# The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-κB

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**Background:** The inducible, higher eukaryotic transcription factor NF-κB is activated by a variety of stimuli. Several lines of evidence have suggested that reactive oxygen intermediates (ROIs) serve as messengers for most if not all of these stimuli. To identify the relevant ROI species and to gain more direct evidence for an involvement of ROIs as messengers, we investigated whether changes in the levels of enzymes that control intracellular ROI levels affect the activation of NF-κB.

**Results:** Cell lines stably overexpressing the $H_2O_2$-degrading enzyme catalase were deficient in activating NF-κB in response to tumor necrosis factor α (TNF) or okadaic acid. The catalase inhibitor aminotriazol restored NF-κB induction. In contrast, stable overexpression of cytoplasmic Cu/Zn-dependent superoxide dismutase (SOD), which enhances the production of $H_2O_2$ from superoxide, potentiated NF-κB activation. The level of cytoplasmic NF-κB–IκB complex was unchanged, indicating that synthesis of NF-κB was not affected.

**Conclusions:** Our data show that one ROI species, $H_2O_2$, acts as a messenger in the TNF- and okadaic acid-induced post-translational activation of NF-κB. Superoxide is only indirectly involved, as a source for $H_2O_2$. These data explain the inhibitory effects of many antioxidative compounds on the activation of NF-κB and its target genes. $H_2O_2$ is overproduced in response to various stimuli, and normal levels of catalase appear insufficient to remove it completely. $H_2O_2$ can therefore accumulate and act as an intracellular messenger molecule in the response to pathogens.

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## Introduction

Extracellular stimulation of cells can dramatically alter gene expression in the nucleus. This is fairly well investigated in the case of steroid hormones, which cross the plasma membrane and bind to highly specific receptors in the cytoplasm or nucleus [1]. These receptors are latent DNA-binding proteins that, upon interaction with their hormone ligand, bind their recognition sequences in *cis*-regulatory DNA elements. Ultimately, this initiates the assembly of basic transcription factors on the transcription start sites of target genes, the recruitment of RNA polymerase II and the synthesis of mRNA [2].

Peptide hormones or xenobiotic stimuli use far more complex pathways to transduce signals from the plasma membrane into the nucleus. If cell-surface receptors are involved, second messenger molecules, such as cAMP and lipid metabolites, are frequently produced. These may activate protein kinases or kinase cascades, altering the activity of nuclear transcription factors by phosphorylation [3]. Two well-studied examples are the cAMP-responsive transcription factor, CREB, which is directly modified in the nucleus by the catalytic subunit of cAMP-dependent protein kinase [4], and the growth stimulus-responsive factor Elk-1, which is modified by cascade-controlled MAP kinases [5]. Surface receptor-associated tyrosine kinases have also been shown to modify latent cytoplasmic forms of the so-called STAT transcription factor family directly [6]. The tyrosine-phosphorylated STAT factors then appear in the nucleus, bind to DNA and activate transcription. In all these cases, the transcription factors are activated by a restricted number of specific extracellular stimuli.

Nuclear factor κB (NF-κB) is an inducible transcription factor that is activated by a surprisingly broad panel of conditions [7–9]. In unstimulated cells, this factor resides in a latent form in the cytoplasm [10]. Latency is achieved by association of the DNA-binding NF-κB dimer with an inhibitory subunit, IκB [11]. IκB suppresses DNA-binding and nuclear transport of NF-κB. Upon stimulation of cells, IκB is phosphorylated and proteolytically degraded [12–15]. Both reactions are required for activation [16]. The released NF-κB is then translocated to the nucleus where it initiates transcription of target genes. Five distinct DNA binding subunits of NF-κB, which can homo- and hetero-dimerize to some extent, are currently known [17–19]. Very frequently, NF-κB is composed of p50/p65 heterodimers. There are also a variety of distinct IκB proteins [20], but so far only the

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IκB-α protein has been shown to be degraded in response to NF-κB-inducing stimuli.

NF-κB actively participates in the cytoplasmic transduction of signals to the nucleus, and its activity is essentially controlled by the interaction between IκB-α and the p65 DNA-binding subunit. This simple mode of activation allows NF-κB to be used in many cell types as a rapid genetic switch, activating genes in response to pathogenic stimuli. Among the many proteins that are induced by the concerted action of NF-κB with other transcription factors are cytokines, chemokines, cell adhesion molecules, hematopoietic growth factors and receptors, histocompatibility antigens and acute-phase proteins [7–9]. Although NF-κB may be indispensable as an inducer of many immediate-early inflammatory and immune reactions, it is probably harmful or even fatal in diseases and syndromes that involve an aberrant expression of inflammatory cytokines, such as toxic/septic shock, the graft-versus-host reaction, rheumatoid arthritis and Crohn's disease. Hence, NF-κB and proteins controlling its activation represent attractive targets for novel antiinflammatory and immunosuppressive drugs.

A large number of different viruses and bacteria have been reported to activate NF-κB [7–9]. In some cases, the active components have been identified as lipopolysaccharides, muramyl peptides, pleiotropic viral transactivators or virion proteins. Various kinds of energy-rich radiation can also activate NF-κB. Among the endogenous activation signals are inflammatory cytokines that are produced in response to pathogenic stimulation, such as the tumor necrosis factors (TNFs) α and β and interleukin-1 (IL-1). In T and B cells, antigenic stimulation and many other activating conditions stimulate NF-κB activation [9]. Very recent reports suggested that the neurotoxic Aβ peptide of the Alzheimer amyloid precursor protein [21] and advanced glycation end products (AGEs), which occur in diabetes [22], also activate NF-κB in neurons and endothelial cells, respectively. In view of this variety of pathogen and pathogen-related stimuli, it is hard to imagine much overlap in the receptor signalling pathways. But it also seems very unlikely that each stimulus activates the cytoplasmic NF-κB–IκB complex in a completely different way.

