, cell viability was assessed by the ability of cells to reduce MTT reagent (25). In brief, the medium was changed, and cells were incubated for 2 h with MTT (0.5 mg/ml in PBS). Reduced MTT was dissolved by using dimethyl sulfoxide (DMSO), and the absorbance was read at 570 nm with reference at 630 nm using a Tecan Sunrise (Männedorf, Switzerland) microplate reader.

Mathematical modeling

A simple model formed only by the association-dissociation between free NF- κ B and κ B regions was set up. Equation 1, which describes this chemical equilibrium, was solved in order to (NF- κ B | κ B), yielding Equation 2.

$$K_{eq} = \frac{[(NF - \kappa B | \kappa B)]}{[NF - \kappa B_{free}][\kappa B_{free}]} \tag{1}$$

$$[(NF - \kappa B | \kappa B)] = \frac{1 + K_{eq}[\kappa B_{tot}] + K_{eq}[NF - \kappa B_{tot}] - \sqrt{-4[\kappa B_{tot}][NF - \kappa B_{tot}]K_{eq}^2 + (1 + K_{eq}[\kappa B_{tot}] + K_{eq}[NF - \kappa B_{tot}])^2}}{2K_{eq}} \tag{2}$$

(NF- κ B | κ B) represents the complex formed and ultimately is proportional to the activation of the reporter gene (Eq. 2); [NF- κ B_{free}] = [NF- κ B_{tot}] - [(NF- κ B | κ B)], [κ B_{free}] = [κ B_{tot}] - [(NF- κ B | κ B)], and K_{eq} is the equilibrium constant. We assumed a κ B_{tot} concentration of 10⁻³ μ M and three K_{eq} of 20, 200, and 2,000 μ M⁻¹ to represent the low-, medium-, and high-affinity sequences, respectively. The total nuclear NF- κ B concentration was assumed in the range of 10⁻⁴ to ~4 μ M.

The extended model was developed to introduce competition between the endogenous κ B and plasmidic κ B sites for a coactivator, cooperativity in the binding of NF- κ B to κ B sites, and endogenous luciferase expression in nontreated cells. Therefore, to represent the endogenous DNA, 100 hypothetical κ B sites were set up in the model with the same rate constant for the first binding of NF- κ B to the promoter region (24 μ M⁻¹s⁻¹) and a 10-times increase for the second binding to the promoter region, to simulate cooperative binding. The dissociation rate constant used for the higher-affinity κ B

sequence was 2.0 \times 10/s. The other 99- κ B sites were set up with a dissociation rate constant (k_d) according to the following geometric sequence: k_d for κ Bsite_{n+1} = (k_d for κ Bsite_n) \times 10^(1/25) (with n = 1, ..., 100 and for n = 1, k_d = 2.0 \times 10/s). The competition was restricted to those endogenous κ B sites that have a similar order-of-magnitude affinity to the plasmidic κ B region: for the κ Bh, κ Bm, κ Bi regions, the endogenous regions considered were regions 11 to 36, 30 to 55, and 50 to 75, respectively. The dissociation constants for the low-, medium-, and high-affinity sites in the plasmids were 1.8, 0.3, and 0.05 s⁻¹, respectively. The coactivator had a higher affinity to endogenous κ B sites, with an association constant of 2.4 \times 10⁴ μ M⁻¹s⁻¹, whereas the constant for plasmid DNA was 24 μ M⁻¹s⁻¹, with the same dissociation constant (2 per second). It was assumed that each of the plasmidic κ B concentrations was 5 \times 10⁻⁵ μ M; the coactivator concentration was 5 \times 10⁻⁴ μ M, and the nuclear NF- κ B concentration was 1 \times 10⁻⁴ μ M. Endogenous luciferase expression was modeled assuming (NF- κ B | κ B | co-activator) concentrations of 1.0 \times 10⁻¹¹, 4.8 \times 10⁻¹⁰, and 1.2 \times 10⁻⁹ μ M for the κ Bi, κ Bm, and κ Bh regions, respectively. This model could not be solved analytically; instead, we applied numeric simulation by using the PLAS software (39). The description of the mathematical model can be found in Tables 2, 3, and 4.

Statistical analysis

All data are presented as the average \pm standard deviation.

Results

H₂O₂ modulates NF- κ B translocation by TNF- α

In HeLa cells, H₂O₂ was not able to activate NF- κ B significantly, as shown in the representative experiment of Fig. 1A, unlike the classic NF- κ B activator TNF- α (Fig. 1B). Nevertheless, we recently showed that H₂O₂ modulates

NF- κ B by stimulating the activation caused by TNF- α , provided the H₂O₂ delivery to cells mimics *in vivo* conditions (28). This observation was obtained for a single dose of TNF- α . Here, these studies were extended by characterizing the action of H₂O₂ for a wide range of TNF- α concentrations, including physiologic levels of TNF- α (0.2–1.5 ng/ml) (1) and also doses that are much higher, but that are typically used in experimental settings (e.g., 10 ng/ml) (2, 16, 18, 35). Figure 1B shows a time course of NF- κ B activation by TNF- α at three different concentrations. The typical NF- κ B activation profile was observed, *i.e.*, a fast response to TNF- α exposure and a maximal activation that increased with TNF- α concentration. As described previously (28), H₂O₂ modulation is more significant after 2 h. Thus, analysis of the effect caused by H₂O₂ was performed at 3 h for the various TNF- α concentrations used (Fig. 1C). Figure 1C, middle panel, shows that NF- κ B activation by TNF- α had an hyperbolic dependence on TNF- α concentration with an half-maximal response obtained with a

TABLE 2. DESCRIPTION OF THE ABBREVIATIONS USED IN THE MODEL

Abbreviation	Description
kBsite	DNA endogenous κ B sites (1–100)
kBluc	Plasmidic κ B sites with gene reporter luciferase (kBh, kBm, and kBl)
NFkBnuc	Nuclear NF- κ B that binds to kBsite and kBluc
NFkBnuc2	Species bound to two NF- κ B
kbind	Association constant
kbind2	Association constant for the second binding of NFkBnuc
kdiss	Dissociation constant (k_d)
Coact	Co-activator

