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Separation of oxidant-initiated and redox-regulated steps in the NF-κB signal transduction pathway

(tyrosine phosphorylation/inflammatory cytokines/SRC-kinases/glutathione)

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ABSTRACT **Studies presented here show that overall NF-κB signal transduction begins with a parallel series of stimuli-specific pathways through which cytokines (tumor necrosis factor α), oxidants (hydrogen peroxide and mitomycin C), and phorbol ester (phorbol 12-myristate 13-acetate) individually initiate signaling. These initial pathways culminate in a common pathway through which all of the stimulating agents ultimately signal NF-κB activation. We distinguish the stimuli-specific pathways by showing that the oxidative stimuli trigger NF-κB activation in *only one* of two human T-cell lines (Wurzburg but not Jurkat), whereas tumor necrosis factor α and phorbol 12-myristate 13-acetate readily stimulate in both lines. We propose the common pathway as the simplest way of accounting for the common requirements and properties of the signaling pathway. We include a redox-regulatory mechanism(s) in this common pathway to account for the previously demonstrated redox regulation of NF-κB activation in Jurkat cells (in which oxidants don't activate NF-κB); we put tyrosine phosphorylation in the common pathway by showing that kinase activity (inhibitable by herbimycin A and tyrphostin 47) is required for NF-κB activation by all stimuli tested in both cell lines. Since internal sites of oxidant production have been shown to play a key role in the cytokine-stimulated activation of NF-κB, and since tyrosine kinase and phosphatase activities are known to be altered by oxidants, these findings suggest that intracellular redox status controls NF-κB activation by regulating tyrosine phosphorylation event(s) within the common step of the NF-κB signal transduction pathway.**

The activated form of the nuclear transcription factor NF-κB increases transcription of inflammatory response genes and viral genes, notably human immunodeficiency virus (1). The unactivated form of this transcription factor is sequestered in the cytosol by member(s) of the I-κB family. T-cell stimulation by a wide variety of agents, ranging from phorbol esters (phorbol 12-myristate 13-acetate; PMA) and cytokines [tumor necrosis factor α (TNF-α) and interleukin 1α] to oxidants such as hydrogen peroxide (H_2O_2) and UV irradiation, cause I-κB to dissociate from the NF-κB complex. The release of NF-κB from its inhibitory protein(s) permits the translocation of NF-κB into the nucleus, where it binds to DNA enhancer motifs and regulates transcription of a diverse series of genes (1, 2).

Intracellular redox levels have been shown to regulate NF-κB signal transduction triggered by a variety of stimuli (3, 4). H_2O_2 and UV triggering of NF-κB activation are often used as models of this common redox regulation; however, we show here that the mechanism responsible for triggering by these oxidants is genetically separable from the common, redox-sensitive mechanism that operates in response to all stimuli. We also demonstrate a common requirement for protein tyrosine phosphorylation in NF-κB activation. Since the collective activity of tyrosine kinases and phosphatases stimulated is redox regulated (5–7), we suggest that the redox regulation of these enzymes contributes to the redox sensitivity of the common step of the NF-κB signal transduction pathway.

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EXPERIMENTAL METHODS

Cell Lines And Culture. Patrick Baeuerle (Ludwig Universität, Freiburg, Germany) kindly provided Wurzburg Jurkat T cells (subclone JR), and the Jurkat T-cell line was obtained from the American Type Culture Collection (TIB152). Jurkat κB5.2 clone, derived from TIB152, has been described (8). Cell lines were cultured as described (8).

Cell Stimulation and Reporter Gene Assays. Cells were preincubated with the inhibitors herbimycin A (Biomol, Plymouth Meeting, PA) and tyrphostin 47 (Biomol) and stimulated with H_2O_2 (Fisher), PMA (Sigma), mitomycin C (Mito-C; Sigma), and TNF-α (Cetus) as described in the figure legends. Cells to be UV irradiated were resuspended in PBS containing 1% (wt/vol) glucose and irradiated with a Stratagene Stratalinker model 2400 at 254 nm. After irradiation, cells were pelleted and resuspended in RPMI containing 10% (vol/vol) fetal calf serum. β-Galactosidase reporter gene activity was measured with 4-methylumbelliferyl β-D-galactoside as described (8).