Many conditions activating NF-κB are known to induce oxidative stress. In other words, these conditions increase the production of reactive oxygen intermediates (ROIs) such as superoxide, $H_2O_2$ and secondary reactive compounds. This has been shown for the NF-κB inducers TNF, IL-1, lipopolysaccharide, phorbol esters, UV light, γ radiation, anti-IgM, Aβ peptide, AGE, okadaic acid and anti-CD28 [23–25]. This common effect prompted us to test the involvement of ROIs in two ways. One was to investigate whether different antioxidants inhibit the activation of NF-κB in response to several stimuli. Several studies have shown that they do [23–25]. The second approach was to test chemicals known to induce oxidative stress for their ability to activate NF-κB. In some, but not all, cell lines, NF-κB could be activated by micromolar concentrations of $H_2O_2$ or butylperoxide [24–28]. Compounds generating superoxide, nitric oxide, hypochlorite and singlet oxygen were much less effective or virtually ineffective [24,25], suggesting that NF-κB selectively responds to peroxides. Other investigators observed effects on NF-κB activation upon manipulation of the cellular redox status using compounds that affect the intracellular levels of the antioxidant glutathione (GSH). Treatment of cells with the GSH precursor N-acetyl-L-cysteine (NAC) or L-cysteine suppressed NF-κB activation by TNF and phorbol myristate acetate (PMA) [29,30], whereas the GSH synthesis inhibitor L-buthionine-(SR)-sulfoximine (BSO) potentiated NF-κB activation in response to okadaic acid [31]. All these studies used pharmacological agents of unknown side effects at occasionally very high inhibitory doses, however. Moreover, the use of drugs does not allow one to decipher which ROI species are essential for activation of the transcription factor.

We have taken a novel approach to demonstrate the involvement of ROIs in NF-κB activation and to determine the roles of $H_2O_2$ and superoxide, the two most common intracellular ROI species. The levels of superoxide and hydrogen peroxide can be manipulated by moderately increasing the levels of SOD or catalase. When SOD is overexpressed, more $H_2O_2$ is formed from superoxide; when catalase is overexpressed, $H_2O_2$ is more efficiently detoxified to yield $H_2O$ and $O_2$. We have used the cytosolic form of SOD, Cu/Zn–SOD, because NF-κB activation is known to occur in the cytosol. Catalase is predominantly present in peroxisomes, but because $H_2O_2$ can readily cross membranes, cytosolic $H_2O_2$ has unlimited access to the peroxisomal enzyme. The effects of overexpressed catalase and SOD cannot be determined properly in transiently-transfected cells because there will be variations in transfection efficiencies and plasmid copy numbers. We therefore tested cell lines which were stably transfected with the genes encoding catalase and Cu/Zn–SOD. The levels of overexpression and increased enzymatic activities in these cells were previously characterized in detail [32,33]. Here we show that the overexpression of catalase and SOD both influence NF-κB activation, with opposite effects. $H_2O_2$ appears to induce NF-κB–IκB activation more directly than does superoxide. The data lend strong support to the idea that ROIs are the common messengers in the activation of the pathogen-induced transcription factor NF-κB.

## Results and discussion

### Characteristics of cell lines stably overexpressing catalase and Cu/Zn–SOD

Studies by Amstad *et al.* [32,33] described and characterized clones of the mouse epidermal cell line JB6 clone 41, which were stably transfected with either a human Cu/Zn–SOD or the human catalase gene. Increased levels of the appropriate mRNAs, proteins and enzymatic activities were observed in these cell lines. For the present study, we have chosen clones JB6-SOD15 and

JB6-Cat4 to examine the role of ROIs in the activation of NF-κB. Compared to the parental cell line, these cell lines have 2.3- and 2.6-fold enhanced levels of SOD and catalase activities, respectively. As expected for cells with an increased potential to detoxify $H_2O_2$, the JB6-Cat4 line was less sensitive towards the growth-inhibitory and DNA-damaging effects of ROIs produced by a xanthine/xanthine oxidase (X/XO) system [32]. In contrast, JB6-SOD15 cells were hypersensitive to X/XO-induced oxidative stress, probably because the increased SOD levels produced more $H_2O_2$ from superoxide. A double SOD/Cat transfectant cell line confirmed this; the hypersensitivity towards oxidative stress was relieved by the coexpression of catalase.

To confirm that the cell lines had altered steady-state levels of intracellular $H_2O_2$ as a consequence of catalase or Cu/Zn–SOD overexpression, we analyzed them for the oxidation of the dye 2',7'-dichlorofluorescein diacetate (DCFH) by fluorescence-activated cell sorting (FACS). This dye allows determination of intracellular ROI levels, predominantly detecting peroxides [34]. As expected, JB6-Cat4 cells had reduced levels of $H_2O_2$ while JB6-SOD15 cells had increased ROI levels as compared to JB6 clone 41 cells (Fig. 1).

The levels of overexpressed SOD and catalase in the cell lines described here appear quite low. Similar alterations in the Cu/Zn–SOD levels are known to cause disease in some clinical disorders, however [35]. Overexpression of Cu/Zn–SOD in transgenic mice leads to a Down's syndrome-like phenotype [36]. Aberrant forms of Cu/Zn–SOD are associated with familial amyotrophic lateral sclerosis, also called Lou Gehrig's disease, a lethal neurodegenerative disorder [37]. These syndromes show that the intracellular metabolism of superoxide and $H_2O_2$ is very finely tuned and that any subtle change in the levels of enzymes converting or degrading ROIs has fatal consequences for the organism, mainly affecting the central nervous system. In the present study, we investigated whether the activation of transcription factor NF-κB is affected by relatively small alterations in catalase and SOD enzyme levels.

