ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 7, 2009

DOI: 10.1089/ars.2008.2279

Original Research Communication

Modulation of NF-κB–Dependent Gene Expression by H_2O_2: A Major Role for a Simple Chemical Process in a Complex Biologic Response

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Abstract

We recently observed that H_2O_2 regulates inflammation *via* upexpression of a few NF-κB–dependent genes, while leaving expression of most NF-κB–dependent genes unaltered. Here we test the hypothesis that this differential gene expression depends on the apparent affinity of κB sites in the gene-promoter regions toward NF-κB. Accordingly, cells were transfected with three reporter plasmids containing κB sequences with different affinities for NF-κB. It was observed that the lower the affinity, the higher the range of TNF-α concentrations where H_2O_2 upregulated gene expression. Mathematical models reproduced the key experimental observations indicating that H_2O_2 upregulation ceased when NF-κB fully occupied the κB sites. *In vivo*, it is predicted that genes with high-affinity sites remain insensitive to H_2O_2, whereas genes with lower-affinity sites are upregulated by H_2O_2. In conclusion, a simple chemical mechanism is at the root of a complex biologic process such as differential gene expression caused by H_2O_2. *Antioxid. Redox Signal.* 11, 0000–0000.

Introduction

Hydrogen peroxide (H_2O_2), the most abundant reactive oxygen species *in vivo*, has a central role in cell signaling (15, 31). The tight control of intracellular H_2O_2 levels and mild oxidative properties of H_2O_2 support such a role. H_2O_2 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 H_2O_2 include activator protein-1 (AP-1), hypoxia-inducible factor-1 (HIF-1), NF-E2–related factor-2 (NRF-2), and nuclear factor κB (NF-κB) (14), but the precise molecular oxidative events are not known.

In addition to these global actions on the cellular signaling network, H_2O_2 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 H_2O_2 modulates the activation of only a handful set of genes among 100 genes whose expression is dependent on NF-κB (28). The molecular basis for this selectivity constitutes the topic of this work, and we use the modulation of NF-κB–dependent genes activated by the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) as a test model. NF-κB is a transcription factor with a key regulatory role in inflammation, adaptive immune response and apoptosis. The prototypical NF-κB is a heterodimer composed of two proteins, the p65 and p50 subunits, which remains inactive in the cytosol bound to its inhibitory proteins, the IκBs. After a specific signal, such as the binding of TNF-α to its cell receptors, IκBs are phosphorylated by the IκB kinase (IKK) complex and subsequently degraded by the 26S proteasome. Free NF-κB migrates to the nucleus to activate the target genes. The biologic role of H_2O_2 as a regulator of NF-κB activation is biphasic, because, in HeLa and MCF-7 cells, NF-κB translocation into the nucleus is inhibited by high levels of H_2O_2 (millimolar range), whereas low doses of H_2O_2 (micromolarrange) similar to those found *in vivo*, stimulate this

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translocation (28). Once inside the nucleus, NF-κB binds to the promoter/enhancer regions of the target genes (27), the κB sites, which have a general consensus sequence of GGGR NNYYCC (R is purine, Y is pyrimidine, and N is any base). The modulation of specific genes leaving others unchanged, even with an increase in the levels of nuclear NF-κB, can be achieved in a number of ways. The transactivation potential of NF-κB depends on modifications in the NF-κB 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-κB | κB), which can be changed by modification of only one nucleotide in the κB 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 H_2O_2, causing the upregulation of some genes but not others. However, ultimately, the interaction of NF-κB with κB binding sites is determined by the equilibrium between free NF-κB, free κB sites, and the complex (NF-κB | κB), and can be regarded as a simple chemical process. We hypothesized that for each κB binding site (a) H_2O_2 is able to shift this equilibrium toward a higher degree of κB site occupancy, by increasing NF-κB translocation into the cell nucleus, and (b) the magnitude of this shift is dependent on the affinity of the κB binding site to NF-κB. It is important to note that a single modification of a nucleotide within a κB site can change abruptly its affinity toward NF-κB (38). According to our hypothesis, genes containing low- or medium-affinity κB sites should be positively modulated by H_2O_2, whereas genes with high-affinity κB sites should be mostly insensitive to H_2O_2, because occupancy of their κB 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 κB 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 H_2O_2 to check whether the profile of luciferase expression in the presence of H_2O_2 was dependent on the apparent affinity of the κB sequence toward NF-κB. 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 μg/ml, and L-glutamine, 2 m*M*, 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). H_2O_2 was obtained from Merck & Co., Inc. (Whitehouse Station, NJ). All plasmid reporter vectors were kindly provided by Dr. Helena Soares (Instituto Gulbenkian de Ciência, Portugal).