Nuclear Extracts and Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared by the method of Schreiber *et al.* (9) except that buffer C contained 10% (wt/vol) glycerol. The NF-κB and AP-1 probes and the gel retardation analysis methods have been described (10, 11).

RESULTS

Separation of Oxidant Stimulation and Redox Regulation. The mechanisms that mediate redox regulation and oxidant stimulation of NF-κB activation are resolved by studies with two human T-cell lines, Jurkat and Jurkat JR. We obtained the latter line from P. Baeuerle and provisionally call it Wurzburg (after its city of origin). Its surface marker expression and T-cell receptor rearrangement (shown in Fig. 1) clearly distinguish it from the Jurkat line that is commonly used in the U.S. and is available from the American Type Culture Collection. Surface marker differences (data not

Abbreviations: PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor α; Mito-C, mitomycin C.

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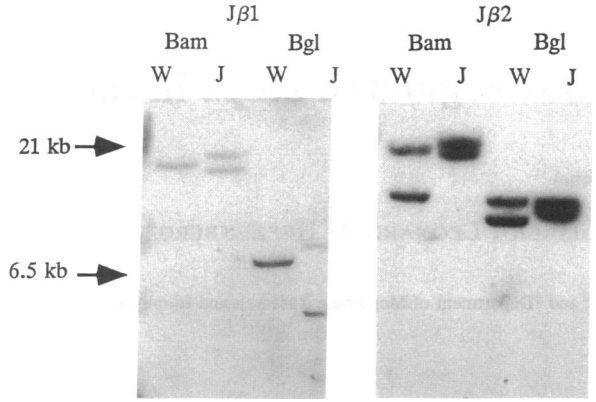

FIG. 1. The T-cell receptor rearrangements of the Wurzburg (W) and Jurkat (J) T-cell lines are distinct. Genomic DNA from the Wurzburg and Jurkat cell lines was analyzed by Southern analysis with either a 0.7-kb *Xba* Jβ1 or a 4.5-kb *Eco*RI Jβ2 labeled genomic probe after restriction digestion with either *Bgl* II or *Bam*HI.

shown) include the following: Jurkat expresses CD2, CD45RA, and high levels of CD3 and the C305 idiotype (12); Wurzburg lacks CD2 and CD45RA, expresses low levels of CD45RO and CD3, and does not express the C305 idiotype.

Baeuerle and colleagues (4) demonstrated that NF-κB is readily activated in the Wurzburg line by stimulation with H_2O_2 and UV. Data in Fig. 2 confirm these findings and show further that another oxidative stimulus, Mito-C (13), also activates NF-κB in Wurzburg. These oxidative stimuli, however, do not detectably activate NF-κB in Jurkat (Fig. 2; also see ref. 14). Thus, the mechanism through which oxidants trigger NF-κB activation in Wurzburg does not function in the Jurkat cell line.

The redox-sensitive mechanism that regulates NF-κB activation by PMA and cytokines, however, is fully functional in Jurkat (and in Wurzburg). Antioxidants block stimulation of NF-κB activation by these agents in Jurkat (3, 14, 15) and in Wurzburg (4); nordihydroguaiaretic acid completely inhibits both PMA and TNF-α stimulations in Jurkat (data not shown); and *N*-acetylcysteine, which acts both as an antioxidant and as a source of cysteine for replenishing intracellular glutathione, blocks NF-κB activation consistently but to a lesser extent (40% for TNF-α stimulation; 75% for PMA stimulation) in both Jurkat and Wurzburg.

Taken together, the above findings demonstrate that NF-κB activation is redox regulated in Jurkat T cells, despite the inability of oxidants to stimulate NF-κB activation in these cells. Thus, two redox-sensitive mechanisms must be involved in NF-κB signal transduction. One, present only in Wurzburg, allows oxidants such as H_2O_2 to trigger NF-κB activation. The other, present both in Jurkat and Wurzburg, allows intracellular redox levels to regulate the extent of NF-κB activation.