TNF- α concentration of 0.324 ± 0.126 ng/ml in the absence of H_2O_2 , and of 0.206 ± 0.092 ng/ml in the presence of $25 \mu M H_2O_2$. The maximal NF- κ B translocation (NF- κ B_{max}) induced by TNF- α was 6.59 ± 0.475 a.u., and 7.49 ± 0.582 a.u. in the presence of H_2O_2 ($p = 0.054$; $n = 4$). If these data are replotted by pairing same-day samples to avoid day-to-day variance, the stimulatory effect of H_2O_2 on TNF- α -dependent NF- κ B activation becomes clearer, being more pronounced for low physiologic TNF- α concentrations (Fig. 1C, lower panel). Altogether, these data indicate that H_2O_2 , at concentrations that can be found *in vivo* in an inflammatory environment (24, 37), is a positive modulator of NF- κ B activation by TNF- α , particularly for physiologic TNF- α concentrations.

TABLE 4. PARAMETER VALUES USED IN THE MODEL

Parameter	Value	Units
kBsite	5.0×10^{-5}	μM
kBluc	5.0×10^{-5}	μM
NFkBnuc	1.0×10^{-4}	μM
Coact	5.0×10^{-4}	μM
kdiss1	2.0×10^{-2}	s^{-1}
kdiss2	$Kdiss1 \times 10^{(1/25)}$	s^{-1}
⋮	⋮	⋮
kdiss100	$Kdiss99 \times 10^{(1/25)}$	s^{-1}
kbind	2.4×10	$\mu M s^{-1}$
kbind2	$kbind \times 10$	$\mu M s^{-1}$
kbindCoact	$2.4E \times 10^4$	$\mu M s^{-1}$
kdissCoact	2.0	s^{-1}
kbindluc	2.4×10	$\mu M s^{-1}$
kbind2luc	$kbindluc \times 10$	$\mu M s^{-1}$
kdissluc	kBh:0.05; kBm:1.8; kBl:0.3	s^{-1}
kbindCoactluc	2.4×10	$\mu M s^{-1}$
kdissCoactluc	2.0	s^{-1}

Effects of transfection: viability and NF- κ B activation

The doses of H_2O_2 and TNF- α used in this work did not damage HeLa cells (not shown). Conditions used for transfection in subsequent studies were also analyzed to check for possible interferences. Transfection decreased cell viability by $\sim 50\%$, when compared with nontransfected cells (Fig. 2). However, the addition of $25 \mu M H_2O_2$ *s.s.*, alone or simultaneously with either 10 or 50 ng/ml of TNF- α , did not lead to a loss of viability of transfected cells when compared with

TABLE 3. SET OF REACTIONS CONSIDERED IN THE MATHEMATICAL MODEL FOR THE ACTIVATION OF NF- κ B

	Reaction	Kinetic law
1	$kBsite1 + NFkBnuc \rightarrow kBsite1NFkBnuc$	$kbind \times kBsite1 \times NFkBnuc$
⋮	⋮	⋮
100	$kBsite100 + NFkBnuc \rightarrow kBsite100NFkBnuc$	$kbind \times kBsite100 \times NFkBnuc$
101	$kBsite1NFkBnuc \rightarrow kBsite1 + NFkBnuc$	$kdiss1 \times kBsite1NFkBnuc$
⋮	⋮	⋮
200	$kBsite100NFkBnuc \rightarrow kBsite100 + NFkBnuc$	$kdiss100 \times kBsite100NFkBnuc$
201	$kBsite1NFkBnuc + NFkBnuc \rightarrow kBsite1NFkBnuc2$	$kbind2 \times kBsite1NFkBnuc \times NFkBnuc$
⋮	⋮	⋮
300	$kBsite100NFkBnuc + NFkBnuc \rightarrow kBsite100NFkBnuc2$	$kbind2 \times kBsite100NFkBnuc \times NFkBnuc$
301	$kBsite1NFkBnuc2 \rightarrow kBsite1NFkBnuc + NFkBnuc$	$kdiss1 \times kBsite1NFkBnuc2$
⋮	⋮	⋮
400	$kBsite100NFkBnuc2 \rightarrow kBsite100NFkBnuc + NFkBnuc$	$kdiss100 \times kBsite100NFkBnuc2$
401	$kBsite1NFkBnuc2 + Coact \rightarrow kBsite1NFkBnuc2Coact$	$kbindCoact \times kBsite1NFkBnuc2 \times Coact$
⋮	⋮	⋮
500	$kBsite100NFkBnuc2 + Coact \rightarrow kBsite100NFkBnuc2Coact$	$kbindCoact \times kBsite100NFkBnuc2 \times Coact$
501	$kBsite1NFkBnuc2Coact \rightarrow kBsite1NFkBnuc2 + Coact$	$kdissCoact \times kBsite1NFkBnuc2Coact$
⋮	⋮	⋮
600	$kBsite100NFkBnuc2Coact \rightarrow kBsite100NFkBnuc2 + Coact$	$kdissCoact \times kBsite100NFkBnuc2Coact$
601	$kBluc + NFkBnuc \rightarrow kBlucNFkBnuc$	$kbindluc \times kBluc \times NFkBnuc$
602	$kBlucNFkBnuc \rightarrow kBluc + NFkBnuc$	$kdissluc \times kBlucNFkBnuc$
603	$kBlucNFkBnuc + NFkBnuc \rightarrow kBlucNFkBnuc2$	$kbind2luc \times kBlucNFkBnuc \times NFkBnuc$
604	$kBlucNFkBnuc2 \rightarrow kBlucNFkBnuc + NFkBnuc$	$kdissluc \times kBlucNFkBnuc2$
605	$kBlucNFkBnuc2 + Coact \rightarrow kBlucNFkBnuc2Coact$	$kbindCoactluc \times kBlucNFkBnuc2 \times Coact$
606	$kBlucNFkBnuc2Coact \rightarrow kBlucNFkBnuc2 + Coact$	$kdissCoactluc \times kBlucNFkBnuc2Coact$