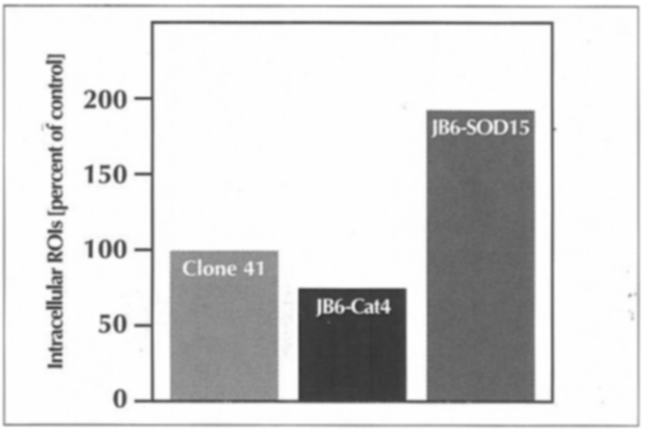


**Fig. 1.** The effect of catalase and Cu/Zn–SOD overexpression on the intracellular levels of $H_2O_2$. Aliquots of $10^4$ cells of the lines JB6 clone 41, JB6-Cat4 and JB6-SOD15 were analyzed by FACS for intracellular levels of peroxides using DCFH [34]. Median values from FACS histograms are shown. The level determined for clone 41 was taken as 100 %.

### Overexpression of catalase suppresses NF-κB activation by TNF

The subclone JB6-Cat4, which overexpresses the $H_2O_2$-degrading enzyme catalase to a level ~2.6-fold greater than that seen in the parental cell line [32,33], was tested for the activation of NF-κB in response to TNF using electrophoretic mobility shift assays (EMSAs). After a 10 min TNF treatment, the parental JB6 clone 41 cells showed a dramatic induction of a novel activity, which binds to a $^{32}$P-labelled oligonucleotide harbouring a high-affinity NF-κB-binding motif (Fig. 2a, compare lanes 1 and 2). The amount of the complex increased slightly over 30 min (lanes 2–4) and slowly decreased thereafter (lanes 5–7). The kinetics were quantified by phosphorimaging (Fig. 2b). A more slowly-migrating complex did not show induction (Fig. 2a). Only the TNF-inducible complex was specific, as it was abrogated by antibodies to the p50 and p65 subunits of NF-κB (data not shown).

In the cell clone JB6-Cat4, activation of NF-κB in response to TNF was significantly impaired. The maximal activation of NF-κB seen between 20 and 30 min reached only 50 % of that seen in the parental cells (Fig. 2a, compare lanes 3 and 10; Fig. 2b). Even more striking, NF-κB activation could not be maintained for longer than 30 min in cells overexpressing catalase (Fig. 2a, compare lanes 5–7 with lanes 12–14). Had we only measured NF-κB activation after 50 or 60 min no induction would have been apparent in JB6-Cat4 cells.

To investigate whether catalase overexpression had an effect on the intracellular level of the inducible NF-κB–IκB complex in the cytoplasm, we treated cytosolic fractions from these cells with desoxycholate (DOC) [10]. DOC can release IκB from NF-κB, allowing the determination of the amount of inducible NF-κB by EMSA. This type of analysis showed that JB6-Cat4 cells had the same amount of inducible NF-κB–IκB complex in the cytosol as did the parental cells (Fig. 3, compare lanes 2 and 4). In a DOC titration experiment, 0.4 % DOC gave maximal cell-free activation of NF-κB in all three cell lines tested (data not shown). Thus, overexpression of catalase impairs not the synthesis but the post-translational activation of NF-κB.

Stable overexpression of catalase might lead to secondary changes in the oxidant defence system of the cell. We therefore examined whether the impairment in the ability of JB6-Cat4 to activate NF-κB was due to the increased catalase activity in the overexpressing cell line, using the catalase inhibitor 3-amino-1,2,4-triazole (AT) [38]. In the presence of increasing amounts of AT, the complete block of NF-κB activation seen after a 60 min TNF treatment (Fig. 4, compare lanes 1 and 2) was