Protein Tyrosine Phosphorylation Is a General Requirement for NF-κB Activation. Karin and colleagues (5), working with the HeLa cell line, have shown that UV stimulation activates Src kinase(s), which phosphorylate particular protein tyrosine residues. This activation is an essential component of the signal transduction pathway leading to activation of NF-κB and AP-1, another transcription factor. The studies that follow confirm the requirement for protein tyrosine phosphorylation in the oxidant stimulation of NF-κB induction and implicate tyrosine phosphorylation events as essential for NF-κB activation, both by oxidative and nonoxidative stimuli. Thus we demonstrate that tyrosine phosphorylation is a general requirement for NF-κB activation in human lymphocytes.

Reporter Gene Studies. Cytokines and PMA stimulate the expression of a stable, integrated reporter gene construct (tri-κB *lacZ* in Jurkat) whose transcription is controlled by a tandem trimer of NF-κB binding sites (8). Two differently acting tyrosine kinase inhibitors, herbimycin A and tyrphostin 47, completely inhibit stimulation of this transcription factor by either PMA or TNF-α (Figs. 3*B* and 4). This complete inhibition is observed after either a short-term, high-dose herbimycin A (4 hr, 10 μg/ml) preincubation or the long-term, low-dose preincubation conditions (18 hr, 0.5–3 μg/ml) used by many laboratories.

Herbimycin A is a widely used tyrosine kinase inhibitor that is attributed to be a selective inhibitor of Src-like kinases (15) by targeting their degradation. It is relatively nontoxic and does not interfere with protein synthesis, PMA/ionomycin stimulation of interleukin 2 production, or signaling via pathways that do not depend on tyrosine kinase activity [e.g., pathways dependent upon serine/threonine kinases or guanine nucleotide-binding-protein-dependent signaling pathways (16–18)]. Furthermore, it only minimally decreases the PMA-signaled induction of the T-cell surface "activation" antigen, CD69 (Fig. 3*B* and ref. 19), although,

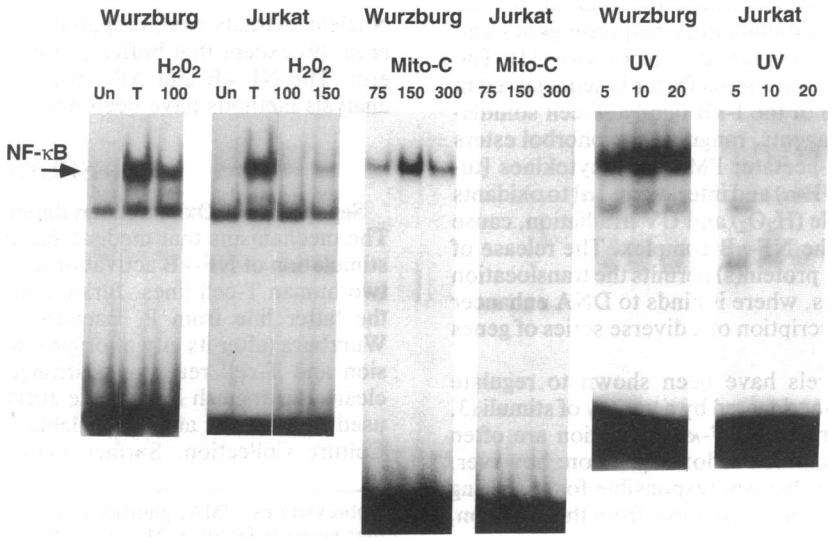

FIG. 2. Jurkat and Wurzburg lines differ in their activation of NF-κB in response to oxidative stimuli. Nuclear extracts were prepared 4 hr after stimulation of Wurzburg or Jurkat cells. Un, unstimulated; T, TNF-α (20 ng/ml); H_2O_2, hydrogen peroxide (100 and 150 μM); UV, UV-C (5, 10, and 20 joules); Mito-C, Mito-C (75, 150, and 300 μg/ml), a third oxidative stimulus. The arrow indicates the inducible NF-κB band.