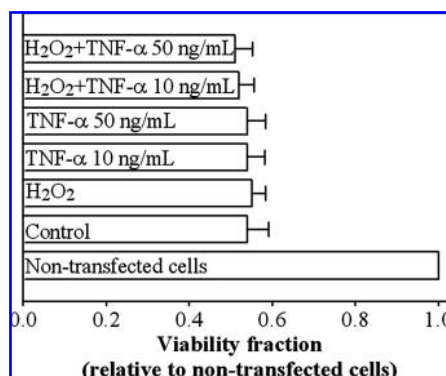
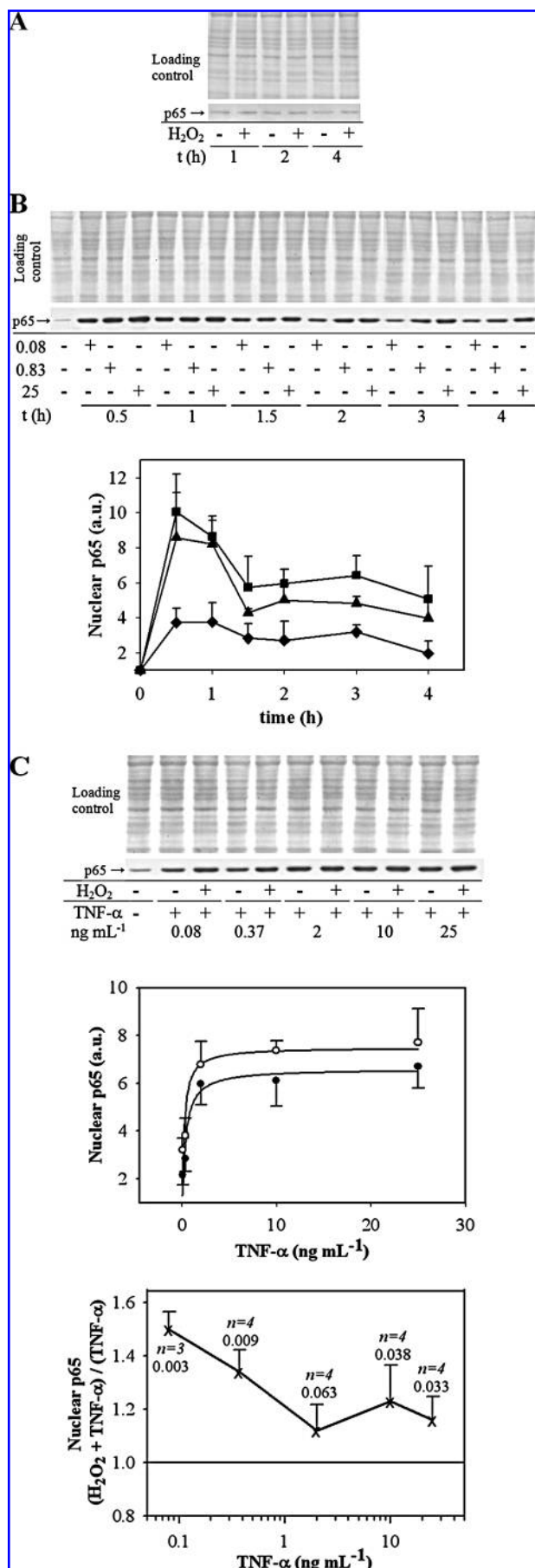


FIG. 2. Viability of transfected cells is not affected by H₂O₂ and TNF-α treatments. Viability was measured with the MTT assay both in nontransfected cells and transfected cells. Viability of transfected cells exposed to 25 μM H₂O₂ s.s. with or without 10 and 50 ng/ml TNF-α is also shown.

nontreated cells (control), which indicates that surviving cells completely recovered from the transfection stress. Moreover, transfected cells were seeded at such a density that the confluence at which incubations with TNF-α and H₂O₂ were carried out was similar to that in experiments with non-transfected cells.

We also checked whether transfection could alter NF-κB activation. Transfection had a slight effect on the basal levels of NF-κB activation, but did not change NF-κB activation in the presence of H₂O₂ or TNF-α or both (not shown).

The transcriptional activity of the reporter gene is dependent on its κB sites

To test our working hypothesis that the modulatory effect of H₂O₂ is dependent on the affinity of NF-κB toward the κB sites in the promoter region, HeLa cells were transfected with three plasmids coupled to a luciferase reporter gene with different κB sequences. The choice for the κB sequences was based on the data provided by Udalova *et al.* (38), who showed that minor changes in the κB region have a large impact on the affinity of NF-κB toward the κB region. In spite of just two nucleotide changes in the κB region of the plasmids

FIG. 1. H₂O₂ increases the translocation of NF-κB to the nucleus, in the presence of TNF-α. HeLa cells were treated for the indicated times with (A) 25 μM H₂O₂ s.s.; (B) 0.08 (◆), 0.83 (▲), and 25 (■) ng/ml TNF-α; and (C) for 3 h with TNF-α alone (●) or (○) simultaneously with H₂O₂ s.s., for the indicated TNF-α concentrations. Representative immunoblots of the nuclear p65 band are presented for each treatment (all n = 4), together with the respective membrane stained with Ponceau S (loading correction). In (B) and (C, upper panel), p65 intensity signal quantification is presented. (C, lower panel) Ratio of the p65 measured in cells incubated with both TNF-α and H₂O₂ over the p65 measured in cells incubated only with TNF-α. Data passed the Kolmogorov-Smirnov normality test and were compared with 1 (no modulation by H₂O₂) by using the one-tailed Student's *t* test, except for the TNF-α concentration of 2 ng/ml. In this latter case, the nonparametric sign test was used instead. The *p* and *n* values are indicated.