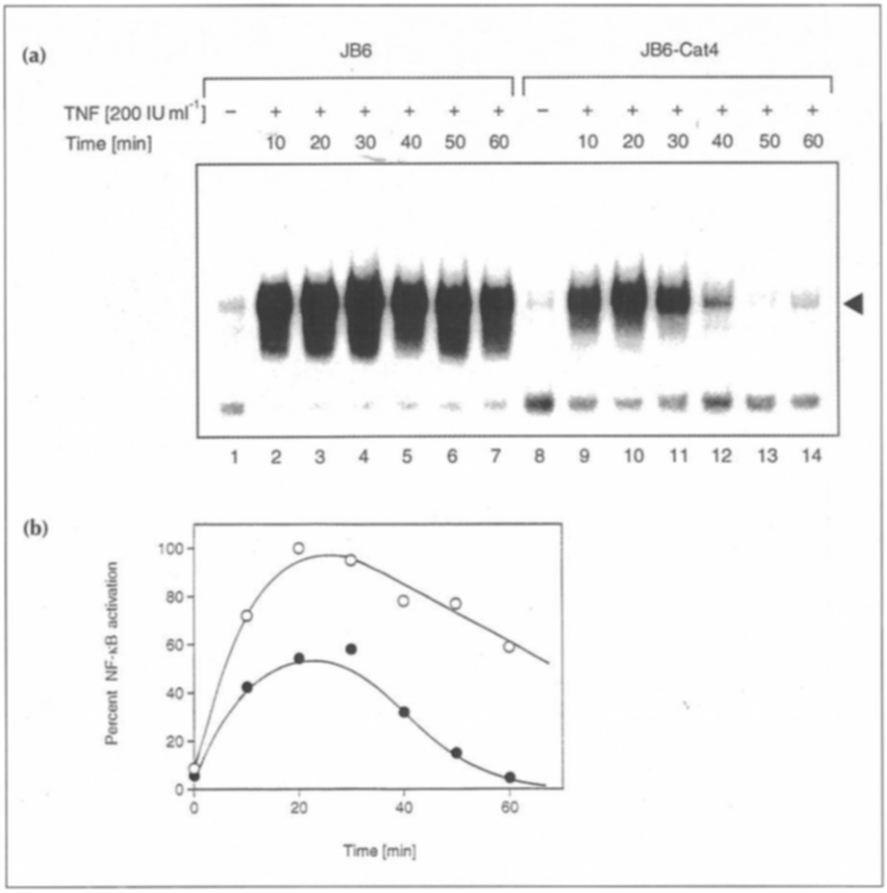


**Fig. 2.** The activation of NF-κB by TNF in cells stably overexpressing catalase. **(a)** Cell cultures of the parental line JB6 clone 41 (lanes 1–7) and of the JB6-Cat4 clone (lanes 8–14) were left untreated (lanes 1 and 8) or were stimulated with 200 IU ml$^{-1}$ TNF for the indicated periods of time (lanes 2–7 and 9–14). Total cell extracts were subsequently prepared and equal amounts of protein analyzed for NF-κB-specific DNA binding activity using EMSA. A filled arrowhead indicates the position of the TNF-inducible protein–DNA complex. A faster-migrating non-specific DNA binding activity is seen at the bottom. Its abundance negatively correlated with the abundance of NF-κB activity. A section of a fluorogram from a native gel is shown. **(b)** Quantitation of the radioactivity in the NF-κB–DNA complexes by β imaging. Open circles, JB6 clone 41 cells; filled circles, JB6-Cat4 cells. The maximal level of NF-κB activity obtained with JB6 clone 41 cells was taken as 100 %.

relieved in a dose-dependent manner (lanes 4–8). The optimal AT dose was between 100 and 800 μM (lanes 4–8). Treatment with 100 μM AT in the absence of TNF did not activate NF-κB in JB6-Cat4 cells (lane 3).

These data clearly show that $H_2O_2$ is required for the activation of NF-κB by TNF. Overexpression of catalase not only decreased the initial rate of NF-κB activation but also reduced the duration of NF-κB induction. This suggests that $H_2O_2$ is important for both the induction and the maintenance of NF-κB activity. The observation that the inhibition by catalase was only partial is explained by the fact that the catalase activity in JB6-Cat4 cells was enhanced only by a factor of 2.6, which might be not be sufficient to remove all newly-produced $H_2O_2$ completely.

Until now, the evidence for ROIs being involved in NF-κB activation was solely based on pharmacological and circumstantial evidence. Although many structurally-unrelated antioxidants have been shown to prevent NF-κB activation in cell and tissue culture systems [24,25], this type of experiment cannot determine which ROI species is the relevant one, nor can one exclude the possibility that the compounds used may have side effects. Experiments using $H_2O_2$ treatments of cells were also less than definitive [27,28]. First, $H_2O_2$ did not activate NF-κB in every cell line tested. Different cell types, however, have different complements of $H_2O_2$-degrading enzymes and may therefore vary in their responses to extracellular $H_2O_2$. Second, the kinetics of NF-κB induction by $H_2O_2$ were rather slow [28]. It is possible that the DNA-binding activity of NF-κB suffers from oxidative damage, which must be repaired before activation can be observed [39]. Enhanced production of superoxide and $H_2O_2$ was observed upon treatment of cells with TNF, phorbol esters, UV light, IL-1 and lipopolysaccharide; all of these conditions are known to induce NF-κB. This observation is consistent with the hypothesis that NF-κB is induced via a common ROI messenger, but also falls short of direct proof.

The molecular biology approach used here allows a causal link to be made between hydrogen peroxide production and NF-κB activation, and is thus superior to the previous pharmacological approaches. We have shown that moderate levels of overexpression of the highly specific enzyme catalase have a profound inhibitory effect on NF-κB activation. This strongly indicates that the $H_2O_2$ produced in response to TNF, presumably generated via changes in the mitochondrial electron flow [40,41], is in fact required for the subsequent activation of NF-κB. It also suggests that the removal of $H_2O_2$ in normal cells is limited by the amount of catalase. This is necessary to allow a certain amount of the ROI to accumulate and act as a messenger molecule.

It is not yet clear how $H_2O_2$ ultimately triggers the removal of IκB from NF-κB in the cytoplasm. It has been suggested [42] that $H_2O_2$ may act by three distinct

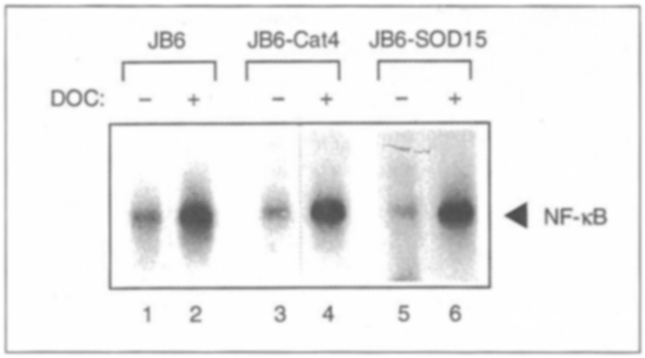


**Fig. 3.** Determination of the amount of NF-κB–IκB complex in the three cell lines used. Cytosol was prepared from JB6 clone 41, JB6-Cat4 and JB6-SOD15 cells and treated with different concentrations of DOC followed by EMSA analysis [10]. A section of a fluorogram from one native gel is shown. As determined by β imaging, all three cell lines contained very similar amounts of DOC-activatable NF-κB activity. Optimal activation was obtained at 0.4 % DOC (lanes 2, 4 and 6). Note that untreated samples (lanes 1, 3 and 5) contained some background activity. Note also that the extract preparation was different from that used in the other figures.

mechanisms: lipid peroxidation, calcium mobilization and/or generation of glutathionedisulfide (GSSG), a mild oxidant which modifies cysteine residues in proteins. GSSG might transiently modify kinases or phosphatases that control the proteolysis of IκB [16].