Cells incubations

HeLa cells were counted and plated ~46 h before the experiment. Fresh medium was added to the cells 1 h before the incubations. H_2O_2 exposure was performed by using the steady-state titration (4); the method is extensively explained in (28). In brief, a steady-state level of H_2O_2 is maintained during the entire assay by adding, simultaneous with an initial dose of H_2O_2, a quantity of glucose oxidase enough to counteract H_2O_2 consumption by cells. All experiments were performed with an initial concentration of 25 μ*M*, ending at ~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 ~3% of total p65. If endogenous NF-κB activation is considered (6), we concluded that contamination of nuclear fraction is negligible.

Plasmid constructs

The κB-reporter plasmids were generated by inserting a minimal promoter in the pGL3-basic vector (Promega, Madison, WI) with BglII (5′ end) and *Hin*dIII (3′ end) restriction enzymes (New England Biolabs, Ipswich, England): 5′– GAT CTGGGTATATAATGGATCCCCGGGTACGCAGCTCA–3′. Three different κB sequences, which were chosen based on the work of Udalova *et al.* (38), were inserted upstream of the minimal promoter, between the *Kpn*I/*Sac*I restriction site, with the following general sequence: 5′–GCT-**κB**-CTGGCTC CT-**κB**-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 μl of Opti-MEM containing a total of 360 ng of DNA

Table 1. κB Sequences Inserted in the Plasmid Construct

Plasmid	*Affinity*	*κB sequence (5′–3′)*
κBl	Low	GGGGACTTCC
κBm	Medium	GGGGATTCCC
κBh	High	GGGAATTTCC

(180 ng of κB experimental plasmid, 9 ng of pRL-SV40 control plasmid, and 171 ng of pGL3-basic plasmid) and 0.9 μl of fugeneHD (Roche, Mannheim, Germany). Cells recovered for 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 κB 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 μl of the complex prepared. After exposure to TNF-α or H_2O_2 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-κB and κB regions was set up. Equation 1, which describes this chemical equilibrium, was solved in order to (NF-κB | κB), yielding Equation 2.

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

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

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

The extended model was developed to introduce competition between the endogenous κB and plasmidic κB sites for a coactivator, cooperativity in the binding of NF-κB to κB sites, and endogenous luciferase expression in nontreated cells. Therefore, to represent the endogenous DNA, 100 hypothetical κB sites were set up in the model with the same rate constant for the first binding of NF-κB to the promoter region ($24\ \mu M^{-1}s^{-1}$) 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 κB sequence was 2.0×10/s. The other 99-κB sites were set up with a dissociation rate constant (k_d) according to the following geometric sequence: k_d for $\kappa Bsite_{n+1} = (k_d$ for $\kappa Bsite_n) \times 10^{(1/25)}$ (with $n = 1, \ldots, 100$ and for $n = 1$, $k_d = 2.0\times10$/s). The competition was restricted to those endogenous κB sites that have a similar order-of-magnitude affinity to the plasmidic κB region: for the κBh, κBm, κBl 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 κB sites, with an association constant of 2.4×10^4 $\mu M^{-1}s^{-1}$, whereas the constant for plasmid DNA was $24\ \mu M^{-1}s^{-1}$, with the same dissociation constant (2 per second). It was assumed that each of the plasmidic κB concentrations was 5×10^{-5} μM; the coactivator concentration was 5×10^{-4} μM, and the nuclear NF-κB concentration was 1×10^{-4} μM. Endogenous luciferase expression was modeled assuming (NF-κB | κB | co-activator) concentrations of 1.0×10^{-11}, 4.8×10^{-10}, and 1.2×10^{-9} μM for the κBl, κBm, and κBh 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 ± standard deviation.