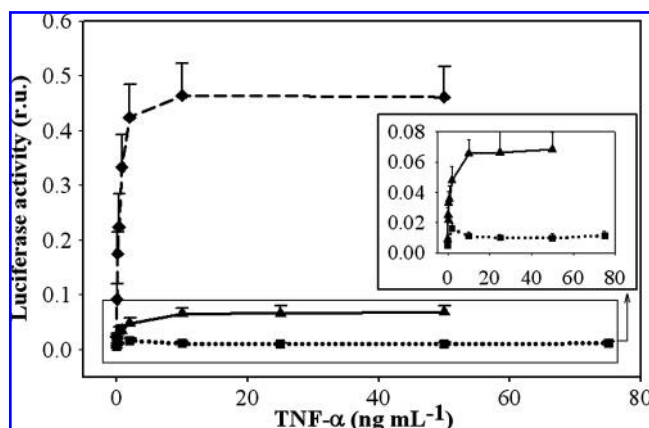


FIG. 3. Luciferase activity is dependent on the κ B reporter plasmid. Cells transfected with κ Bl (■), κ Bm (▲), and κ Bh (◆) reporter plasmids were exposed to increasing concentrations of TNF- α in the range of 0.016–75 ng/ml for 4 h, and luciferase activity was measured (F/R).

used (Table 1), the levels of luciferase activity measured for each plasmid showed large differences (Fig. 3). Taking into account that all other factors were maintained constant, and that the plasmids had similar transfection efficiencies, the differences obtained in luciferase activity were probably a consequence of the apparent affinity of NF- κ B toward that particular κ B site.

The three reporter plasmids were named according to their apparent affinity: κ Bh, κ Bm, and κ Bl for the highest-, medium-, and lowest-affinity plasmids, respectively (Table 1 and Fig. 3). Because nuclear levels of NF- κ B were augmented with increasing TNF- α concentrations (Fig. 1C), the binding of NF- κ B to κ B sites is also expected to increase with TNF- α concentration, until reaching saturation. The luciferase expression of the constructs κ Bm and κ Bh fitted a hyperbolic curve, with similar half-maximal responses (0.390 ± 0.112 and 0.349 ± 0.035 ng/ml, respectively), which suggests that the saturation observed was due not to binding to a κ B region, but to a process occurring at an earlier stage. Moreover, these half-maximal responses were close to those obtained for the p65 nuclear translocation in Fig. 1C, indicating that a limiting step occurs before translocation, possibly at the level of either the TNF- α receptor, IKK activation, or proteasome activity. The maximal level of luciferase was 0.0653 ± 0.004 a.u. for the κ Bm construct and 0.476 ± 0.011 a.u. for the κ Bh construct, and so the apparent affinity of κ Bh was 7 times higher than that of κ Bm. Concerning the κ Bl sequence, a similar hyperbolic behavior was observed, but the error associated with the measurement of low levels of luciferase was too high to allow a meaningful fitting. Even so, by using the maximal value of luciferase activity, it could be inferred that the apparent affinity of this κ B sequence toward NF- κ B was ~ 6 times lower than that of the κ Bm sequence. These results confirm that changing just two nucleotides within the κ B site can originate different transcriptional activities.

H₂O₂ modulates the transcriptional activity of the reporter genes

By assuming that the binding of NF- κ B to the free κ B sites follows a dynamic equilibrium, it should be expected that an

TABLE 5. LUCIFERASE EXPRESSION IS NOT ALTERED BY H₂O₂

Plasmid	Control $\times 10^{-3}$	H ₂ O ₂ $\times 10^{-3}$
κ Bl	3.1 ± 0.2	3.1 ± 0.4
κ Bm	8.4 ± 0.9	8.3 ± 0.8
κ Bh	25.2 ± 8.0	26.3 ± 8.8

Luciferase activity (F/R) for nontreated (control) and H₂O₂-treated (25 μ M s.s.) HeLa cells for 4 h. $n = 4$ for κ Bl, $n = 3$ for κ Bm, and $n = 5$ for κ Bh.

increase of nuclear p65 stimulated by the presence of H₂O₂, in TNF- α -treated cells (Fig. 1), would favor complex formation between free NF- κ B and free κ B sites, therefore increasing luciferase expression. H₂O₂ alone did not induce significantly the luciferase expression (Table 5), which is in agreement with the negligible NF- κ B translocation observed in the presence of H₂O₂ (Fig. 1A). As expected, when added simultaneously with TNF- α , H₂O₂ induced synergistic effects on luciferase expression but, surprisingly, it also induced antagonistic effects (Fig. 4). These opposing effects were dependent on the TNF- α concentration used, with synergistic effects being observed in a wide range of low TNF- α concentrations and antagonistic effects observed in a narrow range of high TNF- α concentrations. Concerning the low-affinity κ Bl construct, H₂O₂ stimulated significantly the luciferase expression for TNF- α concentrations up to 25 ng/ml. This maximal TNF- α concentration when synergistic effects induced by H₂O₂ occurred, decreased to 0.83 ng/ml for the medium-affinity κ Bm construct, and to 0.08 ng/ml for the high-affinity κ Bh construct. An unexpected observation was that for all concentrations of TNF- α studied, maximum synergistic effects in luciferase expression were $\approx 10\%$, whereas H₂O₂ synergistic

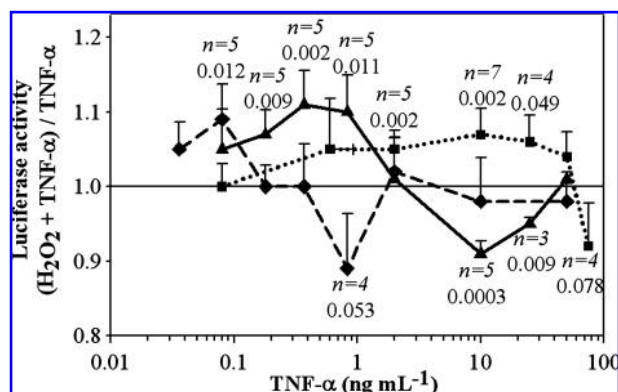


FIG. 4. H₂O₂ modulation of reporter plasmid expression is dependent on both the TNF- α concentration and the κ B sequence on the gene. Cells transfected with κ Bl (■), κ Bm (▲), and κ Bh (◆) constructs were exposed to TNF- α concentrations in the range of 0.016–75 ng/ml with or without of 25 μ M H₂O₂ s.s. for 4 h. The ratio of the luciferase activity measured in the presence and absence of H₂O₂ of same-day paired samples is shown. Experimental data passed the Kolmogorov–Smirnov normality test and were compared with 1 (no modulation by H₂O₂) by using the two-tailed Student's t test. The n and p values are indicated for data at a significance level $< 10\%$.