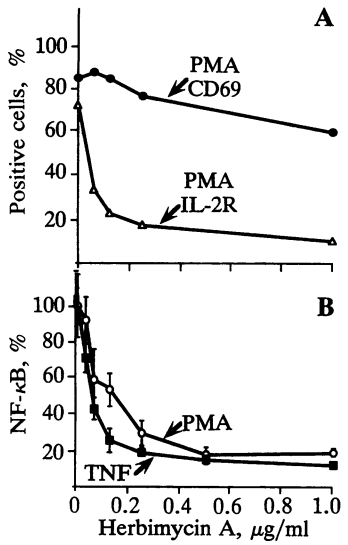

FIG. 3. Herbimycin A specifically blocks NF-κB-dependent transcription. (*A*) Surface expression of PMA-induced activation markers. Jurkat T cells were preincubated with a variety of concentrations of herbimycin A for 18 hr, stimulated with PMA (50 ng/ml) for 16 hr in the continuous presence of herbimycin A, and then analyzed for surface expression of the interleukin 2 receptor (IL-2R) and CD69 by fluorescence-activated cell sorting analyses after staining of the cells with phycoerythrin-labeled monoclonal antibodies. Frequency of cells expressing the indicated surface marker is determined by staining with fluorochrome-conjugated monoclonal antibodies and gating to include cells with associated fluorescence greater than two standard deviations above the median autofluorescence. (*B*) Reporter gene assay for NF-κB activation. Jurkat κB5.2 cells with an integrated construct consisting of three κB sites linked to *lacZ* were preincubated for 18 hr with a range of concentrations of herbimycin A and then stimulated with TNF-α (20 ng/ml) or PMA (50 ng/ml). Cells were lysed, and β-galactosidase activity was assayed as percentage of the *lacZ* activity obtained in the absence of inhibitor (an index of NF-κB-dependent transcription). Error bars indicate the standard deviation of 10 replicates per condition.

as Fig. 3*B* shows, it completely inhibits the NF-κB dependent induction of interleukin 2 receptor surface expression by PMA in Jurkat T cells.

Expression of the NF-κB-controlled reporter gene in Jurkat is also inhibited by a pseudosubstrate tyrosine kinase inhibitor, tyrphostin 47 (20, 21). Preincubation for 30 min with a wide range of tyrphostin 47 concentrations blocks the expression of the tri-κB *lacZ* reporter gene in cells stimulated either with PMA or TNF-α (Fig. 4). Thus, two tyrosine kinase inhibitors with different modes of action block signaling of NF-κB-dependent gene expression.

Gel Retardation Studies. The herbimycin A inhibition of NF-κB-directed gene expression is due to a failure to activate/translocate free NF-κB to the nucleus (Fig. 5). NF-κB bands are readily detectable in gels of nuclear extracts prepared from PMA or TNF-α-stimulated cells; however, these bands are missing in gels of extracts from cells preincubated with herbimycin A (4 hr, 10 μg/ml) prior to stimulation (22, 23).

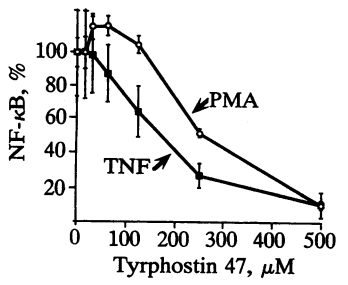

FIG. 4. Tyrphostin 47 specifically blocks NF-κB-dependent transcription. Jurkat κB5.2 cells were preincubated for 30 min with tyrphostin 47 and then stimulated with TNF-α (20 ng/ml) or PMA (50 ng/ml). Analysis of β-galactosidase activity was measured as in Fig. 3.

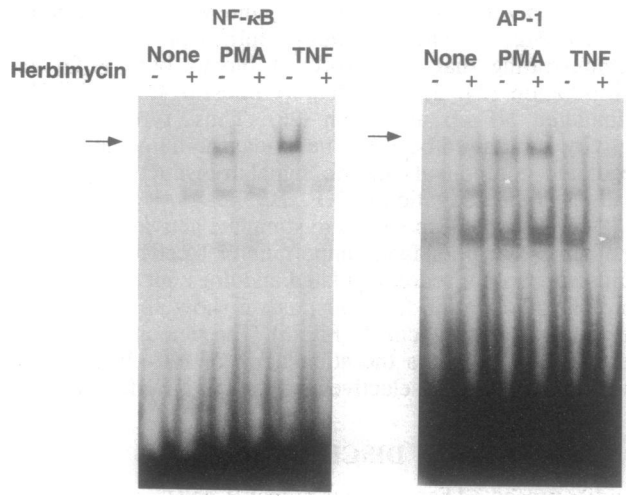

FIG. 5. Herbimycin A inhibits the activation of NF-κB, but not AP-1, in Jurkat T cells. Jurkat T cells were pretreated with tyrosine kinase inhibitor herbimycin A for 4 hr and stimulated with TNF-α or PMA. Gel retardation data are shown for the same extracts analyzed with an NF-κB probe (*Left*) and an AP-1 probe (*Right*). The arrow indicates the inducible band.