Results

H_2O_2 modulates NF-κB translocation by TNF-α

In HeLa cells, H_2O_2 was not able to activate NF-κB significantly, as shown in the representative experiment of Fig. 1A, unlike the classic NF-κB activator TNF-α (Fig. 1B). Nevertheless, we recently showed that H_2O_2 modulates NF-κB by stimulating the activation caused by TNF-α, provided the H_2O_2 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 H_2O_2 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-κB activation by TNF-α at three different concentrations. The typical NF-κB 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), H_2O_2 modulation is more significant after 2 h. Thus, analysis of the effect caused by H_2O_2 was performed at 3 h for the various TNF-α concentrations used (Fig. 1C). Figure 1C, middle panel, shows that NF-κB 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 κB sites (1–100)
kBluc	Plasmidic κB sites with gene reporter luciferase (kBh, kBm, and kBl)
NFkBnuc	Nuclear NF-κB that binds to kBsite and kBluc
NFkBnuc2	Species bound to two NF-κB
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 μM H_2O_2. The maximal NF-κB translocation (NF-κB_{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-κB 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-κB 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	$\text{Kdiss1}\times10^{(1/25)}$	s^{-1}
⋮	⋮	
kdiss100	$\text{Kdiss99}\times10^{(1/25)}$	s^{-1}
kbind	2.4×10	μMs^{-1}
kbind2	kbind×10	μMs^{-1}
kbindCoact	$2.4E\times10^{4}$	μMs^{-1}
kdissCoact	2.0	s^{-1}
kbindluc	2.4×10	μMs^{-1}
kbind2luc	kbindluc×10	μMs^{-1}
kdissluc	kBh:0.05; kBm:1.8; kBl:0.3	s^{-1}
kbindCoactluc	2.4×10	μMs^{-1}
kdissCoactluc	2.0	s^{-1}

Effects of transfection: viability and NF-κB 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 ~50%, when compared with nontransfected cells (Fig. 2). However, the addition of 25 μM H_2O_2 in 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-κB