stimulation of translocation was $\approx 50\%$ for physiologic doses of TNF- α (Fig. 1C). Because H₂O₂ is involved in the activation of other transcription factors, such as AP-1 (29), that are dependent on the same co-activators, one possible explanation may rely on a competition with other transcription factors for the same co-activators and co-repressors. Another possible explanation is based on the activation by H₂O₂ of the early growth-response protein-1 (Egr-1). Egr-1 has a zinc-finger domain that interacts with the p65 subunit, inhibiting NF- κ B-dependent gene expression (7).

To help the interpretation of these results, a minimal model for the binding of NF- κ B to κ B sites based on a simple equilibrium was set up. In this simple model, H₂O₂ stimulated the formation of the complex (NF- κ B | κ B) for a wide range of NF- κ B concentrations. This positive modulation occurred within a wider interval of NF- κ B concentrations for the low-affinity κ Bl sequence than for the high-affinity κ Bh sequence (Fig. 5A). These results can be understood by considering that when low concentrations of NF- κ B are present in the nucleus (*i.e.*, when low concentrations of TNF- α are applied to the cell), the increase of nuclear NF- κ B induced by H₂O₂ increases the formation of the complex (NF- κ B | κ B) (*i.e.*, H₂O₂ has a synergistic effect). For higher nuclear NF- κ B concentrations, the occupancy of κ B sites also increases until they are fully occupied by NF- κ B. At this point, a further increase of nuclear NF- κ B, as caused by H₂O₂, does not increase the occupancy of κ B sites, because the occupancy is already maximal. For higher-affinity κ B sites, the formation of the complex is favored, which means that κ B sites become totally occupied at a lower NF- κ B concentration. Thus, the higher the affinity of NF- κ B toward a κ B site, the lower the concentration of NF- κ B (or TNF- α) at which H₂O₂ stops having a synergistic action.

The minimal model was extended because it was not able to reproduce three important experimental features:

1. The antagonistic effect of H₂O₂ on the transcriptional activity of the reporter genes observed experimentally at higher TNF- α concentrations (Fig. 4). A priori, such behavior could be explained by a competition for a transcriptional co-activator between the κ B sites of the endogenous genes and the κ B sites of the reporter genes bound to NF- κ B. An example of such co-activator is the cyclic AMP response element binding proteins (CREB)-binding protein/adenoviral protein E1A (CBP/p300), indispensable for the activation of transcription by NF- κ B (9). CBP/p300 exists in limiting quantities, and the competition between different promoters can result in some downregulation of gene transactivation (19). Therefore, κ B regions simulating the endogenous genes that compete with the luciferase reporter genes for a hypothetical co-activator were introduced in the model.
2. In the minimal model, a large change in nuclear NF- κ B concentration is needed for the synergistic effect of H₂O₂ to disappear, as opposed to the experimental results in which the transition from synergism to no effect is observed in a narrow range of TNF- α concentrations (Fig. 4). Such steep transitions are often an indication of cooperativity, and it has been shown that NF- κ B binding to DNA involves cooperativity [*e.g.*, between two or more κ B regions within the same gene, as observed in the IL-1 β and iNOS (17,36) promoters, or by the coop-

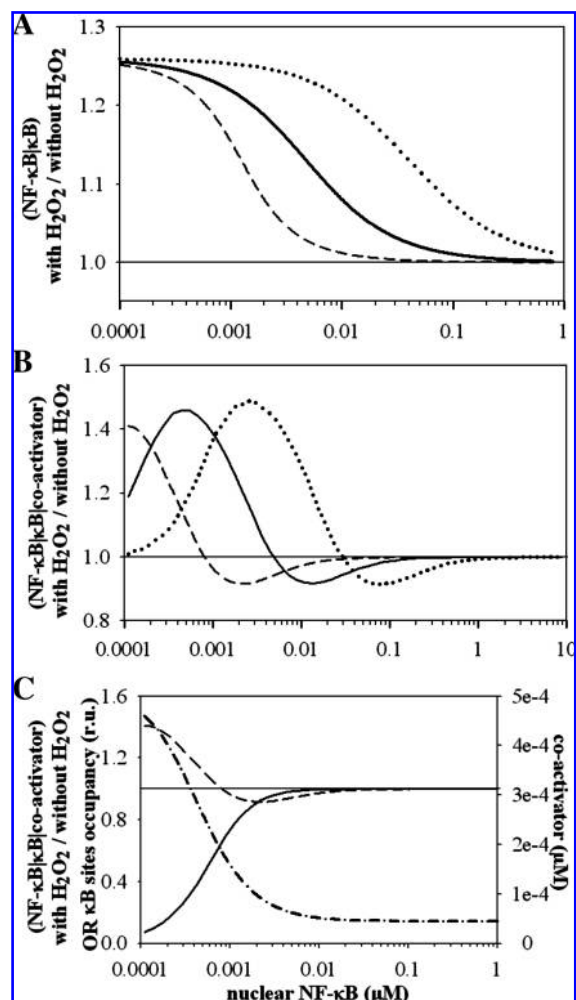


FIG. 5. Mathematical modeling predicts a differential modulation of gene expression by H₂O₂ based on the affinity constants of the κ B regions toward NF- κ B. (A) The simple model in which the concentration of the complex (NF- κ B | κ B) is given by Eq. 2 was used; the ratio of this complex, with and without H₂O₂, is plotted as a function of the NF- κ B concentration, for three different κ B sites (κ Bl, dotted line; κ Bm, solid line; κ Bh, dashed line). The effect of H₂O₂ was modeled by assuming a 26% increase of the nuclear NF- κ B concentration. (B) As in (A), but the extended model with cooperativity in the binding of NF- κ B between two κ B sites within the same gene, competition for a co-activator and basal luciferase expression was used. The ratio between the (NF- κ B | κ B | co-activator) complex with and without H₂O₂ is plotted. (C) In addition to this ratio (dashed line), the concentration of the coactivator (dashed-dotted line) and the occupancy of the κ B sites (solid line) for the κ Bh case are represented. For details, see Materials and Methods.