### Overexpression of Cu/Zn–SOD increases NF-κB activation by TNF

Cytoplasmic $H_2O_2$ metabolism can also be modulated using Cu/Zn–SOD, an isozyme of SOD that is present in the cytosol. This enzyme efficiently converts superoxide into $H_2O_2$, increasing the cytosolic steady state level of $H_2O_2$ (see Fig. 1). Here, we tested the subclone JB6-SOD15, which has a ~2.3-fold increased level of SOD activity compared to the parental JB6 clone 41 [32,33], for NF-κB activation. In this experiment, the parental cells showed similar kinetics of NF-κB activation in response to TNF to those seen in Fig. 1 (Fig. 5a, lanes 1–7), but the NF-κB activation in JB6-SOD15 cells was dramatically accelerated and increased overall (Fig. 5a, lanes 8–14). Maximal stimulation of NF-κB by TNF was reached within only 10 min and reached about four times the level seen in parental cells (lane 9). Thereafter, it steadily declined to the level of parental cells (Fig. 5b). JB6-SOD15 cells have similar amounts of the DOC-activatable cytosolic NF-κB–IκB complex to parental cells (Fig. 3, compare lanes 2 and 6). Thus, moderate overexpression of SOD did not induce synthesis of NF-κB but increased the level and rate of the post-transcriptional NF-κB activation induced by TNF.

The results obtained with the JB6-SOD15 and JB6-Cat4 cell lines support each other. In both cases, enzymes involved in intracellular ROI metabolism were stably overexpressed, but the effects on NF-κB activation were directly opposed. This rules out nonspecific effects related to transfection and selection procedures. Taken together, the data indicate that the ROI species necessary for NF-κB activation is not superoxide, the substrate of the SOD reaction, but its product $H_2O_2$. The inducing effect of SOD overexpression indicates that superoxide, if efficiently converted to $H_2O_2$, can substantially contribute to NF-κB activation. Endogenous SOD levels may thus allow for a physiological modulation of NF-κB activation. Moreover, the data suggest that the conversion of superoxide into $H_2O_2$ is suboptimal, at least in the parental JB6 clone 41 cells studied.

What are the advantages of $H_2O_2$ over superoxide as a messenger? First, due to its uncharged nature $H_2O_2$ can diffuse more easily through membranes than can the superoxide anion. This might facilitate its removal by catalase within peroxisomes or by diffusion out of the cell. $H_2O_2$ may also serve as a hormone-like extracellular stimulus; it is produced at sites of inflammation by neutrophils and macrophages, and could thus activate nearby cells. The second advantage may be its lower reactivity and toxicity. Unlike superoxide, $H_2O_2$ is not a free radical. This increases its diffusion radius within the cytoplasm before decay. Finally, $H_2O_2$ can be completely detoxified in a single enzymatic step into $H_2O$ and $O_2$, whereas superoxide is predominantly degraded via $H_2O_2$, and therefore causes prolonged toxicity.

Under cell-free conditions, the DNA-binding domains of the NF-κB DNA-binding subunits can be inactivated

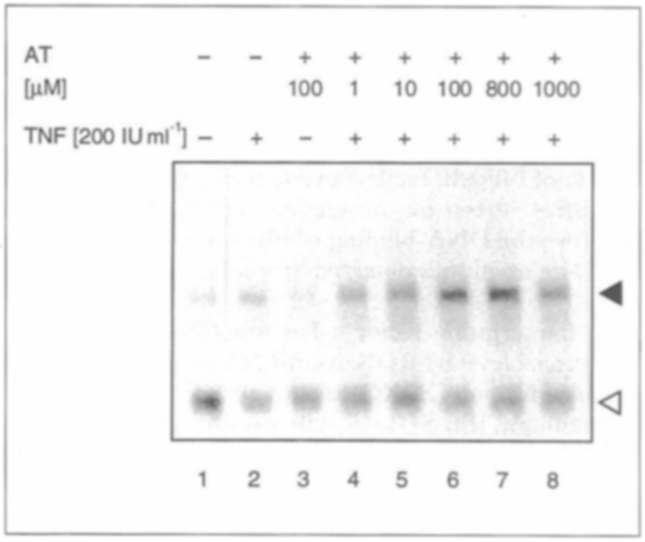


**Fig. 4.** The effect of aminotriazole on the TNF-inducibility of NF-κB in JB6-Cat4 cells. The filled arrowhead indicates the position of the NF-κB–DNA complex. As seen before in Fig. 2, JB6-Cat4 cells showed no induction of NF-κB after a 1 h TNF treatment (compare lanes 1 and 2). The indicated amounts of aminotriazole (AT) were added to the cell cultures 1 h before treatment with 200 IU $ml^{-1}$ TNF (lanes 4–8). The effect of 100 μM AT in the absence of TNF is shown in lane 3. To estimate the effect of AT, compare lanes 3 and 6. A section of a fluorogram from a native gel is shown. The open arrowhead shows the position of a non-specific complex, which is not induced in response to TNF.