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

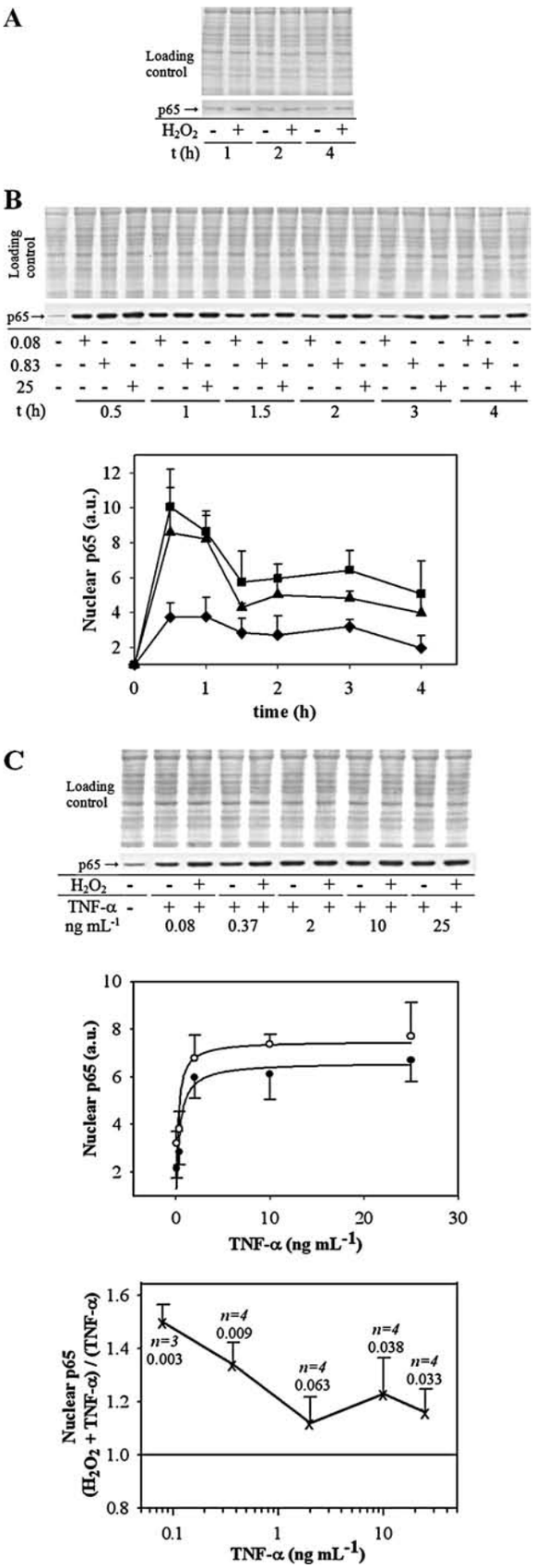

FIG. 1. H_2O_2 increases the translocation of NF-κB to the nucleus, in the presence of TNF-α. HeLa cells were treated for the indicated times with (**A**) 25 μM H_2O_2 s.s.; (**B**) 0.08 (◆), 0.83 (▲), and 25 (■) ng/ml TNF-α; and (**C**) for 3 h with TNF-α alone (●) or (○) simultaneously with H_2O_2 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 H_2O_2 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 H_2O_2) 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.

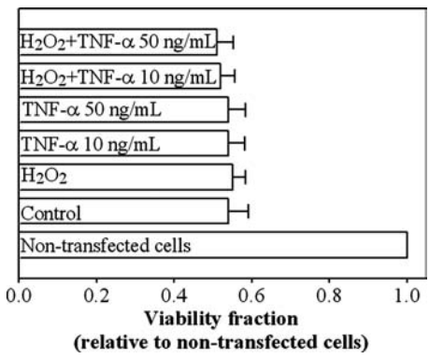

FIG. 2. Viability of transfected cells is not affected by H_2O_2 and TNF-α treatments. Viability was measured with the MTT assay both in nontransfected cells and transfected cells. Viability of transfected cells exposed to 25 μM H_2O_2 s.s. with or without 10 and 50 ng/ml TNF-α is also shown.

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 H_2O_2 were carried out was similar to that in experiments with nontransfected 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 H_2O_2 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 H_2O_2 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

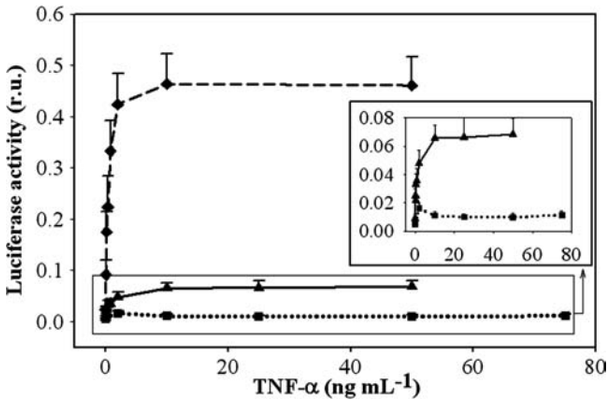

FIG. 3. Luciferase activity is dependent on the κB reporter plasmid. Cells transfected with κBl (■), κBm (▲), and κBh (◆) reporter plasmids were exposed to increasing concentrations of TNF-α in the range of 0.016–75 ng/ml for 4 h, and luciferase activity was measured (F/R).

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-κB toward that particular κB site.