Bomsztyk and colleagues (23), using another herbimycin A treatment protocol that inhibits NF-κB activation by IL-1-α, recently reported that herbimycin A does not inhibit PMA activation of A NF-κB. We also failed to inhibit PMA-stimulated activation of NF-κB with herbimycin A when we used this protocol (10 μg/ml for 1 hr) (data not shown). However, since herbimycin A fully inhibits PMA-stimulated NF-κB activation when cells are pretreated for a longer time (10 μg/ml for 4 hr; see above), we ascribe the lack of inhibition with the Bomstyk protocol to its inability to deplete intracellular tyrosine kinase activity to a level that would limit NF-κB activation by a strong stimulus such as PMA.

Completing the demonstration of the generality of the tyrosine phosphorylation requirements for NF-κB activation are the studies conducted with the Wurzburg cells showing

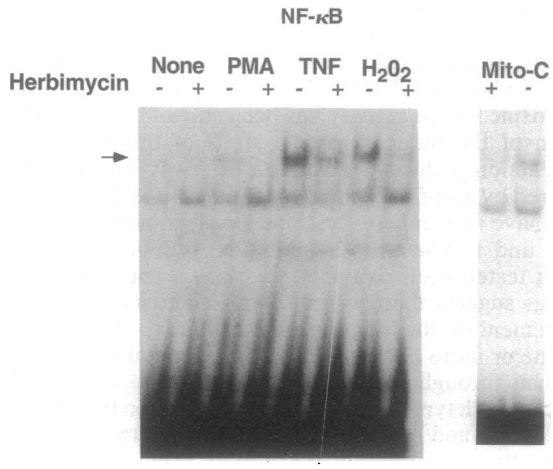

FIG. 6. Herbimycin A blocks activation of NF-κB by oxidants. The oxidant-sensitive Wurzburg cell line was stimulated with PMA (50 ng/ml), TNF-α (20 ng/ml), hydrogen peroxide (150 μM), or Mito-C (300 μM) after a 4-hr preincubation with or without herbimycin A. The arrow indicates the inducible NF-κB band.

that herbimycin A blocks the H_2O_2 and Mito-C activation of NF-κB (Fig. 6). These findings confirm previous findings demonstrating that herbimycin A blocks both H_2O_2 and γ-irradiation (24, 25) and that tyrphostin 47 blocks UV-stimulated NF-κB activation (26). Thus, tyrosine kinase activity is required both for the oxidant-initiated and redox-regulated components of the signal transduction pathway leading to the activation of NF-κB.

PMA has also been shown to stimulate activation of AP-1, another transcription factor important for T-cell function. This stimulation is not redox-regulated and does not involve tyrosine phosphorylation (5). Thus, as we show above, it is not inhibited by herbimycin A (Fig. 5). The demonstration that herbimycin A inhibits the stimulation of NF-κB activation therefore reflects the selective inhibition of NF-κB activation.

DISCUSSION

Studies presented here use two human T-cell lines, Wurzburg and Jurkat, to distinguish the mechanism through which oxidants specifically induce NF-κB signal transduction from the downstream mechanism in which oxidants serve as essential messengers in the signal transduction pathway(s) initiated by all NF-κB inducers. As we have shown, oxidants readily induce NF-κB activation in Wurzburg but do not trigger the induction/activation of this transcription factor in Jurkat; however, nonoxidative molecules such as PMA and TNF induce NF-κB activation in both Wurzburg and Jurkat. Furthermore, although oxidants trigger insufficient signals to activate NF-κB activation in Jurkat, the induction of this factor in both T-cell lines by other stimuli depends upon the generation of intracellular oxidants (27). Taken together, these findings suggest that oxidants, cytokines, and other agents initiate signal transduction by triggering separate, inducer-specific "receptors" and that the signals generated by these inducer-specific receptors ultimately flow through a common, redox-regulated step of the signal transduction pathway to activate NF-κB.