erative binding of the NF- κ B dimer to the κ B region in the promoters]. Therefore, cooperativity for NF- κ B binding to κ B sites was included in the model.

3. Although the experimental results show that the synergism induced by H₂O₂ increases for low TNF- α concentrations, in the minimal model, the synergism is constant in the low range of NF- κ B concentrations. This discrepancy probably occurs both because of

the lack of experimental sensitivity when measuring the very low luciferase expression obtained at very low TNF- α levels and also because in the model, the existence of an endogenous luciferase expression in the absence of TNF- α was not considered. Therefore, this endogenous luciferase expression was introduced in the extended model.

With this improved model, the main discrepancies between experimental and simulation results were eliminated (Fig. 5B). In addition, the model provided a possible interpretation for some of the less-intuitive experimental observations. Concerning the antagonism, at a certain critical NF- κ B level when almost all the κ B sites of the plasmids become occupied, additional NF- κ B will bind to other κ B regions in the endogenous DNA having lower-affinity sequences. The outcome is an increased competition for the transcriptional co-activator and, consequently, its displacement to the endogenous κ B sites, decreasing luciferase expression—antagonism (Fig. 5C). This antagonism disappears if NF- κ B concentrations are further increased, and at high NF- κ B concentrations luciferase activity was not modulated by H₂O₂. According to the model, this recovery was caused by the near saturation of the endogenous κ B regions, which decreases the competition with the transcriptional co-activator. Taken together, the modulation by H₂O₂ of NF- κ B-dependent genes activated by TNF- α was dependent on both the affinity of the κ B region toward NF- κ B and the level of TNF- α , thus providing a rationale for the selective modulation of a subset of genes.

H₂O₂ modulates the transcriptional activity of endogenous genes

Next, the modulation of the expression of endogenous genes by H₂O₂ was evaluated. It should be mentioned that analysis of individual genes can be problematic because it is difficult to estimate the apparent affinity of κ B regions *in vivo* (21–23) and because other effects besides κ B affinity, such as modulation of co-activators or repressors by H₂O₂, also are important to predict how H₂O₂ modulates the expression of a particular gene. Therefore, the approach used was to evaluate the influence of H₂O₂ on the global pattern of expression of 100 NF- κ B-dependent genes induced by TNF- α . The results of the reanalysis of data from (28) show a pattern (Fig. 6) consistent with the apparent affinity of the κ B regions being an important mechanism by which H₂O₂ modulates NF- κ B-dependent gene expression. For genes with low-affinity κ B regions, in which it is expected that the increase of nuclear NF- κ B caused by H₂O₂ is reflected in an increased gene expression, H₂O₂ had a synergistic action on TNF- α -dependent gene expression. This synergism decreased and even transformed into an antagonism for some genes with κ B regions with middle affinity, for which competition with endogenous co-activators is expected to be important. Finally, for genes with high-affinity κ B regions, the modulation effects tend to disappear. This should be expected because of the saturation in κ B regions with NF- κ B and of the lack of competition with co-activators.

Discussion

We provided evidence that a simple chemical equilibrium principle based on the association–dissociation between NF-

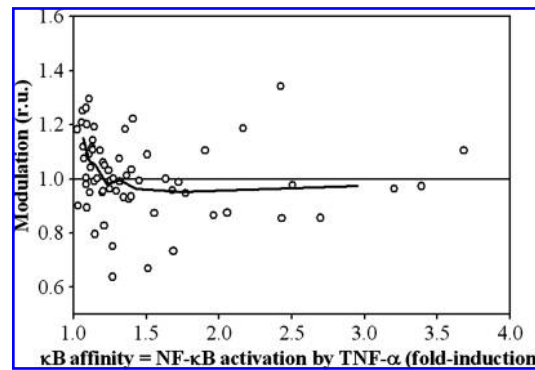


FIG. 6. H₂O₂ modulation of endogenous genes in HeLa cells. Data were taken from (28). In brief, HeLa cells were exposed to 0.37 ng/ml TNF- α and 12.5 μ M H₂O₂ s.s. alone or simultaneously for 6 h. Modulation by H₂O₂ was obtained by calculating the increase of p65 levels in the presence of both TNF- α and H₂O₂ relative to the sum of the individual effects: [(nuclear p65)_{TNF- α +H₂O₂}]/[(nuclear p65)_{TNF- α} + (nuclear p65)_{H₂O₂} - 1]. A value of 1 means that the effects of TNF- α and H₂O₂ are additive; values higher/lower than 1 imply synergism/antagonism between the two agents. The apparent affinity of κ B regions was estimated from the induction of gene expression caused by TNF- α . The rationale was that gene expression is dependent on the apparent affinity of the κ B region; so, on average, genes that are highly activated by TNF- α would tend to have a κ B region with higher apparent affinity than those that are poorly or not activated by TNF- α . A continuous line was obtained by calculating the median of clusters of consecutive eight points, but clusters with other number of points gave identical results. This line reveals a pattern in which synergism is high for genes with low-affinity κ B regions. As the apparent affinity increases, this synergism decreases, reaching even negative values (antagonism) for some genes, and at high apparent affinity, the synergism tends to 1 (no modulation by H₂O₂).