The three reporter plasmids were named according to their apparent affinity: κBh, κBm, and κBl for the highest-, medium-, and lowest-affinity plasmids, respectively (Table 1 and Fig. 3). Because nuclear levels of NF-κB were augmented with increasing TNF-α concentrations (Fig. 1C), the binding of NF-κB to κB sites is also expected to increase with TNF-α concentration, until reaching saturation. The luciferase expression of the constructs κBm and κBh 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 κB 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 κBm construct and 0.476 ± 0.011 a.u. for the κBh construct, and so the apparent affinity of κBh was 7 times higher than that of κBm. Concerning the κBl 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 κB sequence toward NF-κB was ~6 times lower than that of the κBm sequence. These results confirm that changing just two nucleotides within the κB site can originate different transcriptional activities.

H_2O_2 modulates the transcriptional activity of the reporter genes

By assuming that the binding of NF-κB to the free κB sites follows a dynamic equilibrium, it should be expected that an increase of nuclear p65 stimulated by the presence of H_2O_2, in TNF-α–treated cells (Fig. 1), would favor complex formation between free NF-κB and free κB sites, therefore increasing luciferase expression. H_2O_2 alone did not induce significantly the luciferase expression (Table 5), which is in agreement with the negligible NF-κB translocation observed in the presence of H_2O_2 (Fig. 1A). As expected, when added simultaneously with TNF-α, H_2O_2 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 κBl construct, H_2O_2 stimulated significantly the luciferase expression for TNF-α concentrations up to 25 ng/ml. This maximal TNF-α concentration when synergistic effects induced by H_2O_2 occurred, decreased to 0.83 ng/ml for the medium-affinity κBm construct, and to 0.08 ng/ml for the high-affinity κBh construct. An unexpected observation was that for all concentrations of TNF-α studied, maximum synergistic effects in luciferase expression were ≈10%, whereas H_2O_2 synergistic

TABLE 5. LUCIFERASE EXPRESSION IS NOT ALTERED BY H_2O_2

Plasmid	*Control*$\times 10^{-3}$	$H_2O_2 \times 10^{-3}$
κBl	3.1 ± 0.2	3.1 ± 0.4
κBm	8.4 ± 0.9	8.3 ± 0.8
κBh	25.2 ± 8.0	26.3 ± 8.8

Luciferase activity (F/R) for nontreated (control) and H_2O_2-treated (25 μM s.s.) HeLa cells for 4 h. $n = 4$ for κBl, $n = 3$ for κBm, and $n = 5$ for κBh.

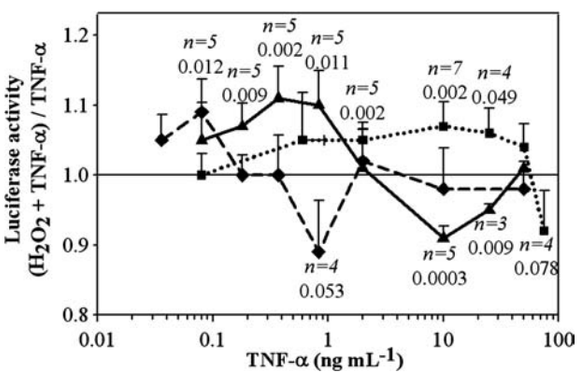

FIG. 4. H_2O_2 modulation of reporter plasmid expression is dependent on both the TNF-α concentration and the κB sequence on the gene. Cells transfected with κBl (■), κBm (▲), and κBh (◆) constructs were exposed to TNF-α concentrations in the range of 0.016–75 ng/ml with or without of 25 μM H_2O_2 s.s. for 4 h. The ratio of the luciferase activity measured in the presence and absence of H_2O_2 of same-day paired samples is shown. Experimental data passed the Kolmogorov–Smirnov normality test and were compared with 1 (no modulation by H_2O_2) 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 ≈50% for physiologic doses of TNF-α (Fig. 1C). Because H_2O_2 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 H_2O_2 of the early growth-response protein-1 (Egr-1). Egr-1 has a zinc-finger domain that interacts with the p65 subunit, inhibiting NF-κB–dependent gene expression (7).