Recent studies show that the cytokine-stimulated production of mitochondria-derived oxidants regulate, but do not initiate, NF-κB activation (28). In essence, these studies show that cytokine-stimulated activation of NF-κB requires superoxide production by mitochondria but that this activation does not occur without additional signaling events. Since the stimulation of mitochondrial oxidant production must occur late in the signal transduction pathway and is necessary, but not sufficient, for NF-κB activation, we propose that it occurs in the common step of the pathway and plays a key role in the redox regulatory activity of this step(s), perhaps, as discussed below, by regulating essential protein tyrosine phosphorylation (Fig. 7).

Tyrosine kinase activity has been shown to be essential to certain of the inducer-specific steps of the NF-κB signal transduction pathway, notably in the early signaling events triggered by anti-CD3 and by oxidants (24, 26, 29). However, as we have shown here, tyrosine kinase inhibitors (herbimycin A and tyrphostin 47) inhibit NF-κB activation by all stimuli tested (i.e., oxidants, cytokines, and PMA). These findings suggest that tyrosine kinase activity is a common requirement for the triggering of NF-κB activation and hence that one or more of these kinases is essential to the passage of signals through the common pathway step. Thus our data implicate both tyrosine kinase activity (protein tyrosine phosphorylation) and redox regulation in this common step.

This idea is attractive because it provides a plausible mechanism through which the redox environment can regulate signal flow through the pathway. Tyrosine kinase activity can be increased by oxidants and decreased by antioxidants. In contrast, tyrosine phosphatase activity, which dephosphorylates the protein tyrosine residues that are phosphorylated by tyrosine kinases, is inhibited by oxidants and protected by antioxidants (5–7). Thus, we suggest that redox-regulating agents such as *N*-acetylcysteine and nordihydroguaiaretic acid, which increase the cell's ability to reduce/inactivate internally generated oxidants, controls NF-κB activation by controlling the extent to which mitochondrial-derived oxidants increase tyrosine phosphorylation.

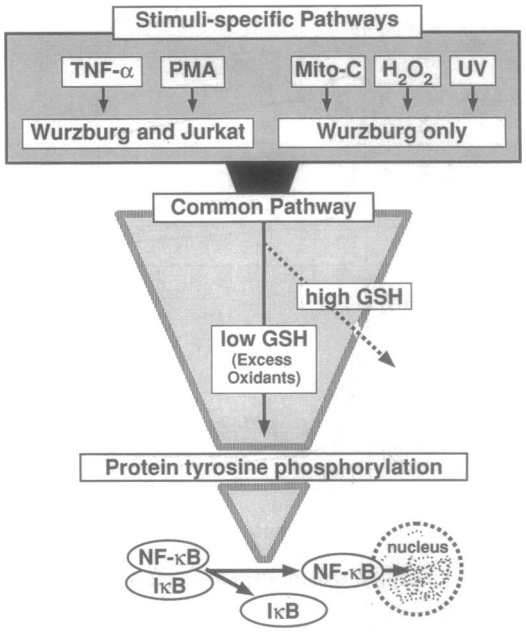

FIG. 7. Proposed location of redox-regulatory and tyrosine phosphorylation events in the stimuli (inducer)-specific and common portions of the overall NF-κB signal transduction pathway in human T cells. GSH, glutathione.

Consistent with this hypothesis, we have recently shown that TNF-α becomes an effective stimulator of overall protein tyrosine phosphorylation in Jurkat T cells when cells are pretreated with the agent buthionine sulfoximine, which depletes glutathione, the principal intracellular redox buffer (30). Tyrosine phosphorylation is barely detectable in phosphotyrosine blots when cells are stimulated at normal glutathione levels; however, blots of extracts from glutathione-depleted cells demonstrate that TNF-α stimulates as much overall tyrosine phosphorylation as anti-CD3. This redox regulation of tyrosine phosphorylation would be readily explained if, as we propose, the signals generated by triggering cells with oxidants, cytokines, and phorbol esters converge on a common pathway that leads initially to generation of mitochondrial oxidants, which in turn increases protein tyrosine phosphorylation and the transmission of signals that stimulate NF-κB activation (Fig. 7).

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