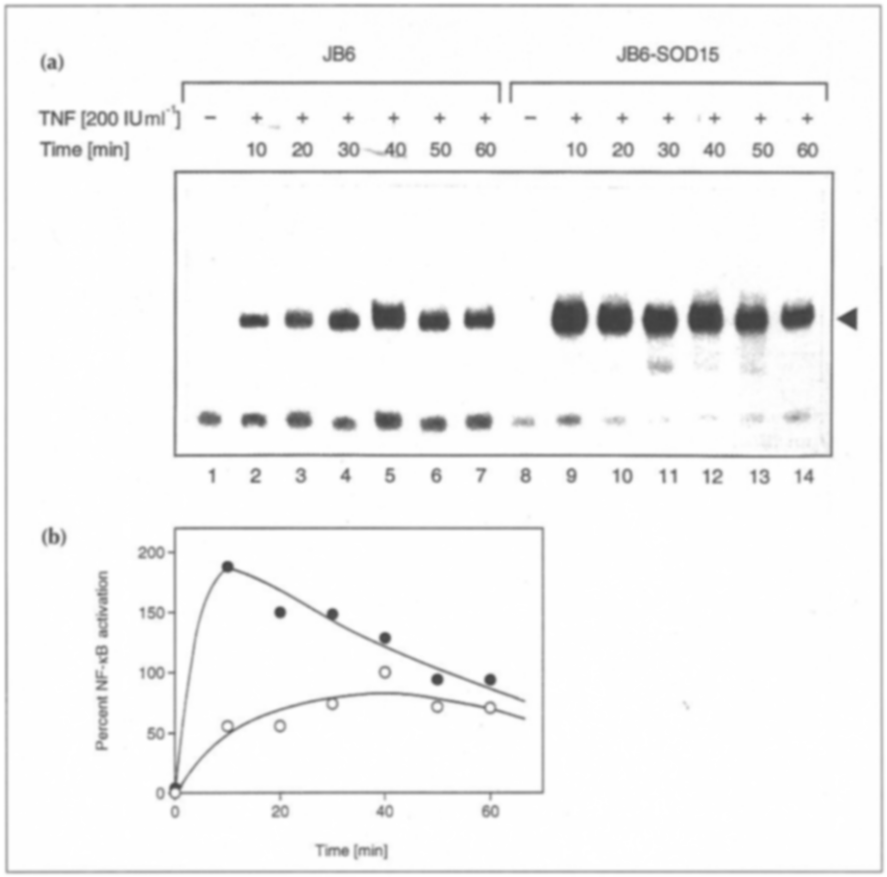


**Fig. 5.** The activation of NF-κB by TNF in cells stably transfected with Cu/Zn–SOD. **(a)** Cell cultures of the parental line JB6 clone 41 (lanes 1–7) and of the SOD-overexpressing line JB6-SOD15 (lanes 8–14) were left untreated (lanes 1 and 8) or were stimulated with 200 IU $ml^{-1}$ TNF for the indicated periods of time. Total cell extracts were prepared and equal amounts of protein analyzed for NF-κB DNA binding activity using EMSA. For details of illustration, see legend to Fig. 2. **(b)** Quantitation of the radioactivity in the NF-κB–DNA complexes by β imaging. Open circles, JB6 clone 41 cells; filled circles, JB6-SOD15 cells. The maximal NF-κB activity seen with JB6 clone 41 cells (at the 40 min time point) was taken as 100 %.

by oxidation of conserved cysteine residues [43,44]. In contrast, in intact cells NF-κB is activated, not inactivated, by prooxidant treatments [27,28]. We consider it very unlikely that overexpression of catalase or SOD directly affected the DNA binding potential of p50 and p65 NF-κB by cysteine oxidation. SOD overexpression, which creates a prooxidant condition by producing more $H_2O_2$ (see Fig. 1), should suppress the DNA-binding ability of NF-κB; catalase overexpression, which relieves oxidative stress by decreasing $H_2O_2$ levels, should improve the DNA-binding of NF-κB. In both cases, the opposite reaction is observed here in intact cells.

The transcription factor c-Fos is activated at the transcriptional level by ROIs. Its mRNA level is dramatically increased upon X/XO treatment of JB6 cells [32]. Intriguingly, JB6-SOD15 cells are strongly deficient for induction of c-Fos by X/XO, suggesting that, in contrast to NF-κB, this factor predominantly requires superoxide for its transcriptional activation. The selectivity of NF-κB for $H_2O_2$ is not without precedent. The bacterial transcription factor oxyR is also specifically activated by $H_2O_2$ [45]. Another bacterial system, soxR/S, is activated by superoxide but not by peroxide [46].

### Suppression of NF-κB activation by exogenous catalase

$H_2O_2$ readily passes through biological membranes. The addition of catalase to the culture medium may thus lower intracellular $H_2O_2$ levels by establishing a steeper efflux gradient of $H_2O_2$ over the plasma membrane. We have added increasing amounts of purified bovine liver catalase to JB6 clone 41 and JB4-SOD15 cells and tested the efficiency of NF-κB activation upon TNF stimulation. In both cell lines, a dose-dependent decrease in NF-κB activation was observed (Fig. 6). Optimal inhibition of NF-κB activation was obtained after preincubation with the enzyme for 30 h. At this point, some catalase may have been internalized. These data support the findings with the catalase-overexpressing cell line JB6-Cat4, and suggest that the primary cause of NF-κB inhibition in the subclone was an increased depletion of $H_2O_2$.

### Catalase and SOD levels also affect NF-κB activation by okadaic acid

We asked whether activation of NF-κB by inducers that are unrelated to TNF is also affected by catalase and SOD levels. JB6 cells did not respond to phorbol esters or IL-1 (data not shown), but the phosphatase IIa inhibitor okadaic acid was a potent activator of NF-κB in this cell line (Fig. 7), as previously found for other cell lines [31]. Unlike TNF, the induction of NF-κB-binding activity in EMSAs by okadaic acid showed a lag phase of 15–20 min (data not shown). The changes in NF-κB activation in response to 400 nM okadaic acid in cell lines overexpressing catalase and SOD were similar to those seen with TNF. In JB6-Cat4 cells, NF-κB activation was reduced, whereas it was potentiated in JB6-SOD15 cells. These data suggest that the use of $H_2O_2$ as a messenger is not restricted to TNF.

There are several additional lines of evidence to suggest that the phosphatase IIa inhibitor okadaic acid is using

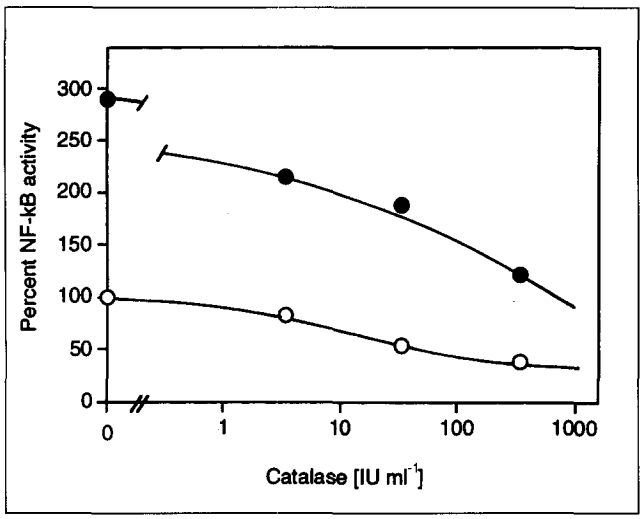


**Fig. 6.** The effect of exogenously added catalase on NF-κB activation by TNF in JB6 clone 41 and JB6-SOD15 cells. Cell cultures were incubated for 30 h with the indicated IU $ml^{-1}$ of purified bovine liver catalase and then induced for 20 min with 200 IU $ml^{-1}$ TNF. Total cell extracts were prepared and equal amounts of protein analyzed for NF-κB activity by EMSA. Open circles, JB6 clone 41 cells; filled circles, JB6-SOD15 cells. The amount of $^{32}P$-radioactivity in the NF-κB–DNA complexes was quantitated by β imaging and is shown plotted in a dose–response curve. The value found for JB6 clone 41 cells under control conditions was taken as 100 %.