To help the interpretation of these results, a minimal model for the binding of NF-κB to κB sites based on a simple equilibrium was set up. In this simple model, H_2O_2 stimulated the formation of the complex (NF-κB | κB) for a wide range of NF-κB concentrations. This positive modulation occurred within a wider interval of NF-κB concentrations for the low-affinity κBl sequence than for the high-affinity κBh sequence (Fig. 5A). These results can be understood by considering that when low concentrations of NF-κB are present in the nucleus (*i.e.*, when low concentrations of TNF-α are applied to the cell), the increase of nuclear NF-κB induced by H_2O_2 increases the formation of the complex (NF-κB | κB) (*i.e.*, H_2O_2 has a synergistic effect). For higher nuclear NF-κB concentrations, the occupancy of κB sites also increases until they are fully occupied by NF-κB. At this point, a further increase of nuclear NF-κB, as caused by H_2O_2, does not increase the occupancy of κB sites, because the occupancy is already maximal. For higher-affinity κB sites, the formation of the complex is favored, which means that κB sites become totally occupied at a lower NF-κB concentration. Thus, the higher the affinity of NF-κB toward a κB site, the lower the concentration of NF-κB (or TNF-α) at which H_2O_2 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 H_2O_2 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 κB sites of the endogenous genes and the κB sites of the reporter genes bound to NF-κB. 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-κB (9). CBP/p300 exists in limiting quantities, and the competition between different promoters can result in some downregulation of gene transactivation (19). Therefore, κB 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-κB concentration is needed for the synergistic effect of H_2O_2 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-κB binding to DNA involves cooperativity [*e.g.*, between two or more κB regions within the same gene, as observed in the IL-1β and iNOS (17,36) promoters, or by the cooperative binding of the NF-κB dimer to the κB region in the promoters]. Therefore, cooperativity for NF-κB binding to κB sites was included in the model.
3. Although the experimental results show that the synergism induced by H_2O_2 increases for low TNF-α concentrations, in the minimal model, the synergism is constant in the low range of NF-κB concentrations. This discrepancy probably occurs both because of the lack of