κ B and κ B sites in the promoter region of genes may explain the selective modulation of some genes by H₂O₂, leaving other genes unchanged. This explanation has predictive power because it is based on the affinity of the promoter regions toward the transcription factor.

Based on our results, several general predictions can be made concerning the role of H₂O₂ as a regulator of inflammation. During inflammation, some of the H₂O₂ generated during phagocytosis leaks from the phagosome. Therefore, cells present at inflammation sites are simultaneously subjected to H₂O₂ and other pro-inflammatory cytokines, such as TNF- α . In the present work, three NF- κ B-dependent reporter genes that simulate three hypothetical genes with different affinities toward NF- κ B were used to investigate the differential activation of genes. Although a broad range of TNF- α concentrations was investigated, in most *in vivo* situations, TNF- α levels are within the range 0.2 and 1.5 ng/ml. We predict that in this range, genes bearing high-affinity sites are probably not modulated by H₂O₂, because TNF- α will probably trigger NF- κ B translocation to the nucleus at high enough levels to occupy high-affinity κ B sites fully. Thus, these genes will be insensitive to further increases in NF- κ B translocation caused by H₂O₂. Conversely, genes containing

medium-affinity κ B sites and, particularly, genes containing low-affinity κ B sites, are predicted to have their expression upregulated by H₂O₂ during inflammation. The analysis of the influence of H₂O₂ on the global pattern of expression of 100 NF- κ B-dependent genes induced by TNF- α performed in HeLa cells supports these predictions. Making concrete predictions to identify specific genes that are expected to be upmodulated or unchanged by H₂O₂ is difficult. First, the affinity of κ B regions is not uniquely defined by the nucleotide sequence in the κ B region. Instead, the context of the neighboring sequence, either in the linear DNA sequence or in the tridimensional spatial organization of DNA, affects the affinity of the sequence toward NF- κ B (21, 22). Thus, the κ B affinities obtained *in vitro* with the isolated sequence cannot be straightforwardly extrapolated to the cellular environment. Second, promoter or enhancer regions of most genes can have more than one κ B site, including the presence of both high- and low-affinity κ B sites in the same gene. For example, Taylor *et al.* (36) showed that the different κ B regions within the inducible nitric oxide synthase (iNOS) promoter have variable importance for the gene expression in response to TNF- α . A vital κ B site for iNOS expression exists, three with regulatory effects and another one with no apparent importance for the overall expression. In our context, this latter κ B site might be a good candidate for regulation by H₂O₂, which would probably cause an increased expression of iNOS.

Analysis of the affinities of κ B sites toward NF- κ B could also be very useful when investigating the NF- κ B misregulations that have been implicated in the development of human diseases, especially chronic inflammation, autoimmune diseases, and cancer. Genetically inherited or somatic mutations in the NF- κ B family members that affect NF- κ B activation have been identified and are at the origin of several disorders (11). Other mutations associated with the NF- κ B signaling, such as polymorphisms within κ B sites of NF- κ B-target genes, have been correlated with human disease. Rossouw *et al.* (33) identified a significant association between a polymorphism within the NF- κ B binding site of the interferon γ (IFN- γ) promoter and the susceptibility to tuberculosis, probably because of the decreased levels of IFN- γ expression. Similarly, Kang *et al.* (20) identified one polymorphism within the NF- κ B binding site of the cyclooxygenase-2 (COX-2) gene, which leads to changes in COX-2 expression and has been associated with increased risks of bladder cancer. In this context, understanding the regulation of NF- κ B-dependent gene expression by H₂O₂ is important because H₂O₂ is present in the inflammatory environment of NF- κ B-related diseases, and the resulting gene expression will be a function of both the polymorphism itself and the subsequent regulation by H₂O₂. According to our hypothesis, if a polymorphism decreases the affinity of a high-affinity κ B site toward NF- κ B, the stimulatory role of H₂O₂ will be potentiated. This could help maintain the expression level of the gene (*i.e.*, to maintain a normal situation and to avoid the development of diseases), but it would also make gene expression dependent on oxidative stress. If the polymorphism increases the affinity of κ B sites toward NF- κ B, the H₂O₂ regulatory role will be attenuated or even lost, disrupting the fine regulation of the gene, potentially triggering the pathology. To account for the regulation of NF- κ B-dependent genes by H₂O₂, mechanisms

other than κ B affinity should be considered. In general, a vast array of co-repressors and co-activators regulate transcription (32). Concerning H₂O₂, extensive work on the role of oxidative stress in chronic obstructive pulmonary disease and asthma (26) has implicated oxidants in the activation of HAT and in the inhibition of HDAC. A putative protective role, helping with the resolution of inflammation, can become harmful for the organism if the oxidants doses are higher or the exposure is longer or both.

In summary, although it has long been assumed that the affinity of transcription factors to the promoter region of the gene can be one of the elements that control the intensity of the expression, the consequences of this affinity in the differential gene expression induced by an agent have remained unnoticed. Here we provided for the first time conclusive proof of principle that a simple chemical mechanism like the equilibrium between unbound and bound transcription factor to the promoter region of a gene can explain differential gene expression. Application of this principle to the regulation of NF- κ B-dependent genes by H₂O₂ predicts that H₂O₂ will modulate the expression of low- to medium-affinity NF- κ B-target genes, leaving high-affinity genes unchanged.

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Author Disclosure Statement

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References

1. 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.
2. Anderson MT, Staal FJ, Gitler C, Herzenberg LA, and Herzenberg LA. Separation of oxidant-initiated and redox-regulated steps in the NF-kappaB signal transduction pathway. *Proc Natl Acad Sci U S A* 91: 11527–11531, 1994.
3. 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.
4. Antunes F and Cadenas E. Cellular titration of apoptosis with steady state concentrations of H₂O₂: submicromolar levels of H₂O₂ induce apoptosis through Fenton chemistry independent of the cellular thiol state. *Free Radic Biol Med* 30: 1008–1018, 2001.
5. Bosisio D, Marazzi I, Agresti A, Shimizu N, Bianchi ME, and Natoli G. A hyper-dynamic equilibrium between promoter-bound and nucleoplasmic dimers controls NF-kappaB-dependent gene activity. *EMBO J* 25: 798–810, 2006.
6. Carlotti F, Dower SK, and Qvarnstrom EE. Dynamic shuttling of nuclear factor kappa B between the nucleus and cytoplasm as a consequence of inhibitor dissociation. *J Biol Chem* 275: 41028–41034, 2000.