$H_2O_2$ to activate NF-κB. First, treatment of HeLa cells and fibroblasts with submicromolar concentrations of okadaic acid caused greatly increased production of superoxide and $H_2O_2$ (K.N.S., P.A.B. and B. Meier, manuscript in preparation). Second, maximal NF-κB activation at 0.9 μM okadaic acid coincided with the maximal production of ROIs. Third, the antioxidant pyrrolidine dithiocarbamate inhibits NF-κB activation by okadaic acid. Fourth, in some cell lines, $H_2O_2$ and the glutathione inhibitor BSO were found to potentiate the effects of okadaic acid [31]. Thus, it appears that both TNF and okadaic acid use $H_2O_2$ as a messenger, explaining the effects of SOD and catalase.

Our model for the activation of NF-κB given these data is presented in Fig. 8. We believe that both TNF and okadaic acid induce an increased production of superoxide. Superoxide is not directly involved in NF-κB activation, but serves as a source of $H_2O_2$. This is shown by the fact that overexpression of Cu/Zn–SOD, which reduces the amount of superoxide in the cytosol, increases rather than decreases NF-κB activation. This observation also suggests that superoxide is a predominant source for $H_2O_2$, but does not rule out other sources. Several enzymes can be induced to produce superoxide. For example, plasma-membrane-bound forms of NADPH oxidases, which can be activated by phorbol esters, are now known to be wide-spread, and not restricted to neutrophils and macrophages in their expression [47]. Other enzymes inducibly generating oxidative stress are cyclooxygenases, lipoxygenases and xanthine oxidase [42]. Mitochondria are a major source of ROIs, and there is evidence that ROI production by mitochondria is required for the cytotoxic and gene inductive effects of TNF [40,41]. Further studies will be required to decipher which of these ROI-producing enzymes mediate the effects of the various treatments that activate NF-κB.

Another important issue for future studies will be to investigate how widely $H_2O_2$ is used as a messenger of NF-κB activation. Clearly, this ought to be examined with many more inducers and distinct cell types. The analysis of cells stably transfected with catalase or SOD, as exemplified in the present study, provides a very specific, though laborious approach. Such analyses may be complemented by experiments directly determining the induction of oxidative stress as well as pharmacological experiments using different antioxidants.

A recent study has shown that $H_2O_2$ is used as a pathogen-induced messenger in plant cells [48]. $H_2O_2$ production upon injury was induced by inhibition of catalase, mediated by binding of the primary messenger salicylate to the enzyme. There is circumstantial evidence that $H_2O_2$ also serves as a messenger in the response to a number of pathogenic stimuli in cells from higher vertebrates [49]. These stimuli both cause oxidative stress and activate NF-κB, which in turn activates the expression of numerous genes involved in the response to the pathogen. Our results link $H_2O_2$ induction to NF-κB activation; if $H_2O_2$ is also used in animals as a general pathogen-induced messenger, the fact that a wide range

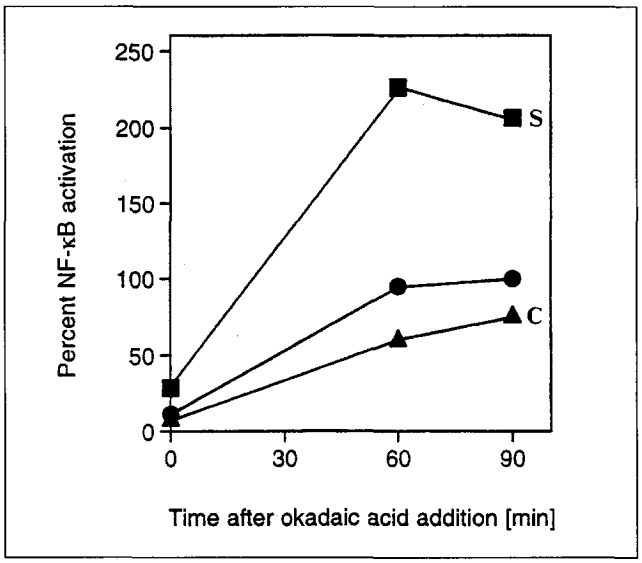


**Fig. 7.** The kinetics of NF-κB activation by okadaic acid in the three cell lines used. Cell cultures from JB6 clone 41, JB6-Cat4 and JB6-SOD15 cells were treated for the indicated periods of time with 400 nM okadaic acid. Total cell extracts were prepared and equal amounts of protein analysed by EMSA for NF-κB DNA binding activity. The radioactivity in NF-κB–DNA complexes was quantitated by β imaging. The value found for JB6 clone 41 cells after 60 min was taken as 100 %. Circles, JB6 clone 41 cells; triangles, JB6-Cat4 cells (C); squares, JB6-SOD15 cells (S).