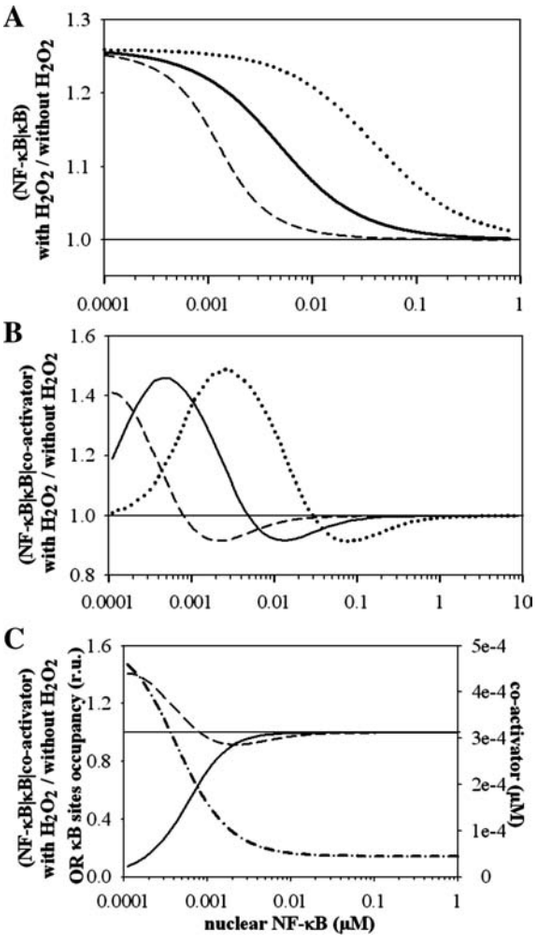

FIG. 5. Mathematical modeling predicts a differential modulation of gene expression by H_2O_2 based on the affinity constants of the κB regions toward NF-κB. (**A**) The simple model in which the concentration of the complex (NF-κB | κB) is given by Eq. 2 was used; the ratio of this complex, with and without H_2O_2, is plotted as a function of the NF-κB concentration, for three different κB sites (κBl, *dotted line*; κBm, *solid line*; κBh, *dashed line*). The effect of H_2O_2 was modeled by assuming a 26% increase of the nuclear NF-κB concentration. (**B**) As in (**A**), but the extended model with cooperativity in the binding of NF-κB between two κB sites within the same gene, competition for a co-activator and basal luciferase expression was used. The ratio between the (NF-κB | κB | co-activator) complex with and without H_2O_2 is plotted. (**C**) In addition to this ratio (*dashed line*), the concentration of the coactivator (*dashed–dotted line*) and the occupancy of the κB sites (*solid line*) for the κBh case are represented. For details, see Materials and Methods.

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-κB level when almost all the κB sites of the plasmids become occupied, additional NF-κB will bind to other κB 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 κB sites, decreasing luciferase expression—antagonism (Fig. 5C). This antagonism disappears if NF-κB concentrations are further increased, and at high NF-κB concentrations luciferase activity was not modulated by H_2O_2. According to the model, this recovery was caused by the near saturation of the endogenous κB regions, which decreases the competition with the transcriptional co-activator. Taken together, the modulation by H_2O_2 of NF-κB–dependent genes activated by TNF-α was dependent on both the affinity of the κB region toward NF-κB and the level of TNF-α, thus providing a rationale for the selective modulation of a subset of genes.

H_2O_2 modulates the transcriptional activity of endogenous genes

Next, the modulation of the expression of endogenous genes by H_2O_2 was evaluated. It should be mentioned that analysis of individual genes can be problematic because it is difficult to estimate the apparent affinity of κB regions *in vivo* (21–23) and because other effects besides κB affinity, such as modulation of co-activators or repressors by H_2O_2, also are important to predict how H_2O_2 modulates the expression of a particular gene. Therefore, the approach used was to evaluate the influence of H_2O_2 on the global pattern of expression of 100 NF-κB–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 κB regions being an important mechanism by which H_2O_2 modulates NF-κB–dependent gene expression. For genes with low-affinity κB regions, in which it is expected that the increase of nuclear NF-κB caused by H_2O_2 is reflected in an increased gene expression, H_2O_2 had a synergistic action on TNF-α–dependent gene expression. This synergism decreased and even transformed into an antagonism for some genes with κB regions with middle affinity, for which competition with endogenous co-activators is expected to be important. Finally, for genes with high-affinity κB regions, the modulation effects tend to disappear. This should be expected because of the saturation in κB regions with NF-κB and of the lack of competition with co-activators.

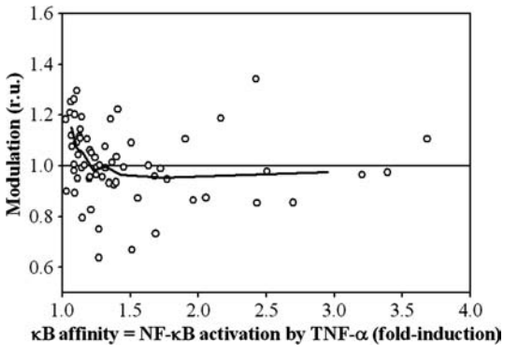

FIG. 6. H_2O_2 modulation of endogenous genes in HeLa cells. Data were taken from (28). In brief, HeLa cells were exposed to 0.37 ng/ml TNF-α and 12.5 μM H_2O_2 s.s. alone or simultaneously for 6 h. Modulation by H_2O_2 was obtained by calculating the increase of p65 levels in the presence of both TNF-α and H_2O_2 relative to the sum of the individual effects: $[(\text{nuclear p65})_{\text{TNF-}\alpha+\text{H2O2}}]/[(\text{nuclear p65})_{\text{TNF-}\alpha} + (\text{nuclear p65})_{\text{H2O2}} - 1]$. A value of 1 means that the effects of TNF-α and H_2O_2 are additive; values higher/lower than 1 imply synergism/antagonism between the two agents. The apparent affinity of κB 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 κB region; so, on average, genes that are highly activated by TNF-α would tend to have a κB 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 κB 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 H_2O_2).