7. Chapman NR and Perkins ND. Inhibition of the RelA(p65) NF-kappaB subunit by Egr-1. *J Biol Chem* 275: 4719–4725, 2000.
8. Chen K, Vita JA, Berk BC, and Keaney JF Jr. c-Jun N-terminal kinase activation by hydrogen peroxide in endothelial cells involves SRC-dependent epidermal growth factor receptor transactivation. *J Biol Chem* 276: 16045–16050, 2001.
9. Chen LF and Greene WC. Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* 5: 392–401, 2004.
10. Chen L, Fischle W, Verdin E, and Greene WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science* 293: 1653–1657, 2001.
11. Courtois G and Gilmore TD. Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene* 25: 6831–6843, 2006.
12. Delaunay A, Isnard AD, and Toledano MB. H₂O₂ sensing through oxidation of the Yap1 transcription factor. *EMBO J* 19: 5157–5166, 2000.
13. Denu JM and Tanner KG. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* 37: 5633–5642, 1998.
14. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47–95, 2002.
15. Forman HJ. Use and abuse of exogenous H₂O₂ in studies of signal transduction. *Free Radic Biol Med* 42: 926–932, 2007.
16. 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.
17. Hiscott J, Marois J, Garoufalos J, D'Addario M, Roulston A, Kwan I, Pepin N, Lacoste J, Nguyen H, and Bensi G. Characterization of a functional NF-kappaB site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Mol Cell Biol* 13: 6231–6240, 1993.
18. 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.
19. Kamei Y, Xu L, Heinzl T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, and Rosenfeld MG. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85: 403–414, 1996.
20. 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.
21. Koch O, Kwiatkowski DP, and Udalova IA. Context-specific functional effects of IFNGR1 promoter polymorphism. *Hum Mol Genet* 15: 1475–1481, 2006.
22. Kuprash DV, Udalova IA, Turetskaya RL, Kwiatkowski D, Rice NR, and Nedospasov SA. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J Immunol* 162: 4045–4052, 1999.
23. Leung TH, Hoffmann A, and Baltimore D. One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers. *Cell* 118: 453–464, 2004.
24. 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.
25. McGahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, Nishioka WK, and Green DR. The end of the (cell) line: methods for the study of apoptosis in vitro. *Methods Cell Biol* 46: 153–185, 1995.
26. Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bouter 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.
27. Moynagh PN. The NF-kappaB pathway. *J Cell Sci* 118: 4589–4592, 2005.
28. Oliveira-Marques V, Cyrne L, Marinho HS, and Antunes F. A quantitative study of NF-kappaB activation by H₂O₂: relevance in inflammation and synergy with TNF-alpha. *J Immunol* 178: 3893–3902, 2007.
29. Rahman I, Marwick J, and Kirkham P. Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappaB and pro-inflammatory gene expression. *Biochem Pharmacol* 68: 1255–1267, 2004.
30. Rasband WS. *Image J*. Bethesda, Maryland: U. S. National Institutes of Health, 1997.
31. Reth M. Hydrogen peroxide as second messenger in lymphocyte activation. *Nat Immunol* 3: 1129–1134, 2002.
32. Rosenfeld MG, Lunyak VV, and Glass CK. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* 20: 1405–1428, 2006.
33. Rossouw M, Nel HJ, Cooke GS, van Helden PD, and Hoal EG. Association between tuberculosis and a polymorphic NF-kappaB binding site in the interferon gamma gene. *Lancet* 361: 1871–1872, 2003.
34. Sandberg EM and Sayeski PP. Jak2 tyrosine kinase mediates oxidative stress-induced apoptosis in vascular smooth muscle cells. *J Biol Chem* 279: 34547–34552, 2004.
35. 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 U S A* 87: 9943–9947, 1990.
36. Taylor BS, de Vera ME, Ganster RW, Wang Q, Shapiro RA, Morris SM, Jr., Billiar TR, and Geller DA. Multiple NF-kappaB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J Biol Chem* 273: 15148–15156, 1998.
37. 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.
38. Udalova IA, Mott R, Field D, and Kwiatkowski D. Quantitative prediction of NF-kappaB DNA-protein interactions. *Proc Natl Acad Sci U S A* 99: 8167–8172, 2002.
39. Voit EO and Ferreira A. *Computational analysis of biochemical systems*. Cambridge: Cambridge: Cambridge University Press, 2000.
40. Wang D and Baldwin AS Jr. Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. *J Biol Chem* 273: 29411–29416, 1998.
41. Zheng M, Aslund F, and Storz G. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279: 1718–1721, 1998.

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Abbreviations Used

AP-1 = activator protein-1
COX-2 = cyclooxygenase-2
CBP/p300 = cyclic AMP response element binding
proteins (CREB)-binding protein/
adenoviral protein E1A
DMSO = dimethyl sulfoxide
H₂O₂ = hydrogen peroxide
HAT = histone acetyltransferase
HDAC = histone deacetylases
HIF-1 = hypoxia-inducible factor-1
IKK = I κ B kinase
IFN- γ = interferon- γ
iNOS = inducible nitric oxide synthase
 κ Bl = low-affinity κ B site
 κ Bm = medium-affinity κ B site
 κ Bh = high-affinity κ B site
MTT = 3-(4,5-dimethylthiazol-2-yl)-
2,5-diphenyltetrazolium bromide
NF- κ B = nuclear factor κ B
(NF- κ B/ κ B) = NF- κ B and κ B site complex
NRF-2 = NF-E2-related factor-2
s.s. = steady-state
TNF- α = tumor necrosis factor- α

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