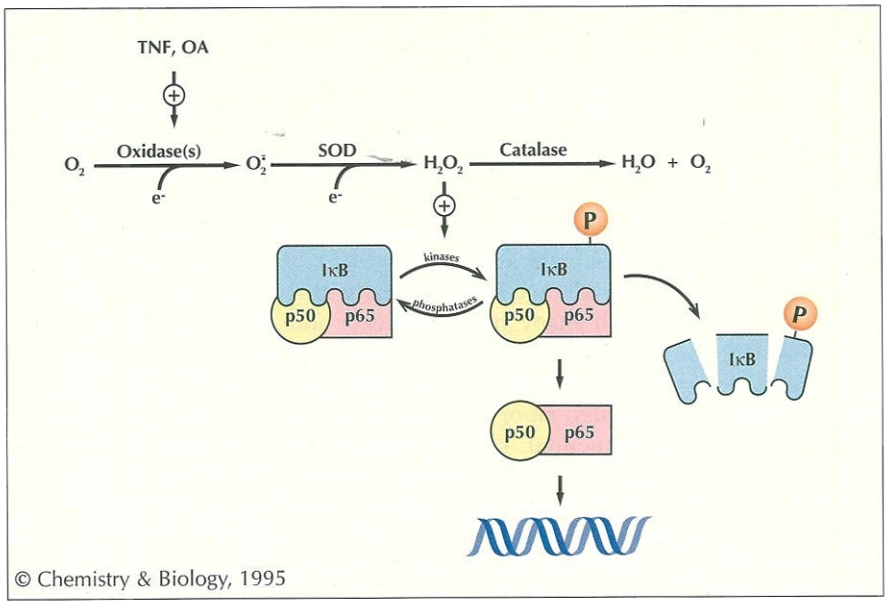


**Fig. 8.** A model for NF-κB activation. TNF or okadaic acid (OA), or other stimuli, induce the production of superoxide anion ($O_2^-$) and subsequently $H_2O_2$. $H_2O_2$, perhaps via glutathione disulfide, may then either activate kinases or inhibit phosphatases, increasing the phosphorylation of IκB. IκB is then degraded, releasing the NF-κB p50/p65 dimer to bind to DNA.

of stimuli that are mostly pathogenic can all activate NF-κB would be easily explained.

## Significance

**NF-κB is an important mediator of the host defence against pathogens. It is also an extreme example of a transcription factor that responds to extracellular stimuli to give altered patterns of nuclear gene expression. Most transcription factors are activated by very specific stimuli, such as hormones. The cytoplasmic latent form of NF-κB is activated by many different stimuli, most of which are pathogenic, including many bacteria and viruses, different kinds of energy-rich radiation, inflammatory cytokines and oxidants. This variety of inducers raises the question of whether NF-κB itself responds to many distinct signal transduction pathways, or whether the pathways converge earlier, by forming a common pathogen-induced messenger molecule.**

**Circumstantial evidence has suggested that reactive oxygen intermediates may be involved in the activation of NF-κB. Here, we have used cell lines that overexpress either $H_2O_2$-degrading or superoxide-degrading enzymes to show that $H_2O_2$, but not superoxide, can act as a second messenger in the response to both TNF and okadaic acid stimulation. We have thus shown that the reactive oxygen intermediates are not simply the undesired side products of oxidizing reactions but are important as pathogen-induced messenger molecules. Using the approach described here, it should also be possible to examine whether $H_2O_2$ acts as a messenger in other cellular responses, and whether there are any situations in which superoxide itself acts as a messenger.**

## Materials and methods

### *Cell culture and treatments*

The mouse epidermal cell line JB6 clone 41, the catalase overexpressing subclone JB6-Cat4 and the Cu/Zn–SOD-overexpressing subclone JB6-SOD15 were described and characterized previously [32,33]. Cells were grown in Minimal Essential Medium (MEM) plus 10 % fetal calf serum, 1 % (w/v) penicillin/streptomycin (all purchased from GIBCO Laboratories, Grand Island) and 50 ng $ml^{-1}$ $Na_2SeO_3$ (Sigma Chemicals, St. Louis).

For activation of NF-κB, cells were treated for the indicated periods of time with 200 IU $ml^{-1}$ of human recombinant TNF-α (Boehringer, Mannheim) or 400 nM of the sodium salt of okadaic acid (Calbiochem). For catalase inhibition, cells were incubated with the indicated concentrations of 3-amino-1,2,4-triazole (AT) 1 h before stimulation with 200 IU $ml^{-1}$ TNF. Treatment of JB6 clone 41 and JB6-SOD15 cells with purified catalase from bovine liver (Boehringer, Mannheim) was performed for 30 h at final enzyme concentrations of 3–333 IU $ml^{-1}$ followed by stimulation with 200 IU $ml^{-1}$ TNF for 20 min.

### *FACS analysis*

Cells (1 x $10^6$) in phosphate-buffered saline (PBS) were incubated with 5 μM DCFH diacetate (Fluca) in dimethylformamide for 10 min at 37 °C. Aliquots of 1 x $10^5$ cells were then scanned on a FACSSORT (Beckton Dickinson) with excitation and emission settings of 495 and 525 nm, respectively. Histograms were analyzed with the software program Lysis II.

### *Cell extracts*

To monitor the activity state of cellular NF-κB, total cell extracts rather than nuclear extracts were prepared and used for electrophoretic mobility shift assays. Cell extracts were prepared by resuspending PBS-washed cell pellets with a high-salt buffer containing the non-ionic detergent Nonidet P-40, as described previously [50]. Protein concentrations in supernatants of cell lysates were determined by an assay based on the Coomassie Brilliant Blue reaction (Bio-Rad).

### *DOC treatment*

To determine the amount of NF-κB–IκB complex, cytosolic fractions were prepared from JB6 clone 41 and JB6-SOD15

cells and treated with final concentrations of 0.2, 0.4 and 0.8 % (w/v) sodium desoxycholate (DOC) (Merck), followed by addition of Nonidet P-40, as described previously [10]. Optimal activation of NF-κB as detected by EMSA was seen using 0.4 % DOC.

### *Electrophoretic mobility shift assays*

Aliquots of cell extracts containing equal amounts of protein were used in EMSAs. The DNA binding conditions for NF-κB have been described in detail elsewhere [51]. In the present study, between 10 and 15 μg of total protein were used for a binding reaction. Binding reactions contained 10 000 cpm (Cerenkov counting) of an end-$^{32}$P-labelled double-stranded oligonucleotide with a high-affinity NF-κB-binding motif (Promega). DNA binding reactions were analyzed on native 4 % polyacrylamide gels. Dried gels were exposed to Kodak XR5 films. The radioactivity in NF-κB–DNA complexes was quantitated by β imaging (Molecular Dynamics imager).

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