Discussion

We provided evidence that a simple chemical equilibrium principle based on the association–dissociation between NF-κB and κB sites in the promoter region of genes may explain the selective modulation of some genes by H_2O_2, 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 H_2O_2 as a regulator of inflammation. During inflammation, some of the H_2O_2 generated during phagocytosis leaks from the phagosome. Therefore, cells present at inflammation sites are simultaneously subjected to H_2O_2 and other pro-inflammatory cytokines, such as TNF-α. In the present work, three NF-κB–dependent reporter genes that simulate three hypothetical genes with different affinities toward NF-κB 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 H_2O_2, because TNF-α will probably trigger NF-κB translocation to the nucleus at high enough levels to occupy high-affinity κB sites fully. Thus, these genes will be insensitive to further increases in NF-κB translocation caused by H_2O_2. Conversely, genes containing medium-affinity κB sites and, particularly, genes containing

low-affinity κB sites, are predicted to have their expression upregulated by H_2O_2 during inflammation. The analysis of the influence of H_2O_2 on the global pattern of expression of 100 NF-κB–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 H_2O_2 is difficult. First, the affinity of κB regions is not uniquely defined by the nucleotide sequence in the κB 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-κB (21, 22). Thus, the κB 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 κB site, including the presence of both high- and low-affinity κB sites in the same gene. For example, Taylor *et al.* (36) showed that the different κB regions within the inducible nitric oxide synthase (iNOS) promoter have variable importance for the gene expression in response to TNF-α. A vital κB 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 κB site might be a good candidate for regulation by H_2O_2, which would probably cause an increased expression of iNOS.

Analysis of the affinities of κB sites toward NF-κB could also be very useful when investigating the NF-κB 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-κB family members that affect NF-κB activation have been identified and are at the origin of several disorders (11). Other mutations associated with the NF-κB signaling, such as polymorphisms within κB sites of NF-κB–target genes, have been correlated with human disease. Rossouw *et al.* (33) identified a significant association between a polymorphism within the NF-κB 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-κB 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-κB–dependent gene expression by H_2O_2 is important because H_2O_2 is present in the inflammatory environment of NF-κB–related diseases, and the resulting gene expression will be a function of both the polymorphism itself and the subsequent regulation by H_2O_2. According to our hypothesis, if a polymorphism decreases the affinity of a high-affinity κB site toward NF-κB, the stimulatory role of H_2O_2 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 κB sites toward NF-κB, the H_2O_2 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-κB–dependent genes by H_2O_2, mechanisms other than κB affinity should be considered. In general, a vast array of co-repressors and co-activators regulate transcription (32). Concerning H_2O_2, 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-κB–dependent genes by H_2O_2 predicts that H_2O_2 will modulate the expression of low- to medium-affinity NF-κB-target genes, leaving high-affinity genes unchanged.

Acknowledgments

This work was supported by grant POCTI/BCI/42245/2001 from FCT–Portugal. V.O.M. acknowledges the fellowship SFRH/BD/16681/2004 from FCT–Portugal. Drs. Cyrne and Antunes contributed equally to this work.

Abbreviations

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; H_2O_2, hydrogen peroxide; HAT, histone acetyltransferase; HDAC, histone deacetylases; HIF-1, hypoxia-inducible factor-1; IKK, IκB kinase; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; κBl, low-affinity κB site; κBm, medium-affinity κB site; κBh, high-affinity κB site; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; (NF-κB | κB), NF-κB and κB site complex; NRF-2, NF-E2–related factor-2; s.s., steady-state; TNF-α, tumor necrosis factor-α.

Disclosure Statement

No competing financial interests exist.

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Date of first submission to ARS Central, September 4, 2008; date of final revised submission, November 12, 2008; date of acceptance, November 14, 2008.